RELATIONSHIP BETWEEN HUMAN GENITAL EPITHELIAL CELLS, SQUAMOUS METAPLASIA AND HPV16-MEDIATED ONCOGENESIS

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By

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ABSTRACT

The stratified squamous epithelium (SSE; for abbreviations, see lists on page xviii) in the transformation zone (TZ) of human uterine cervix is metaplastic in nature, and the TZ is the most common site for carcinomas related to infection by human papillomavirus type 16 (HPV16). This study attempted to elucidate the mechanisms for the high incidence of HPV16-mediated malignancy in the metaplastic SSE.

In vitro cultured normal human keratinocytes (HKC) from foreskin (HFK) and ectocervix (HEC), and epithelial cells from human endocervix (HEN) were reconstructed into epithelia using an *in vivo* nude mice model. While HKC from both HFK and HEC formed well-differentiated SSE, HEN formed an epithelium displaying morphological features of immature metaplastic SSE, which was substantiated by the expression patterns of cytokeratins (CKs). These results established the endocervical origin of metaplastic SSE in the TZ.

HEN immortalized by HPV16 genomic DNA (HEN16) formed highly dysplastic lesions, whereas lesions formed by the immortalized HKC (HKC16) displayed low grade dysplastic changes in the same *in vivo* system. The CK expression patterns in lesions from both types of cells supported the pathological features. Northern and Southern blot analyses for the immortalized cells cultured *in vitro* failed to reveal any significant differences between the HPV16-immortalized cells in the viral DNA status, the expression of the viral E7 and E5 oncogenes, and the expression of the cellular protooncogenes c-*myc* and H-*ras*. Therefore, E7 expression in the *in vivo* implants from the immortalized cells was examined. *In situ* hybridization assays showed that, while E7 expression was limited to the basal cells of the HKC16 lesions, E7 was expressed throughout the HEN16 lesion. The compartmentalized E7

expression in the HKC16 lesions was not due to a defunct transcription mechanism, since the cells that did not express E7 did transcribe CK1. These results suggested that HKC, but not HEN, possess a cell type-specific mechanism to repress HPV16 oncogene expression, and this mechanism is functional only upon undergoing programmed squamous differentiation.

In the *in vivo* system, there may have been uncontrollable variables, such as cytokines induced by surgical trauma or by immune responses to the lesions. To exclude a possible involvement of such factors in the putative repression function for HPV E7, the immortalized cells were tested in the organotypic (raft) culture system. Raft culture is an *in vitro* epithelia reconstruction system, which allows control over the epithelial reconstruction conditions. The morphology and E7 expression of the raft lesions were similar to those in the *in vivo* implants, indicating that this HPV repression function is independent of *in vivo* factors.

The epithelial-specific expression of HPV16 genes, including that of the E7 gene, is controlled by the long control region (LCR) in the HPV16 genome. Thus, the role of the HPV16 LCR in the compartmentalized expression of E7 was evaluated. Immortalized cell lines were established by *in vitro* transfection of the $pSV_2$1667 plasmid, which contained the HPV16 E6 and E7 genes regulated by the SV40 early promoter. The immortalized cells all contained integrated viral oncogenes and expressed E7 at a comparable level in *in vitro* monolayer cultures. Furthermore, the viral oncogenes appeared to retain the functional heterologous control elements, since transforming growth factor β (TGF-β) repressed E7 expression in the cells immortalized by HPV16 genomic DNA, but not in those immortalized by $pSV_2$1667. The signal transduction pathway for TGF-β was functional in all the cell lines, because c-*myc* expression showed normal response to TGF-β. Unexpectedly, the raft lesions

from the $pSV_2$1667-immortalized HEN and HKCs showed pathological features, E7 expression patterns, and 5-AZ responses very similar to those of their respective HPV16 genomic DNA-immortalized counterparts. These results indicated that the squamous differentiation-associated cellular mechanism responsible for the compartmentalized E7 expression in the HKC lesions is not dependent on the HPV16 LCR.

5-azacytidine (5-AZ), a DNA demethylation agent, decompartmentalized E7 expression in the raft of the immortalized HFK, which was accompanied with increased severity of dysplastic changes. Treatment of normal HEC with 5-AZ induced selective changes in the patterns of CK expression. Therefore, the compartmentalized E7 expression in the HKC lesions may result from a cellular mechanism that is functional for gene repression at a global level in differentiating HKC and is mediated by DNA methylation.

To test the clinical relevance of the above observations, expression of the HPV16 E7 oncogene was examined by *in situ* hybridization in natural premalignant lesions occurring at the cervical metaplastic SSE (CINs) and vulval native SSE (VINs). The status of HPV16 DNA and permissiveness for viral vegetative replication in the lesions were evaluated by assessing viral DNA amplification and transcription of the viral L1 structural gene with DNA and RNA *in situ* hybridization assays, respectively. In the VINs, E7 was expressed in the bottom layers of lesions, with viral DNA amplification and late gene expression in the differentiated upper layers. In contrast, E7 was expressed in the CINs throughout the lesions. Furthermore, in the immature metaplastic SSE, viral DNA replication occurred in the bottom cells, without the expression of the viral L1 gene, in contrast to the upper layer viral DNA amplification in the mature metaplastic SSE. Thus, HPV16 infection in immature metaplastic

SSE may represent a form of non-vegetative infection, and the expression of the HPV16 oncogenes and possibly the amplification of viral DNA were dysregulated.

In summary, my studies constitute the first systematic and experimental dissection of the relationship between squamous metaplasia, cervical cancer and HPV16 infection. The results showed that metaplastic SSE derives from HEN, that viral oncogene expression in HEN and HKC in conditions allowing squamous differentiation is distinct, and that this difference is specific for the cell types but not for the HPV16 LCR. The results from clinical samples revealed that HPV16 infection may undergo a distinct life cycle in the cervical immature metaplastic SSE, which is featured by persistent viral oncogene expression and dysregulated viral DNA amplification. Because persistent expression of the HPV16 oncogenes and viral DNA integration are common features of HPV16-containing cervical cancers, this state of non-vegetative infection may be important for the unusual susceptibility of the cervical metaplastic epithelium to HPV16-mediated oncogenesis.

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A	=	Adenosine
5-AZ	=	5-azacytidine
AIM	=	Atypical immature metaplasia
AP-1	=	Activator protein-1
ATP	=	Adenosine triphosphate
B cells	=	B lymphocytes
Bcl-2	=	B-cell lymphoma/leukemia factor 2
BL	=	Burkitt's B cell lymphoma
bp	=	Base pair
BPV	=	Bovine papillomaviruses
BRK	=	Baby rat kidney
BRL	=	Bethesda Research Laboratories
C	=	Cytosine
C-terminus	=	Carboxyl-terminus
CAK	=	CDK activating kinase
CAT	=	Chloramphenicol acetyl transferase
CDI	=	CDK inhibitors
CDK	=	cyclin dependent serine/threonine protein kinase
cDNA	=	Complementary DNA
CIF	=	Cellular interfering factor
CIN	=	Cervical intraepithelial neoplasia
CIAP	=	Calf intestinal alkaline phosphatase
CIS	=	Carcinoma *in situ*
CK	=	Cytokeratin
CKII	=	Casein kinase II
cpm	=	counts per minute
CR1	=	E1A conserved region 1
CR2	=	E1A conserved region 2
CRPV	=	Cottontail rabbit papillomavirus
DNA	=	Deoxyribonucleic acid
DNA-PK	=	DNA-activated protein kinase
DNase I	=	Deoxyribonuclease I
E1-BS	=	E1 binding site
E1A	=	Adenovirus early region 1A
E2-BS	=	E2 binding site
EBNA	=	EBV nuclear antigen
EB	=	Ethidium bromide
EBV	=	Epstein-Barr virus
EDTA	=	Ethylene diamine tetracetic acid

EGF	=	Epidermal growth factor
EN1667	=	HEN immortalized by the construct $pSV_2$1667
FH2	=	HFK immortalized by the construct $pSV_2$1667
G	=	Guanosine
G_0	=	Gap 0 phase of mitotic cycle
G_1	=	Gap 1 phase of mitotic cycle
GRE	=	Glucocorticoid-progesterone response element
HBV	=	Hepatitis B virus
HEC	=	Human ectocervical epithelial cells in culture
HEC16	=	HEC immortalized by HPV16 genomic DNA
HEN	=	Human endocervical epithelial cells in culture
HEN16	=	HEN immortalized by HPV16 genomic DNA
HFK	=	Human foreskin epithelial cells in culture
HFK16	=	HFK immortalized by HPV16 genomic DNA
HKC	=	Human keratinocytes in culture
HKC16	=	HKC immortalized by HPV16 genomic DNA
HHV-6	=	Human herpesvirus-6
HIV	=	human immunodeficiency virus
HKC	=	Human keratinocytes in culture
HLA	=	Human leukocyte antigen
hMSH2	=	Human mismatch repair gene
HNPCC	=	Hereditary nonpolyposis colorectal cancer
HPV	=	Human papillomavirus
HSV	=	Herpes simplex virus
Ig	=	Immunoglobulin
IGF	=	Insulin-like growth factor
INI	=	Irreversible non-vegetative infection
kb	=	Kilo base pair
kDa	=	Kilo dalton
KGM	=	Keratinocyte growth media
KRF	=	Keratinocyte-specific transcriptional activator
LCR	=	Long control region
M	=	Mitosis phase of mitotic cycle
M_1	=	Mortality stage 1
M_2	=	Mortality stage 2
MAP	=	Mitogen activated protein
MHC	=	Major histo-compatibility complex
mRNA	=	Messenger RNA
MSPF	=	Methylation-sensitive papillomavirus transcription factor
N-terminus	=	Amino-terminus
NF-IL6	=	Nuclear factor for interleukin 6 expression

NMU	=	Nitrosomethylurea
nt	=	Nucleotide
ORF	=	Open reading frame
PBS	=	Phosphate buffered saline
PCNA	=	Proliferating cell nuclear antigen
PCR	=	Polymerase chain reaction
PDGF	=	Platelet derived growth factor
PKC	=	Protein kinase C
PLC-γ	=	Phospholipase C-γ
Pol	=	Polymerase
PP1	=	Protein phosphatase 1
PP2A	=	Protein phosphatase 2A
pRb	=	Protein of the retinoblastoma gene
RA	=	Retinoic acid
Rb	=	Retinoblastoma gene
RNA	=	Ribonucleic acid
S	=	DNA synthesis phase of mitotic cycle
SC	=	squamo-columnar
SCC	=	Squamous cell carcinoma
SCE	=	Simple columnar epithelium
SDS	=	Sodium dodecyl sulfate
SH2	=	*Src* homology domain 2
SH3	=	*Src* homology domain 3
SIL	=	Squamous intraepithelial lesion
SSE	=	Stratified squamous epithelium
SV40	=	Simian virus 40
T Ag	=	Large tumour antigen
T	=	Thymidine
TAF	=	TBP-associated factor
TBP	=	TATA box binding protein
TGF	=	Transforming growth factor
TNF	=	Tumour necrosis factor
TNI	=	Transitional non-vegetative infection
TZ	=	Transformation zone
U	=	Uridine
VIN	=	Vulval intraepithelial neoplasia
VLP	=	Virion-like particles
XS2	=	HEC immortalized by the construct $pSV_2$1667

CHAPTER 1

GENERAL INTRODUCTION:

MALIGNANCY AND DNA TUMOUR VIRUSES

Viruses are believed to be involved in 15% of human cancers (zur Hausen, 1991a). The majority of the known virus-linked malignancies are associated with infections by DNA tumour viruses. DNA tumour viruses established for human cancers include human papillomaviruses (HPV), Epstein-Barr virus (EBV), and hepatitis B virus (HBV). Certain types of HPVs have been firmly established for their involvement in genital cancers, especially cervical carcinoma, one of the most common neoplasms in the world (Parkin *et al.*, 1988; Parkin *et al.*, 1993). EBV, the first viral etiological agent established for human cancers, is associated with several human malignancies including Burkitt's lymphoma (BL). Our understanding in DNA virus-mediated oncogenesis, especially at the level of molecular biology, has greatly benefited from the studies of the so-called small DNA tumour viruses, such as simian virus 40 (SV40), even though they are irrelevant to naturally occurring malignancy (Addison *et al.*, 1989). Studies on these model viruses have provided important information about the mechanisms of oncogenesis by DNA viruses.

1.1 Tumour cells

Malignancy is a disease characterized by uncontrolled cell growth that no longer responds to intra- as well as inter-cellular regulation. After years of intensive studies, the main framework of the oncogenic process has emerged, although the exact mechanism(s) is still not clear (Rhim, 1991; Vogelstein and Kinzler, 1993).

1.1.1 Phenotypes of tumour cells

Pathologically, tumour cells manifest themselves in characteristic cellular morphologies and abnormal relationships with each other and the normal cells. The former include abnormal nuclear figures and increased nucleus/cytoplasm ratios, and the latter involve cell division and outgrowth inappropriate to their location and differentiation status (Lewin, 1994; Alberts, 1994).

When tumour cells are cultured *in vitro*, these features are usually retained, and presented in corresponding phenotypes. Generally, tumour cells *in vitro* show less growth factor-dependence, less anchorage-dependence, as well as less inhibition by cell-cell contact than their normal counterparts. Since these characteristics are distinct, and obviously developed from those of their normal precursor cells, they are referred to as being "transformed." Furthermore, while normal somatic cells have a limited life span in culture, tumour cells can usually grow infinitely or are "immortal" in the same condition. Transformed and immortal phenotypes are believed to be hallmarks of tumour cells.

The relationship between the transformed and immortal phenotypes is important for understanding the mechanisms of oncogenesis. In realization of this importance, great efforts have been made to study the mechanisms for immortalization and transformation using *in vitro* cultured cells as models. Experiments with rodent cells have provided valuable information on the nature of transformation. Rodent cells can become immortal spontaneously at a reproducible and predictable frequency. For mice, the frequency is approximately 10^{-5}. The nature of the spontaneous immortalization of rodent cells is not known. The immortal rodent cells can subsequently acquire transformed phenotypes, including tumorigenicity, by

being treated with chemical carcinogens or introduced with oncogenes or activated protooncogenes (see Section 1.1.5). The acquisition of tumorigenicity is regarded as being "fully" transformed. However, *in vitro* transfection of certain combinations of oncogenes, or treatment with certain carcinogens, can also fully transform rodent cells without their undergoing the intermediate stages. For example, while primary rodent cells stop dividing after 30 days in culture, the cells treated with 4-hydroxyaminoquinoline-1-oxide can continue dividing, and exhibit fully transformed phenotypes after 3 months in culture (Kuroki and Sato, 1968). Infection by small DNA tumour viruses, or *in vitro* transfection of their viral oncogenes, can also transform rodent cells (Chang, 1986). Thus, immortalized and transformed phenotypes cannot be clearly separated in rodent cells.

In contrast, human cells cultured *in vitro* acquire the immortal and transformed phenotypes independently and at much lower frequencies. Since human cells are the subject of the current project, immortalization and transformation of human cells are introduced in more detail.

1.1.2 Immortalization

Human cells are very resistant to transformation (Shay *et al.*, 1991). Spontaneous immortalization, or transformation, of human cells is a very rare event. In fact, so far there are only three reports, and one of these was established in the condition of elevated culturing temperature (Boukamp *et al.*, 1988; Kuroki and Huh, 1993). The oncogenes and carcinogenic chemicals that are transforming for rodent cells show virtually no transforming or immortalizing activity for human cells. The nature of the difference between human and

rodent cells for their susceptibility to transformation is unknown. Only the persistent expression of certain viral oncogenes of DNA tumour viruses has been shown predictably to immortalize human cells (Pope *et al.*, 1968; Chang *et al.*, 1982; Rhim *et al.*, 1985; Christian *et al.*, 1987; Durst *et al.*, 1987b; Shay and Wright, 1989; Wright *et al.*, 1989). The efficiency for immortalizing human cells is usually rather low, with the exception of the EBV-infected B lymphocytes (B cells). Except their infinite growth potential under suitable conditions, no other marker or behaviour is characteristic for the immortalized human cells.

Little is known about the mechanism for immortalization. The current working hypothesis regards immortalization as a recessive event, resulting from compromised functions of a dominant mechanism for senescence control (Shay *et al.*, 1991; Stamps *et al.*, 1992; Kuroki and Huh, 1993). Senescence is defined as a permanent resting state of normal somatic cells after they undergo a definite number of cell divisions (Hayflick and Moorhead, 1961). The control of senescence is believed to involve a group of genes and to function with a genetically programmed process that is closely associated with the length of the chromosome telomere (Wright and Shay, 1992). The telomere is gradually shortened for each cell division, and its length is regulated by telomerase (Counter *et al.*, 1992), an enzyme responsible for the synthesis of telomere DNA. In normal human somatic cells, telomerase activity is not detectible, while it is in tumour cells. Based on data from *in vitro* immortalization studies, senescence control is hypothesized to consist of two mortality stages, M1 and M2 (Hayflick and Moorhead, 1961; O'Brien *et al.*, 1986; Shay and Wright, 1989). These two stages are believed to be activated in tandem. Upon reaching a definite number of cell divisions, M1 is switched on to arrest cells in G_0. In case M1 fails to stop cell cycling

under certain conditions, M2 is activated to enforce cellular arrest (see Section 1.2.1).

The immortalized cells may show no or minimal phenotypic changes from their normal counterparts and they are able to undergo differentiation under proper conditions (Lechner and Laimins, 1991; Durst *et al.*, 1991). Therefore, senescence control has been rationalized to be an independent system that serves as an ultimate mechanism for a multicellular organism to protect itself from uncontrolled cell growth. Thus, theoretically, malfunction of senescence control alone imposes no immediate neoplastic risk on the organism, since these cells remain subject to controls by other mechanisms such as differentiation and programmed cell death or apoptosis (Hockenbery, 1995) (see Section 1.2.3.3). These cellular functions can effectively control cell growth. However, immortalization may be a key step in oncogenesis, since it allows mutations required for transformed phenotypes to accumulate. It is obvious that a functional senescence program would force cells that have completed certain rounds of replications to withdraw from cycling, and thus prevent the potentially tumorigenic cells from expanding to a biologically significant population.

The above statements are mostly hypothetical. None of the genes directly involved in senescence control has been identified, although cell fusion experiments with SV40-immortalized cells suggested that at least four complementary gene groups may be involved in senescence control (Pereira-Smith and Smith, 1988; Whitaker *et al.*, 1992; Chen *et al.*, 1993).

1.1.3 Transformation

The immortalized human cells can further acquire transformed phenotypes. Full

transformation can be induced by radiation, treatment with chemical carcinogens, or transfection with certain oncogenes or activated protooncogenes (Rhim *et al.*, 1986; Thraves *et al.*, 1990; Abbas *et al.*, 1991; Rhim *et al.*, 1985). Since the growth of transformed cells still requires continuous expression of the viral genes that induce immortalization, transformation may require secondary genetic changes that are different from those required for immortalization. Because sequential transformation can be achieved by introducing different oncogenes, transformation could be caused by different pathways. The immortalized cells can also develop transformed phenotypes after prolonged culture *in vitro*, indicating that the mechanism mediating immortalization may also induce transformation, if given sufficient time. It should be noted because mutations for immortalization and transformations are acquired as independent events (refer to Section 1.1.5), mortal cells may also show transformed phenotypes but are obviously mortal (Stamps *et al.*, 1992).

Transformation can be, and has been, more vigorously defined biologically, genetically, and biochemically. Tumorigenicity in immuno-deficient animals is usually accompanied by acquirement of other criteria of transformation, such as anchorage-independent growth *in vitro*, but acquisition of these *in vitro* transformed phenotypes is not necessarily accompanied with tumorigenicity (Boukamp *et al.*, 1988; Fusenig *et al.*, 1990). The central hallmark of transformed phenotypes is the enhanced growth potential, which is resultant from genetic changes that are generally different from those required for immortalization. Mutations responsible for transformation are found to be extremely diverse, involving genes regulating many aspects of cell biology, such as growth, differentiation, and apoptosis (Boettiger, 1989; Cross and Dexter, 1991; Hockenbery, 1995). The most studied

aspects are the genes for growth factors, receptors, and their downstream elements, which are important constituents in signal transduction pathways for intercellular communications (Cross and Dexter, 1991). These signal transduction pathways impinge on a wide spectrum of cellular functions, and one of them is control of cell replication (see Section 1.2.3.1).

Transformed phenotypes are thought to be directly responsible for the neoplastic risk to the organism. The cells with transformed phenotypes lose their response to physiological inhibitory and differentiation signals, and escape apoptosis that should be activated when cell growth is not appropriate to physiological conditions. As a result, these cells undergo uncontrolled proliferation and invade into and metastasize to locations that they are not supposed to reside (Aznavoorian *et al.*, 1993). However, these cells cannot expand into significant populations unless senescence control is also compromised.

1.1.4 Immortalization and transformation as secondary events

Mutations involved in immortalization and transformation are often secondary to other disrupted cellular functions, such as those controlling cell cycle regulation, genomic integrity, and DNA repair (Yokota and Sugimura, 1993). Several genes have been found to encode products that are central components in these mechanisms. The retinoblastoma (Rb) gene, which was identified due to its high mutation/deletion rate in juvenile retinoblastoma, encodes the protein pRb that plays a key role in cell cycle progression (Riley *et al.*, 1994). p53, which is mutated in nearly 50% of human malignancies, is involved in cell cycle control, genome integrity control, DNA repair and apoptosis (Oliner, 1993). The human mismatch repair gene (hMSH2), which has a high mutation rate in hereditary nonpolyposis colorectal cancer

(HNPCC), encodes a mismatch-DNA binding protein that is an important component for DNA mismatch repair (Cleaver, 1994; Jiricny, 1994). Malfunction of any functions mentioned above may cause an increased incidence of cellular mutation. If mutations are introduced into cellular genes involved in mechanisms controlling cellular mortality and growth, corresponding phenotypes will concur. Thus, although the defunct cellular controls leading to immortalization and transformation may be fundamentally different, the mutations responsible for immortalization and transformation may be derived from the same mechanism(s) that renders a higher incidence of mutations.

1.1.5 Multistep nature of oncogenesis

All the mechanisms summarized above involve numerous genes that function interactively and at multiple levels, constructing independent control circuits that may overlap functionally and/or constituently. This substantiates the well-established fact that oncogenesis is a multistep process (Vogelstein and Kinzler, 1993). Cellular genes involved in oncogenesis may be protooncogenes encoding proteins that promote cell growth, or tumour suppressor genes involved in inhibiting cell growth. The best examples for the former are c-*ras* and c-*myc*. C-*ras* encodes G proteins mediating signal transduction pathways for peptide growth factors, and c-*myc* is the gene for a transcription factor involved in cell growth regulation. The tumour suppressor genes include pRb and p53, which will be discussed in Section 1.2.3.

Malfunction of the protooncogenes and tumour suppressor genes may result from inherited mutations, such as the mutations in the Rb gene in juvenile retinoblastoma, and mutations in the hMSH2 gene in HNPCC. Alternatively, functions of these cellular genes may

be sabotaged by acquired mutations, such as the point mutations in the K-*ras* found in colon cancers and the c-*myc* translocations in lymphomas. In addition, more relevant to this thesis, functions of cellular genes may be mimicked or/and disrupted by products of exogenous genes introduced by viral infections. Retroviruses may carry viral oncogenes that are transducted mutant or activated cellular protooncogenes (Bouton and Parsons, 1993). DNA tumour viruses encode oncogenic proteins that are required by viral reproduction and interfere with functions of cellular protooncogenes and/or tumour suppressor genes (see Section 1.2.3). Finally, functions of cellular protooncogenes and tumour suppressor genes may also be disrupted by mutations caused by viral DNA integration, which is part of the normal life cycle for the retroviruses, but is not for the DNA tumour viruses.

1.2 Models of viral oncogenesis: SV40 and EBV

SV40 and EBV represent the best studied DNA tumour viruses. The principles of their oncogenic mechanisms may also be applicable to other DNA tumour viruses.

1.2.1 SV40-mediated oncogenesis is specific for non-vegetative infection

SV40 exemplifies the oncogenic mechanisms of the small DNA tumour viruses, and has been intensively studied as a model because of its simple genome composition (Acheson, 1981; Topp *et al.*, 1981; Fried and Prives, 1986). In its natural host, the rhesus monkeys, SV40 is not tumorigenic, even though it is carried in high concentrations in kidneys. SV40 is not oncogenic for the cultured cells that support virus productive life cycles, or are permissive for viral vegetative infection. However, it is tumorigenic in certain non-natural

hosts, such as hamsters, and can transform cultured cells that are non-permissive for virus vegetative infection. This phenomenon underscores the importance of the genetic properties of the host cell in relation to virus-mediated oncogenesis (Fried and Prives, 1986).

Restricted by its small size, SV40 carries a genome encoding only a limited number of genes for the "early" and "late" proteins. The former include the regulatory proteins and the latter the structural proteins for the virion capsid. Thus, SV40 depends heavily on cellular mechanisms for virus reproduction, and replicates by literally "hijacking" and modifying these cellular functions. In vegetative infection, such as in African green monkey cells, SV40 genes are expressed in programmed sequences (Acheson, 1981). Expression of the early genes occurs prior to viral DNA replication in the early phase of infection. One of the early genes is the large tumour antigen (T Ag), which directs the proceeding of the viral life cycle. SV40 T Ag first modulates transcription of cellular genes to promote cell proliferation, preparing the cellular environment for the late stages of virus replication. Accumulation of SV40 T Ag triggers the entry of the virus life cycle into the late phase by initiating viral DNA amplification and stimulating late gene expression. At the same time SV40 T Ag represses its own transcription (Fanning, 1992). The completion of the program for vegetative infection leads to virion assembly and the death of the host cells.

In non-permissive cells, SV40 cannot complete the replicative cycle. Viral early genes are expressed, but viral DNA amplifies poorly and the expression of late genes is not activated, resulting in a state of persistent non-productive or non-vegetative infection (Topp *et al.*, 1981). The mechanism for the abortive viral life cycle is not understood completely. An incompatible relationship between the cellular environment and viral genetic composition

is suspected, since cell hybrid experiments showed that the permissiveness for vegetative infection is a dominant phenomenon. Cells from certain species, such as humans, are semi-permissive for SV40 vegetative infection. For semi-permissive cells, few cells in the cell population support virus vegetative replication. SV40 infection in the rest of the cells is persistently non-vegetative. The mechanism(s) that determines the semi-permissiveness is unknown, but was suggested due to either inadequate amount of cellular factors required for vegetative replication, or a semi-compatible relationship between cellular and viral factors (Fried and Prives, 1986).

The non-vegetative SV40 infection in human cells may lead to immortalization of the infected cells at a low frequency (Chang, 1986). The process of immortalization can be regarded as consisting of two phases (Shay *et al.*, 1991). First, the cells infected with SV40 have an extended life span of approximately 20 doublings compared with the mock infected cells. Interpretation of this phenomenon is that cells expressing certain viral genes are able to overcome the M1 phase of the senescence control (refer to Section 1.1.2), while the non-infected cells are susceptible to the arrest by the M1 phase upon reaching a certain number of divisions. Second, the infected cells enter "crisis," a phenomenon characterized by halted cell division, karyotypic abnormalities and abortive mitosis. The nature of this crisis is also unknown. The crisis was proposed to represent the M2 phase of the senescence control, which cannot be overcome by viral functions (Shay *et al.*, 1991; Stamps *et al.*, 1992). At frequencies of less than 10^{-9}, individual clones in the infected human cells survive the crisis, and they show faster cycling than the cells in crisis, and gradually become the dominant population. The post-crisis cells are regarded as being immortal, since they can be cultivated

indefinitely in suitable conditions. The post-crisis cells are aneuploid in karyotypes and usually have integrated viral genes randomly dispersed in the cellular genome. This suggests that the surviving immortalized cells have undergone genetic changes including recombination.

The viral DNA sequence encoding SV40 T Ag is always selectively retained in the cells immortalized by SV40 infections. Indeed, SV40 T Ag is also always expressed in cells undergoing the process of immortalization and in those established for immortalization (Chang, 1986; Rhim, 1991, Shay *et al.*, 1991; Stamps *et al.*, 1992; Kuroki and Huh, 1993). This suggests that the function of SV40 T Ag be important for immortalization. In fact, the SV40 T Ag gene has been established as the major viral oncogene, since transfection of T Ag alone is sufficient for inducing immortalization of human cells, and the immortalization caused by T Ag transfection follows events similar to those by persistent non-vegetative infections. Studies showed that the immortalizing/transforming capacity of SV40 T Ag can be partially attributed to its ability to introduce mutations into cellular genomes, as a result of its functions in promoting cell cycle progression, sabotaging cellular mechanisms for DNA repair and genome integrity maintenance, and stimulating the signal transduction pathways for growth factors (Chang, 1986; Rhim, 1991; Shay *et al.*, 1991; Manfredi and Prives, 1994) (see Section 1.2.3). It should be noted that immortalizing the host cell is not an inherent part of the normal life cycle of SV40, and the integrated viral genomes generally cannot be used as templates for viral DNA amplification. Consistent with the multistep model for oncogenesis, the SV40-immortalized cells may show minimal or no other phenotypic changes (Lechner and Laimins, 1991), but can subsequently obtain transformed phenotypes. Thus, SV40-mediated oncogenesis is closely associated with a persistent state of non-vegetative infection, which

may result from incompatible interactions between the cellular environment and viral elements.

Studies on oncogenesis by other small DNA tumour viruses indicated that they all share features similar to those of SV40. As SV40, these DNA tumour viruses carry transforming or/and immortalizing genes that are expressed in the early phases of virus vegetative infection. The molecular mechanisms for the immortalizing/transforming viral genes will be discussed in more detail in Section 1.2.3.

1.2.2 EBV-mediated malignancy is related to cell differentiation

EBV is a member of the γ herpes viruses and is responsible for BL and undifferentiated squamous cell carcinomas (SCC) of the nasopharynx (zur Hausen, 1991a). The oncogenesis of BL by EBV has been studied intensively due to its clinical importance. These studies provided important virological information on the relationship between DNA virus-mediated oncogenesis and the physiological status of the infected cells, although our understanding of the molecular mechanisms for the EBV-mediated oncogenesis is much limited in comparison to that of the small DNA tumour viruses (Klein, 1993; Klein, 1994; Niedobitek and Young, 1994).

B cells undergo programmed differentiation events for maturing from precursors to effectors (Abbas *et al.*, 1991). During maturation, the stem cells of B cells undergo somatic site-specific recombination at the immunoglobulin loci, becoming resting mature B cells. Upon contacting specific antigens, B cells are stimulated to proliferate and undergo somatic mutations, producing early response antibodies. Some of these early response B cells may

withdraw from cycling and become resting memory cells. Upon being stimulated again by the specific antigen, memory cells commence cycling once more for clonal expansion, which gives rise to differentiated non-cycling plasma cells and new memory cells.

EBV infects B cells via the specific CR2 complement receptor, which is present on all the B cells (Klein, 1994). EBV infection in B cells may be classified into three types of infection states: resting latent infection, cycling latent infection, and lytic infection. All the resting B cells and the majority of the cycling B cells support only EBV latent infections, in which only the eleven viral latency genes are expressed from the viral genome carrying approximately 100 genes. The only viral latency gene expressed in the latently infected resting B cells is the EBNA-1 gene from a specific promoter. It encodes a transcription factor involved in maintaining viral genomes in episomal form. When the resting B cells are activated to cycle, the pattern of viral gene expression changes. In the latently infected cycling B cells, the other latency genes are activated, and are expressed together with EBNA-1 from an alternative promoter. Although still largely unknown, the biological function of the viral latency proteins is hypothesized to drive the latently infected cells to undergo clonal expansion, increasing the pool of the EBV-infected cells. Only 10^{-3}-10^{-6} of the latently infected cycling B cells enter the late phase of the viral life cycle by successfully expressing the downstream viral genes, including those required for viral DNA amplification and encoding structural proteins. The mechanism(s) triggering the lytic infection is not clear, although conditional expression activation of the viral gene BZLF-1 has been identified as the switch (Taylor *et al.*, 1989; Baichwal and Sugden, 1994). Its activation is possibly associated with a certain physiological differentiation status of B cells. Consistent with this notion,

certain antibodies, which induce B cell differentiation by stimulating the membrane-bound immunoglobulins of the B cells, have been found to activate EBV vegetative replication (Baichwal and Sugden, 1994). Thus, the programs for EBV gene expression and the states of EBV infection are possibly associated with the differentiation status of the host cells.

Oncogenesis by EBV infection resides on the functions of the EBV latency genes, which are expressed in the latently infected cycling B cells at the early stages of EBV infection. These latency genes encode proteins that have been shown to be potent for promoting cell cycle progression, sabotaging cellular mechanisms for DNA repair and genome integrity maintenance, and stimulating signal transduction pathways for growth factors (Ring, 1994). Thus, the latency proteins are oncogenic in nature, since they may induce cellular genetic mutations as a side effect for activating cell proliferation (see Section 1.2.3). Consistently, studies showed that *in vitro* infecting B cells with EBV induces immortalization of the infected cells and the immortalizing functions have been attributed to the EBV latency genes (Sugden, 1989).

The cellular mutations induced by the latency proteins may compromise cellular functions important for immortality, growth, and apoptosis, which are all involved in oncogenesis. In normal individuals, the latently infected cycling cells with compromised cellular control mechanisms impose no immediate neoplastic threat to the host. That is because all the latency proteins, except EBNA-1, are highly immunogenic. Thus, expression of these latency proteins on one hand stimulates cell proliferation and causes cellular mutations, while on the other hand, these oncoproteins provoke the immune system and result in effective clearance of the infected cells by cytotoxic T cells. However, some cells may

escape the immuno-surveillance system when they differentiate into resting memory cells. In these cells, the virus expresses only EBNA-1, which has poor immunogenicity and thus does not induce effective immuno-reaction. At the stage of resting latent infection, new mutations are unlikely to be introduced, since the cells are not replicating and expression of the viral oncogenic latency genes is switched off. However, when the latently infected resting cells are reactivated in response to physiological stimuli, the virus at the same time switches on *de novo* expression of all the viral latency genes including the oncogenic ones, which drives the cells to actively proliferate again, introducing more mutations into cellular genomes (Klein, 1993).

In immuno-competent individuals, BL manifests only when the mutations accumulated during the stage of latent cycling infection are sufficient to sabotage important cellular functions including the one responsible for arresting memory B cells at G_0 (Klein, 1989; Klein, 1994; Niedobitek and Young, 1994). When the latently infected, mutation-carrying, and cycling B cells differentiate into memory cells, the accumulated mutations may result in failure in arresting the cells from cycling, and instead the infected cells continue to proliferate, with a phenotype of the resting cells. However, since these cells possess the phenotypes of resting B cells, the viral gene expression program is switched to expressing only the non-immunogenic EBNA-1, allowing the neoplastic cells escape the surveillance of the immune system. This scenario is consistent with the clinical finding that BL cells are in fact proliferating B cells with memory cell phenotypes.

Thus, in conditions of repeated B cell activation, the infected cells tend to be more susceptible to EBV-mediated oncogenesis, since the frequent expression episodes of the

oncogenic EBV latency proteins in the cycling B cells may subject the host cells to increased mutation accumulation. This may explain why BL has a high incidence in individuals with chronic malaria infections (Niedobitek and Young, 1994). Oncogenesis of BL by EBV has other unique features. Unlike the small DNA tumour viruses, EBV induces immortalization of the *in vitro* infected B cells at an astonishingly high efficiency of 80-100%. In addition, EBV genomes are maintained in a low copy number as episomes in the immortalized cells. These distinct features of EBV-mediated immortalization and tumorigenicity suggest that certain EBV latency functions may specifically disrupt the M2 phase of senescence control in B cells. Furthermore, certain functions of the EBV latency proteins may also interfere with the cellular mechanisms responsible for somatic recombination and mutations, which are inherently active in the B cells. This may render the infected B cells prone to illegitimate recombination and mutations. In fact, the majority of the epidemic BL cases possess a translocation that juxtaposes the c-*myc* protooncogene to the positive influence of the immunoglobulin (Ig) enhancer, and 60% of them have mutations in the tumour suppressor p53 gene (Sugden, 1989).

In summary, EBV-mediated oncogenesis is closely associated with the differentiation-dependent expression of the EBV latency genes. In addition, the highly efficient immortalization of B cells mediated by EBV infection indicates the complicity in the oncogenesis mediated by DNA tumour viruses.

1.2.3 Unifying molecular mechanisms for DNA tumour virus-mediated oncogenesis

Studies showed that the immortalizing and/or transforming activities of the DNA

tumour viruses can be attributed to individual viral genes, and these viral oncogenes interfere with several important cellular control circuits for cell replication.

1.2.3.1 Normal cell cycle is controlled by promoting and inhibiting functions

Cell cycle is the process by which genomic DNA is duplicated and divided into daughter cells. It is controlled strictly and coordinately by multiple cellular mechanisms to ensure genetic fidelity as well as stability. In multicellular organisms, the cell cycle must also be coordinated with signals from other cells in the organism (Hunter and Pines, 1991; Hunter and Pines, 1994).

Current concepts of cell cycle regulation recognize the importance of various specific cyclin-dependent serine/threonine protein kinases (CDKs), which are a family of key regulators for cell cycle controls (Sherr, 1994). The substrates of CDKs include the well-established pRb tumour suppressor and its homologous proteins, such as p107 and p130. The proteins of the pRb family share structural and functional features (Riley *et al.*, 1994). For instance, they all contain a pocket structure containing the binding sites for some other important cell cycle regulators, such as E2F and cyclins. E2F and its homologs are a family of transcription factors important for the activation of genes responsible for cell division (La Thangue, 1994). The pRb family of proteins binds to the E2F family factors, and modulates the transacting function of the E2F factors.

CDKs are activated at specific times during cell cycles to trigger an avalanche or cascade of reactions, driving the cell cycle through successive checkpoints that restrict cell cycle progression. The most important for cellular DNA replication are the late G_1 restriction

point, the G_1/S transition checkpoint and the S phase checkpoint. CDKs are regulated for their activity and substrate specificity by binding to various protein regulators, including cyclins and specific CDK inhibitors (CDI) (Peter and Herskowitz, 1994). In addition, phosphorylation at specific sites on CDKs by specific kinases, such as CDK activating kinases (CAK) or CDK inhibiting kinases, is also important for CDK activity (Solomon, 1994).

The extracellular mitogenic signals converge on the CDK-centred circuit for promoting cell proliferation. For example, the peptide and protein growth factors function through signal transduction pathways emitting from the plasma membrane to the nucleus (Fantl *et al.*, 1993; van der Geer and Hunter, 1994). Upon binding to these ligands, the cognate receptors on the plasma membrane undergo dimerization. This dimerization activates the intrinsic protein tyrosine kinase activity of the receptors, resulting in auto-phosphorylation of the specific tyrosine sites in the cytoplasmic domains of the receptors. These phosphorylated tyrosines have high specific affinity for particular proteins containing the *src* homology domain 2 (SH2), and binding with these proteins transmits mitogenic signals to various downstream chain reactions. The SH2-containing proteins include those possessing domains with various catalytic activities, and those possessing the *src* homology domain 3 (SH3) (Schlessinger, 1993). The former are well exemplified by the phospholipase C-γ (PLC-γ), which activates the protein kinase C (PKC)-mediated signal transduction pathway by hydrolysing phosphatidylinositol 4,5-diphosphate to diacylglycerol, the physiological activator of PKC (Panayotou and Waterfield, 1993). Another, and one of the best studied, signal transduction pathway, involves the products of the *ras* protooncogenes (Schlessinger, 1993; Maruta and Burgess, 1994). As a result of ligand binding to its cognate receptor protein

kinase, such as EGF binding to the EGF receptor, the *ras* proteins are recruited to the activated receptor by Grb2 and SOS proteins (Maruta and Burgess, 1994). Grb2 is an adaptor protein containing SH2 as well as SH3 domains. The SH2 domain of Grb2 binds to the specific phosphorylated tyrosines on the cytoplasmic domain of the EGF receptor, while the SH3 domains bind to SOS, the nucleotide-releasing factor for *ras* proteins. SOS then promotes the conversion of the inactive *ras*-CDP to the active *ras*-GTP. The latter recruits and activates c-*raf*, the mitogen activated protein (MAP) kinase kinase kinase, triggering the MAP kinase signal transduction pathway, which is composed of a series of serine/threonine kinases (Cook and McCormick, 1994; Hall, 1994; Daum *et al.*, 1994). The signals from the cell surface are relayed to the nucleus by cascades of protein phosphorylations. The final substrates of the chain reactions include transcription factors that are encoded by protooncogenes important for the regulation of cell replication. Phosphorylation for the transcription factors such as c-*myc*, c-*fos*, and c-*jun* activates their potential for transcription transactivation, and for NF-κB/I κB phosphorylation activates and induces the translocation of the factor from cytoplasm to the nucleus. Therefore, activation of these transcription factors that regulate the expression of cyclins and activity of CDKs completes the signal transduction from the peripheral to the central control circuits for cell replication (Hunter and Pines, 1991; Hunter and Pines, 1994).

To ensure genetic fidelity as well as stability, the progression of the cell cycle is monitored by multiple cellular mechanisms. In case of irregular activities, these mechanisms serve either to stop cell cycle progression via CDI at checkpoints, and/or to activate DNA repair functions to repair the DNA damage that may occur as a result of dysregulated and

incoordinate cell cycles. Mechanisms for apoptosis may be activated under conditions beyond normal control, leading to the suicide of the uncontrollable cells that are potentially neoplastic to the organism. Another well-established tumour suppressor, p53, plays important roles in these aspects of cell cycle control (White, 1994).

Thus, cyclins, CDI and CDK kinases are the central sensors/effectors for the signal transduction pathways that respond to intra- or extra-cellular mitogenic signals. CDKs integrate these signals, and translate them into "on" or "off" switches, in the form of the specificity and activity of their protein kinase. The switching of CDKs is relayed by the pRb family proteins to the E2F family transcription factors, which regulate the expression of genes directly involved in cell cycle control.

1.2.3.2 Viral oncoproteins disrupt cellular controls for cell cycle progression

Studies showed that viral oncoproteins disrupt the control of cell cycle progression, resulting in incoordinate cell division. As a result of such incoordination, mutations such as translocations, deletions and duplications are introduced into the cellular genome. Importantly, viral oncoproteins also sabotage the cellular mechanisms for DNA repair and apoptosis. Failure of these mechanisms would allow the accumulation of cellular mutations, which subsequently impair the cellular mechanisms for senescence control and the signal transduction pathways for intercellular communication. In addition, products of some viral oncogenes can directly interfere with growth factor signal transduction pathways by themselves. The combined effects of viral oncogenes and cellular mutations would finally lead to totally uncontrolled cell growth- the hallmark of tumour cells.

1.2.3.2.1 Defunct pRb functions compromise the late G_1 restriction point

The late G_1 checkpoint or restriction point is particularly important for controlling cell cycle (Sherr, 1994). Based on the integrated signals from both the intra- and extra-cellular environments, irreversible commitment to DNA replication is made at this checkpoint. The pRb tumour suppressor is a very important factor for regulating the G_1 checkpoint (Wiman, 1993). The phosphorylation status of pRb determines the biological activity of pRb. pRb can be de-phosphorylated by protein phosphatase 1 (PP1) and phosphorylated by CDKs. The hypo-phosphorylated pRb binds to E2F, and such binding is believed to be relieved by pRb phosphorylation.

The key regulators at the restriction point are cyclins D1-D3 (Hunter and Pines, 1994). The level of cyclin Ds is elevated in response to mitotic signals from growth factor stimulation. In response to this stimulation, cyclin Ds form complexes with CDK4 or CDK6, a prerequisite for the kinase activity of the CDKs. CDK4 and CDK6 kinase activities are also regulated by CAK, which activates the CDKs by phosphorylating them at specific threonine residues. CAK itself is a complex comprising cyclin H and CDK7, and the activity of CAK may be regulated by cAMP-mediated signal transduction pathways. In addition, CDK4 and CDK6 are also under the regulation of CDIs, such as p15, p16, p21, p27. CDI p15 is involved in the signal transduction of transforming growth factor β (TGF-β), an important cytokine for regulating cellular differentiation and tissue homeostasis (Hannon and Beach, 1994). The expression of p21 is regulated by the p53 tumour suppressor. CDIs may function by competing with cyclins for CDK binding or by inhibiting cyclin-CDK kinase. These two modes of functioning are exemplified by p16 and p21, respectively.

In the presence of activating signals (*e.g.*, serum mitogens) and in the absence of inhibitory signals (*e.g.*, low cell density), phosphorylated CDK4 and CDK6 become bound with cyclin Ds, and are activated for the CDK kinase activity as a result. It has been shown that cyclin D1 directs CDK4 to phosphorylate the E2F-bound pRb, inducing E2F release from the complex, possibly as a result of a conformation change in the phosphorylated pRb (La Thangue, 1994). The free E2F subsequently activates genes important for DNA replication, promoting the entry of a new round of cell cycle. Consistently, a defunct Rb function by mutations, or an overridden Rb function by overexrpressed E2F, induces inappropriate DNA synthesis (Morgenbesser *et al.*, 1994; Qin *et al.*, 1994). The progression through the late G_1 restriction point appears to be an irreversible commitment for DNA replication, since removal of serum cannot reverse this process.

Perhaps in reflection of its key role in the cell cycle control, pRb is targeted by viral oncoproteins encoded by several well-known DNA tumour viruses. For example, adenovirus E1a, SV40 T Ag and EBV latency protein EBNA-5 bind to pRb (Whyte *et al.*, 1989; Ring, 1994; Ludlow, 1993). Significantly, the pRb-binding viral oncoproteins of the small DNA tumour viruses show conserved motifs for pRb binding, and such binding involves only the hypo-phosphorylated pRb, which is the pRb form that complexes with and inhibits E2F (Chellappan *et al.*, 1992). Adenovirus E1a or SV40 T Ag compromises the E2F-binding activity of the hypo-phosphorylated pRb (Bagchi *et al.*, 1990; Hiebert *et al.*, 1991). Under the effect of pRb-binding viral oncoproteins, E2F transcription factors are released from the pRb-mediated inhibition, free to activate downstream functions important for DNA replication (Farnham, 1993). Thus, viral oncoproteins may potentially promote cell cycle progression by

lowering the threshold for the G_1 checkpoint, regardless of the external environment and the physiological condition of the cell.

1.2.3.2.2 Defunct p107 functions compromise the G_1/S transition and S phase *checkpoints*

Cyclin E and A are involved in the G_1/S transition and S checkpoints, respectively (Sherr, 1994; Peter and Herskowitz, 1994). Cyclin E, which is an activator for CDK2 kinase activity, is expressed after cyclin D and complexes with CDK2. Activation of cyclin E-CDK2 is thought to initiate the G_1/S transition, the actual onset of DNA replication. After the G_1/S transition, cyclin E is quickly degraded and CDK2 forms complexes with cyclin A. Cyclin A-CDK2 is required for the cell cycle progression through the S phase. Similar to CDK4 and CDK6, CDK2 kinase is also inhibited by the p21 CDI, the expression of which is regulated by the p53 tumour suppressor.

The cyclin E- and the cyclin A-CDK2 complexes are able to interact with p107, which has a sequence and function homologous to pRb (Dyson *et al.*, 1993; Sherr, 1994). Like pRb, p107 also binds to E2F. Although it is possible that p107 may sequester and release E2F in a similar way to pRb, the relationship between the p107-E2F complexing and the E2F activity is more complex than that between the pRb-E2F complexing and E2F activity. Cyclin-CDK2 may bind to the p107-E2F complex and modify E2F function, rather than simply freeing it from the complex (Moran, 1993). This could be achieved by phosphorylating E2F or other factors interacting with E2F. Indeed, a complex containing E2F, p107, cyclin A, and CDK2 can be detected in the S phase cells, (Dyson *et al.*, 1993) and kinase activities can be detected

in this complex (Arroyo *et al.*, 1993; Devoto *et al.*, 1992).

SV40 T Ag and the pRb-binding oncoproteins of other small DNA tumour viruses also interact with p107 (Fanning, 1992; Chellappan *et al.*, 1992). It is possible that T Ag binding also disrupts the E2F-p107 complex, and releases free E2F. Alternatively, the viral oncoproteins may function not only by releasing free E2F, but also by modifying the substrate specificity of the CDK2-cyclin complex. Nevertheless, the interaction between the viral oncoproteins of DNA tumour viruses and p107 should promote transversing the G_1/S and S checkpoints.

1.2.3.3 Viral oncoproteins sabotage cellular controls for genomic integrity maintenance, DNA repair and apoptosis

The DNA replication induced by viral oncoproteins is obviously not compatible with the normal physiological condition of the host cell. This could trigger cellular mechanisms to correct such a condition. One system is the recently identified p53-mediated functions. Inappropriate DNA synthesis induced by defunct pRb functions or overexrpressed E2F has been shown to result in p53-dependent cell cycle arrest and apoptosis (Morgenbesser *et al.*, 1994; Qin *et al.*, 1994). p53 is a nuclear phosphoprotein and a transcription factor that transactivates the promoters/enhancers containing specific p53 binding sites (Zambetti and Levine, 1993; Oliner, 1993). In addition, p53 also globally represses the TATA box-containing promoters that have no p53 binding site. p53-mediated repression may involve p53 binding to the TATA box binding protein (TBP), and this binding may be bridged by TBP-associated factors (TAFs) (Thut *et al.*, 1995). The genes activated by p53 include those for

the p21 CDI, GADD45, MDM2 and p53 itself. The expression activation of the p21 CDI by p53 is particular significant (El-Deiry *et al.*, 1993). As mentioned above, p21 binds to and inhibits a wide variety of cyclin-CDK complexes, such as CDK4, CDK6 and CDK2. The former two are involved in the late G_1 checkpoint, while the latter is implicated in the G_1/S transition and the S checkpoint. In addition, p21 inhibits DNA replication directly by binding to the proliferating cell nuclear antigen (PCNA) (Li *et al.*, 1994). PCNA is a subunit of DNA polymerase δ, which is involved in DNA replication and DNA repair. Moreover, GADD45, expression of which is also activated by p53, binds to PCNA and enhances its involvement in DNA excision repair (Smith *et al.*, 1994). Thus, on one hand, p53 can effectively arrest cell cycle progression and cellular DNA replication via p21, and, on the other hand, it activates the DNA repair mechanism via GADD45.

p53 is also involved in at least one pathway, and possibly more, for apoptosis, a genetically programmed cell death control for maintaining tissue homeostasis (Collins and Lopez Rivas, 1993; Martin *et al.*, 1994; Oltvai and Korsmeyer, 1994; Anderson, 1993). The mechanism for the involvement of p53 in apoptosis is not clear, although it has been shown that p53 may activate the expression of the Bax gene, and may repress the protooncogene Bcl-2, resulting in changes in the ratio of Bcl-2 and Bax (Selvakumaran *et al.*, 1994; Miyashita *et al.*, 1994; Miyashita and Reed, 1995). The former is a apoptosis repressor, and is involved in the p53-mediated, as well as the p53-independent, apoptosis pathways (Wang *et al.*, 1993). The latter represses apoptosis by forming a hetero-complex with Bcl-2 (Korsmeyer, 1995). The expression of Fas, which belongs to the tumour necrosis factor (TNF) receptor family and is involved in apoptosis, may also be subject to p53 regulation

(Own-Schaub *et al.*, 1995). Thus, p53 not only arrests cell cycling and activates the DNA repair mechanism, but also triggers apoptosis, possibly in case of damage beyond salvage. It should be noted that apoptosis may also occur via p53-independent pathways (Donehower *et al.*, 1992; Clarke *et al.*, 1993).

The expression of p53 seems to be controlled by complex feedback circuits. For example, MDM2 inactivates p53 by binding to p53 (Momand *et al.*, 1992), while the MDM2 gene is activated by p53 (Barak *et al.*, 1993). In addition, p53 expression is regulated by its own positive feedback at the transcription level. However, p53 function seems to be more predominantly controlled at post-translational levels. Activity of p53 may fluctuate with cell cycles, since p53 phosphorylation at Ser315 enhances its degradation, and this site is a target of the CDK2 *in vitro* (Lin and Desiderio, 1993). In addition, p53 is also a substrate of the DNA-activated protein kinase (DNA-PK), which phosphorylates p53 at specific serine sites (Anderson, 1993). The regulatory subunit of DNA-PK, the Ku protein, has high binding affinity for double-stranded free DNA ends. Interestingly, mutation in the Ser15 phosphorylation site increases the half life of p53 (Fiscella *et al.*, 1993). Thus, DNA-PK/Ku was suggested to serve as the sensor and activator for the p53-mediated genome control mechanism. In addition, the C-terminus of p53 has been shown to be able to recognize damaged DNA and to influence DNA repair mechanism by interacting with RPA and TFIIH (Jayaraman and Prives, 1995; Lee *et al.*, 1995). Both pathways may also be involved in the regulation of p53 functions.

Obviously, for counteracting the inhibitory function of p53, DNA tumour viruses encode oncoproteins that specifically target p53. Adenovirus E1b, SV40 T Ag and EBV

latency protein EBNA-5 bind to, and inactivate p53 (Linzer *et al.*, 1979; Kress *et al.*, 1979; Linzer and Levine, 1979; Ludlow, 1993; Ring, 1994; Sontag *et al.*, 1993). Thus, through interacting with p53, the viral oncoproteins disable negative controls over cell cycle progression. At the same time, the viral oncoproteins also block DNA repair and apoptosis pathways, allowing mutations to occur and accumulate.

1.2.3.4 Viral oncoproteins interfere with signal transduction pathways for growth factors

Viral oncoproteins encoded by DNA tumour viruses may also stimulate cell proliferation by interfering with the peripheral signal transduction pathways that mediate intercellular communications for coordinating cell growth in multicellular organisms. These viral oncoproteins may interfere with the function of certain peptide or protein growth factors, their receptors, and other protein elements downstream in the signal pathways.

SV40 T Ag can up-regulate the expression of the insulin-like growth factor (IGF), thus stimulating cell growth by activating the IGF receptor tyrosine kinase in an autocrine manner (Baserga, 1994). Similarly, the EBNA-2 latency protein of EBV enhances the expression of CD23, a B cell growth factor receptor (Ring, 1994). Thus, these viral oncoproteins serve to create persistent peripheral mitogenic signals. These signals may cooperate with the pRb-binding viral oncoproteins to drive the cells to enter cycling. In addition, mitogenic signals may also contribute to preventing apoptosis, since entering cell cycles in the absence of mitogenic signals may trigger apoptosis.

1.3 Malignancy and papillomaviruses

1.3.1 Brief history

Although it was as early as 1842 when Rigoni-Stern (1842) suspected that the etiological factor(s) for cervical malignancy was infectious in nature, it required more than 150 years to confirm this hypothesis beyond any reasonable doubt.

In 1949, papillomavirus was observed electron-microscopically in the benign tumour papillomas (Strauss *et al.*, 1949). Then it was established that the benign papillomas induced by cottontail rabbit papillomavirus (CRPV) can progress into malignant tumours, and this malignant progression occurs more frequently to infections in a non-natural host (Syverton, 1952; Ito and Evans, 1961). The etiological role of papillomavirus infection in oncogenesis for humans was only firmly established with the advent of molecular biology. First, DNA sequences homologous to papillomaviruses were detected in 1974 in cervical cancer by low stringent nucleic acid hybridization (zur Hausen *et al.*, 1974). This result was correctly interpreted to indicate that papillomavirus is a family of viruses, and some of them may impose a particular risk on the host to develop malignant tumours. Confirming this prediction in 1983, a new type of papillomavirus, HPV16, was molecularly cloned from a biopsy sample of cervical cancer, and was shown to be prevalent in a substantial percentage of cervical cancers (Durst *et al.*, 1983; Boshart *et al.*, 1984). A new era for studies on DNA virus-mediated oncogenesis began (zur Hausen and de Villiers, 1994).

1.3.2 Methodology for studying oncogenesis by HPVs

The major problem hindering papillomavirus research has been the difficulties

propagating papillomavirus in experimental systems. Due to this reason, research on papillomaviruses has relied mainly on molecular biological and non-conventional virological methods.

1.3.2.1 Experimental studies

Rodent cells, and, more recently, human cells, have been extensively used in research on papillomavirus. Viral gene expression and DNA amplification can be studied by transient experiments using *in vitro* transfection of viral DNA. In addition, *in vitro* transfection of viral DNA, alone or cotransfected with other oncogenes, has also been used to immortalize or transform cultured cells to test the oncogenic activities of the viral genes. Studies on the cell lines established from *in vitro* immortalization/transformation assays, as well as cell lines established from naturally occurring infected tissues, have provided the majority of the information on papillomavirus-mediated oncogenesis. The effect of viral activities on the host cells has been studied with cells cultured in *in vitro* monolayer, and more recently in conditions allowing squamous differentiation. Epithelia reconstruction systems in either *in vitro* or *in vivo* conditions have been developed or adapted for this purpose. Further, transgenic mice carrying papillomavirus transgenes have also been utilized for studying papillomavirus-mediated oncogenesis.

1.3.2.2 Studies on naturally occurring papillomavirus infections

Clinical samples with papillomavirus infections have been extensively used for epidemiological studies on the prevalence of HPV infection and HPV-mediated oncogenesis.

Most of the important data concerning virological and oncogenic aspects of papillomaviruses have been derived from *in situ* studies on naturally occurring papillomavirus lesions. This is the golden standard test for the relevance of the data from experimental systems.

1.3.3 Virology of papillomaviruses

1.3.3.1 General aspects of papillomaviruses

Although the polyomaviruses and papillomaviruses were initially grouped together in the papovavirus family (papovaviridae), recent studies showed that papillomaviruses are fundamentally distinguished from the former in many aspects. Now, papillomaviruses are recognized as a separate subfamily (papillomavirinae) in papovaviridae (Murphy and Kingsbury, 1990). Members in papillomavirinae are small, nonenveloped, icosahedral viruses with an average diameter of 55 nm. In cesium chloride, the virions have a density of 1.34 g/ml. Each virion carries a single molecule of double-stranded, closed circular supercoiled genomic DNA of 7500-8,000 base pairs, packaged in a protein capsid consisting of 72 capsomeres (Howley, 1990).

Papillomavirus infections are widespread in higher vertebrates and characterized by their infection specificities. First, papillomavirus infections are strictly species-specific. Second, the vegetative life cycles of papillomaviruses can only be completed in stratified squamous epithelium (SSE). Only few papillomaviruses, such as bovine papillomavirus type 1 (BPV1) have been found to infect fibroblasts. Nevertheless, BPV1 infection in fibroblasts is non-vegetative. Third, papillomavirus infections also show some selectivity for anatomical locations.

1.3.3.2 Classification of papillomaviruses

The most basic classification for papillomaviruses is according to the host species that they infect. BPV and HPV infect bovines and humans, respectively. Cross-species infection occurs rarely, if ever. Papillomaviruses are subclassified into types according to their genomic DNA sequences, based on liquid hybridization experiments under stringent conditions (Pfister and Fuchs, 1994). Two isolates of papillomaviruses are regarded as separate types if they show less than 50% DNA homology. More than 70 types of HPV have been identified (de Villiers, 1994). For practical reasons, HPVs are also classified into cutaneous, mucocutaneous and mucosal, based on the target tissues of preference (Pfister and Fuchs, 1994). For example, HPV6, 16, and 31 preferentially infect mucosa, especially in genitalia, while HPV1 and HPV8 infects skin. Based on the relative tendency of the infected tissue to malignant progression, HPV can also be classified as being high-, intermediate- or low-risk (Lorincz *et al.*, 1992). For example, HPV16 and HPV31 are regarded as the high and intermediate-risk types, respectively, since they are found often to be associated with malignant tumours (carcinomas or cancers). HPV6 and HPV11 are the low-risk types that are associated with benign tumours (warts or condylomata) and rarely found in cancers.

1.3.3.3 Target tissue for papillomavirus vegetative infection

SSE is the only tissue that is found to support vegetative replication of papillomaviruses. SSE provides an extensive protective covering for the body surface, and body cavities that directly lead to the external environment (Fuchs, 1990). The basal germinal cells of SSE, which are closest to the stroma, are competent to divide, producing overlying

suprabasal cells that are permanently withdrawn from cycling and committed to programmed terminal differentiation (see Section 1.3.5.1) (Barrandon and Green, 1987; Rochat *et al.*, 1994). The program for the squamous differentiation directs a fundamental change in gene expression, which is characterized by the sequential activation of certain genes and concurrent repression of many others. The cells approaching the epithelial surface are terminally differentiated, and are finally shed off from the epithelial surface. Thus, SSE maintains a precisely balanced state of tissue homeostasis, which is characterised by controlled proliferation of basal cells to replenish the cells leaving the basal layer and undergoing terminal squamous differentiation.

SSE covering different anatomical regions are similar in respect of the programmed squamous differentiation. However, these SSE can be distinguished by distinct features. For example, the epidermal SSE is keratinizing, while the mucous SSE is non-keratinizing. In addition, these different types of SSE also express characteristic constituent structural proteins.

One type of the proteins that are expressed differentially in different SSE is cytokeratin (CK), which is expressed in association with the process of squamous differentiation. CK is a family of intermediate filament proteins that are important constituents of the cytoskeleton in eukaryotic epithelial cells (Fuchs, 1993). They provide a stabilizing framework within the cells, and may also be involved in intracellular transportation and gene regulation (Traub, 1985). Different type CKs are classified based on their isoelectric pH and immunologic properties. According to the former, CKs can be divided into two classes, class I (acidic) and class II (basic to neutral). Generally, these two classes are expressed in pairs,

combining a class I and a class II protein. Immunologically, human CKs can be identified as at least 20 types, and they are numbered as CK1-20. CK expression is tissue-specific. Different types of epithelia express distinct combinations of CKs. Simple epithelia usually express the so-called simple epithelial CKs, such as CK18, which is usually not expressed in the stratified epithelia. CK expression is also differentiation-dependent in SSE. The basal cells of both the keratinizing and non-keratinizing SSE express the basal type CK5 and 14. However, the upper layer cells undergoing squamous differentiation suppress the transcription of the basal type CKs, and activate the expression of CK1 and 10 in keratinizing SSE, or CK4 and CK13 in non-keratinizing SSE (Fuchs, 1988).

The molecular mechanism for squamous differentiation is not clear (Fuchs, 1990). Inspired by the finding that myogenesis is controlled by the expression of a family of master genes, the myo-D family of transcription factors, genes in association with squamous differentiation have been cloned for analysing the control sequences and searching for the possible master factors related to squamous differentiation. Although some sequences have consistently been found in the control region of these genes, it seems that these motifs are binding sites for ubiquitous rather than unique factors. One of the motifs is the TTTGGCTT so-called CK motif, which binds to transcription factor NF1 (Blessing *et al.*, 1987; Jones *et al.*, 1987). So far no specific transcription factors, or sequence motifs suggestive of such factors, have been well established for squamous differentiation and expression of CK genes. The nature of the stem cells that continuously give rise to the differentiated cells is not known. The SSE basal germinal cells have been reported to contain at least three subpopulations of cells (Barrandon and Green, 1987). Those expressing high levels of integrin are believed to

represent the stem cells capable of cycling to replenish the offspring cells that will commit themselves to terminal differentiation (Jones and Watt, 1993). Equally unknown is the relationship between differentiation commitment and the mechanism for the cell cycle withdrawal.

1.3.4 Molecular biology of papillomaviruses

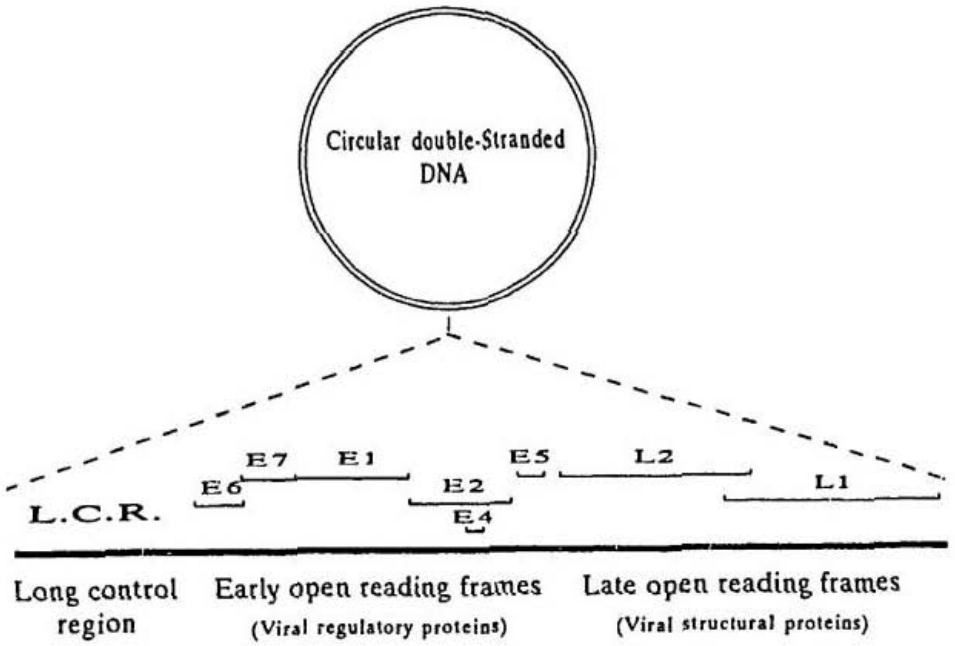
HPV16, the subject of the current study, will be the focus for discussion wherever data is available. In the cases in which information on HPV16 is unavailable, data on other papillomaviruses will be discussed instead.

1.3.4.1 Genome organization of papillomaviruses

The nomination of papillomavirus genes follows the convention established for BPV1, of which the genome was first completely sequenced and analysed (Pfister and Fuchs, 1994).

Along the 7904 bp circular genomic DNA of HPV16 (Figure 1.1), a sequence of approximately 850 bp has no open reading frames (ORFs). This region contains numerous binding sites for transcription factors, indicating that it serves as the control region for the transcription of viral genes. Hence it was named the long control region (LCR). Eight ORFs are clustered in the remaining sequences. All the ORFs are located in only one strand of the DNA, and cDNA analysis confirmed that only one strand of the viral genomic DNA is transcribed. Selected transcripts of HPV16 are shown in Figure 1.2. The coding region can be divided into early and late regions according to the positions of two polyadenylation signal sequences. The ORFs immediately 3' to the LCR encode the early (E) regulatory proteins.

Figure 1.1 **Genome organization of HPV16, modified according to Bernard and Apt (1994)**



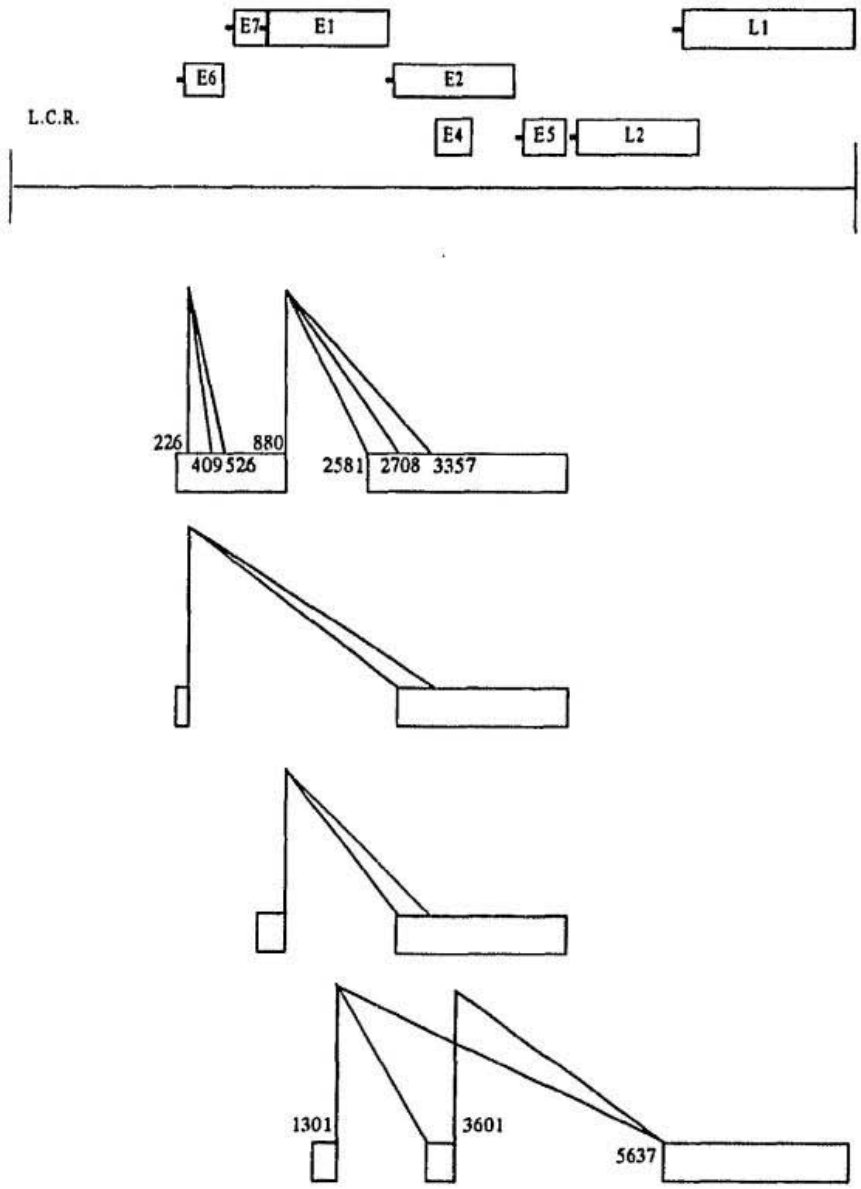
HPV16 DNA is found in over 50% of cervical carcinomas

Viral E6 and E7 genes are preferentially retained and expressed in cancer cells.

HPV16 Genome Organization

Figure 1.2 Schematic presentation of HPV16 mRNA splicing, modified according to Fuchs and Pfister (1994)

Shown are selected transcripts of HPV16 (Smotkin and Wettstein, 1986; Doorbar *et al.*, 1990; Nasseri *et al.*, 1991; Rohlfs *et al.*, 1991; Sherman *et al.*, 1992; Sherman and Alloul, 1992). Vertical lines indicate splice donor sites; slant lines point to acceptor sites. Open boxes indicates ORFs in viral genome, and tippled boxes indicate ORFs potentially encoded by the transcripts.


E7
E1
L1
E6
E2
L.C.R.
E4
E5
L2
Potential products
226
880
409 526
2581
2708
3357
E6, E7, E1^E4, E2, E5
E2, E5
E1^E4, E2, E5
1301
3601
5637
L1

The early genes use the polyadenylation signal immediately downstream of the early ORFs. Downstream of the early genes are the late (L) L1 and L2 genes encoding viral structural proteins. The structural genes use a separate polyadenylation signal located downstream of the late genes and 5' to the LCR.

As for other related viruses, the genome organization of HPV16 shares remarkable similarity to that of other papillomaviruses. However, although most of the ORFs and control elements are conserved among papillomaviruses, their presence, location in the genome, sequence homology and functions may vary. This variation reflects the adaptive evolution of papillomaviruses to different target hosts and tissues, and underscores the differential oncogenic potential among HPVs.

1.3.4.2 HPV genes and control elements

1.3.4.2.1 E6 ORF and E6 protein

The full length E6 ORF of HPV16 encodes a 151 amino acid protein with a molecular weight of approximately 16-18 kDa. It is a basic, zinc binding protein localized in the nuclear and non-nuclear membrane fractions (Androphy *et al.*, 1987). The four Cys-X-X-Cys motifs in the carboxyl terminus (C-terminus) mediate binding to zinc, and are involved in nuclear translocation (Barbosa *et al.*, 1989; Kanda *et al.*, 1991).

In *in vitro* conditions, HPV E6 protein has been found to bind the p53 tumour suppressor (Werness *et al.*, 1990). Unlike SV40 T Ag, which binds and sequesters p53 from functioning, p53 binding by E6 results in enhanced degradation of p53 (Scheffner *et al.*, 1990; Lechner *et al.*, 1992). The binding between p53 and E6 is mediated by the cellular protein E6-

AP, which is a ubiquitin ligase in the ubiquitin proteolysis pathway (Huibregtse *et al.*, 1991; Huibregtse *et al.*, 1993a; Huibregtse *et al.*, 1993b; Scheffner *et al.*, 1994; Shkedy *et al.*, 1994). Significantly, E6 proteins from high-risk HPVs show a higher activity for p53 degradation than those from low-risk HPVs, indicating that the difference in the biochemical nature of E6 proteins may underlie the oncogenic potential of the HPVs. The low p53 degradation rate mediated by the low risk E6 proteins may be due to either their low affinity for p53 (Scheffner *et al.*, 1990), or their low activity for the subsequent degradation step (Crook *et al.*, 1991b). In consistence with the *in vitro* E6 function for p53 binding and degradation, expression of HPV E6 *in vivo* has been correlated with a reduced half-life of p53 (Crook *et al.*, 1991b; Lechner *et al.*, 1992; Band *et al.*, 1993), inhibited p53-mediated transcription (Lechner *et al.*, 1992; Mietz *et al.*, 1992; Lechner and Laimins, 1994; Crook *et al.*, 1994; Pim *et al.*, 1994; Mansur *et al.*, 1995) and compromised p53-dependent G_1 arrest (Kessis *et al.*, 1993; Foster *et al.*, 1994).

HPV E6 proteins have also been shown to transactivate the heterologous adenovirus E2 promoter (Sedman *et al.*, 1991; Desaintes *et al.*, 1992), although it has not been shown that E6 is transacting on its homologous viral promoters (see Section 1.3.4.2.8.). The E6 domain required for transactivation is distinct from the one for p53 interaction, and the transactivation capacity is similar between the high- and low-risk HPVs (Crook *et al.*, 1991b). The biological significance of E6 transactivation for virus replication and its role in viral oncogenesis are not clear. Very recently, HPV16 E6 protein has been shown to bind a calcium-binding protein *in vitro* and this binding is specific for the high-risk, but not the low-risk, HPVs (Chen *et al.*, 1995). Since calcium is an important second messenger involved in

cell differentiation, mitosis, and other cellular functions, HPV E6 proteins possibly exert effects on a wide spectrum of cellular functions during virus life cycle.

HPV16 E6 ORF contains one splice donor site at nt 226 and two acceptor sites at nt 409 and nt 526. Differential usage of these splice sites inside the E6 ORF produces different E6 transcripts with the full-length E6, and differentially shortened E6* and E6** (Smotkin *et al.*, 1989). In addition, the single donor site can also be used to splice to acceptor sites at nt 2078 5' to E2 ORF (Nasseri *et al.*, 1991) or at nt 3357 5' to the E5 ORF (Doorbar *et al.*, 1990), producing transcripts containing an E6*** leader sequences for the E2 or E5 ORFs. It is not clear whether the shortened E6* and E6** ORFs encode any products (Roggenbuck *et al.*, 1991). It is generally accepted that splicing within the E6 ORF serves mainly to facilitate efficient translation of the downstream ORFs, although it has been shown that translation of the E7 ORF can be reinitiated on the transcripts with the full length E6 ORF (Sedman *et al.*, 1991; Tan *et al.*, 1994). The E6 ORFs of the low-risk HPVs, such as HPV6 and HPV11, do not contain similar splicing sites in the E6 ORF. In contrast, the E6 ORFs of these HPVs contain a promoter from which E7 transcription is initiated (Smotkin *et al.*, 1989). E6 is a viral oncogene for HPV16 (see Section 1.3.5.3.1).

1.3.4.2.2 E7 ORF and E7 protein

The E7 ORF of HPV16 encodes a single 100 amino acid protein with a molecular weight of 14 kDa. It is acidic, highly phosphorylated and located in the nucleus (Sato *et al.*, 1989b). The amino terminus (N-terminus) contains two regions homologous to the conserved regions 1 (CR1) and 2 (CR2), domains of the adenovirus E1A protein (Phelps *et al.*, 1988).

At the C-terminus of CR2, there is one casein kinase II (CKII) recognition site, which is responsible for the phosphorylation of serines 32 and 33. In addition, the C-terminus of the E7 protein contains two Cys-X-X-Cys motifs that mediate binding to zinc. This domain is also important for the stability and dimerization of the E7 proteins (Edmonds and Vousden, 1989; Phelps *et al.*, 1992; McIntyre *et al.*, 1993).

Like the oncoproteins E1A of adenovirus and SV40 T Ag, the E7 proteins of the high-risk HPVs, but not the low-risk ones, bind to the hypo-phosphorylated form of pRb (Dyson *et al.*, 1989). However, unlike E1A and T Ag, HPV E7 binding to pRb involves the CR2 region, but not the CR1 region. In addition, the E7 C-terminus may also be involved in interacting with pRb (Munger *et al.*, 1991; Huang *et al.*, 1993). The E7 proteins of high-risk HPVs also bind to p107 in a N-terminal domain that is overlapping with, but distinct from, the one for pRb binding (Davies *et al.*, 1993; Ciccolini *et al.*, 1994). The HPV16 E7 protein may disrupt the complex between hypo-phosphorylated pRb and E2F through steric effects, relieving E2F from the pRb sequestration, since the binding sites on pRb for E7 and E2F are not overlapping (Munger *et al.*, 1989b; Imai *et al.*, 1991; Munger *et al.*, 1991; Chellappan *et al.*, 1992; Huang *et al.*, 1993; Wu *et al.*, 1993). The increased availability of E2F would up-regulate the expression of the genes related to cell cycle progression. In contrast to E1A, the binding of HPV E7 protein to p107 does not result in freeing E2F from the complex, and a complex containing E7, E2F, cyclin A and CDK2 can be detected in the cells expressing E7 (Pagano *et al.*, 1992; Arroyo *et al.*, 1993; Lam *et al.*, 1994). Thus, E7 may not only interfere with the binding between pRb and E2F, but also modify E2F function in the complex containing p107 (Pagano *et al.*, 1992). E7 can also directly bind to cyclin A-CDK complex,

without the mediation of pRb or p107 (Tommasino *et al.*, 1993). Consistent with the important roles of the pRb and E2F proteins in cell cycle control, HPV16 E7 induces cellular DNA synthesis in *in vivo* assays (Sato *et al.*, 1989a; Banks *et al.*, 1990; Rawls *et al.*, 1990; Blanton *et al.*, 1992), and mediates an up-regulated expression of B-*myb*, a transcription factor encoded by a protooncogene and involved in cell cycle progression (Lam *et al.*, 1994). This up-regulated expression of B-*myb* involves the binding at the E2F site in the B-*myb* promoter by a complex containing p107.

E7 proteins of all HPVs exhibit a similar trans-activating potential for the adenovirus E2 promoter (Phelps *et al.*, 1988; Munger *et al.*, 1991). This activity is dependent on the E2F binding sites in the adenovirus E2 promoter, suggesting the involvement of E2F in this function. Consistent with this notion, the trans-activating activity of E7 is susceptible to mutations in the pRb binding region (Edmonds and Vousden, 1989; Phelps *et al.*, 1992). Trans-activation requires the E7 C-terminus, which is interchangeable between the high- and low-risk HPVs for trans-activating abilities (Munger *et al.*, 1991). This is in contrast to the pRb-binding N-terminal domain of E7, which is not interchangeable for pRb binding between high- and low-risk HPVs. HPV E7 has not been reported to be transactivating for its homologous promoters.

It is still controversial whether or not E7 phosphorylation is important for E7 function (Phelps *et al.*, 1988; Barbosa *et al.*, 1990). High-risk HPV E7 does seem to be a better substrate for CKII phosphorylation, and phosphorylation at these sites is important for transformation/immortalization (see Section 1.3.5.3.1) (Barbosa *et al.*, 1990). E7 is the major viral oncogene for HPV16 (see Section 1.3.5.3.1).

1.3.4.2.3 E1 ORF and E1 protein

The full length E1 ORF of papillomavirus encodes a nuclear protein of 600-650 amino acids. Most information on the E1 protein was from studies on BPV1 and HPV11. BPV E1 is a DNA-binding phosphoprotein, which has a phosphorylation site for the CDK2- $p34^{cdc2}$ (Lentz *et al.*, 1993). The E1 protein has a DNA-dependent ATPase activity and is an ATP-dependent helicase (Seo *et al.*, 1993; Yang *et al.*, 1993). Both activities are dependent on the ATP binding site at the C-terminus. The E1 oligomers bind specifically to the E1 binding sites (E1-BS) in the replication origin of the papillomavirus genome, and the binding is greatly facilitated by the presence of the viral E2 proteins (Lu *et al.*, 1993; Bream *et al.*, 1993). E1 is involved in DNA amplification of papillomaviruses and shares some structural and functional features with SV40 T Ag, which is also involved in initiating viral DNA amplification. For DNA replication of papillomaviruses, see Section 1.3.4.2.9.

BPV E1 represses its homologous E6 promoter (Le Moal *et al.*, 1994; Zemlo *et al.*, 1994; Vande Pol and Howley, 1995). Because mutations in HPV16 E1 are associated with increased immortalizing efficiency (Romanczuk and Howley, 1992), and HPV16-mediated immortalization is dependent on the efficient expression of the E6 and E7 genes (Munger *et al.*, 1989a; Smits *et al.*, 1990), HPV16 E1 has also been suspected to be trans-repressing for the E6 promoter.

The HPV16 E1 ORF contains two splice donor sites at nt 880 and 1301, which may alternatively splice to the nt 2581 or nt 2708 acceptor sites 5' to the E2 ORFs, or the nt 3357 acceptor site inside the E4 ORF (Cornelissen *et al.*, 1990; Doorbar *et al.*, 1990; Rohlfs *et al.*, 1991; Sherman and Alloul, 1992; Sherman *et al.*, 1992).

1.3.4.2.4 E2 ORF and E2 protein

HPV16 E2 ORF encodes a full length E2 protein (McBride *et al.*, 1991), and possibly a truncated E2 containing only the C-terminus (E2C) due to the presence of a splice acceptor (Doorbar *et al.*, 1990). The full length E2 is a nuclear DNA-binding phosphoprotein with a molecular weight of approximately 43-48 kDa (McBride *et al.*, 1991). It consists of three functionally and structurally distinct segments (Giri and Yaniv, 1988). The amino- and carboxyl- regions are well conserved among the papillomaviruses. The former are responsible for trans-acting viral gene transcription, and the latter for DNA binding and E2 dimerization. The two regions are linked by a "hinge" segment, which is poorly conserved among the papillomaviruses. HPV16 contains four copies of the E2 binding site (E2-BS) in the LCR, which are palindromic $ACC(N)_6GGT$ and $ACC(N)_6GTT$ sequences. BPV E2 protein binds to E2-BS as dimers, and each monomer makes contact with the major groove and resides on the same side of the DNA helix (Dostatni *et al.*, 1988).

E2 is a regulatory protein that regulates viral gene expression *in trans* (Barsoum *et al.*, 1992; Sherman and Alloul, 1992; Storey *et al.*, 1992; Tan *et al.*, 1992; Dong *et al.*, 1994; Tan *et al.*, 1994). The consensus of opinion about the HPV E2 is that, in the natural context of the HPV enhancer, E2 acts as a repressor for the E6 promoter through binding to the E2-BSs located adjacent to the TATA box, possibly disrupting the adjacent SP1 binding (Bernard *et al.*, 1989; Gloss and Bernard, 1990; Romanczuk *et al.*, 1990; Tan *et al.*, 1992; Tan *et al.*, 1994). However, E2 mutants with disruptions in these two E2-BSs only partially derepress the P97 promoter, suggesting the involvement of additional mechanisms for E2-mediated

repression. In addition, similar to BPV E2, the HPV16 E2 protein has been shown to transactivate an artificial LCR (Ushikai *et al.*, 1994). This function is dependent on the binding of E2 to the E2-BS and requires cooperation by AP1, Oct1 and NF1 transcription factors (Gauthier *et al.*, 1991). HPV16 E2C, which contains only the C-terminus of the E2 protein, has unknown function (Doorbar *et al.*, 1990). The BPV counterpart of E2C acts as a competitive repressor for the full length E2 protein, which itself is a trans-activator for the BPV LCR (Barsoum *et al.*, 1992). Similar observation has also been made on HPV11 (Chin *et al.*, 1988). These data suggest that HPV E2 may also function as trans-activator in certain unidentified circumstances (Bouvard *et al.*, 1994b). Interestingly, the E2 protein of HPV8 has been shown to transactivate the viral late promoter (Stubenrauch *et al.*, 1992; May *et al.*, 1994b). Since E2 represses the transcription of the early genes, E2 may be involved in switching viral gene expression from the early to late stages during vegetative infection.

E2 is also involved in viral DNA amplification. The N-terminal domain of BPV E2 protein interacts with the E1 protein, stimulating and stabilizing E1 binding to the replication origin on viral DNA (Mohr *et al.*, 1990). Studies with HPV11 and HPV31b confirmed that E2 is important for the origin-dependent amplification of viral DNA (Frattini and Laimins, 1994; Kuo *et al.*, 1994).

1.3.4.2.5 E4 ORF and E1^E4 protein

The E4 ORF of papillomaviruses overlaps with the central part of the E2 ORF, and is the most divergent among the papillomaviruses (Doorbar *et al.*, 1989). The E4 ORF itself has no translation initiation codon. cDNA sequences containing the E4 ORF show that

HPV16 has a splice acceptor at nt 3357, which is spliced to the donor site at nt 880, producing an E1^E4 message composed of a small portion from the 5' end of E1 ORF and a major portion from the E4 ORF (Doorbar *et al.*, 1990). The E1^E4 protein is a 17 kDa phosphoprotein localized in the cytoplasm in a close relationship with the CK network (Grand *et al.*, 1989; Crum *et al.*, 1990; Roberts *et al.*, 1994). HPV16 E1^E4 was shown to associate with CKs, resulting in the collapse of the intermediate filament network (Doorbar *et al.*, 1991). Mutation analysis with the HPV16 E1^E4 protein indicated that the N-terminus of E4 is important for CK association, and a C-terminal domain is required for disrupting the CK network (Roberts *et al.*, 1994). This data suggests that the E1^E4 protein may be related to the escape of mature virions from the host cells (see Section 1.3.4.3).

1.3.4.2.6 E5 ORF and E5 protein

The E5 ORF shares also low homology among papillomaviruses, and some cutaneous HPVs, such as HPV5 and 8, do not have this ORF (Bubb *et al.*, 1988). Structural analysis predicts that the E5 protein contains three transmembrane domains and is highly hydrophobic. The HPV16 E5 protein has been shown to be associated with a 16 kDa H^+-ATPase (Finbow *et al.*, 1991; Conrad *et al.*, 1993). The H^+-ATPase is involved in the acidification of endosomes, a condition required for ligand-induced down-regulation of growth factor receptors. Cells expressing HPV16 E5 have increased tyrosine kinase activity for the epidermal growth factor (EGF) receptor and reduced degradation of EGF (Straight *et al.*, 1993), possibly as a result of the abrogated H^+-ATPase activity by HPV16 E5. In addition to binding to the H^+-ATPase, HPV6 and BPV E5 proteins have been shown to be associated directly with the

tyrosine kinase receptors for EGF and the platelet-derived growth factor (PDGF), respectively (Goldstein *et al.*, 1991; Goldstein *et al.*, 1992; Conrad *et al.*, 1993; Conrad *et al.*, 1994). Consistent with its biochemical activity, HPV16 E5 stimulates the growth of human keratinocytes (Gu and Matlashewski, 1995)and this stimulation is enhanced by EGF (Leechanachai *et al.*, 1992; Straight *et al.*, 1993). E5 is a major oncogene for BPV1, but not HPVs (see Section 1.3.5.3.1).

1.3.4.2.7 L1 and L2 ORFs and proteins

L1 is the most highly conserved ORF among the papillomaviruses. It encodes a large nuclear glycosylated protein of 55 kDa (Zhou *et al.*, 1991a; Zhou *et al.*, 1993b). L2 ORF is less conserved, and encodes a nonspecific DNA-binding protein with a molecular weight of 76 kDa (Zhou *et al.*, 1994). Artificial expression of these two ORFs in different systems showed that the L1 protein alone is sufficient of forming virion-like particles (VLP), although the assembly of VLP is greatly enhanced by the coexpression of L1 and L2 proteins (Zhou *et al.*, 1991b; Hagensee *et al.*, 1993; Zhou *et al.*, 1993a). Significantly, coexpressed L1 and L2 package DNA of appropriate size into the VLP. Studies with such VLPs have suggested that L1 and L2 are involved in virus entry (Muller *et al.*, 1995) and viral immunogenicity (Zhou *et al.*, 1992; Hagensee *et al.*, 1994).

1.3.4.2.8 *Cis*-acting promoter and enhancer elements of HPV16

The LCR of HPV16 contains a promoter, a DNA replication origin, a composite enhancer and a segment of unknown function. The promoter is located at the 3' end of the

HPV16 LCR, in front of the E6 ORF (Smotkin and Wettstein, 1986). It has been referred to as the early promoter, E6 promoter or P97 promoter. The existence of a promoter in front of the E6 ORF is a feature shared by all the papillomaviruses. The HPV16 P97 promoter includes a TATA box 39 bp upstream of the start codon of the E6 ORF, and its activity greatly depends on the integrity of an SP1 factor site 36 bp upstream of the TATA box. The promoter region contains two E2-BSs. Binding by E2 at these sites has been shown to displace the SP1 factor and the general transcription factor TFIID, thus repressing the activity of the P97 promoter (Tan *et al.*, 1992; Tan *et al.*, 1994). Transcripts initiated from this promoter have been found to be potentially able to encode all the HPV16 early genes (Doorbar *et al.*, 1990). An equivalent promoter in HPV31b has been reported to initiate the transcripts for the L1 ORF constitutively (Hummel *et al.*, 1995).

The 400 bp sequence upstream of the P97 promoter has been established to contain a composite enhancer that can modulate the P97, or a heterologous, promoter in an epithelial-specific manner (Chong *et al.*, 1991). Numerous binding sites for both cellular and viral transcription factors have been found in this enhancer. The cellular transcription factors that are well established to bind the enhancer are ubiquitous, and include NF1, TEF1 and TEF2, AP1, YY1, Oct1, *et al.* (Bernard and Apt, 1994). Significantly, glucocorticoid/progesterone responsive elements (GREs), the binding sites for the ligand-bound steroid hormone receptors have been found in the LCRs of HPV16, 18 and 11 (Klock *et al.*, 1988; Chan *et al.*, 1989; Mittal *et al.*, 1993a). These GREs have been shown to mediate the up-regulation of viral gene expression in response to glucocorticoid and progesterone (Chan *et al.*, 1989; Mittal *et al.*, 1993a). Although the HPV18 enhancer has been found to have the binding site for a putative

keratinocyte-specific transcription factor, the keratinocyte-specific transcriptional activator (KRF-1) (Mack and Laimins, 1991), no binding sites for KRF-1 have been identified for HPV16 (Hoppe-Seyler and Butz, 1994).

The replication origin of HPV16 is also located within the enhancer and contains two more E2-BS, which are involved in DNA replication (see Section 1.3.4.2.9). The 5' portion of the LCR upstream of the enhancer has unknown function for HPV16. However, in cutaneous HPVs and animal papillomaviruses, differentiation-specific promoters for the late structural genes have been found in this region (Baker and Howley, 1987; Wettstein *et al.*, 1987; Stubenrauch *et al.*, 1992; Barksdale and Baker, 1993).

Besides the P97 promoter, which is the only promoter firmly established for HPV16, the existence of other promoters has been suggested in HPV16 genome by various studies. *In situ* hybridization studies using subgenomic probes (Higgins *et al.*, 1992b) and cDNA clone analysis (Doorbar *et al.*, 1990) have suggested another promoter inside the E7 ORF. Activation of this promoter seems to be dependent upon squamous differentiation, and this promoter potentially controls the expression of the early, as well as the late genes (Doorbar *et al.*, 1990). Another promoter, suggested by cDNA cloning, seems to be located at the 5' end of the E1 ORF, and this promoter may initiate transcription of the L1 gene (Doorbar *et al.*, 1990; Rohlfs *et al.*, 1991). These two promoters have not been thoroughly characterized.

Unlike HPV16 and HPV18, which initiate the E6 and E7 transcripts from the E6 promoter located 5' to the E6 ORF, HPV6 and 11 have a promoter inside the E6 ORF for initiating E7 gene expression (Smotkin *et al.*, 1989). HPV18 has been suggested to have promoters in front of the E2 ORF, within the E2 ORF and at the end of L2 ORFs (Karlen and

Beard, 1993). For BPV, HPV1, and HPV8, which are all cutaneous papillomaviruses, the late promoter for viral L1 and L2 structural genes seems to be located within the 5' portion of the LCR (Baker and Howley, 1987; Palermo Dilts *et al.*, 1990; Stubenrauch *et al.*, 1992). Although transcripts from these late promoters span the early and the late ORFs, the mature transcripts contain only the late ORFs, as a result of alternative splicing and polyadenylation site usage.

1.3.4.2.9 Replication of papillomavirus DNA

Most information on papillomavirus DNA replication has been derived from studies on BPV and HPV11. Transient and cell-free replication systems showed that the origin for viral DNA replication contains an AT-rich region and a putative E1 binding site (E1-BS), which is flanked by two E2-BSs (Chiang *et al.*, 1992a; Dong *et al.*, 1994). Viral DNA replication is bidirectional, and produces θ form intermediates during replication (Auborn *et al.*, 1994). Studies of BPV showed that viral E1 and E2 proteins are required for viral DNA replication, in addition to cellular factors such as DNA polymerase α/primase, DNA polymerase δ/PCNA, and topoisomerases I and II (Kuo *et al.*, 1994). Although the E1 protein binding at the E1-BS alone is sufficient for viral DNA replication, the efficiency of viral DNA amplification is dependent on the integrity of the E2-BSs, and is proportional to the number of E2-BSs (Lu *et al.*, 1993). The N-terminal domain of the BPV E2 protein interacts with the E1 protein, stimulating and stabilizing E1 binding at the origin (Mohr *et al.*, 1990). Thus, it has been suggested that the E2 protein is the primary origin-recognition protein, and its binding to the E2-BSs facilitates E1 binding to E1-BS. The E1 protein in turn

recruits cellular factors to assemble the initiation complex for viral DNA replication.

Supplemented with viral E1 and E2 proteins, papillomavirus DNA amplification can occur in all the cell types tested (Chiang *et al.*, 1992b), including those of humans and mice, and of epithelial and fibroblast origins. Thus, viral DNA amplification seems not to be the restriction for the species- and tissue-specificity of papillomavirus vegetative replication.

1.3.4.3 Differentiation-dependent and tissue-specific gene expression and life cycles of papillomaviruses

SSE is the only tissue found to support vegetative replication of papillomaviruses, and the gene expression of papillomaviruses is in association with the squamous differentiation of SSE. This feature of papillomavirus infection was first indicated by electron-microscopic studies on HPV-infected lesions, showing that virions can only be observed in the upper layer differentiated cells of SSE (Strauss *et al.*, 1949). *In situ* hybridization studies confirmed that viral DNA amplification and gene expression are differentiation-dependent. In basal cells, few copies of viral DNA are present and the late genes are not expressed (Nuovo *et al.*, 1991b). With the onset of terminal squamous differentiation, viral E2 and E1 genes are increasingly expressed in the upper layer differentiating cells (Stoler *et al.*, 1992). The accumulation of E2 protein in the nucleus is correlated with the amplification of viral DNA (Li *et al.*, 1988; Sekine *et al.*, 1989). In the terminally differentiated cells, E1^E4, which is believed to be involved in virion escape, is the most highly expressed viral message (Crum *et al.*, 1990). Accompanied by the accumulation of E1^E4 protein is the expression of the viral late genes, occurring in the upper layer differentiated cells (Crum *et al.*, 1990).

Although it is well established that viral DNA amplification and late gene expression occur in the upper layer differentiating cells of SSE, the pattern for the expression of the E6 and E7 viral genes is intriguingly paradoxical from the virological point of view. Based on the biochemical properties of their products, as introduced in Section 1.3.4.2.1 and 1.3.4.2.2, the E6 and E7 proteins logically should serve to promote cell proliferation. Since the onset of the late phase of the papillomavirus life cycle is activated in the upper layer cells, which are permanently withdrawn from cycling, it is also reasonable to expect that the E6 and E7 genes to be expressed in the bottom layer cells, which are competent for cycling. In addition, the position of these two genes in the papillomavirus genome, which is located immediately downstream of the E6 promoter (see Figure 1.1), is also consistent with the view that E6 and E7 should be expressed at the earliest phase during the infection. However, overwhelming data from studies on many HPVs, including HPV6, 11, 16 and 18, suggest that the E6 and E7 genes be actively expressed in the upper layer cells, overlapping with other viral genes (Crum *et al.*, 1988; Durst *et al.*, 1992; Stoler *et al.*, 1992; Bohm *et al.*, 1993). Only one study on HPV6 showed that the E6-E7 expression is, consistent with theoretical predictions, expressed in the bottom layer proliferating cells (Iftner *et al.*, 1992). Since these two viral early genes, especially the E7 gene, may play important roles in HPV-mediated oncogenesis (see Section 1.3.5.3.1), it is urgent to address this question.

The mechanisms for the differentiation-dependent activities of papillomavirus are unclear and the knowledge we have is fragmentary. Differentiation-dependent viral DNA amplification may be explained by differentiation-dependent activation of the expression of E1 and E2 proteins, which are important for viral DNA amplification (Chiang *et al.*, 1992b).

The promoter inside the E7 ORF of HPV31b, which is immediately upstream of the E1 and E2 ORFs, has been found to initiate transcription of E1^E4 and E5 in a manner dependent on squamous differentiation (Pray and Laimins, 1995). An equivalent promoter was also suspected in HPV16 (Higgins *et al.*, 1992b). However, it is not clear how this promoter achieves the differentiation-dependency, and which viral *cis* elements modulate this promoter. Differentiation-dependent expression of the papillomavirus late genes may also be due to differentiation-dependent activation of specific promoters. For cutaneous HPVs and BPV1, which have their late promoters at the 5' end of the LCR, such a switching may be provided by the differentiation-dependent expression of the E2 gene (Baker and Howley, 1987; Palermo Dilts *et al.*, 1990; Stubenrauch *et al.*, 1992). In the case of HPV31b, which is closely related to HPV16, differentiation-dependent expression of the late genes is not only due to the differentiation-dependent activation of the promoter that is also used for initiating the expression of E1^E4 (Pray and Laimins, 1995), but also the differentiation-dependent alternative splicing that removes the E1^E4 ORF (Hummel *et al.*, 1995). It has been shown that in BPV and HPV 16, a short splice site-like sequence in the 3'-untranslated region of the late messages determines the stability of the late messages or/and the usage of polyadenylation signals in a differentiation-dependent manner (Baker and Noe, 1989; Kennedy *et al.*, 1990; Furth and Baker, 1991; Kennedy *et al.*, 1991; Furth *et al.*, 1994).

Thus, the mechanism for the SSE-specific infection of papillomaviruses is not clear. Since papillomavirus DNA amplification is indiscriminate of cell types in cell-free replication systems, this specificity is unlikely due to differential existence of cellular factors required for viral DNA replication (Yang *et al.*, 1991; Chiang *et al.*, 1992b; Kuo *et al.*, 1994). In addition,

experiments showed that the VLPs assembled from the L1 and L2 proteins of HPV16 or HPV11 can mediate DNA entry into various types of cells (Muller *et al.*, 1995). This nonspecific entry for the VLPs of HPV16 and HPV11 is consistent with the fact that BPV1 cannot only infect SSE, but also stromal fibroblasts, although the latter do not support BPV1 vegetative infection (Lambert *et al.*, 1988). Thus, it is possible that tissue-specific papillomavirus infection is determined by tissue-specific expression of viral genes, rather than restrictions on virus entry, or on amplification of viral DNA. Indeed, the HPV16 enhancer is active only in cell lines that express CK and inactive in the others of different linage (Chong *et al.*, 1991). Consistent with this view, it has also been shown that, while the HPV16 early promoter is active in epithelial cells, it has only minimal activity in normal fibroblasts (Smits *et al.*, 1988; Smits *et al.*, 1990). However, no epithelial-specific cellular transcription factors have been established to be responsible for this selectivity of HPV16 infection. Thus, it seems that the interplay between differential concentrations of ubiquitous cellular factors may determine the epithelia-specific activities of the papillomavirus promoter and enhancer elements.

Although studies on the specificity of papillomavirus infections have focused mostly on the selective activation of viral promoter/enhancers, tissue-specific and differentiation-dependent viral activities could also be achievable by regulations at post-transcriptional levels, such as alternative utilization of splice sites and polyadenylation signals. The importance of post-transcriptional control for the differentiation-dependent expression of papillomavirus genes is also suggested by the complex scheme for the splicing of HPV16 transcripts (refer to Figure 1.2).

1.3.5 Oncogenesis by papillomaviruses

Epidemiological and experimental data strongly indicate that certain types of papillomaviruses are etiological agents for the oncogenesis of genital cancers, especially cervical carcinomas (Broker and Botchan, 1986; Burghardt, 1986; Matlashewski, 1989; zur Hausen, 1994; Vousden *et al.*, 1995).

1.3.5.1 Histology of human genital epithelia and pathology of genital squamous cell carcinoma

Most genital malignancies are squamous cell carcinomas (SCC) derived from the epithelia lining genitalia including the uterine cervix, vagina, vulva and penis. The composition of genital epithelia in most genital regions, in comparison with that in the cervix, is relatively simple. The vagina and labia minora of the vulva are covered with non-keratinizing SSE, while the labia majora of the vulva and most parts of the penis are covered with keratinizing SSE (Jenson and Lancaster, 1990). All these type SSE are collectively referred to as native SSE. As introduced in Section 1.3.3.3, these SSE undergo programmed squamous differentiation, and the CK composition in their epithelial compartments is changed in association with terminal differentiation. The epithelial lining in the cervix is complex and dynamic (Figure 1.3A). The cervix may comprise two distinct regions. The ectocervix is lined with non-keratinizing SSE, which is continuous with that in the vagina. The endocervix is covered with a single-layered simple columnar epithelium (SCE). The junction where these two type epithelia adjoin is called squamo-columnar junction (SC junction). Under certain physiological and pathological conditions, a third type of epithelium, metaplastic SSE, appears

Figure 1.3 Stages of metaplasia and CINs

A: Diagrammatic presentation of the histology of the uterine cervix. The top sketch presents a cervix with two regions, where SCE is immediately adjacent to native SSE. The bottom sketch shows a cervix with three regions, where SCE transits to native SSE via metaplastic SSE.

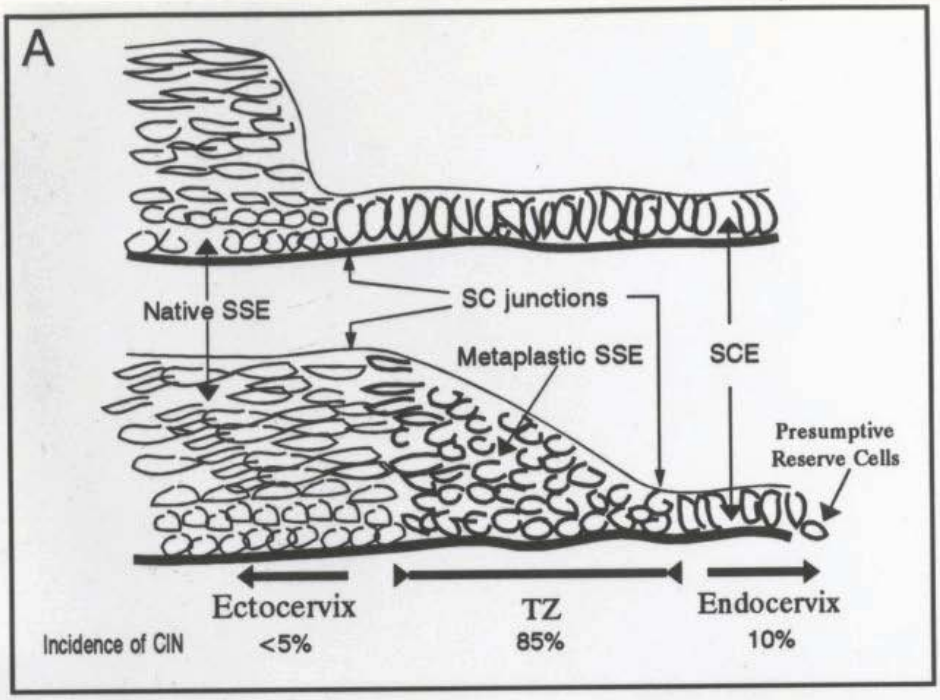
B: Morphological stages of naturally occurring metaplastic SSE, copied from Coleman and Evans (1988) with permission. SCE is indicated by the arrowhead, immature metaplasia by the solid arrow, mature metaplasia by the open arrow and SC junction by the black snowflake. The metaplastic nature of the epithelium was attested by the presence of the underlying cervical gland, as indicated by the solid triangle. Note the relationship between the epithelia: immature metaplasia and mature metaplasia are proximal to and distal from SCE or SC junction, respectively.

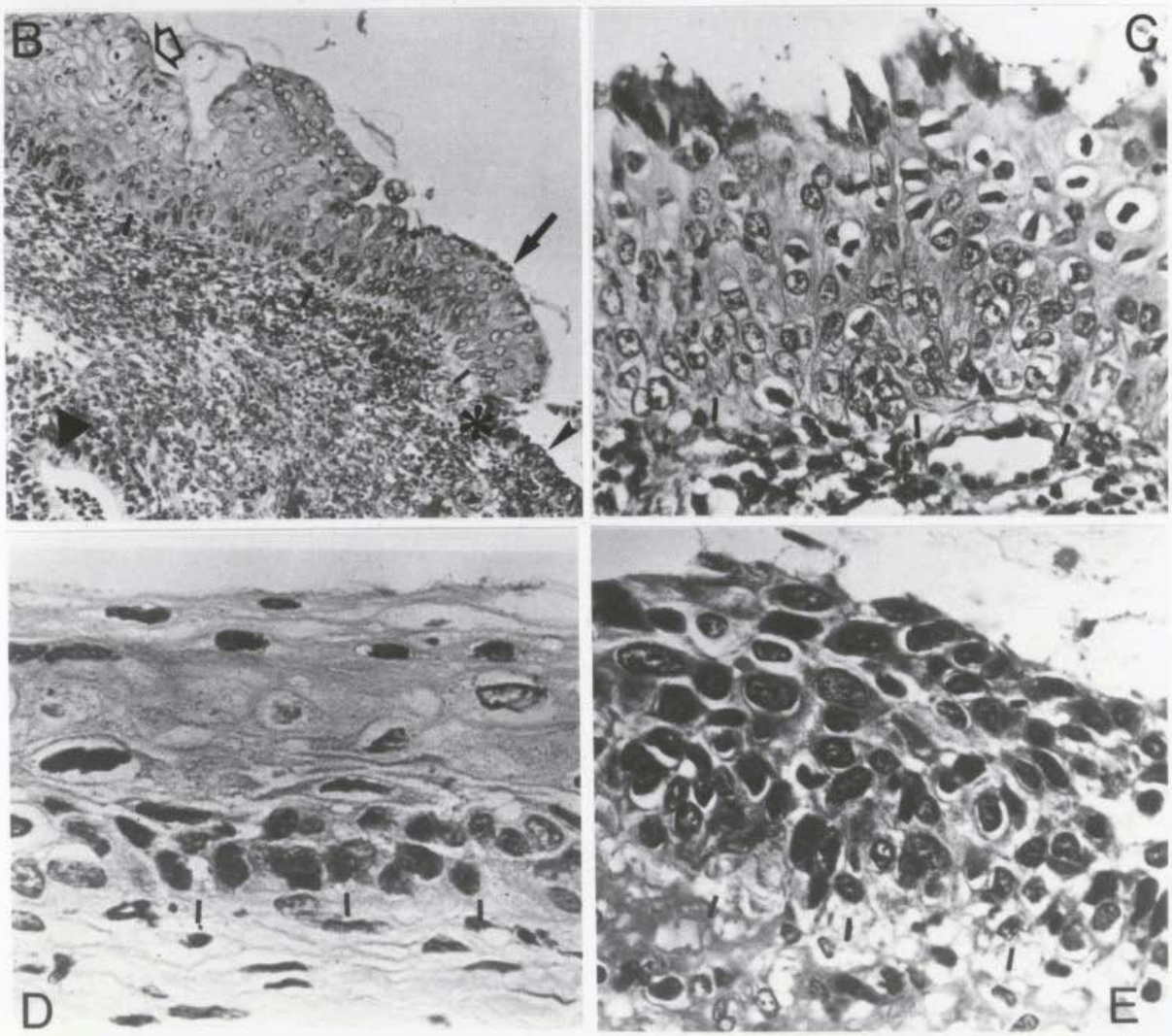
C: Morphology of naturally occurring AIM, copied from Fu and Reagan (1989) with permission. Note the remnant of SCE on the surface of the lesion.

D: Naturally occurring low degree dysplasia, copied from Coleman and Evans (1988) with permission. Atypical cells are limited to the low one third of the lesion and the lesion maintained mature squamous differentiation.

E: Naturally occurring high degree dysplasia, copied from Coleman and Evans (1988) with permission. Atypical cells are throughout the lesion and no mature squamous differentiation is present in the lesion.

Figures B-E were H&E staining. The magnifications were 250 X and 380 X, respectively for B and C, and D and E were 400 X. The basal membrane is indicated by black bars.


A
Native SSE
SC junctions
Metaplastic SSE
SCE
Presumptive
Reserve Cells
Ectocervix
TZ
Endocervix
Incidence of CIN
<5%
85%
10%


B
C
D
E

between ectocervical SSE and endocervical SCE. Clinically, the region occupied by metaplastic SSE is recognized as the transformation zone (TZ) by colposcopy, which is an important pathological landmark (Burghardt, 1986). The histology of the cervical lining is shown in Figure 1.3B. The histogenesis of the cervical metaplastic SSE will be introduced in more detail in Section 3.1.1.

The current concept on genital malignancy recognizes the well-founded assumption that female genital malignancies develop from progressive premalignant lesions that are referred to as intraepithelial neoplasia. This notion has been widely accepted for cervical carcinomas. The premalignant lesions of cervical carcinomas, cervical intraepithelial neoplasias (CINs), are morphologically graded in progressive severity as CIN I for mild dysplasia, CIN II for moderate dysplasia, or CIN III for severe dysplasia/carcinoma *in situ* (CIS) (Richart, 1973). Other benign lesions such as condyloma (warts) without dysplastic changes and atypical immature metaplasia (AIM) are regarded as separate categories (see Section 5.2.2.3) (Fu and Reagan, 1989a). In CIN I, the distribution of dysplastic (cancerous-like) cells is limited to the bottom one third of the SSE lesion, while dysplastic cells occupy the full thickness of the lesion in CIN III. The new Bethesda system classifies CIN I and condylomatous benign lesions as low grade squamous intraepithelial lesions (SILs), and CIN II and CIN III/CIS as high grade SILs (National Cancer Institute Workshop, 1989). The premalignant lesions at each stage may regress, persist, or progress into higher grade dysplasias and/or invasive carcinomas. Typical histological pictures of AIM, low and high grade dysplasias are shown in Figure 1.3C, D and E, respectively. Similarly to CINs, this conceptional framework of continuous progression of the premalignancy to malignancy has

also been applied to other genital carcinomas. Vulval intraepithelial neoplasias (VIN), vaginal intraepithelial neoplasias (VaIN), and penile intra-epithelial neoplasia (PIN) have also been graded accordingly, although these SILs in native SSE have not been studied as extensively and intensively as CINs.

The attention given to CINs is well grounded. More than 95% of female genital CIS lesions occur in the cervix, while only 3% of them are located in the valva and vagina. More significantly, 97% of the CINs involve metaplastic SSE in the TZ, while only 3% of the CINs occur in the cervical native SSE (Abdul-Karin *et al.*, 1982; Fu *et al.*, 1988) (refer to the caption in Figure 1.3A). Consistent with these observations, one to two thirds of cervical CIS lesions are expected to progress into invasive carcinomas over 10 to 15 years in the absence of medical intervention, while only 6% of the vulval and vaginal CIS lesions will progress (Jenson and Lancaster, 1990). Interestingly, and for unknown reasons, VINs at diagnosis are usually high grade premalignant lesions, although VINs are much less common than CINs and more rarely develop into invasive carcinomas than CINs (Crum, 1992). Thus, metaplastic SSE may not only be the most common tissue in which premalignant lesions occur, but may also be more prone to progression. Indeed, it has long been suspected that metaplastic SSE is particularly susceptible to certain oncogenic agents (Burghardt, 1986).

1.3.5.2 HPV as etiological agents for genital carcinomas

The etiological role of HPV infection in cervical carcinomas has been firmly established based on epidemiological studies. More than 90% of cervical cancers contain HPV DNA, while the incidence of HPV infection in the cervices is approximately 10% in the

normal cervices (zur Hausen and de Villiers, 1994). HPV infection has also been implicated in other genital cancers, such as those in the vulva and penis (Pilotti *et al.*, 1989; Higgins *et al.*, 1992c). HPV16 is the most common HPV found in cervical cancers, with a detection rate of 50% in HPV-related cervical cancers. Consistent with the concept that female genital carcinomas develop as a result of the progression of low grade premalignant neoplasia, HPV DNA is also detectible in premalignant lesions. One study showed that 36% of the HPV16 infections are low grade CINs and the majority of the rest are high grade CINs (Nuovo *et al.*, 1990). In contrast to the high-risk HPVs, the low-risk HPVs, such as HPV6 and 11, are rarely detected in high grade premalignant lesions and genital cancers. Since only 30% of vulval and penile carcinomas contain HPV infections (Pilotti *et al.*, 1989; Nuovo *et al.*, 1991a; Higgins *et al.*, 1992c), it has been suggested that other mechanisms independent of HPV infection may also be involved in the oncogenesis of these genital cancers.

1.3.5.3 Mechanisms of HPV-mediated oncogenesis

1.3.5.3.1 HPV oncogenes

The oncogenic potential of papillomaviruses is attributable to the viral oncogenes E7, E6 and possibly E5 (Stoppler *et al.*, 1994). The biochemical and virological aspects of their products have been introduced in Section 1.3.4.2. Most HPV-related cancers contain only integrated viral DNA. Although viral DNA is integrated into the cellular genome randomly with some preference for chromosome fragile sites (Popescu and DiPaolo, 1989; Smith *et al.*, 1992), the integrated viral DNA always retains intact E6 and E7 genes (Boshart *et al.*, 1984; Schwarz *et al.*, 1985; Pater and Pater, 1985; Yee *et al.*, 1985). In the HPV-related cancers

and cell lines derived from them, the viral E6 and E7 genes are always expressed (Schwarz *et al.*, 1985; von Knebel Doeberitz *et al.*, 1988) and the expression of these two genes are related to cell growth (von Knebel Doeberitz *et al.*, 1988). These data suggest that HPV E6 and E7 genes are important for HPV-mediated oncogenesis.

Since papillomaviruses cannot be propagated by conventional virological methods, the oncogenic potential of HPV has been studied mostly by the *in vitro* transfection of papillomavirus genomic or subgenomic DNA to test the immortalizing/transforming ability in cultured rodent or human cells. E6 and/or E7 of the high-risk HPVs, but not the low-risk HPVs, transforms immortal rodent cells such as NIH 3T3 cells (Matlashewski *et al.*, 1988; Pater and Pater, 1986; Kanda *et al.*, 1988; Pater *et al.*, 1988; Vousden *et al.*, 1988; Watanabe and Yoshiike, 1988; Tanaka *et al.*, 1989; Pater *et al.*, 1990). In addition, in cooperation with certain activated oncogenes such as EJ-*ras*, c-*myc*, or mutant p53 (Phelps *et al.*, 1988; Crook *et al.*, 1988; Storey *et al.*, 1988; Chesters and McCance, 1989; Crook *et al.*, 1991a), the E7 genes of the high-risk, but not the low-risk HPVs, are able to transform primary rodent cells. The HPV E6 gene has also been shown to transform rodent cells. In cooperation with or without the activated *ras*, the E6 of HPV16, but not of HPV6, has been shown to transform primary or immortal rodent cells (Sedman *et al.*, 1991; Storey and Banks, 1993).

More relevantly, the oncogenic potential of the E6 and E7 genes of high-risk HPVs can be demonstrated with human cells. In its natural genomic context, the DNA of high-risk HPVs immortalizes human keratinocytes but not fibroblasts in a low but predicable frequency (Durst *et al.*, 1987b; Pirisi *et al.*, 1987; Kaur and McDougall, 1988; Smits *et al.*, 1990). Similar to the SV40 T Ag gene, the genomic DNA of high-risk HPVs increases the life span

of the transfected cells, and clonal immortalized cells emerge after the transfected cells undergo a phase of crisis. Low-risk HPVs neither increase the cell life span of, nor immortalize, human cells. However, the transfected cells are induced to proliferate transiently, as for the high-risk HPVs (Pirisi *et al.*, 1987; Woodworth *et al.*, 1989). Thus, analogous to SV40 T Ag, the oncoproteins of the high-risk HPVs may surpass the hypothetical M1 phase of senescence control, while their counterparts of the low-risk HPVs do not. Not coincidentally, and also similarly to SV40-induced immortalization, HPV-mediated immortalization was found to be correlated with the length of the chromosome telomeres (Klingelhutz *et al.*, 1994) and to be associated with genetic changes in the cellular genome (Chen *et al.*, 1993). Both the E6 and E7 genes are required to induce efficient immortalization of human cells, while mutations in either gene result in loss or reduction of immortalizing activity (Schlegel *et al.*, 1988; Munger *et al.*, 1989a). However, under the control of a strong heterologous promoter, the E7 gene of HPV16 alone also showed immortalizing activity for keratinocytes, although the immortalization efficiency is greatly enhanced with the cooperation of the E6 gene (Halbert *et al.*, 1991). The oncogenic potential of the E6 gene was demonstrated by *in vitro* immortalization assays with human mammary cells. HPV16 E6, but not E7, is immortalizing for human mammary epithelial cells (Band *et al.*, 1991; Band *et al.*, 1993; Shay *et al.*, 1993).

The oncogenic potential of the HPV E7 proteins is correlated with their pRb-binding affinities, and this correlation is best demonstrated in the *in vitro* transformation assays using rodent cells. Transformation of rodent cells requires the N-terminal pRb-binding domain in the E7 gene, but not the C-terminal domain (Munger *et al.*, 1991). The importance of pRb binding for rodent cell transformation was corroborated by studies showing: that the E7 genes

of low-risk HPVs have lower pRb-binding activity and low transforming ability than those from the high-risk ones (Gage *et al.*, 1990); that the high-risk HPV E7 mutants defective in pRb binding show abrogated transforming activity (Munger *et al.*, 1991; Heck *et al.*, 1992); and that a single nonhomologous amino acid inside the pRb-binding domain is responsible for the differential transforming potentials between the high and low-risk HPVs (Sang and Barbosa, 1992b). However, the E7 domains involved in the immortalization of human cells are less clear. One study showed that the E7 immortalizing potential depends on the integrity of the C-terminal domain, rather than the pRb-binding domain (Jewers *et al.*, 1992). The significance of this study remains to be evaluated.

The oncogenic potential of the E6 oncoproteins is closely correlated with their p53-binding ability. Indeed, dominant negative p53 mutants can substitute for E6 to cooperate with HPV16 E7 for more efficient immortalization of human cells (Sedman *et al.*, 1992). HPV18 E6 mutants with reduced p53-binding ability displayed a compromised transforming ability in rodent cells (Pim *et al.*, 1994). In the cell lines containing high-risk HPVs, p53 level is usually 5-fold lower than in the normal cells (Lechner *et al.*, 1992). Under conditions inducing cellular DNA damage, such as UV irradiation, E6 expressing-cells fail to be arrested at G_0 (Kessis *et al.*, 1993; Slebos *et al.*, 1994). In addition, expression of the E6 genes of high-risk HPVs, but not low-risk ones, disrupts the expression of genes dependent on p53 transactivation (Lechner *et al.*, 1992; Mietz *et al.*, 1992; Gu *et al.*, 1994). Furthermore, HPV-positive cervical cancers contain wild type p53 genes, while many HPV-negative cervical cancers have mutant p53 (Scheffner *et al.*, 1991; Helland *et al.*, 1993).

Although BPV E5 is the major oncogenic gene, HPV E5 can only weakly induce

anchorage-independent growth of immortal rodent cells (Leechanachai *et al.*, 1992; Pim *et al.*, 1992; Cohen *et al.*, 1993; Straight *et al.*, 1993) and has not been demonstrated to be immortalizing or transforming for human cells. Consistent with the biochemical features of the E5 protein, which interferes with the down-regulation of growth factor receptors, the transforming activity of the HPV16 E5 gene in rodent cells is dependent on the EGF receptor and EGF (Leechanachai *et al.*, 1992). The E5 of HPV16 has been shown to be potentially capable of enhancing the expression of viral oncogenes, and cooperating with the E7 gene to promote cell proliferation via the MAP kinase pathway (Bouvard *et al.*, 1994a; Gu and Matlashewski, 1995). Thus, HPV16 E5 may contribute to oncogenesis. However, E5 may be involved in oncogenesis only at an early stage, since most HPV-mediated cancers contain integrated viral DNA and the E5 ORF is often deleted in cancer cells.

1.3.5.3.2 Roles of HPV oncogenes in oncogenesis

Based on the biochemical and biological property of the HPV E6 and E7 proteins, a picture, albeit fragmentary, has emerged for the mechanism responsible for HPV-mediated oncogenesis. The most provocative, and perhaps oversimplified interpretation for the oncogenic potential of the HPV16 E7 and E6 proteins is their interactions with the pRb/p107 and p53 cell cycle regulators. Analogous to SV40 T Ag, the E7 protein may promote the entry and progression of cell cycles by disrupting at least two checkpoints in the cell cycle, and the E6 protein may compromise cell cycle inhibitory controls mediated by p53. Therefore, the viral oncoproteins are capable of driving the infected cells to undergo cellular DNA replication. Since the viral oncoprotein-driven cell cycling is not in coordination with the

physiological state of the cells and is dysregulated in itself, it would incur a high mutation rate. Because the multifunctional p53 is also involved in DNA repair and apoptosis, the E6-mediated p53 degradation may result in the accumulation of cellular mutations. Indeed, the expression of HPV16 E7 and E6 has been shown to enhance cellular DNA synthesis (Banks *et al.*, 1990; Rawls *et al.*, 1990; Blanton *et al.*, 1992), and to induce chromosomal abnormalities in *in vivo* assays (Hashida and Yasumoto, 1991; White *et al.*, 1994). This is also consistent with the observation that the immortalized human cells are all aneuploid in karyotypes (Durst *et al.*, 1987b; Kaur and McDougall, 1988).

The importance of HPV viral oncogenes may not only reside in introducing cellular mutations, but also in other functions of the viral oncogenes. Similar to the SV40-immortalized human cells, the HPV-immortalized and -transformed cells require continuous expression of the viral E6 and E7 oncogenes. Conditional expression of the viral oncogenes or treatment with antisense RNA against HPV E6 and E7 genes inhibits the growth of the immortalized or HPV-containing cancer cells, and induces senescence in *in vitro* as well as *in vivo* condition (von Knebel Doeberitz *et al.*, 1988; von Knebel Doeberitz *et al.*, 1992; von Knebel Doeberitz *et al.*, 1994). This phenomenon fits well into the two phase theory for senescence control, which predicts that viral oncoproteins are constantly required for suppressing the M_1 phase of senescence control, unless mutations subsequently introduced into the M_1 pathway waiver this requirement. In addition, the continuous expression of the mitogenic viral oncoproteins may be required to promote cell cycling.

Therefore, the molecular mechanism of HPV-mediated oncogenesis shares great similarity to that of other DNA tumour viruses, in that viral oncoproteins contribute directly

and indirectly to oncogenesis. HPV oncoproteins directly contribute to oncogenesis by promoting cell replication and perhaps partially abrogating the cellular senescence control, while indirectly they render a higher rate of mutations. The mutation accumulation in the cellular genome may be restrictive for the process of oncogenesis. This is consistent with the fact that HPV oncogenes induce immortalization at a statistically low frequency. Because the immortalized cells may show a few, or no transformed phenotypes (Durst *et al.*, 1991; Woodworth *et al.*, 1990b), immortalization induced by HPV oncogenes must be critical for oncogenesis. Indeed, the acquisition of immortality would allow more mutations to accumulate. In fact, if cells with any number of mutations or growth advantages remain subject to an intact senescence control, the eventual activation of the senescence program would halt the proliferation of the transformed cells and thus prevent the cells from expanding to a biologically significant population.

1.3.5.3.3 Multistep nature of HPV-mediated oncogenesis

Human cells immortalized by HPV can subsequently obtain transformed phenotypes including tumorigenicity. Full transformation of HPV-immortalized cells has been achieved by artificial introduction of activated protooncogenes such as *ras* (Durst *et al.*, 1989; DiPaolo *et al.*, 1989), treatment with carcinogenic chemicals such as nitrosomethylurea (NMU) (Garrett *et al.*, 1993; Shin *et al.*, 1994), and subsequent transfection of the mutagenic genomic fragment of herpes simplex virus type 2 (HSV2) (DiPaolo *et al.*, 1990). In addition, two cell lines, both immortalized by HPV18, acquired fully transformed phenotypes after prolonged cultivation in *in vitro* culture (Hurlin *et al.*, 1991; Pecoraro *et al.*, 1991). Thus, the

viral functions resulting in immortalization may also induce full transformation in a time-dependent manner. The molecular events responsible for the subsequent transformation have not been determined. However, the subsequent transformation of the HPV-immortalized cells is presumed to be due to newly introduced cellular genomic mutations, since viral oncogene expression does not correlate with malignant transformation. Indeed, cytogenetic analysis showed that, in the two cases of NMU-induced tumorigenic transformation of the HPV18-immortalized cells (Garrett *et al.*, 1993), acquisition of tumorigenicity was associated with allele deletion of the DCC gene, which encodes a cell adhesion molecule and is a tumour suppressor gene (Klingelhutz *et al.*, 1993). Thus, HPV-mediated oncogenesis is also a multistep process. This is consistent with the clinical observation that most HPV infections do not lead to malignancy, and the incubation time from initial HPV infection to malignancy is usually decades (Ponten *et al.*, 1995).

1.3.5.3.4 Progression of HPV-mediated oncogenesis and HPV oncogene expression

Even for high-risk HPVs, only a small proportion of the infected lesions develop into malignancy (Schiffman, 1994). Therefore, the most important and intriguing question concerning HPV-mediated oncogenesis is, "What causes an HPV-infected benign lesion to progress into advanced malignancy?" Because E6 and E7 are the major viral oncogenes required for initiating the oncogenic process and maintaining the oncogenic phenotype, their expression was speculated to be critical for the progression of the benign infections. This expectation seems to be consistent with the differential expression patterns of the viral oncogenes observed in the low and high grade HPV lesions. In the former, the viral

oncogenes were found to be expressed mainly in the differentiating SSE cells and weakly or not at all in the proliferating basal cells, while they are expressed extensively and actively in all the epithelial compartments of the latter (Durst *et al.*, 1991; Durst *et al.*, 1992). Another prominent feature of HPV-mediated cancers is that the status of viral DNA in the lesion is correlated with the lesion's malignant severity. Most, if not all, of the premalignant lesions seemed to contain episomal viral genome and support vegetative infections, while viral DNA is often integrated into the cellular genome in cancers, which are usually non-vegetatively infected (Boshart *et al.*, 1984; Pater and Pater, 1985; Schwarz *et al.*, 1985; Yee *et al.*, 1985). Therefore, the progression of HPV-induced neoplastic lesions may be closely related to dysregulated expression of the viral oncogenes and the changed status of viral DNA.

1.3.5.3.4.1 Dysregulated viral functions and viral oncogene expression

Dysregulated expression of the E6-E7 oncogenes may result from disrupted HPV16 E2 functions, since E2 may act as a repressor for the E6 promoter that controls the expression of E6-E7 oncogenes (Bernard *et al.*, 1989; Gloss and Bernard, 1990; Romanczuk *et al.*, 1990; Tan *et al.*, 1992; Tan *et al.*, 1994). When mutations were introduced into the E2 ORF of HPV16, the immortalization efficiency was markedly increased in *in vitro* immortalization assays (Romanczuk and Howley, 1992). The interpretation was the E2 mutations result in derepressed expression of the E6-E7 oncogenes. Therefore, enhanced viral oncogene expression resultant from a disrupted E2 ORF may contribute to the transition from the low to high grade premalignant lesions. Consistent with this notion, E2 ORFs are often disrupted in malignant lesions containing integrated viral genomes (Schwarz *et al.*, 1985), and it is not

expressed in many cancers (Sang and Barbosa, 1992a; Krajinovic *et al.*, 1993). It is conceivable that a derepressed expression of the E6-E7 oncogenes could occur only if the cells are devoid of episomal viral DNA, since the E2 protein expressed from the episomal genome may suppress viral oncogene expression from the integrated viral genome. However, since the cells expressing a high level of viral oncogenes may have a growth advantage, the cells containing integrated viral DNA and not expressing E2 may be selected for among the infected cells. This is consistent with the fact that the majority of HPV-related cancers contain only integrated HPV DNA. However, there are observations that are not consistent with a negative regulation of viral oncogenes by E2. Indeed, an HPV16 construct with a mutation in the E2 ORF was shown to have a reduced immortalizing efficiency (Storey *et al.*, 1992), and overexpression of E2 was also reported to increase HPV16 transformation efficiency in rodent cells (Lees *et al.*, 1990). It has also been reported that approximately 30 percent of HPV16-mediated cancer cases may contain only episomal viral DNA (Matsukura *et al.*, 1989; Cullen *et al.*, 1991). Therefore, although it is a very attractive model, the involvement of E2 in the progression of HPV-mediated oncogenesis is more complex than it is thought.

Dysregulated expression of the E6-E7 oncogenes may result from mutations in the viral LCR. The LCR of HPVs contains numerous binding sites for cellular, as well as viral, transcription factors that modulate viral oncogene expression (see Section 1.3.4.2.8). Mutations in these binding sites can potentially result in dysregulated expression of viral oncogenes. HPV16 (May *et al.*, 1994a) and HPV18 (Bauknecht *et al.*, 1992) LCRs have binding sites for YY1, a transcription factor that has been shown to be either trans-activating or -repressing, dependent on the composite context of the binding sites (Shi *et al.*, 1991). The

number of DNA helical turns between the YY1 binding sites and the promoter element also contributes to the "yin-yang" feature of YY1, indicating the importance of chromosomal structure. In several cases of cancers containing only episomal HPV16, the YY1 binding sites in the LCR were found to be either deleted or mutated (Dong *et al.*, 1994). *In vitro* experiments showed that abrogation of the YY1 binding sites in the LCR results in enhanced expression of the viral E6-E7 oncogenes (Bauknecht *et al.*, 1992; May *et al.*, 1994a; Bauknecht *et al.*, 1995).

1.3.5.3.4.2 Dysregulated cellular functions and viral oncogene expression

Dysregulated expression of the HPV oncogenes may be secondary to dysregulated cellular functions, rather than directly resultant from defunct viral function. For example, compromised cellular functions that repress HPV expression may lead to an increased expression of the HPV oncogenes and progression of the lesions.

The prediction that there exist cellular functions to suppress the expression of HPV oncogenes was derived from cell fusion experiments. The cell hybrids from HPV-harbouring cancer cells and normal cells showed suppressed tumorigenicity in athymic mice (Standbridge *et al.*, 1982). The repressing karyotype was attributable to chromosome 11, since the same non-tumorigenic phenotype can be achieved by introducing a single normal chromosome 11 into the HPV-harbouring cancer cells (Srivatsan *et al.*, 1986). Studies with karyotype analysis and restriction enzyme fragment length polymorphism on these hybrid cells suggest that chromosome 11 may be involved in HPV18 oncogene repression (Srivatsan *et al.*, 1986). The suppressed tumorigenicity of the hybrid cells was associated with the extinction of the

expression of viral oncogenes at the transcription level (Bosch *et al.*, 1990; Rosl *et al.*, 1991). *In vitro* treatment of the cells with 5-azacytidine (5-AZ), a DNA demethylation agent, resulted in suppression of viral oncogenes in non-tumorigenic, but not in tumorigenic, cells (Rosl *et al.*, 1988). These results were interpreted to indicate that, in non-tumorigenic cells, but not in the tumorigenic cells, the expression of the HPV oncogene is repressed by a dominant cellular function located on chromosome 11. This cellular function was presumed to be carried out by a putative cellular interfering factor(s) (CIF), and the integrity of this CIF function was believed to be important for the progression of HPV-mediated oncogenesis (zur Hausen, 1990). The inactive CIF function in the non-tumorigenic cells *in vitro* was speculated to be due to DNA hypermethylation, and this DNA methylation-mediated suppression of the CIF was believed to be removed *in vivo* by default. However, no cellular genes have been so far found to qualify for CIF, and the relationship between the speculative CIF function and HPV vegetative infection is unknown. This topic will be further discussed in Section 4.1.2.

Experiments using human fibroblast cells with an abnormal karyotype supported that chromosome 11 may indeed encode a function suppressing HPV oncogene expression (Smits *et al.*, 1988; Smits *et al.*, 1990; Smits *et al.*, 1992a; Smits *et al.*, 1992b; Smits *et al.*, 1993). *In vitro* transfection of HPV16 DNA usually fails to immortalize normal human fibroblasts. However, human fibroblasts with a large deletion in the short arm of chromosome 11 are susceptible to transformation by HPV16 DNA transfection. Consistently, HPV expression is minimal in the normal human fibroblasts, while viral expression is enhanced in fibroblasts with such a deletion (Smits *et al.*, 1988). Significantly, the abnormal fibroblasts also showed an increased expression of the 55 kDa inhibitory subunit of the protein phosphatase 2A (PP2A)

(Smits *et al.*, 1992b). This 55 kDa protein can substitute the SV40 small t antigen for enhancing the transforming ability of SV40 T Ag (Smits *et al.*, 1992b). Because the SV40 small t antigen is able to activate the expression of HPV16 oncogenes via a specific domain in the HPV16 LCR (Smits *et al.*, 1992b; Smits *et al.*, 1993) and PP2A suppresses HPV16 gene expression through TBP and the P97 early promoter (Smits *et al.*, 1990), the 55 kDa PP2A inhibitor may be regarded as a factor derepressing the HPV16 oncogenes. Therefore, the deleted region in chromosome 11 was hypothesized to encode a factor that represses the expression of the 55 kDa PP2A subunit. Abrogation of this factor may result in overexpression of the 55 kDa PP2A, down-regulation of the PP2A function, and derepression of HPV oncogenes.

Cellular transcription factors may negatively regulate HPV expression. It is logical that a compromised function of these inhibitory factors may up-regulate viral oncogene expression and promote malignant progression. The Oct-1 and the nuclear factor for interleukin 6 expression (NF-IL6) transcription factors, both of which bind in the HPV16 and HPV18 LCRs, were shown to repress HPV oncogene expression (Hoppe-Seyler *et al.*, 1991; Butz and Hoppe-Seyler, 1993; Kyo *et al.*, 1993). YY1 is another negative transcription factor, which has several binding sites in HPV16 as well as HPV18 LCRs (May *et al.*, 1994a; Dong *et al.*, 1994). Interestingly, in the presence of an uncharacterized cellular factor that binds to a switch sequence near the YY1 site in the HPV16 LCR, YY1 becomes an activator for HPV oncogene expression (Bauknecht *et al.*, 1995). Conceivably, down-regulated expression of Oct-1 or NF-IL6, or up-regulated expression of the YY1 switch factor, may result in increased expression of viral oncogenes. The clinical relevance of these observations remains

to be tested *in vivo*.

Several cytokines have been shown also to repress HPV oncogene expression. TGF-β (Woodworth *et al.*, 1990a; Braun *et al.*, 1992), tumour necrosis factor α (TNF-α) (Villa *et al.*, 1992; Malejczyk *et al.*, 1994; Kyo *et al.*, 1994; Rosl *et al.*, 1994), and EGF (Yasumoto *et al.*, 1991) are able to repress HPV expression, although the signal transduction pathway and transcription factors involved are not known. TGF-β is involved in the differentiation of keratinocytes and epithelial homeostasis control, and TNF-α participates in inflammation and immunity. Interestingly, TGF-β, EGF and TNF-α have been reported to repress viral expression in the non-tumorigenic HPV-immortalized cells, while their tumorigenic derivatives are resistant (Braun *et al.*, 1992; Kyo *et al.*, 1994). Retinoic acid (RA), which regulates epithelial cell growth and differentiation, represses expression of HPV16 and HPV18 oncogenes in immortalized cells and cancer cell lines (Agarwal *et al.*, 1991; Bartsch *et al.*, 1992; Pirisi *et al.*, 1992; Khan *et al.*, 1993a; Merrick *et al.*, 1993; Agarwal *et al.*, 1994; Creek *et al.*, 1994). The effect of RA on HPV expression seems to be indirect, since no RA binding sites for RA receptors are found in the HPV LCR. In contrast, repression of HPV16 oncogene expression by the cytokine interleukin-6 appears to be specific for the HPV promoter via a NF-IL6 binding site in the LCR (Kyo *et al.*, 1993). Repression of HPV expression was also observed for interferon α and γ, leukoregulin, interleukin-1 (Woodworth *et al.*, 1992; Kyo *et al.*, 1994). Therefore, defunct signal transduction pathways for these inhibitory cytokines may result in an increased expression of viral oncogenes, leading to lesion progression.

The potential dysregulation of cellular functions discussed above represent

pathological conditions resultant from mutations introduced by HPV activities. Alternatively, changes in the cellular environment that lead to dysregulated expression of viral oncogenes may also be physiological. One possible scenario is the physiological fluctuation of hormonal factors. Glucocorticoid and progesterone hormones are able to enhance the efficiency of HPV16-mediated transformation of rodent cells (Pater *et al.*, 1988; Crook *et al.*, 1988), and to up-regulate the expression of HPV16 oncogenes in cervical keratinocytes in a manner dependent on the GREs in the HPV LCR (Mittal *et al.*, 1993a). Thus, certain physiological conditions such as the menstrual cycle and pregnancy, or artificial conditions such as usage of birth control pills, may affect the expression of HPV oncogenes and result in increased risk for lesion progression.

1.3.5.3.4.3 Progression of HPV-induced lesions and viral DNA integration

The common occurrence of viral DNA integration in the HPV-mediated cancers, in contrast to the predominantly episomal viral DNA in low grade premalignant lesions, suggests that viral DNA integration is an important event for HPV-mediated oncogenesis. The mechanism for viral DNA integration is unknown. Because viral DNA integration is not required and is, in contrast, detrimental for viral reproduction, it must occur as a mishap to the viral life cycle, and possibly as an inevitable outcome from an incompatible or dysregulated relationship between the cellular environment and viral activities. This is consistent with the fact that viral DNA integration is a common feature, with few exceptions, for the oncogenesis mediated by other small DNA tumour viruses. However, viral DNA integration is not necessarily obligatory for oncogenesis, as indicated by the existence of HPV16-

mediated cancers containing only episomal viral DNA (Matsukura *et al.*, 1989; Cullen *et al.*, 1991). Consistently, the *in vitro* immortalized human cell lines, all of which contain integrated viral DNA, most often display benign phenotypes in reconstructed lesions (Durst *et al.*, 1991). In addition, the expression pattern of HPV16 oncogenes in the reconstructed lesions does not resemble the one observed in the naturally occurring HPV-mediated cervical cancer (Durst *et al.*, 1991). The expression of viral oncogenes is limited to the proliferating, undifferentiated basal cells in the artificial lesions, whereas it tends to be expressed throughout the entire lesion in the naturally occurring HPV-induced high grade CIN (Durst *et al.*, 1992; Stoler *et al.*, 1992). The restricted expression of viral oncogenes has been interpreted as being due to the intact function of the hypothetical CIF in the immortalized cells. Therefore, the events for viral DNA integration and enhanced expression of viral oncogenes are separable, and the derepressed expression of viral oncogenes may occur secondarily to other genetic events rather than viral DNA integration.

However, viral DNA integration does represent a fundamental transition for the nature of HPV infection, since integrated viral DNA stably perpetuates an abnormal non-vegetative relationship with the host and this relationship may favour malignant progression of HPV-induced lesions. As discussed in the last two sections, viral DNA integration may lead to dysregulated expression of the viral oncogene as a result of dysregulated functions of the viral and cellular *cis*- or *trans*-acting elements. Integration of the viral genome may also subject the expression of viral oncogenes to other mechanisms for gene regulation that are inherent in the cellular genome. *Cis*-juxtaposed cellular enhancers in the vicinity of the integrated viral oncogenes may interfere with the functions of viral control elements. Indeed, viral gene

expression in cell lines containing integrated HPV DNA with functional GRE sites responds differently to steroid treatment, indirectly indicating that the expression of viral genes is differentially regulated by flanking cellular enhancers or silencers (von Knebel Doeberitz *et al.*, 1991). The mRNA encoding viral oncogenes in CaSki cells, which also contain integrated HPV16 DNA, was shown to contain a cellular polyadenylation signal (Smits *et al.*, 1991). Furthermore, since the integrated viral genome becomes one physical part of the cellular genome, expression of the viral genes from the integrated genome is inevitably affected by local chromatin structure or/and epigenetic modification of the flanking cellular DNA. Indeed, the majority of the integrated viral genes in CaSki cells is suppressed, and such a suppression appears to involve chromatin structure (Rosl *et al.*, 1993).

A *cis*-juxtaposed viral enhancer may also interfere with the function of cellular control elements, resulting in dysregulated expression of cellular genes. Methylation of cellular DNA flanking integrated viral DNA has been shown to be correlated with viral DNA integration (Gallego *et al.*, 1994). At least in two well-documented cases, HPV DNA was found to be integrated in the vicinity of the protooncogene c-*myc*, which are accompanied with an overexpression of this protooncogene (Durst *et al.*, 1987a). However, although cervical cancers may indeed be associated with increased expression of c-*myc* in some cases (Riou *et al.*, 1987; Riou, 1988), the prevalence, frequency, and specificity of this phenomenon remain in question (Ikenberg *et al.*, 1987; Tate *et al.*, 1994).

1.3.5.3.5 Roles of other co-factors in HPV-mediated oncogenesis

Co-factors have been suspected to be involved in HPV-mediated oncogenesis to

explain the long incubation time for HPV-mediated oncogenesis. Consistent with this view, genomic DNA of human herpesvirus-6 (HHV-6) has been detected in a small percentage of HPV16-associated CINs and cervical carcinomas (Chen *et al.*, 1994b). Interestingly, it has also been shown that HHV-6 infects human cervical epithelial cells *in vitro*, and HHV-6 infection enhances the expression of HPV oncogenes (Chen *et al.*, 1994a). Other factors that are mutagenic themselves may also play roles in HPV-mediated oncogenesis. Smoking has been suspected to be involved in anogenital oncogenesis (Holly *et al.*, 1989; Winkelstein, 1990). Consistently, studies showed that NMU, one of the major tobacco-specific nitrosamines, converts HPV-immortalized human cells to malignancy (Garrett *et al.*, 1993).

Another important factor influencing oncogenesis is the immune function of the host. Infection of the human immuno-deficiency virus (HIV) may compromise cell-mediated immunity, which is an important function protecting against HPV16 infections (Chen *et al.*, 1992a; Chen *et al.*, 1992b). Indeed, cervical carcinomas now are recognized as one of the early indications for HIV infections. Although it is not clear whether HIV infects cervical epithelial cells *in vivo*, the regulatory *tat*1 protein of HIV-1 has been reported to transactivate the early promoter of HPV16 (Vernon *et al.*, 1993). In addition, individuals with certain HLA types were reported to be susceptible to cervical carcinomas (Wank and Thomssen, 1991). Analogous results were also obtained from studies using CRPV-induced rabbit malignancy as a model system (Han *et al.*, 1995). This phenomenon can be well explained by the finding that HPV16 E7 and E6 oncoproteins are tumour rejection antigens inducing immuno-reactions mediated by cytotoxic T-cells and activated macrophages, and presentation of these viral antigens are MHC-restricted (Banks *et al.*, 1991; Chen *et al.*, 1991; Chen *et al.*, 1992a;

Chen *et al.*, 1992b).

1.3.5.4 Current opinion of the natural history of HPV infection and HPV16-mediated malignancy

Because DNA virus-mediated oncogenesis is a result of dysregulated interaction between viral activities and cellular environment, it is important to understand the relationship between the natural course of HPV infection and the natural history of HPV-mediated oncogenesis. Since the life cycle of HPV16 cannot be duplicated in experimental system, the scenario constructed from available data is largely fragmentary and mainly speculative (Figure 1.4).

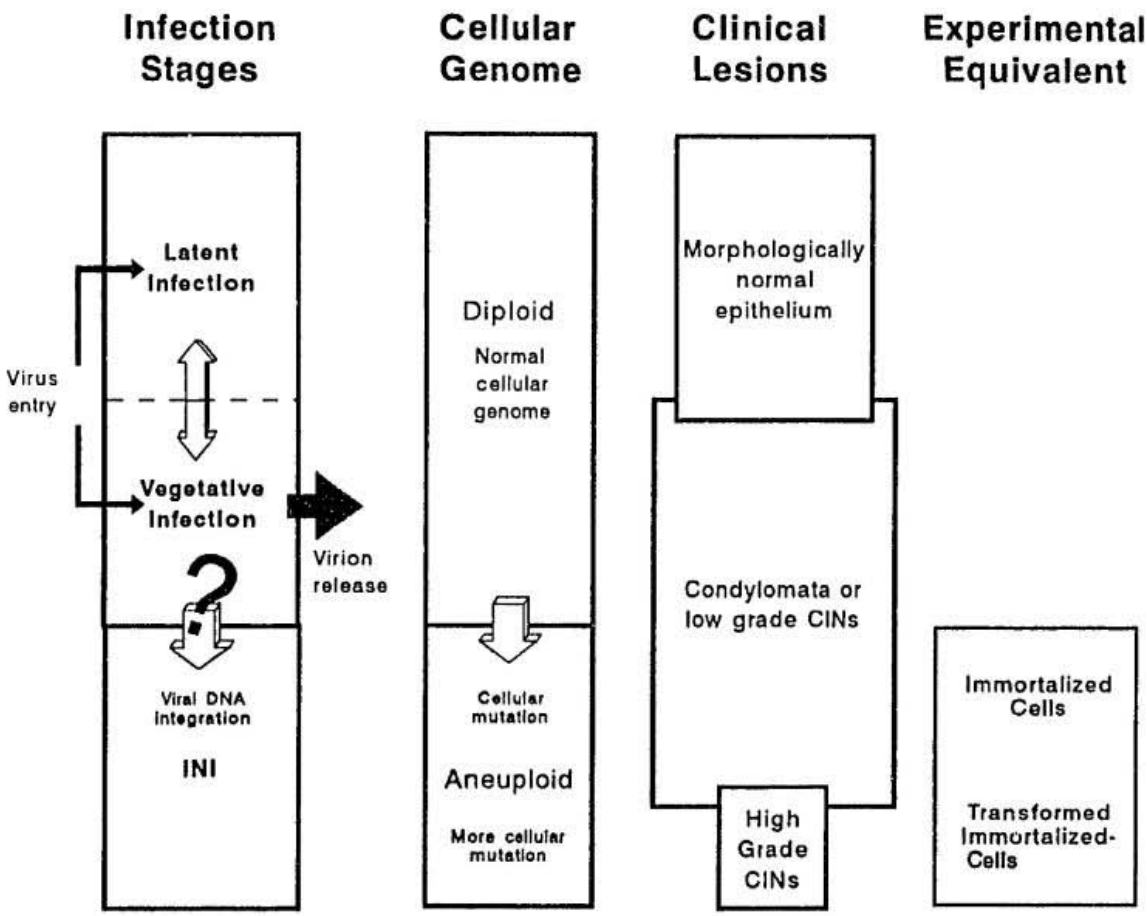
Almost nothing is known about the process of the papillomavirus entry into host cells. The establishment of HPV16 infection in SSE is possibly initiated by virus entry at the basal cells that are exposed by micro-traumas via a specific cellular receptor. The receptor(s) for HPV appears to be ubiquitously present on the surface of different cell and species types (Muller *et al.*, 1995). Based on the high incidence of HPV-mediated carcinomas in the cervix, the cells at the cervical SC junctions, which are basal types and are exposed directly to the external environment, are proposed to provide a receptive entry site for HPV infection (see Figures 1.3A and B) (Burghardt, 1986; zur Hausen and de Villiers, 1994).

To explain the positive HPV detection in approximately 10% healthy population, it is hypothesized that HPV infection does not necessarily manifest symptoms clinically and pathologically. This state of latent infection was first proposed by Steinberg *et al.* (1983). Viral activity was postulated to be minimal in latent infection, since viral DNA and RNA

Figure 1.4 Schematic presentation of the current understanding on HPV infection and HPV-mediated oncogenesis

Virus entry may be followed by either a latent or vegetative infection. These two infection states are believed to be interconvertible. However, the virus reproduction can only be achieved in a vegetative infection and not in the latent infection. In addition, a lesion with integrated viral DNA, which represents a state of irreversible non-vegetative infection, or INI, is thought to derive from certain vegetative infections. The question mark placed between the vegetative infection and INI indicates the doubt about the prevalence of such a transition.

The relationship among the infection stages of HPV16, the status of cellular genome, clinical manifestation and established experimental models is also illustrated on a hypothetical basis. Cells in lesions with a latent or vegetative infection are most likely diploid. Viral DNA integration definitely causes mutation in the cellular genome, and is possibly associated with an aneuploid karyotype. While the latent infections may not manifest themselves pathologically and clinically, vegetative infection causes condylomata and low grade CINs. The immortalized human cells and their tumorigenic derivatives represent INIs for the virological point of view, although oncologically they represent two identities.


Infection Stages
Cellular Genome
Clinical Lesions
Experimental Equivalent
Latent Infection
Virus entry
Vegetative Infection
Virion release
Viral DNA Integration
INI
Diploid
Normal cellular genome
Cellular mutation
Aneuploid
More cellular mutation
Morphologically normal epithelium
Condylomata or low grade CINs
High Grade CINs
Immortalized Cells
Transformed Immortalized-Cells

transcripts are not readily detectible by the current *in situ* assays (Ferenczy *et al.*, 1985; Nuovo *et al.*, 1988; Jenson and Lancaster, 1990; Hildesheim *et al.*, 1994). A similar state of papillomavirus infection exists in a CRPV model system (Amella *et al.*, 1994). In this model, artificially induced latent CRPV infection can convert to vegetative infection after chronic physical or chemical stimulation. In latent infection, viral DNA may be replicated synchronously with cellular DNA in the basal cells at a low episomal copy number (Ferenczy *et al.*, 1985; Jenson and Lancaster, 1990).

In contrast to latent infection, vegetative infection of HPV should require programmed viral activities that are dependent on the program of squamous differentiation of the host cells. At this stage, the infected tissues manifest themselves as benign or low grade dysplastic lesions that are characterized by a diploid karyotype and episomal viral DNA (Fu and Reagan, 1989a; zur Hausen, 1991b). Since latent and vegetative infections do not both contain integrated viral DNA, these two stages should be inter-convertible.

For unknown reasons, HPV DNA becomes integrated into cellular genomes through unknown mechanisms. Because the integrated viral DNA can no longer be used as the template for progeny virus reproduction, HPV infection in cells with integrated viral genomes can be regarded as an irreversible non-vegetative infection (INI). Phenotypes of the INI lesions should be dependent on the combinations of multiple conditions, including the expression of viral oncogenes and the disruptions in the cellular functions. Thus, INI may manifest as low grade premalignant lesions, if viral oncogenes remain to be repressed and the cellular mutations resulting from viral DNA integration do not impair important cellular functions. Since the expression of viral oncogenes is advantageous for cell growth, the cells

that persistently express viral oncogenes are constantly selected for and cellular mutations are continuously introduced. These cells may develop into high grade dysplasia and malignancy, when they accumulate sufficient cellular mutations and are not eliminated by the immune system in time. Obviously, INI cannot regress to either latent or vegetative infections. Theoretically, INI cannot derive from latent infections, since viral activities in latent infection is assumed to be minimal (Ferenczy *et al.*, 1985; Nuovo *et al.*, 1988; Jenson and Lancaster, 1990). Therefore, based on the above argument, INI is presumed to develop from vegetative infections, a notion that has never been closely scrutinized.

1.4 Hypothesis and objectives

1.4.1 Working hypothesis

As reviewed in Section 1.3.5.1, cervical metaplastic SSE in the TZ is the site that is most often involved in female genital carcinomas. The mechanism behind this well-established pathological phenomenon is not clear. Based on the involvement of HPV infection in oncogenesis, it has been suggested that the metaplastic SSE may be more susceptible to HPV infection, since the exposed basal cells at SC junctions may provide an easy access for HPV infection (zur Hausen and de Villiers, 1994). This might be an oversimplified explanation and other mechanism may also be implicated. Indeed, HPV infection also occurs in native SSE covering the vulva and penis. While premalignant lesions in native SSE are highly associated with HPV infection, the malignant lesions are not (Andersen *et al.*, 1991, Higgins *et al.*, 1992c). In contrast, both the premalignant and malignant lesions in the cervical metaplastic SSE have been established firmly to be associated with HPV infection (Fu and Reagan,

1989b). These data suggest that the metaplastic SSE in the TZ may be more susceptible to HPV16-mediated oncogenesis (Burghardt, 1986; Jenson and Lancaster, 1990).

The high incidence of HPV16-mediated malignancy in the TZ is possibly due to a special relationship between the cervical metaplastic SSE and HPV16 infection. This prediction was based on the knowledge gained from studies on the oncogenesis mediated by other DNA tumour viruses. As reviewed in Section 1.2, SV40- and EBV-mediated oncogenesis is a result of an incompatible cellular environment for the virus vegetative life cycle. For SV40, the oncogenic non-vegetative state is due to infection in the non-natural host cells. For EBV, the oncogenic non-vegetative infection is associated with the transient, physiological phenotypic differentiation of the host cell. Experiments on CRPV also suggested that non-vegetative infection is important for the oncogenesis mediated by papillomaviruses (Syverton, 1952; Ito and Evans, 1961; Wettstein, 1987; Lin *et al.*, 1993). CRPV infects the natural host cottontail rabbits vegetatively, but it infects the non-natural host domestic rabbits semi-vegetatively. While the CRPV-induced benign papillomas in cottontail rabbits progress into carcinomas at a rate of approximately 25% during an 8-14 month interval, those in domestic rabbits become carcinomas at a rate of nearly 80%. Therefore, at least theoretically, HPV vegetative infections should rarely develop into INI and cause malignant transformation, since transformation may be associated with disruptions in the program for squamous differentiation that is depended upon by HPV reproduction. Indeed, from the evolutionary point of view, papillomavirus is a symbiotic parasite that successfully coevolves with mammals. To effectively minimize the damage to the host and economically utilize biological resources, papillomaviruses replicate in differentiating cells of the SSE, which are -

programmed to be replaced periodically. Thus, there should be a strong selective pressure against the transforming potential of HPVs, since transforming the host cells represents a failure for virus reproduction. Therefore, all the arguments support a hypothesis that HPV16 infection in cervical metaplastic SSE may possess some features of non-vegetative infection and thus predispose the infected tissue to oncogenesis.

1.4.2 Objectives of the current project

The objective of this project was to test the hypothesis that metaplastic SSE possesses special properties for HPV16 infection, which facilitates the progression of a benign HPV16 lesion into malignancy. Understanding the mechanism for the high incidence of HPV16-mediated malignancy in the metaplastic SSE could be beneficial for developing novel medical approaches to block HPV-mediated cervical cancers at the early steps of the oncogenesis.

Three approaches were taken with the objective of addressing this issue. In the first and second part, *in vitro* cultured human genital epithelial cells were studied as models, using *in vivo* and *in vitro* epithelia reconstruction systems. In the third part, HPV16 infections in the naturally occurring preneoplastic lesions were studied, to test the clinical relevance of the findings made from the experimental studies.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Unless specified, general chemicals and immunochemicals were purchased from Sigma, biochemical reagents from Bethesda Research Laboratories (BRL), and materials for tissue cultures from BRL.

The [α^{32}P]dCTP and [α^{32}P]UTP were purchased from Amersham, and [α^{35}S]UTP was from DuPont. Sequenase Version 2.0 kit was from United States Biochemical.

The nick translation kit for labelling short DNA sequences was from BRL. The random priming kit was from Amersham.

The cloning vector BlueScript II+ was from Stratagene. The eukaryotic expression vector pSV_2dHFR was from American Type Culture Collection (ATCC). The plasmids 4/1EX.1 and λ-LMC41 were gifts to M.M.Pater and A.Pater from F.X. Bosch at Universitat Heidelberg, Germany, and R. Dalla-Fevara at Columbia University, USA.

The cell lines W12 and 3T3 J2 were gifts to M.M. Pater and A. Pater from M. Stanley at University of Cambridge, UK and L.A. Laimins at University of Chicago, USA, respectively. The cell line CaSki was from ATCC. Silicone plastic was from Dow Corning. Nude mice (*nu/nu*, NIH) were purchased from NIH.

Nitrocellulose paper was from Schleicher &. Schuell. Oligonucleotides were custom synthesized by General Synthesis and Diagnostic, Toronto. X-ray film and autoradiography emulsion NTB-2 were from Kodak. Micrography was made with a Leitz Diaplan microscope.

2.2 Methods

2.2.1 Cell culture and transfection

Cultures of human endocervical and ectocervical epithelial cells, HEN and HEC, respectively, were derived from normal cervical specimens obtained from hysterectomies performed for the benign condition of myoma of the uterus. Cultures of human foreskin epithelial cells, HFK, were derived from normal foreskins obtained from neonatal circumcision. The primary HEN and HEC were prepared as described by Turyk *et al.* (1989) and primary HFK was as by Boyce and Ham (1985) and Pirisi *et al.* (1987). All the primary cultures and derived cell lines were maintained in keratinocyte growth medium (KGM). The primary cells were passed upon being 70% confluent and the cell lines when 90% confluent. CaSki cells were cultured in Dulbecco's modified Eagle's medium plus 10% FCS. The cryo-preserved W12 cells were briefly passed two passages in KGM before being tested *in vivo*.

For transfection, primary 70% confluent cells were harvested and transfected with 5 μg DNA with lipofectin (BRL) following the supplier's instructions.

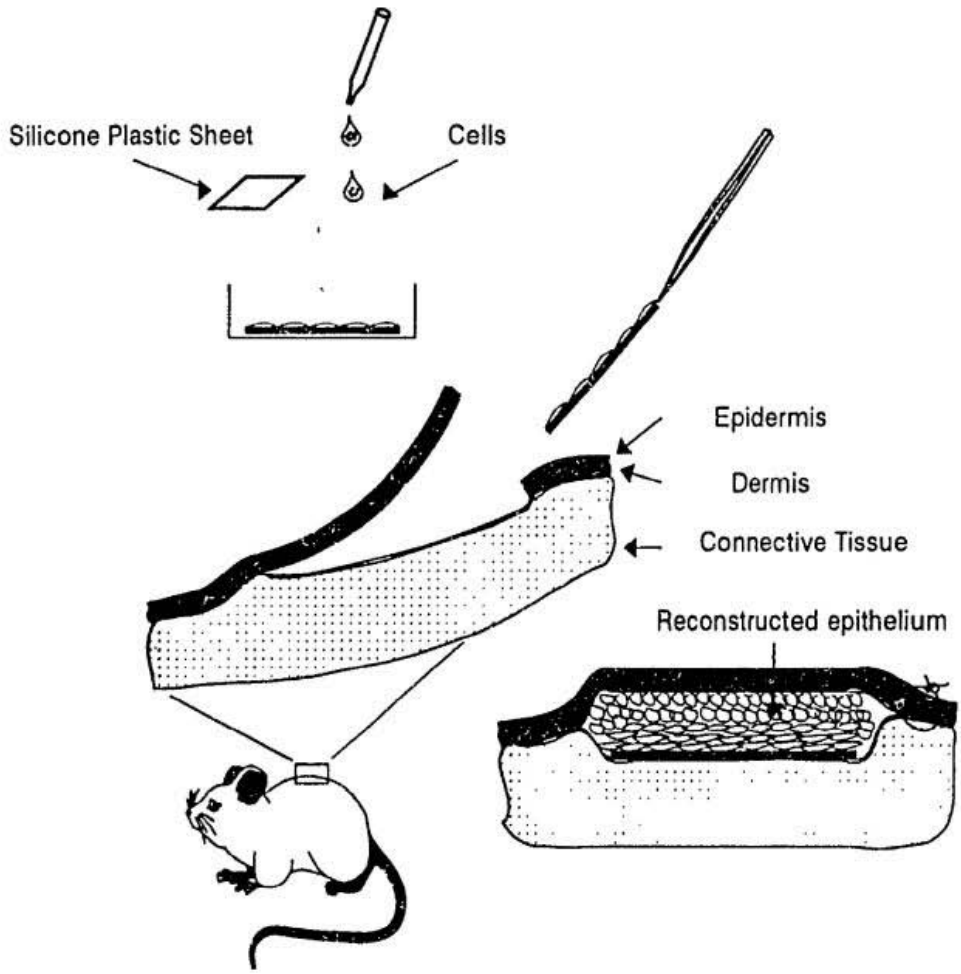
For examining gene expression in response to TGF-β, cells of 80% confluence were treated with TGF-β1 (BRL) at a concentration of 5 ng/ml with 0.2% BSA in KGM for 24 hours before total RNA was collected.

2.2.2 *In vivo* epithelium reconstruction

In vivo epithelial reconstruction was modified from the one described by Barrandon *et al.* (1988). The procedures are diagrammatically presented in Figure 2.1. Trypsinized cells of 10^6 were seeded onto a piece of 1.5 X 1.5 cm^2 silicone plastic sheet in a 35-mm diameter

Figure 2.1 Schematic presentation of the *in vivo* epithelia reconstruction system in nude mice

In vivo epithelial reconstruction was modified from the one described by Barrandon *et al.* (1988). Trypsinized cells were seeded onto a piece of silicone plastic sheet in a petri dish. After overnight attachment, the silicone sheet with the cells attached was implanted beneath a skin flap surgically produced on the back of a nude mouse. The skin flap was closed by surgical staplers or suturing. Blood supply can only reach the human epithelial sheet from one side, while the other side is blocked by the plastic sheet.



In Vivo Epithelium
Reconstruction Sytem
(*in vivo* implantation)

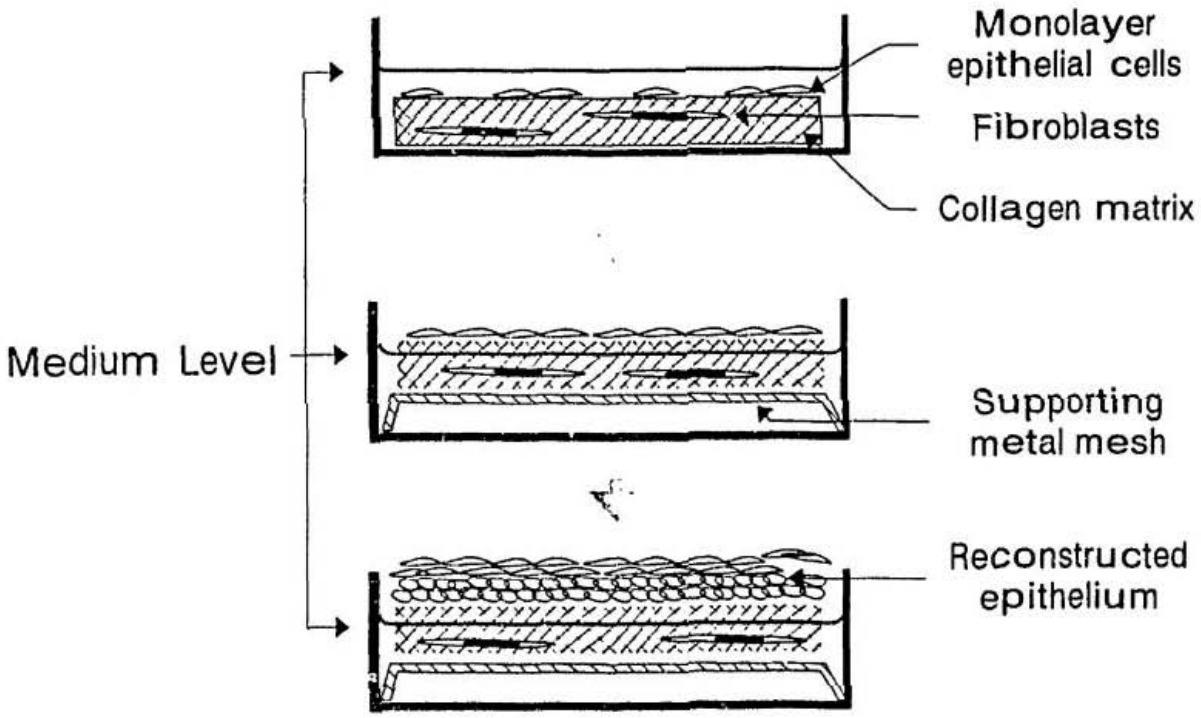
petri dish. After overnight attachment, the cells, together with the silicone sheet was implanted beneath a skin flap surgically produced on the back of nude mice. The skin flap was closed by surgical staplers or suturing. All the surgical procedures were carried out in sterile conditions and experimental animals were anaesthetized by ether inhalation. Care was taken to avoid excessive tissue trauma. The tissues were recovered after 4-10 days and the reconstructed lesion together with the overlying mouse skin was fixed immediately in 4% paraformaldehyde in PBS (pH 7.2).

2.2.3 *In vitro* epithelium reconstruction with organotypic raft culture

In vitro epithelial reconstruction followed the method described by Rader *et* al. (1990). The procedures are diagrammatically presented in Figure 2.2. Briefly, the raft matrix gel was prepared in a 25-mm diameter multi-well plate and was made of eight volumes of Type I collagen (Collaborative Research), one volume of 10 X E-media, and one volume of 10 X raft buffer. The 10 X E-media consisted of 75% DMEM and 25% F12 media in a solution containing 1.8×10^{-3} M adenine, 0.05 mg/ml insulin, 0.05 mg/ml transferrin, 4 ng/ml hydrocortisone, 1 nM cholera toxin and 0.05 mg/ml 3,3',5-triiodo-L-thyronine. The raft buffer contained 2.2% $NaHCO_3$, 4.77% N-[2-hydroxyethyl]piperazine-N'-[1-ethanesulfonic acid], and 0.04 N NaOH. The rafts contained 1.5×10^5/ml 3T3 J2 cells. Epithelial cells of 3×10^5 were seeded onto the raft. When the cells reached confluency, the gel was lifted onto the air-media interface with a metal support. Media was changed every two days. The reconstructed epithelium was recovered after 10 days and was fixed immediately.

Figure 2.2 Schematic presentation of the *in vitro* epithelia reconstruction system (raft culture system)

In vitro epithelial reconstruction followed the method described by Rader *et al.* (1990). The "raft" is a gel matrix made of collagens and contains fibroblast. Epithelial cells were seeded onto the raft. When the cells reached confluency, the raft, with the monolayer cells, was lifted onto the air-media interface with a metal support. Nutrients can only reach the epithelial sheet from one side, via the raft.



In Vitro Epithelium Reconstruction
System
(organotypic raft culture)

Q. Sun

For experiments with 5-AZ, the epithelial cells were treated with 0.2 μM 5-AZ in monolayer culture for three passages. The treated cells were reconstructed in raft culture as above in the presence of 2 μM or 20 μM of 5-AZ.

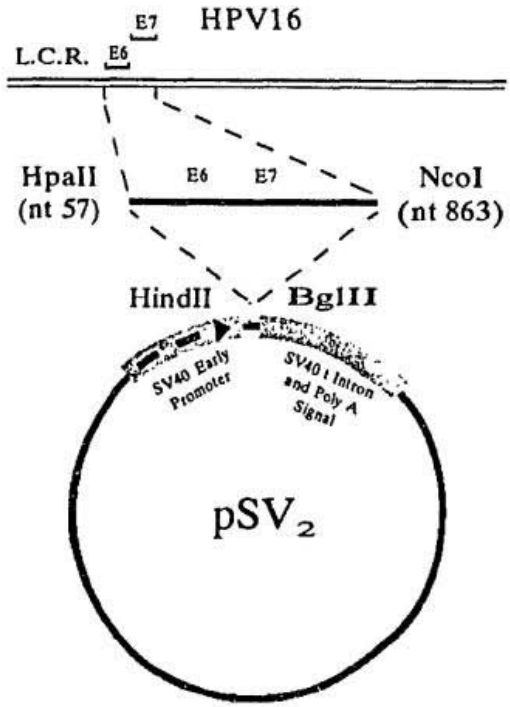
2.2.4 Plasmid construction

The plasmids were constructed following protocols described by Sambrook *et al.* (1989). Plasmid pBS-E7-Δ9 was modified from the plasmid pBS-E7-2, in which the HPV 16 HpaII-SSpI fragment (nt 502-720) is cloned into pBlueScript KS+ (Belaguli *et al.*, 1992). In pBSE7-Δ9, the sequence between KpnI-PstI sites in the BlueScript KS+ multiple cloning region was deleted. Plasmid pSK-LEPΔHK was constructed by inserting the HPV16 EcoRI-PstI fragment (nt 6818- 7007) into BlueScript SK+II, and by deleting the HindIII-KpnI fragment in the vector multiple cloning region. The plasmids $pSV_2$1667 and $pSV_2$1606 were constructed by replacement of the dHFR gene (HindIII-BglII) in pSV_2dHFR (Subramani *et al.*, 1981) with the HPV16 sequences containing E6-E7 ORFs (HpaII-NcoI fragment, nt 57-866) and E6 ORF alone (HpaII-NsiI fragment, nt 57- 566). Figure 2.3 shows the map of $pSV_2$1667.

2.2.5 Probes and probe labelling

For blot analysis, DNA probes were labelled with [α^{32}P]dCTP by random priming reaction following the instruction of a kit supplier. The specific activity was approximately 5 X 10^8 cpm/ug DNA. The HPV16 E5 probe was the HPV16 AccI-RsaI fragment (nt 3956-

Figure 2.3 Schematic presentation of the recombinant plasmid $pSV_2$1667



Construct $pSV_2$1667

4108). The c-*myc* probe was the SstI fragment of the plasmid λ-LMC41 (Dalla Favera *et al.*, 1982). The γ-actin probe was the 2200 bp BamHI fragment of cDNA. The c-Ha-*ras* probe was the 6.6 kb BamHI fragment of EJ-*ras* (Tabin *et al.*, 1982).

For *in situ* hybridization to detect HPV16 DNA, the genomic HPV16 DNA excised from the cloning vector was labelled with Biotin-14-dATP by nick translation reaction following the instruction of the kit supplier (BRL).

RNA probes, or riboprobes, were labelled with isotopes by *in vitro* transcription reaction (Sambrook *et al.*, 1989). The plasmid pBSE7-Δ9 contained the HPV 16 HpaII-SSpI fragment (nt 502- 720) in the pBlueScript KS+ vector. Plasmid 4/1EX.1 contains the SalI-XhoI (nt 735-1263) fragment of human cytokeratin 1 (CK1) gene in BlueScript+ (Knapp and Franke, 1989; Durst *et al.*, 1991). The plasmid pSK-LEPΔHK contained the HPV16 EcoRI-PstI fragment (nt 6818- 7007) in the BlueScript SKII+ vector. For Northern blots, the riboprobe was labelled with [α^{32}P]UTP. For *in situ* hybridization, the probe was labelled with [α^{35}S]UTP. The *in vitro* transcription reaction contained 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 50 mM DTT, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM ATP, 75 μCi [α^{32}P]UTP or [α^{35}S]UTP, and 25 units T3 or T7 RNA polymerase. Upon incubating the reaction at 37 °C for 60 minutes, 5 μg Poly A and 1 unit DNase I (Promega) were added to eliminate the DNA template. The unincorporated isotope was removed by ethanol precipitation twice and the probes were preserved in 0.1 M DTT at -70 °C. The size of the CK1 riboprobe prepared from the plasmid 4/1EX.1, was reduced by heating the probe at 60 °C for 72 minutes in 40 mM $NaHCO_3$ and 60 mM Na_2CO_3 (Cox *et al.*, 1984).

2.2.6 Southern blot analysis

High molecular weight DNA was isolated from 80-90% confluent cells in culture as described by Sambrook *et al.* (1989). DNA of 10 µg was restricted with various restriction enzymes as recommended by the supplier. DNA was separated on 1% agarose and was transferred to nitro-cellulose membrane by the capillary method. The blots were hybridized with one of the ^{32}P-dCTP labelled-DNA probes in a hybridization buffer containing 5 X 10^6 cpm/ml probe, 1% non-fat milk, 1 mM EDTA (pH 8.0), 0.43 M NaH_2PO_4, 1% SDS and 0.5 mg/ml sonicated salmon sperm DNA. The hybridization was carried out at 65 °C overnight. Washing was performed at the same temperature as the hybridization with 0.1 X sodium saline citrate and 0.1% sodium dodecyl sulfate (SDS). For repetitive probing, the membranes were incubated twice with 0.4 N NaOH at 45 °C and the blots were reprobed as above.

2.2.7 Northern blot analysis

Total cellular RNA was isolated from 70-80% confluent cells in culture as described by Sambrook *et al.* (1989). RNA was separated on 1% agarose gel and transferred to nitrocellulose membrane. HPV 16 E7 mRNA was detected by a ^{32}P-labelled riboprobe prepared by *in vitro* transcription from pBSE7-Δ9. Detection of the mRNAs for HPV16 E5, c-*myc* and c-H-*ras* used ^{32}P-labelled DNA probes. The hybridization buffer contained 1% non-fat milk, 1 mM EDTA (pH 8.0), 0.43 M NaH_2PO_4, 1% SDS and 1 mg/ml sonicated salmon sperm DNA. The hybridization temperature was 72 °C for the riboprobe, and 68 °C for the DNA probes. The blots were hybridized in a shaker overnight. Washing was performed at the same temperature as the hybridization with 0.1 X SSC and 0.1% SDS. For

reprobing, the blots were washed with boiling solution containing 0.1% SDS and 0.1 X SSC for three times and reprobed as above.

2.2.8 Routine histo-pathological preparation

The paraformaldehyde fixed tissues were dehydrated with serial ethanol, embedded in paraffin, and sectioned as routine (Zeller and Zogers, 1992).

2.2.9 Cytokeratin staining with indirect immunofluorescence and immunohistochemistry assays

Indirect immunofluorescence staining was as described previously with minor modifications (Zeller and Zogers, 1992). The unstained sections prepared for histology were deparaffined and rehydrated. They were treated with 0.25% trypsin (Sigma) in 27 mM $CaCl_2$, and 50 mM Tris-HCl, (pH 7.5) at 37 °C for 30 min for antibodies from Sigma or 2 hours for antibody from ICN. The sections were first incubated at 4 °C overnight with one of the following mouse anti-cytokeratin monoclonal antibodies as the first antibody: CK18 (CY-90), CK13 (Ks-1A3) and CK10 (K8.60) (Sigma), or CK19 (Ks.19.1, ICN) with dilutions recommended by the suppliers. For indirect immunofluorescence staining, the sections were then incubated with FITC-labelled goat anti-mouse IgG as the second antibody for 1 hour at room temperature. The sections were washed with PBS, (pH 7.4), for 15 minutes twice between each incubation. For immunohistochemistry staining, the sections were incubated with biotin-labelled goat anti-mouse IgG (Vector Laboratories) as the second antibody at room temperature for 60 minutes, followed by incubation with horse-radish peroxidase-

conjugated streptavidin (Vector Laboratories). The complex was detected with 0.4 mg/ml 3-amino-9-ethylcarbazole (Dako) and 1.5% H_2O_2 in 50 mM acetate buffer (pH 5.0). Controls for experimental conditions was provided by substitution of PBS for the first antibodies.

2.2.10 *In situ* hybridization assays for mRNA

The reconstructed tissues were fixed in 4% paraformaldehyde in PBS (pH 7.4) and 2 mM $MgCl_2$ for 30 minutes and preserved in 70% ethanol at 4 °C until routine processing for paraffin embedding and sectioning. The archival clinical samples had been fixed in 10% formalin. Seven μm thick sections were mounted on glass slides pretreated with 2% Silane (Sigma) in acetone. The sections were dewaxed in xylene and rehydrated in graded ethanol. After being saturated with water, the sections were first treated with 0.2 N HCl for 5 minutes, digested in 10 μg/ml proteinase K in water for 5 minutes, and then incubated with 0.1 M triethanolamine-HCl (pH 8.0) and 0.25% acetic anhydride for 5 minutes twice. Selected samples were also treated with 100units/ml DNase I (Promega) in 50 mM Tris-HCl (pH 7.5) at 37 °C for 60 minutes. After being equilibrated in a prehybridization solution containing 50% formamide, 10 mM Tris-HCl (pH 7.5), 0.6 M NaCl, 1 mM EDTA, 100 μg/ml heparin, 50 mM DTT, 0.5 mg/ml salmon sperm DNA, 0.5 mg/ml *E. coli* tRNA at 50 °C for 30 minutes twice, the tissue sections were covered with 15 μl hybridization solution, which had been denatured at 70 °C for 10 minutes. The hybridization buffer contained 50% formamide, 10 mM Tris-HCl (pH 7.5), 0.6 M NaCl, 1 mM EDTA, 100 μg/ml heparin, 50 mM DTT, 0.5 mg/ml salmon sperm DNA, 0.5 mg/ml *E. coli* tRNA, 10% PEG 8000, 1 X Denhardt's solution and 40,000 cpm/μl riboprobe. Hybridization was carried out at 50 °C for 48 hours

(Stoler, 1990).

Washing was done first in 2 X SSC at 50 °C for 30 minutes, then in the digestion solution containing 20 μg/ml RNase A, 0.5 M NaCl, and 0.01 M DTT at 37 °C for 30 minutes, subsequently in 50% formamide, 2 X SSC and 0.01 M DTT at 50 °C for 30 minutes twice, and finally in 1 X SSC, 0.02 M DTT, 0.07% sodium pyrophosphate at 50 °C for 2 X 30 minutes. The dried sections were coated with NTB-2 emulsion. After being autoradiographed for 14-21 days, the slides were developed in D-19 developer. The slides were slightly counterstained with H&E stains. The sensitivity and specificity of the *in situ* hybridization assays were constantly evaluated and controlled by examining the detection in reference samples from W12, CaSki, and HaCat cells (Zeller and Zogers, 1992). These three cells contained episomal, integrated and no HPV16 DNA, respectively (Pater and Pater, 1985; Boukamp *et al.*, 1988; Stanley *et al.*, 1989).

2.2.11 *In situ* hybridization assays for DNA

The archival clinical samples had been fixed in 10% formalin. Seven μm thick sections were dewaxed in xylene and rehydrated in graded ethanol. After being saturated with water, the tissue sections were boiled in a microwave oven (Citizen, model JM55821) at full power for 5 minutes (Van den Brink *et al*, 1990). The tissue sections were then digested in 40 μg/ml DNase-free proteinase K solution in PBS for 20 minutes. After being rinsed twice in PBS, the sections were refixed in 4% paraformaldehyde for 1 minute. The tissue sections were then dehydrated through an ethanol series, air-dried, and then covered with 15 μl hybridization solution containing 2 X SSC, 0.1 M $NaPO_4$, 1 X Denhardt's solution, 10% dextran sulfate

and 50% formamide with a final probe concentration of 0.1 μg/ml. The sections with hybridization solution were denatured at 100 °C for 10 minutes. Hybridization was carried out at 42 °C for 48 hours (Troncone *et al.*, 1992).

The tissue sections were washed twice in 2 X SSC and twice in 0.2 X SSC, 0.1% SDS at 37 °C for 30 minutes. After being treated in the blocking solution containing 10% BSA in 0.1 M Tris-HCl (pH 7.5), 0.1 M NaCl, 2 mM $MgCl_2$ and 0.05% Triton X-100, the sections were incubated first with 2 μg/ml streptavidin and then with 1 μg/ml alkaline phosphatase-conjugated biotin (Vector Laboratories) in 0.1 M Tris-HCl (pH 7.5), 0.1 M NaCl, 2 mM $MgCl_2$ and 0.05% Triton X-100 for 60 and 30 minutes, respectively. The complex was detected by a chromogenic solution containing 330 μg/ml nitroblue tetrazolium and 170 μg/ml 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 50 mM $MgCl_2$ (Zeller and Zogers, 1992).

2.2.12 PCR amplification of HPV16 sequence from biopsy tissue

Two pieces of 10 μm thick sections were dewaxed in xylene, and rinsed in absolute ethanol. The air dried sections were digested overnight at 37 °C in 50 μl lysis buffer containing 100 mM Tris-HCl (pH 8.0), 4 mM EDTA, 0.45% NP-40, 0.45% Tween 20, and 0.4 mg/ml proteinase K. The digested samples were boiled for 10 minutes and extracted twice with phenol and chloroform. Three μl of lysate were used in a PCR reaction containing 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100, 250 mM dNTPs, 1.5 mM $MgCl_2$, and 0.6 unite Taq DNA polymerase (Promega) and 0.33 μM HPV16-specific primers with a total volume of 50 μl. The primers were 5'- GCAAGCAACAGTTACTGCGACGT-3' and

5'-GCAACAAGACATACATCGACCGG-3', which flank a 323 bp sequence in the HPV16 E6 ORF (Ferre and Garduno, 1988). Each PCR-reaction cycle included 95 °C for 1 minute, 56 °C for 1 minute and 72 °C for 2 minutes, totalling 35 cycles. After being separated on a 1.2% agarose gel the amplified DNA fragment was stained by ethidium bromide and/or analysed by Southern blot. SiHa and HeLa cells, which contain HPV16 and HPV18 DNA, respectively, were used as controls.

2.2.13 Soft agar assays

The 0.35% overlying and 0.7% underlying gels for soft agar assays were made with low melt point agarose (Sigma) in KGM. A two fold underlying agarose solution was prepared by melting the low melt point agarose (Sigma) at 60 °C in KGM without the supplementing EGF, insulin and bovine pituitary extract. After being cooled to 40 °C, the 2 X underlying agarose solution was mixed with an equal volume of prewarmed KGM containing 2 X supplements. Three ml of the final underlying agarose solution was dispensed into 50-mm diameter plates, and solidified at 4 °C. For overlying agarose gels, exponentially growing immortalized cells were trypsinized, collected by centrifugation, and suspended in KGM with 2 X supplements. The cell suspension was mixed with an equal volume of 2 X overlying agarose solution without the supplements, and the mixture was poured over the solidified underlying gel. After the gel was solidified in 4 °C, ordinary KGM was added to the petri dish. Medium was changed daily for seven days, and every 5 days afterwards. SiHa and normal primary cells were used as positive and (negative controls, respectively. Formation of colonies was examined after six weeks.

2.2.14 Determination of *in vitro* cell generation time

Cells of 1 X 10^5 were seeded into triplicate 35 mm diameter plates. The cells were trypsinized, collected by centrifugation and counted with a haemocytometer every two days for six days.

2.2.15 Tumorigenicity assays in nude mice

Cells of 1 X 10^7 were subcutaneously injected into the back of nude mice, and three mice were tested for each cell line. After a 3-6 month period, the animals were sacrificed and dissected for tumour formation and metastasis.

CHAPTER 3

IN VIVO MODELS

FROM CULTURED HUMAN GENITAL EPITHELIAL CELLS

3.1 Introduction

Most viral infections show tissue specificity. Thus, the most relevant system to study viral pathogenesis is the natural target for the particular virus. As introduced in Section 1.3.5.1, HPV16 infections mostly induce SSE lesions in genital regions, and seem to be more oncogenic in cervical metaplastic SSE than those in other genital epithelia. To elucidate the mechanisms responsible for this phenomenon, it is necessary to understand the physiological features of genital epithelia and to investigate virological features of HPV16 infections in these epithelia.

3.1.1 Cervical metaplastic SSE

As introduced also in Section 1.3.5.1, external genitalia are covered with native SSE. In contrast, the epithelial lining in the uterine cervix is complex and dynamic (refer to Figure 1.3A). The ectocervix is lined with native SSE, which is continuous with the non-keratinizing SSE in vagina. The general characteristics of SSE have been introduced in Section 1.3.3.3.

The endocervix is covered with SCE. The cervical SCE consists of at least four types of cells, including mucus-secreting cells, ciliated cells and two other types of cells with unknown functions (Kudo *et al.*, 1991). The progenitor cells for cervical SCE is unknown. It has been suggested that some scattered subcolumnar reserve cells give rise to cervical SCE.

Cells in cervical SCE express the so-called simple epithelial CKs, such as CK8, 18, 7, 19 and 20, in addition to the basal cell CKs, such as CK14 and 15 (Smedts *et al.*, 1993b). Although CK19 was believed to be a simple epithelial CK, studies with new monoclonal antibodies have shown that it is also expressed in the basal cells of some non-keratinizing and special keratinizing SSE, such as the SSE covering ectocervix and foreskin. The expression patterns of selective CKs in cervical epithelia are summarized in Figure 3.1A.

Cervical metaplastic SSE is located between ectocervical SSE and endocervical SCE. Metaplasia may appear in three morphological stages (refer to Figure 1.3A and B, and its legend) (Fu and Reagan, 1989a). In the stage of reserve cell hyperplasia, a single layer of primitive cuboidal cells, referred to as reserve cells, appears between endocervical epithelial cells and the basal membrane. Although these reserve cells show higher nucleus/cytoplasm ratios than normal SCE cells, their enlarged nuclei are uniform in size, and do not show active or abnormal mitoses. This suggests that the hyperplastic reserve cells represent benign and reactive proliferation. The proliferating reserve cells displace the columnar epithelial cells upwards, forming a multilayered epithelium called immature metaplasia. The immature metaplasia consists of cells resembling parabasal cells of native SSE and shows no mature squamous differentiation anywhere in the stratified epithelium. Schell (1969) has shown that cells competent for DNA synthesis are distributed throughout the immature metaplastic SSE. In contrast, the DNA-synthesizing cells in native SSE are restricted in the lowest two layers of the epithelium. Mature metaplasia develops from immature metaplastic SSE and has distinct basal, parabasal, intermediate and superficial layers. Morphologically, a well-developed mature SSE is indistinguishable from native SSE, but it can be identified by the

underlying cervical glands beneath metaplastic SSE.

With the increasing knowledge of CKs, attempts have been made to elucidate the histogenesis of squamous metaplasia using the patterns of CK expression as marker systems (Fuchs, 1988). CK expression in metaplastic SSE is dynamic and complex, in association with specific stages of metaplastic SSE (Figure 3.1A) (Gigi-leitner *et al.*, 1986; Smedts *et al.*, 1993b). The successive transitions from reserve cell hyperplasia to immature metaplasia and mature metaplasia are accompanied with characteristic changes in the patterns of CK expression (Smedts *et al.*, 1993b). At the stage of reserve cell hyperplasia, the proliferating reserve cells express simple epithelial CKs, such as CK18 and CK19. Upon developing into immature metaplastic SSE, CK13, the major differentiation marker for non-keratinizing SSE, is increasingly expressed. In contrast, expression of CK18 is diminishing, while CK19 expression becomes restricted (Smedts *et al.*, 1993a). In the maturing metaplastic SSE, CK18 expression is suppressed, while the expression of CK19 becomes limited to the bottom layer cells, and CK13 is compartmentalized in the differentiating upper layer cells. In mature metaplasia, CK10, the marker for native SSE, is also expressed in a pattern similar to CK13, but with lower levels of expression.

The origin of the reserve cells, and their relationship with columnar cells have been controversial for nearly a century. In 1910, Meyer assessed the morphology of fetal and adult cervices, to propose that reserve cells derive from inward growing basal cells of native SSE (Fu and Reagan, 1989a). Also based on studies of morphological observations of biopsy samples, but with the help of staining for mucin production, Carmichael and Jeaffreson (1941) postulated that reserve cells are derived from undifferentiated embryonic stem cells that are

Figure 3.1 Summarized patterns of CK expression in human genital epithelia and in artificial tissues reconstructed *in vivo* from cultured human genital epithelial cells

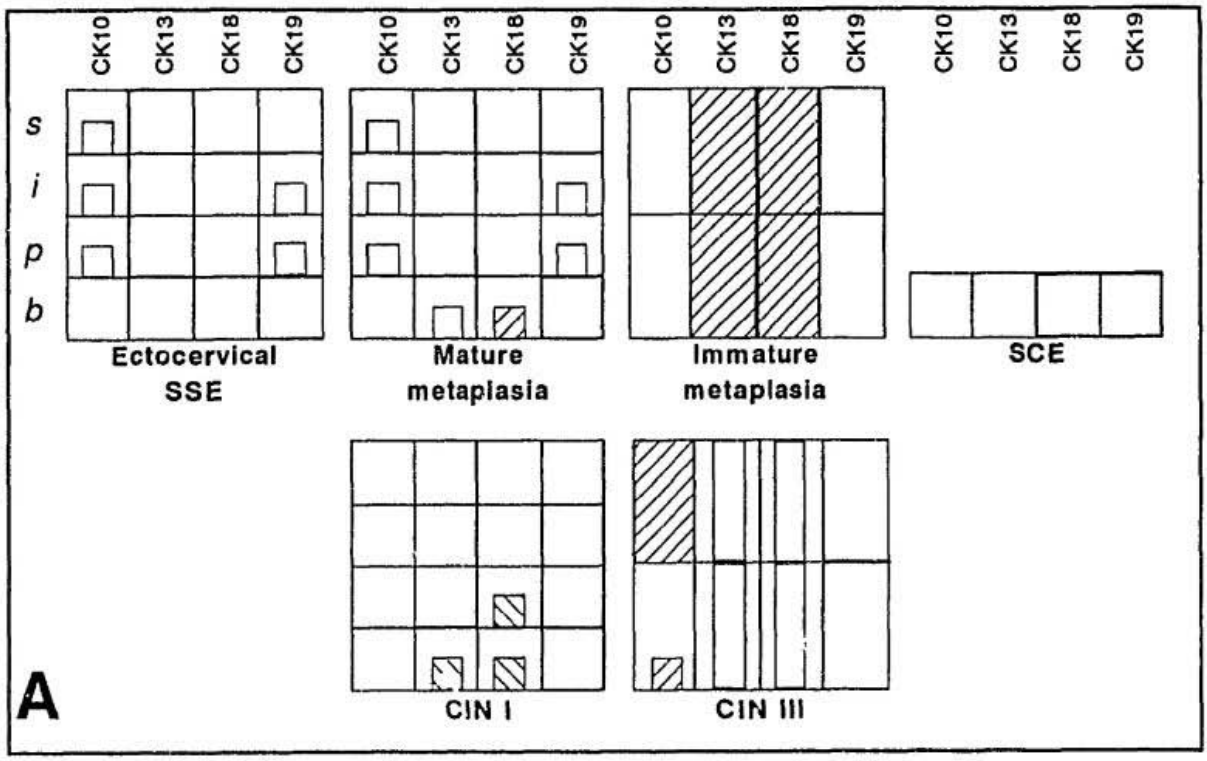
A: Schematic presentation of CK distribution patterns in cervical epithelia as detected by antibodies against CK10, CK13, CK18 and CK19.

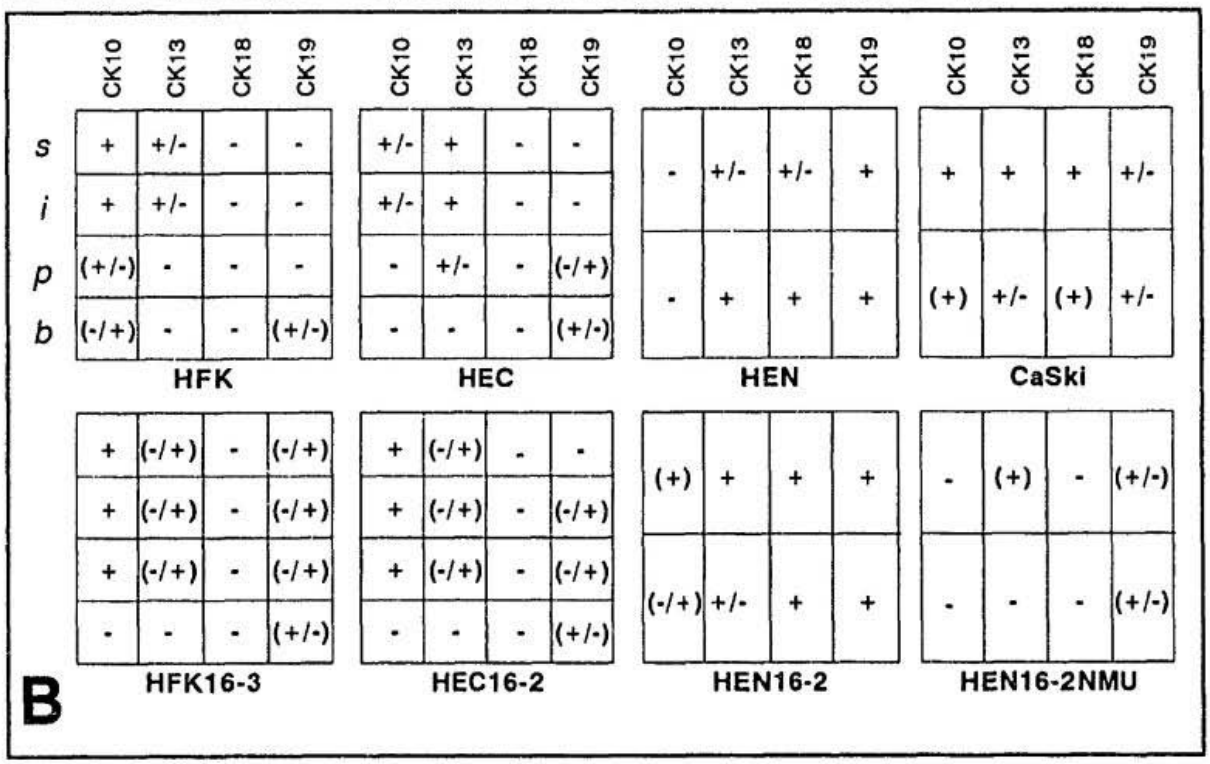
Based on Smedts *et al.* (1990) and modified and updated according to Smedts *et al.* (1992; 1993).

Abbreviations: *b*= basal cells; *p*= parabasal cells; *I*= intermediate cells; *s*= superficial cells. Marks: Open squares: negative reaction; Fully filled squares: homogeneous positive reaction; Short bars: positive reaction in minority cells; Long bars: positive reaction in majority cells; Solid bars: strong positive reaction; Stippled bars: weak positive reaction.

B: Summary of CK expression in the *in vivo* reconstructed tissues

Abbreviations: *b*= basal cells; *p*= parabasal cells; *I*= intermediate cells; *s*= superficial cells. Marks: "-": negative reaction; "+": homogeneous positive reaction; "-/+": positive reaction in minority cells; "+/-": positive reaction in majority cells; "(+)" indicates weak expression.


CK10
CK13
CK18
CK19
s
i
p
b
Ectocervical SSE
Mature metaplasia
Immature metaplasia
SCE
CIN I
CIN III
A


CK10 CK13 CK18 CK19
s
i
p
b
HFK
+ +/- - -
+ +/- - -
(+/-) - - -
(-/+) - - (+/-)
HEC
+/- + - -
+/- + - -
- +/- - (-/+)
- - - (+/-)
HEN
- +/- +/- +
- + + +
CaSki
+ + + +/-
(+) +/- (+) +/-
HFK16-3
+ (-/+) - (-/+)
+ (-/+) - (-/+)
+ (-/+) - (-/+)
- - - (+/-)
HEC16-2
+ (-/+) - -
+ (-/+) - (-/+)
+ (-/+) - (-/+)
- - - (+/-)
HEN16-2
(+) + + +
(-/+) +/- + +
HEN16-2NMU
- (+) - (+/-)
- - - (+/-)
B

scattered below the columnar cells in the cervix. The reserve cells and their derivative squamous metaplastic cells both express mucin, which is also produced in the endocervical SCE cells. These authors suggest that the precursor of the reserve cells may be bi-potent for columnar, as well as metaplastic, differentiation. Some studies indicate that the reserve cells may originate from the stroma. Song (1964) and Reid *et al.* (1967) observed stromal cells migrating through basal membrane in metaplastic SSE, which was interpreted as giving rise to cervical epithelium. Similar observations were also reported by Lawrence and Shingleton (1980) using electron microscopy. However, based on observations in *in toto* organ culture, Schurch *et al.* (1978) suggested that metaplasia is a result of a direct transformation of cells in the columnar epithelium. Consistent with this view, the subcolumnar reserve cells cannot be seen in the normal endocervical SCE, and they are observed only in the stage of hyperplasia (Burghardt, 1986). Indeed, an autoradiography study showed that not all endocervical SCE cells are terminally differentiated, and some endocervical SCE cells with differentiated morphologies are competent to replicate (Hiersche and Nagl, 1980).

The mechanism underlying squamous metaplasia is unclear. Squamous metaplasia has been suggested to represent a physiological transdifferentiation process for epithelial tissue to remodel in response to a changed environment (Fu and Reagan, 1989b). The extended histogenic course of metaplasia, as reflected by the existence of various metaplastic stages, suggests that this transdifferentiation is progressive and may require cell proliferation. The factors that induce metaplasia are currently unknown. However, exposure to an acidic vaginal environment, trauma, chronic physical or/and chemical stimulation, and inflammation have been suggested. In experimental animals, cervical squamous metaplasia has been induced by

exogenously administrated estrogen or vitamin A (De Luca *et al.*, 1994; Tannous Khuri *et al.*, 1994; Arbeit *et al.*, 1995).

In summary, due to technical difficulties, knowledge on metaplastic SSE has been mainly derived from direct examination of clinical samples. No well-established experimental system has been available for studying metaplastic SSE. Thus, the cellular and molecular mechanism of metaplasia has not been elucidated. Due to the pathological significance of metaplastic SSE, in which more than 95% of cervical carcinomas occur, it is imperative, to investigate the nature of squamous metaplasia.

3.1.2 Objective

Recent progress in tissue culture techniques have made it possible to cultivate HKC from foreskin and ectocervix, and HEN from endocervix in *in vitro* condition (Turyk *et al.*, 1989). In the light of a possible involvement of cervical metaplastic SSE and HPV16 infection in cervical cancer, our laboratory has shown that *in vitro* transfection of HPV16 genomic DNA cannot only immortalize HKC, as reported by others, but can also immortalize HEN (Tsutsumi *et al.*, 1992; Sun *et al.*, 1993). The question arising from these observations was "What were the phenotypes of the immortalized HEN?" Because the immortalized HKC formed SSE lesions similar to natural SILs upon being tested in conditions allowing differentiation (McCance *et al.*, 1988; Waggoner *et al.*, 1990; Woodworth *et al.*, 1990b; Durst *et al.*, 1991), another question was, "Does any phenotypic difference exist between the immortalized HEN and HKC?" Indeed, because premalignant cells and their malignant derivatives largely retain the general phenotypes of their normal parental cells, possibly the

HPV16-immortalized HEN represented cells with glandular phenotypes, as a reflection of their SCE origin. Consistently, although less frequently than in SCC, HPV16 is also implicated in cervical adenocarcinomas (Higgins *et al.*, 1992a), which are believed to be derived from cervical SCE and are usually more malignant than SCC. Thus, it was significant to comparatively examine the phenotypic and pathological features of the immortalized HEN and HKC.

To address these questions, the cultured cells need to be tested in conditions similar to those *in vivo*. Therefore, the normal and HPV16-immortalized HEN, HFK and HEC were reconstructed into epithelia in a nude mice system. Then, the phenotypes of the different genital epithelial cells immortalized by HPV16 were compared.

3.2 Results

The *in vivo* system that has been used successfully by others to reconstruct epithelia from HKC, the Barrandon's system, involves implanting a monolayer epithelial sheet into nude mice (Barrandon *et al.*, 1988). However, due to the particular nature of HEN, it was not feasible to use Barrandon's original method to reconstruct epithelia from HEN, since the epithelial sheet formed by HEN was too fragile to be manipulated surgically (data not shown). In addition, treating the cells with a high concentration of calcium, which is required by the original method, is undesirable for the current study, since calcium strongly induces squamous differentiation, and this could potentially affect the phenotype of the subject cells (Bikle and Pillai, 1993). To circumvent these problems, a modified *in vivo* system was developed in the current study, which allowed reconstruction of epithelia from HEC, HFK, as well as HEN,

without prior treatment of the cells with calcium (Figure 2.1).

3.2.1 *In vivo* reconstructed epithelia from cultured normal genital epithelial cells

The epithelial cells from normal foreskins and cervices can be cultured *in vitro* in KGM for approximately 10 passages, until their growth stops (Boyce and Ham, 1983; Boyce and Ham, 1985; Pillai *et al.*, 1988; Tsutsumi *et al.*, 1992). While the HKC from both the foreskin and ectocervix had a characteristic cobble stone-like morphology for all passages in monolayer culture, HEN displayed a distinct morphological transition with passage (Figure 3.2). The initial clones from the endocervical biopsies consisted of cells with a pleomorphic epithelial morphology. With continuing passage, the culture became dominated by keratinocyte-like cells. The CK expression patterns of HKC and HEN in monolayer culture show only some quantitative, rather than qualitative differences (Tsutsumi *et al.*, 1992). As suggested by Turyk *et al.* (1989), the morphological transition of cells in HEN culture indicated that the HEN may undergo a phenotypic transformation *in vitro*. Thus, the *in vivo* equivalent of the *in vitro* HEN cannot be ascertained. To clarify this question, it was necessary to reconstruct the normal cultured cervical epithelial cells into epithelial tissues first.

3.2.1.1 Normal HKC and HEN implants morphologically resembled well-differentiated SSE and immature metaplastic SSE, respectively

The HFK and HEC developed into well-differentiated SSE upon being implanted into nude mice for eight days (Figures 3.3*a* and *b*, respectively). The reconstructed epithelia showed distinctive cellular compartmentalization. The basal layer consisted of cubical cells

Figure 3.2 Morphology of HEN, HEC and HFK in *in vitro* monolayer

Top row: Normal primary culture at passage 1

Middle row: Normal primary culture at passage 4

Bottom row: Immortalized genital epithelial cell by HPV16 genomic DNA.

The initial growing clones were squared in the passage 1 cells. The endocervical clones consisted of cells with pleomorphic epithelial morphology, which assumed morphologies similar to cobble stone-like cells at later passages, while the HKC from both the foreskin and ectocervix had a characteristic cobble stone-like morphology at both the early and late passages. The immortalized cells had no distinct features from each other, or from their respective normal counterparts in late passages.

The magnifications were 325 X.

Phase contrast micrography.

HEN
HEN16-2
HEC
HEC
HEC16-2
HFK
HFK
HFK16-3

with scarce cytoplasm, and the cells in the suprabasal layers became flattened and showed squamous differentiation, as indicated by the existence of inter-cellular bridges (desmosomes), hallmarks for the phenotype of squamous differentiation. Typical inter-cellular bridges are well illustrated in Figure 3.3*b*. These morphological features are consistent with those of the native SSE.

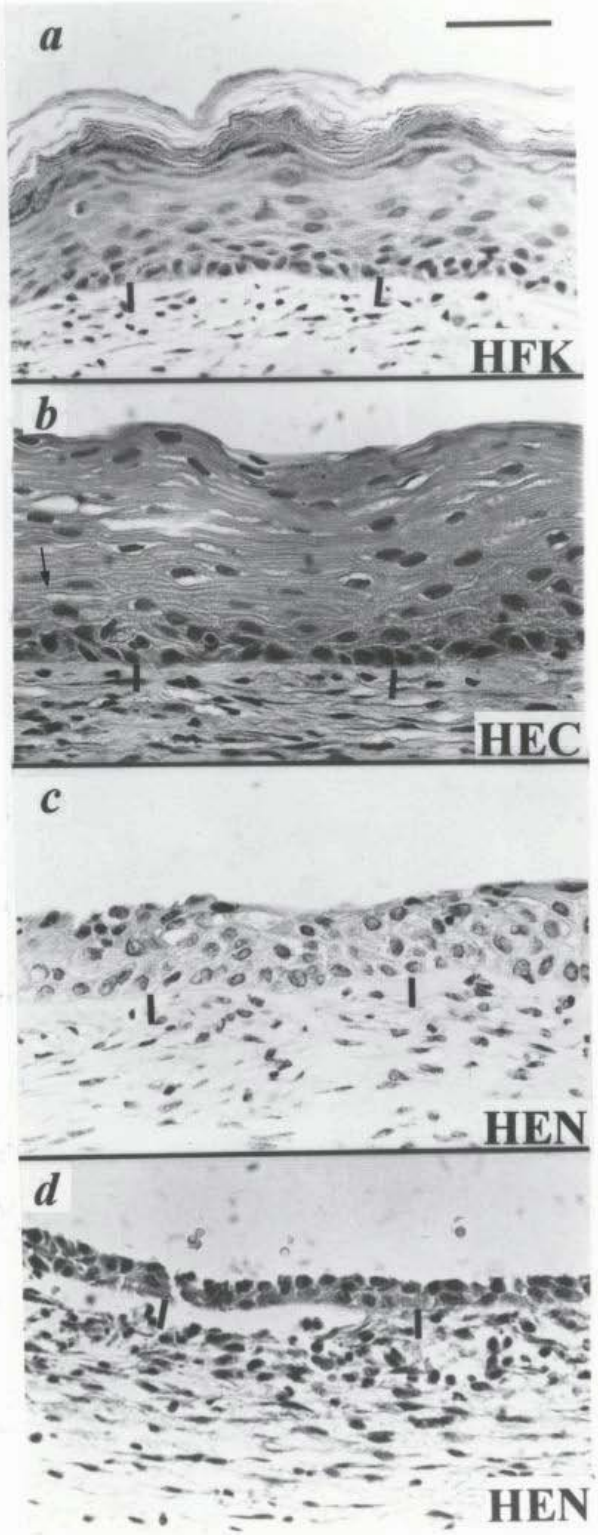
In contrast, the cultured HEN was reconstructed under the same conditions into a stratified epithelium composed of cells with similar sizes and contours (Figure 3.3*c*). In the HEN implant, the nucleus/cytoplasm ratio was relatively high, the nuclei were generally uniform in size and shape, and no atypical features were seen. Inter-cellular bridges can be observed between some cells, although they were less conspicuous than those in the reconstructed epithelia from HKC. Since only individual cells in the upper superficial layer showed a flattened cellular contour, the reconstructed SSE from HEN did not reach mature squamous differentiation. These morphological features are consistent with those for cervical immature metaplasia (Fu and Reagan, 1989b). In order to examine the phenotypes of the cells giving rise to the reconstructed metaplastic SSE, and to assure that the reconstructed SSE originated from the implanted single cellular sheet, the HEN implant was also recovered from the nude mice after a four day period (Figure 3.3*d*). The developing HEN implant displayed two layers of cells that were cubical in cellular contour and had very high nucleus/cytoplasm ratios. No cells with typical endocervical columnar morphology were observed, and no cell migration from the mouse stroma was seen, although the mouse stroma showed some infiltration of inflammatory cells.

Figure 3.3 ***In vivo*** **reconstructed epithelia from HFK, HEC and HEN**

a and *b:* HFK and HEC developed into well-differentiated SSE upon being implanted into nude mice for 8 days, respectively. Arrow in Figure *b* indicates typical serrated structures of the inter-cellular bridges (desmosomes). *c*: HEN implant recovered after 8 days. The implant was composed of cells with similar sizes and contours. *d*: HEN implant recovered after 4 days.

The magnifications were 250 X. H&E staining.

Basal membrane was indicated by black bars.


a
HFK
b
HEC
c
HEN
d
HEN

3.2.1.2 Normal HKC and HEN implants expressed CKs characteristic of native and metaplastic SSE

The implants from cultured HKC and HEN, which morphologically were consistent with native SSE and immature metaplastic SSE, respectively, were further characterized by examining the patterns of CK expression in the reconstructed tissue with immunofluorescence assays (Figure 3.4).

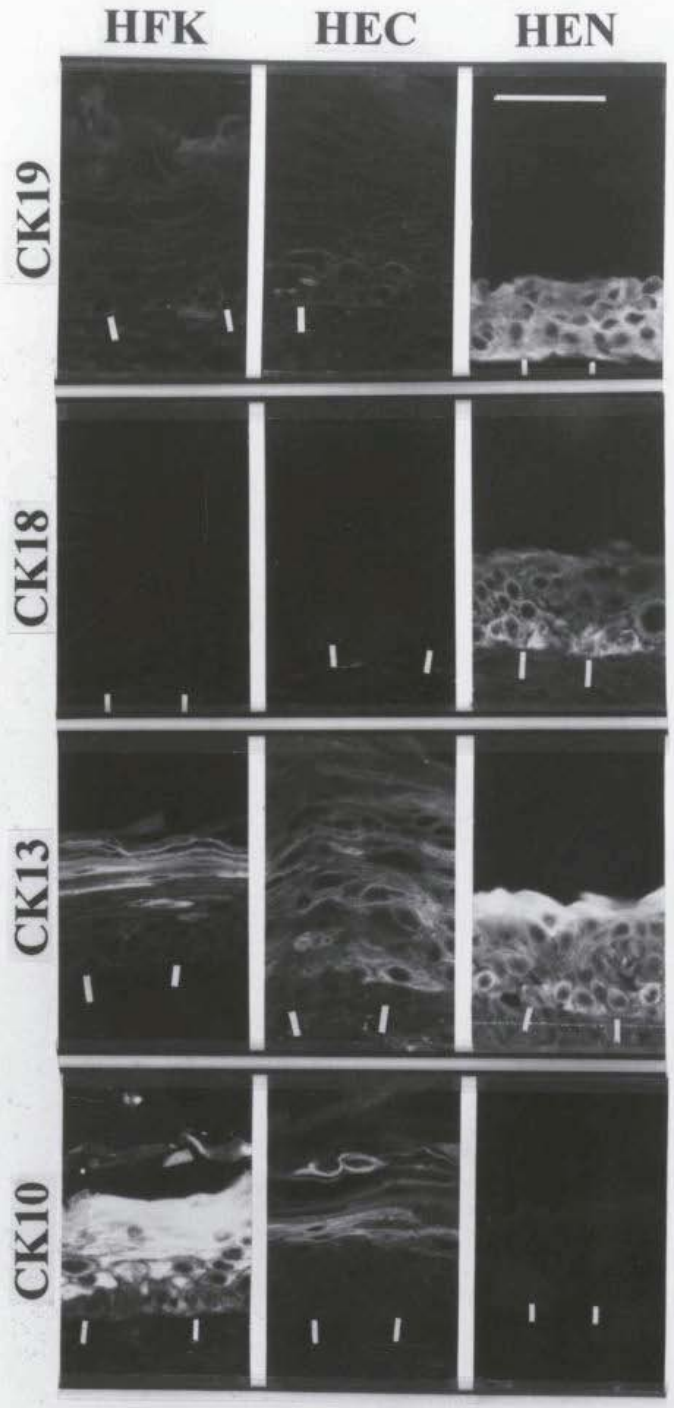
Based on the published data on the patterns of CK expression in naturally occurring SSE and metaplasia, which were summarized in Figure 3.1A, expression of four CKs was determined to be discriminating (Smedts *et al.*, 1993b). The simple epithelial CK18 has been shown to be expressed in cervical SCE, but not in native SSE from both the foreskin and ectocervix. CK19 is expressed in SCE and is also weakly expressed in the basal cells of the foreskin and basal/parabasal cells of the ectocervical SSE. Both CK18 and CK19 are expressed in metaplastic SSE with different patterns, dependent on the maturity of metaplasia. In immature metaplasia, CK18 and CK19 are extensively expressed, often throughout the epithelia. However, CK18 is not detectible, whereas CK19 becomes restricted, in the bottom cells of mature metaplasia. CK10 and CK13, the markers for terminal squamous differentiation in foreskin and ectocervical SSE, are expressed in the differentiating squamous cells, and not in the basal cells, of SSE. In contrast, SCE expresses neither CK10 nor CK13. CK13 is extensively expressed in immature metaplasia, but becomes restricted in the upper layer differentiating cells when metaplasia matures. CK10 becomes only weakly detectible in the very mature metaplastic SSE that resembles native SSE. Thus, the combinations of CK expression patterns in the implants should be indicative of their origin and differentiation

Figure 3.4 Indirect immunofluorescence detection of CK expression in the *in vivo* reconstructed epithelia from HFK, HEC and HEN

The most prominent difference in the epithelia reconstructed from HKC and HEN was the expression pattern of CK18. HFK and HEC implants were negative, while the HEN implant was positive for the CK18-specific antibody.

The magnifications were 250 X.

Basal membrane was indicated by white bars.


HFK
HEC
HEN
CK19
CK18
CK13
CK10

status.

The HEN implant was positive for the CK18-specific antibody (Figure 3.4). The staining in the basal layer was uniform, while it became heterogeneous in the upper layer cells. The antibody against CK19 stained all the cells in the *in vivo* HEN implants. The antibody for CK13 also stained cells throughout the HEN implant. The CK10 antibody showed a negative staining for the HEN implant.

For the HKC implants, CK18 staining was negative for both HFK and HEC implants (Figure 3.4), in contrast to the positive staining in the HEN implant. While CK10 and CK13 were both positive in the superficial layers of HFK and HEC implants, the former was highly expressed in the HFK implant, while the latter was more prominent in the HEC implant. CK19 was only weakly detectible in HKC implants. While it was mostly observed in the basal cells of the HFK implant, CK19 was also sporadically expressed in suprabasal layers of the HEC implant.

These data from HEN and HEC implants were in good agreement with those described by Smedts *et al.* (1993b) for squamous metaplasia and native ectocervical SSE, respectively. The CK expression patterns in the reconstructed *in vivo* epithelia from the normal cells were summarized in Figure 3.1B.

3.2.2 *In vivo* reconstructed lesions from HPV16-immortalized genital epithelial cells

Three immortalized cell lines have been established in this laboratory from HFK, HEC and HEN by *in vitro* transfection of cloned HPV16 genomic DNA. HFK16-3, HEC16-2 and HEN16-2 were derived from HFK, HEC and HEN, respectively (Sun *et al.*, 1993; Tsutsumi

et al., 1993). These cell lines contained HPV16 DNA and expressed viral E6 and E7 oncogenes. Their immortality has been confirmed by continuous *in vitro* culture for more than 12 months. Similar to their normal parental cells, these immortalized cells all have similar epithelial morphology in *in vitro* monolayer culture (Figure 3.2, bottom panels).

Since their parental cells formed SSE with different properties *in vivo*, the three immortalized derivatives may possess different pathological features *in vivo* as well. To test this possibility, the immortalized cells were also reconstructed into epithelia with the same *in vivo* system.

3.2.2.1 Differential *in vivo* dysplastic morphologies in implants from HPV16-immortalized HKC and HEN

To assure that the *in vivo* system was able to faithfully reconstruct cancerous lesions, CaSki cells, the cell line established from a cervical carcinoma, were tested (Figure 3.5). The *in vivo* lesion from CaSki was a multilayered epithelium with cells showing almost no morphological compartmentalization and mature squamous differentiation. The cells in the lesion showed obvious variation in size and shape. The nuclei were also irregular in shapes, and their size varied greatly. The nucleus/cytoplasm ratio was high. The pathological features of the CaSki *in vivo* lesion were consistent with those for a high grade SIL and in agreement with the original clinical pathological diagnosis.

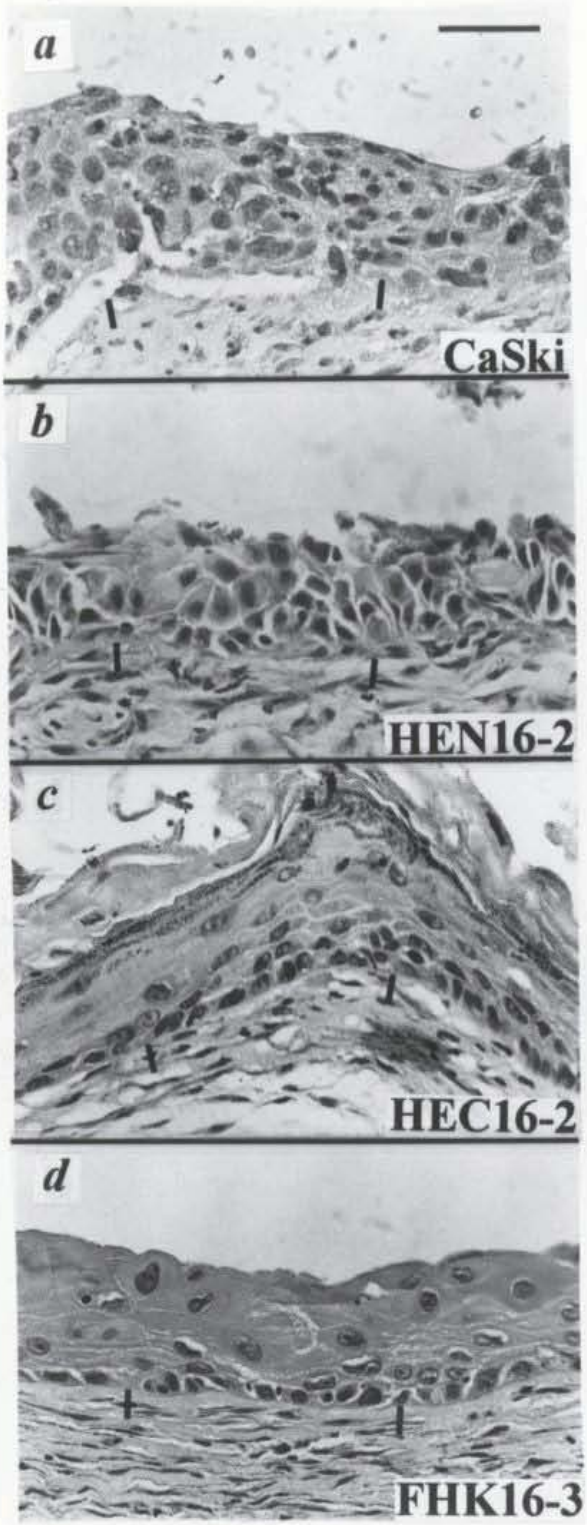
Similar to normal HEN, the HEN16-2 also formed a multilayered epithelium (Figure 3.5*b*). The entire thickness of the epithelium showed no morphological cellular compartmentalization. The cells showed irregular nuclei with significant variations in size and

Figure 3.5 Morphology of the *in vivo* lesions reconstructed from the HPV16-immortalized HKC, HEN and CaSki

The lesion from CaSki and HEN16-2 cells showed the pathological features of a high grade dysplasia, while HEC16-2 and HFK16-3 formed stratified lesions that did not show typical dysplastic changes.

The magnifications were 250 X. H&E staining.

Basal membrane was indicated by black bars.


a
CaSki
b
HEN16-2
c
HEC16-2
d
FHK16-3

shape, and the nucleus/cytoplasm ratio was considerably high. Some typical inter-cellular bridges were observed, indicating the squamous phenotype of the HEN16-2 *in vivo* lesion, although the disorganized cell arrangement did not show any sign of mature terminal squamous differentiation. Serial sections were examined and no indication of invasions could be found. In all the features, the morphology of HEN16-2 was consistent with that for a high grade dysplasia (refer to Figure 1.3E), and was comparable to that of CaSki cervical SCC (Figure 3.5*a*), but was distinct from that of normal HEN (Figure 3.3*c*).

In contrast, HEC16-2 and HFK16-3 formed stratified lesions with obvious squamous differentiation (Figure 3.5*c* and *d*). Both lesions showed cuboidal basal cells and horizontally arranged enlarged suprabasal cells with enriched cytoplasm. The cells also showed prominent inter-cellular bridges, consistent with typical squamous differentiation. The nuclei were generally regular in shape and showed almost no atypical changes. Nevertheless, the upper layers in both lesions showed some disorganization (Figures 3.5*c* and *d*), in comparison with those in their normal counterparts (Figures 3.3*a* and *b*). The histological features of the immortalized HEC are analogous to those of the low grade dysplasias (refer to Figure 1.3D).

3.2.2.2 Differential *in vivo* CK expression in HPV16-immortalized HKC and HEN

The patterns of CK expression in clinical cervical premalignancies have been studied extensively, in hope of finding prognostic markers for cervical premalignant lesions. It has been well documented that expression of the simple epithelial CKs, such as CK8/18, is often associated with the severity of squamous neoplastic lesions (refer to Figure 3.1A) (Smedts

et al., 1990; Ivanyi *et al.*, 1990; Moll *et al.*, 1983; Bobrow *et al.*, 1986; Syrjanen *et al.*, 1988; Lindberg and Rheinwald, 1989). Thus, CK expression in the reconstructed lesions was examined by immunofluorescence (Figure 3.6), to test the correlation between the pathological features and CK expression patterns in the reconstructed *in vivo* lesions from HEN16-2, HEC16-2 and HFK16-3. In addition, since the CKs that are believed to be prognostic for malignancy are constitutively expressed in cervical SCE, a major concern regarding the prognostic value of the CK markers is whether the expression of the simple epithelial CKs may in fact reflect the cell origin of the lesion, rather than the severity of malignancy. Since the *in vitro*-immortalized cells had known origins, the CK expression patterns in the reconstructed *in vivo* lesions should be also of significance in addressing this issue.

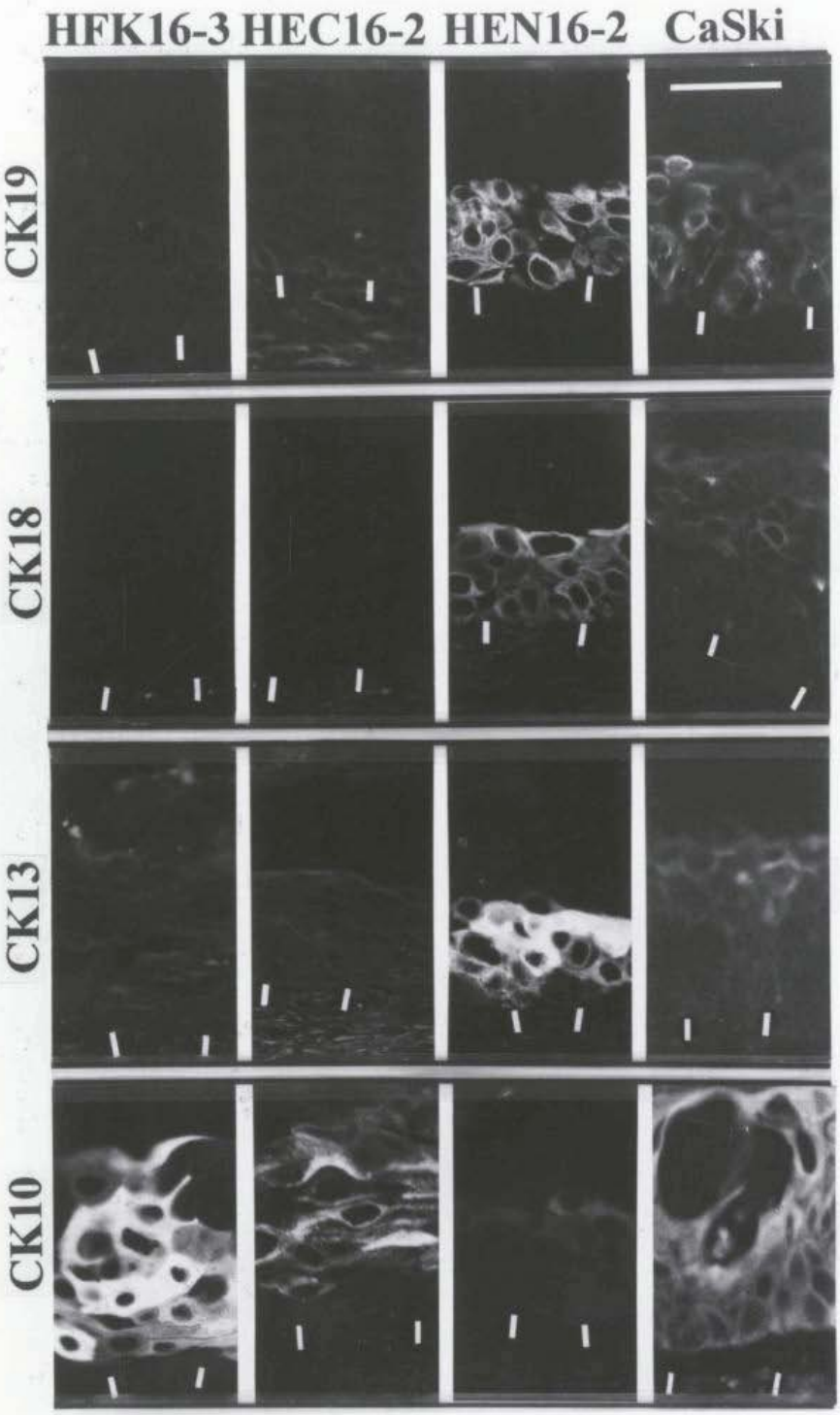
In the HEN16-2 implant, CK18 and CK19 were detectible throughout the lesion, while CK13 was expressed in most cells except a few basal layer cells (Figure 3.6). CK10, which was not detectible in the normal HEN implant (Figure 3.4), was weakly expressed in the superficial cells (Figure 3.6). Significantly, the CaSki *in vivo* lesion displayed a CK expression pattern very similar to that in HEN16-2 (Figure 3.6). For example, the CaSki lesion expressed CK18 and CK19 throughout the lesion, and was also positive for CK10 and CK13, although CK10 was expressed throughout the lesion and CK13 tended to be more compartmentalized in the upper layer than the HEN16-2 lesion. In contrast to the HEN16-2 and CaSki lesions, both HEC16-2 and HFK16-3 were negative for CK18 (Figure 3.6), consistent with the clinical finding that CK18 is not often expressed in low grade CIN. In contrast to the normal HKC implants, the immortalized HKC *in vivo* lesions weakly expressed

Figure 3.6 Indirect immunofluorescence analysis of CK expression in the *in vivo* reconstructed lesions from HEN16-2, HEC16-2 and HFK16-3

The most prominent difference in the *in vivo* lesions reconstructed from the immortalized HKC and HEN was CK18 expression. The HEC16-2 and HFK16-3 lesions were negative, while the HEN16-2 lesion and the CaSki lesion were positive for the CK18-specific antibody.

The magnifications were 250 X.

Basal membrane was indicated by white bars.


HFK16-3
HEC16-2
HEN16-2
CaSki
CK19
CK18
CK13
CK10

CK13, although strong expression of CK10 was retained in the upper layers. In HFK16-3, the weak staining for CK19 became sporadically distributed in the basal and suprabasal cells.

These results showed that the general patterns of CK expression in the lesions from the immortalized HEN and HKC remained distinct from each other, as did their normal counterparts. Although their respective patterns were consistent with their normal counterparts, there were some subtle differences between them.

In the immortalized HEN lesion, CK18 was more homogeneously expressed than in the normal HEN implant, consistent with the clinical finding that CK18 is expressed more often and more extensively in the high than low grade CINs (Smedts *et al.*, 1990; Ivanyi *et al.*, 1990). In addition, CK13 tended to be more compartmentalized in the upper layers than in the normal HEN implant. Further, CK10, which was not expressed in the normal HEN implant, was expressed weakly in the upper layer cells of the HEN16-2 lesion. This suggested that the squamous differentiation in the HEN16-2 *in vivo* lesion was more mature than in the normal HEN implant. For the immortalized HKC *in vivo* lesions, CK13 showed reduced expression in both HEC16-2 and HFK16-3 lesions, and CK19 in HFK16-3 lesion was no longer confined to the basal cells. These differences in the CK expression patterns between the epithelia from normal cells and the lesions from their immortalized derivatives suggested that CK expression can be disrupted in the course of HPV16-mediated oncogenesis, although the main features may be retained. The CK expression patterns in the *in vivo* lesions from the immortalized cells are summarized in Figure 3.1B.

3.2.3 Differential *in vivo* viral E7 expression in HPV16-immortalized genital epithelial cells

Besides expressing simple epithelial CKs, naturally occurring high grade CINs usually feature more extensive expression of HPV16 oncogenes than the low grade ones. Indeed, the progression from low to high grade CINs has been suggested to be associated with enhanced and sustained expression of HPV16 oncogenes (Durst *et al.*, 1991). In addition, the experiments on CK expression described above indicated that the normal, as well as the immortalized, HEN implants may possess a status of differentiation distinct from the those in HKC implants. Since HPV gene expression and virus replication are closely associated with squamous differentiation, the HPV16 oncogenes may be expressed differently in native and metaplastic SSE.

To address this possibility, the expression pattern of HPV16 E7 oncogene was examined in the *in vivo* lesions reconstructed from the HPV16-immortalized cells using *in situ* hybridization assays (Figure 3.7 and 3.8). The HPV16 E7-specific riboprobe was labelled with ^{35}S and was detected by emulsion autoradiography. The signals were presented in the form of deposited silver grains, which can be viewed under the microscope by brightfield and darkfield illumination. Under the latter condition, the signal is presented as obvious white dots in a dark background, helping to emphasize the distribution of silver grains, especially when the signal is weak. Under brightfield illumination, the signal is presented as black grains in the bright background, facilitating revelation of the relationship between the signal and cellular or tissue compartments. The E7-specific probe that was used had also been used in the Northern blot (Section 3.2.4) and RNase protection assays (Belaguli *et al.*, 1992), in which this probe specifically detected mRNA containing HPV16 E7 ORF.

Figure 3.7 **RNA *in situ* hybridization assays for the HPV16 E7 mRNA in the *in vivo* lesions** reconstructed from CaSki, W12 and HaCat reference cells, for experimental controls

The CaSki lesion expressed E7 throughout the epithelium (a and b). The W12 *in vivo* lesion compartmentalized E7 expression in the bottom 2-3 layers (c and d). The E7-specific riboprobe did not detect any signal in the *in vivo* implant from HaCat cells (e and f), which do not contain HPV16.

The magnifications were 250 X.

Basal membrane was indicated by triangles.

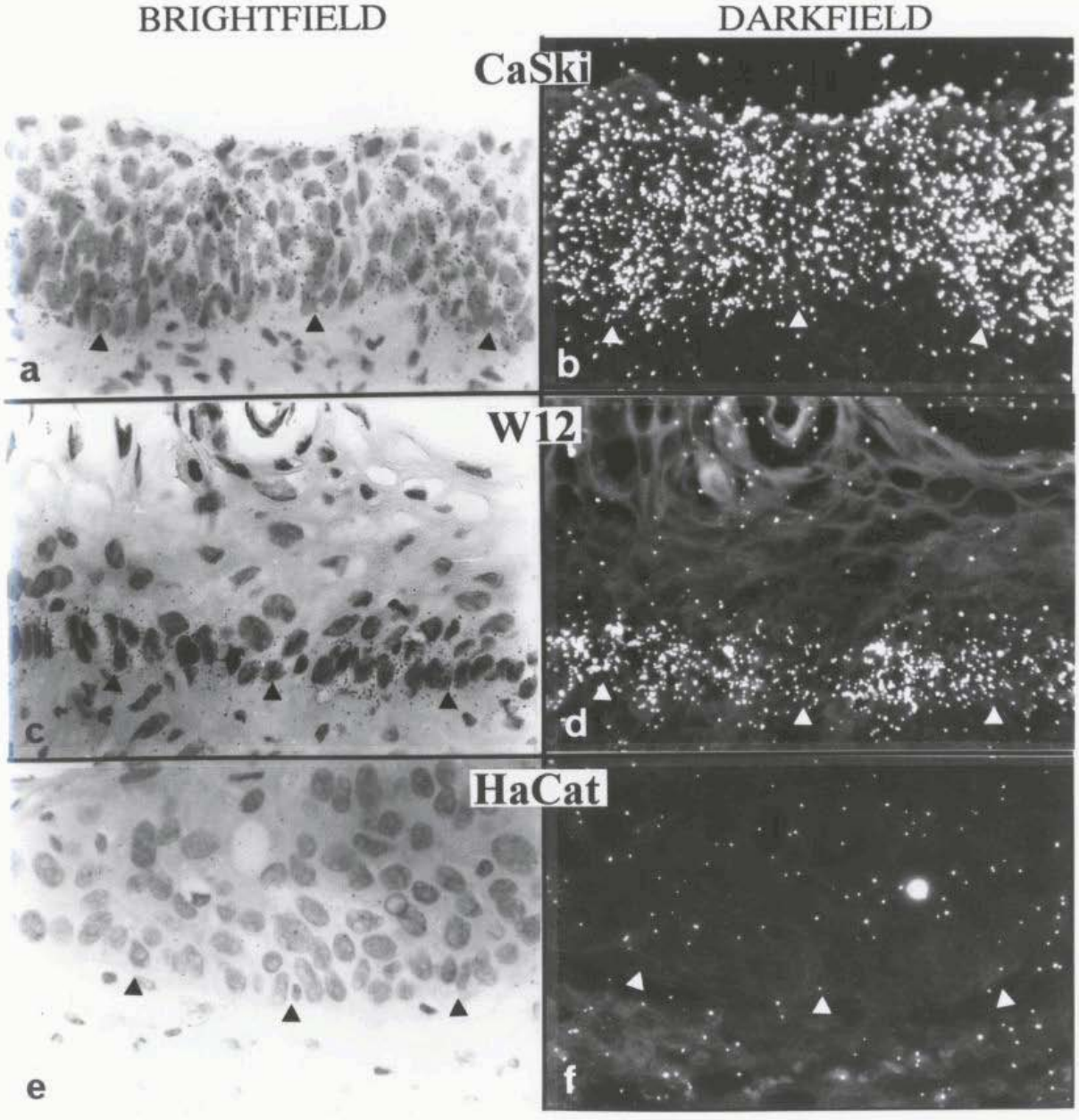
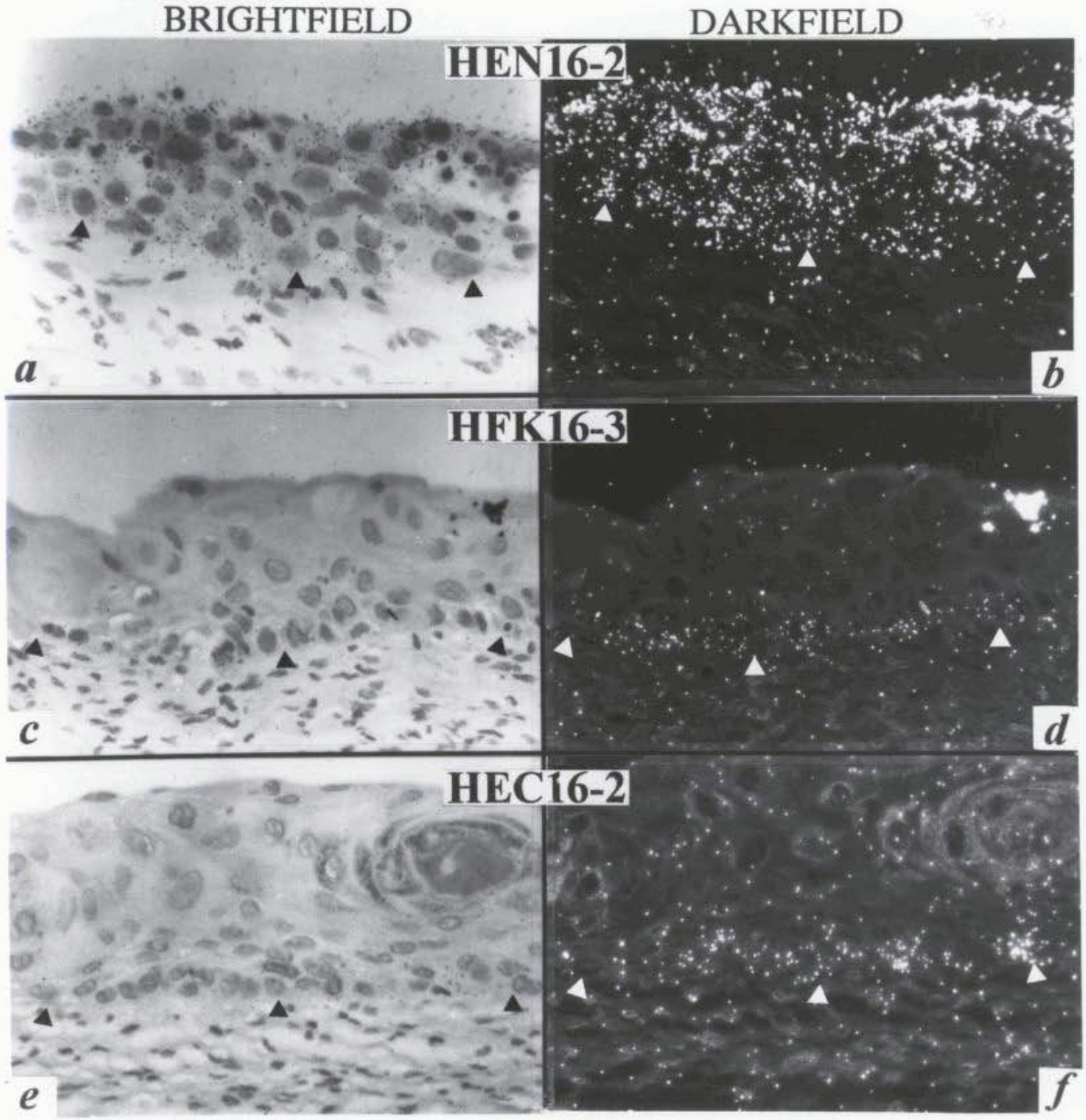

BRIGHTFIELD
DARKFIELD
CaSki
a
b
W12
c
d
HaCat
e
f

Figure 3.8 RNA *in situ* hybridization assays for the HPV16 E7 mRNA in the *in vivo* lesions reconstructed from HEN16-2, HFK16-3 and HEC16-2

The HEN16-2 lesion expressed E7 throughout the lesion (a and b). The HEC16-2 (c and d) and HFK16-3 (e and f) lesions expressed very weak signals that were confined within the basal layer and distributed unevenly among the basal cells.

The magnifications were 250 X.

Basal membrane was indicated by triangles.


BRIGHTFIELD
DARKFIELD
HEN16-2
a
b
HFK16-3
c
d
HEC16-2
e
f

As a first step and controls, HPV16 E7 expression was examined in lesions formed by two cell lines established from naturally occurring HPV16 lesions. CaSki cells contain integrated HPV16 (Pater and Pater, 1985) and W12 cells contain episomal HPV16 DNA (Stanley *et al.*, 1989). *In vivo*, the CaSki cells and W12 cells formed lesions morphologically consistent with those of their original biopsies, cervical SCC and low grade CIN, respectively (Figures 3.7a and c). In the CaSki *in vivo* lesion, E7 was expressed throughout the lesion and the distribution of signals was quite uniform (Figures 3.7a and b). In contrast, E7 signals were compartmentalized within the bottom 2-3 cell layers in the W12 *in vivo* lesion, although the E7-expressing cells appeared to express E7 mRNA at a similar level (Figures 3.7c and d). As a negative control, the same probe did not detect any signal above that of the underlying stroma in the *in vivo* lesion from the HaCat cells (Figures 3.7e and f), a spontaneously immortalized cell line not containing HPV16 (Boukamp *et al.*, 1988).

Weak E7 signals were detected in the lesions formed by HEC16-2 and HFK16-3 (Figure 3.8c d, e and f). Unlike in the CaSki and W12 *in vivo* lesions, signals in HEC16-2 and HFK16-3 were confined within the basal layers and distributed unevenly among the basal cells (Figure 3.8d and f), suggesting that the E7 message may not be expressed at the same level even in the cells in the same epithelial compartment. In contrast, in the HEN16-2 *in vivo* lesion (Figure 3.8a and b), E7 signals were detected throughout the lesion (Figure 3.8b). The distribution of signals in the HEN16-2 lesion was also uneven, indicating that E7 expression among the cells varied.

The compartmentalized expression of the E7 viral gene in the immortalized HKC *in vivo* lesions may have resulted from cell death in this particular epithelial compartment.

Alternatively, all cellular transcription in the suprabasal cells may have been switched-off indiscriminately. To clarify such possibilities, the adjacent serial sections were examined for the pattern of CK1 expression (Figure 3.9). CK1 is expressed in SSE cells undergoing terminal squamous differentiation (Knapp and Franke, 1989). While very low, or no CK1 signals were detectible in the basal layer that contained E7-expressing cells, the immortalized HKC *in vivo* lesions strongly expressed CK1 mRNA in the suprabasal layers that were negative for E7 mRNA (Figure 3.9c and e). Thus, the failure of E7 expression in the upper layer of the HKC *in vivo* lesions was due to the suppression of the HPV16 E7 oncogene. This suppression was apparently accompanied by concurrent activation of the program for squamous differentiation, since the differentiation-dependent expression of CK1 in the HEC *in vivo* lesions showed a reverse and mutually exclusive relationship with the expression of E7.

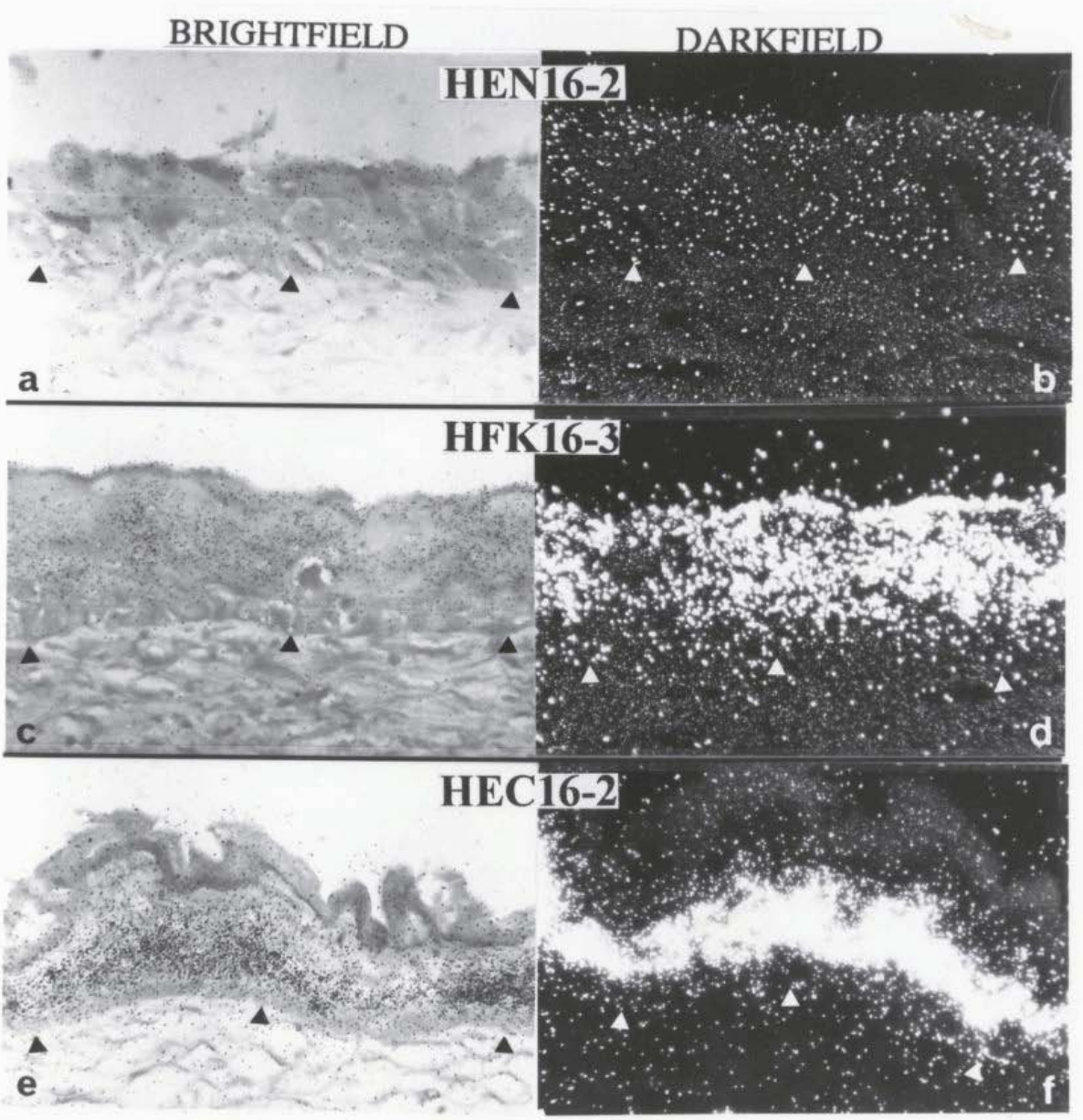
The CK1 gene was expressed at a very low level in the HEN16-2 *in vivo* lesion (Figure 3.9a and b). This is consistent with the immunofluorescence data for CK10 detection (Figure 3.6), since CK1 and CK10 are generally expressed in pairs. However, unlike the CK10 protein, which was detected in the upper layers of the HEN16-2 *in vivo* lesion, CK1 mRNA was diffusely expressed without obvious compartmentalization (Figure 3.9b). Because the expression of CK1/10 is normally activated only in the differentiating SSE cells, the diffuse, non-compartmentalized expression of CK1 indicated that the squamous differentiation in the HEN16-2 lesion was dyscoordinated.

Figure 3.9 RNA *in situ* hybridization assays for the CK1 mRNA in the *in vivo* lesions reconstructed from HEN16-2, HFK16-3, and HEC16-2

Diffuse CK1 signals were detected at a low level in the *in vivo* HEN16-2 lesion (a and b). CK1 was strongly expressed in the suprabasal cells of the HFK16-3 (c and d) and HEC16-2 (e and f) lesions. Note that the basal cells, which were seated on the basal membrane, did not or expressed very weakly the CK1 mRNA in the *in vivo* lesions of HFK16-3 and HEC16-2.

The magnifications were 250 X.

Basal membrane was indicated by triangles.


BRIGHTFIELD
DARKFIELD
HEN16-2
a
b
HFK16-3
c
d
HEC16-2
e
f

3.2.4 HPV16 genome status in the immortalized cells

The differential patterns of the E7 expression in the HPV16-immortalized HKC and HEN lesions may have resulted from differences in status of HPV16 DNA. HPV16 infections containing episomal and integrated viral DNA were suggested to have different expression patterns of viral oncogenes (Durst *et al.*, 1991). Furthermore, since integration is always accompanied with disruptions in the viral genome, the disrupted integrity of the viral genome may result in dysregulated expression of viral genes in the immortalized cells (von Knebel Doeberitz *et al.*, 1991).

To address these possibilities, the status of viral DNA in the immortalized cells was examined by Southern blot analysis with the full length HPV16 DNA probe (Figure 3.10, A panel). To identify HPV16 sequences, the restriction endonucleases BamHI, XbaI, NcoI, and combined BamHI/PstI were used to digest DNA isolated from the immortalized cells and SiHa cells. SiHa is a cervical SCC cell line containing a single copy of HPV16 DNA (Pater and Pater, 1985). The XbaI restriction enzyme has no sites in the HPV16 genomic DNA, NcoI and BamHI have a single site, and PstI has multiple sites. XbaI produced bands larger than 12 kb in all the cell lines, indicating that HPV16 DNA was integrated into cellular DNA. Furthermore, BamHI or NcoI produced a single or double bands in SiHa and HEN16-2 and none of them were approximately 8 kb, consistent with the viral DNA being integrated as a single copy. In contrast, BamHI produced a major fragment of approximately 8 kb and two minor bands in HEC16-2 and HFK16-3, suggesting that the integrated viral DNA contained tandem repeated multiple copies. PstI/BamHI produced the characteristic HPV16 fragments

Figure 3.10 Southern blot analysis of HPV16 and c-H-*ras* DNA sequences in HEN16-2, HEC16-2 and HFK16-3

The restriction endonucleases used are labelled on the top, and the molecular weight markers were indicated on the sides of the figures.

A and **B**: hybridization with the HPV16 probe.

C: panel A rehybridized with the EJ-*ras* probe.

A
B
C
SiHa
HEC16-2
HEN16-2
HFK16-3
HPV16
BamHI
BamHI/PstI
NcoI
XbaI
8 kB
12 kB
8 kB
2.8 kB
1.8 kB
1.0 kB
HPV16 BamHI/PstI
HEN16-2 BamHI/PstI

of 2.8, 1.8, and 1.0 kb bands, together with two smaller bands, in HFK16-3 and HEC16-2. These three bands were also intact in the HEN16-2, which can be seen more clearly from a blot from another experiment with an increased amount of DNA and longer exposure time for autoradiography (Figure 3.10B). Since these three bands span from nt 7009 to nt 4757 in HPV16 genome, the LCR, E6, E7, E1, E2, and E5 sequences should be intact in the integrated viral DNA in all three immortalized cell lines.

The DNA loaded for each sample was calibrated with the DNA dosage of c-Ha-*ras* as an internal control (Figure 3.10C). The blot membrane used for Figure 3.10A was stripped of the probe for HPV16 and then was reprobed with a c-Ha-*ras* probe. The c-H-*ras* probe hybridized to a single 7.7 kb band in BamHI-digested DNA, which was consistent with the expected genomic sequence of H-*ras* (Riou *et al.*, 1988).

To test whether the integrated HPV16 LCR sequence remained in control of viral gene expression, E7 expression in the immortalized cells was examined for the response to TGF-β by Northern blot assays (Figure 3.11). Other workers have already shown that this cytokine represses HPV16 E7 expression in HPV16-immortalized cells (Woodworth *et al.*, 1990a; Pietenpol *et al.*, 1991; Braun *et al.*, 1992). The riboprobe used for *in situ* hybridization assays was used in a Northern blot for E7 detection. Thus, this experiment also provided information on the specificity for the E7-specific probe. The E7-specific riboprobe detected one major band of 1.7 kb in all the three cell lines (Figure 3.11a). HEN16-2 and HEC16-2 expressed the E7 oncogene at a similar level *in vitro*, while the two cervical cell lines expressed a higher level of the E7 message than HFK16-3 did. Nevertheless, TGF-β repressed E7 expression in all the cell lines significantly.

Figure 3.11 Northern blot analysis of *in vitro* expression of HPV16 E7, c-*myc*, and γ-*actin in the immortalized cells in response to* TGF-β1

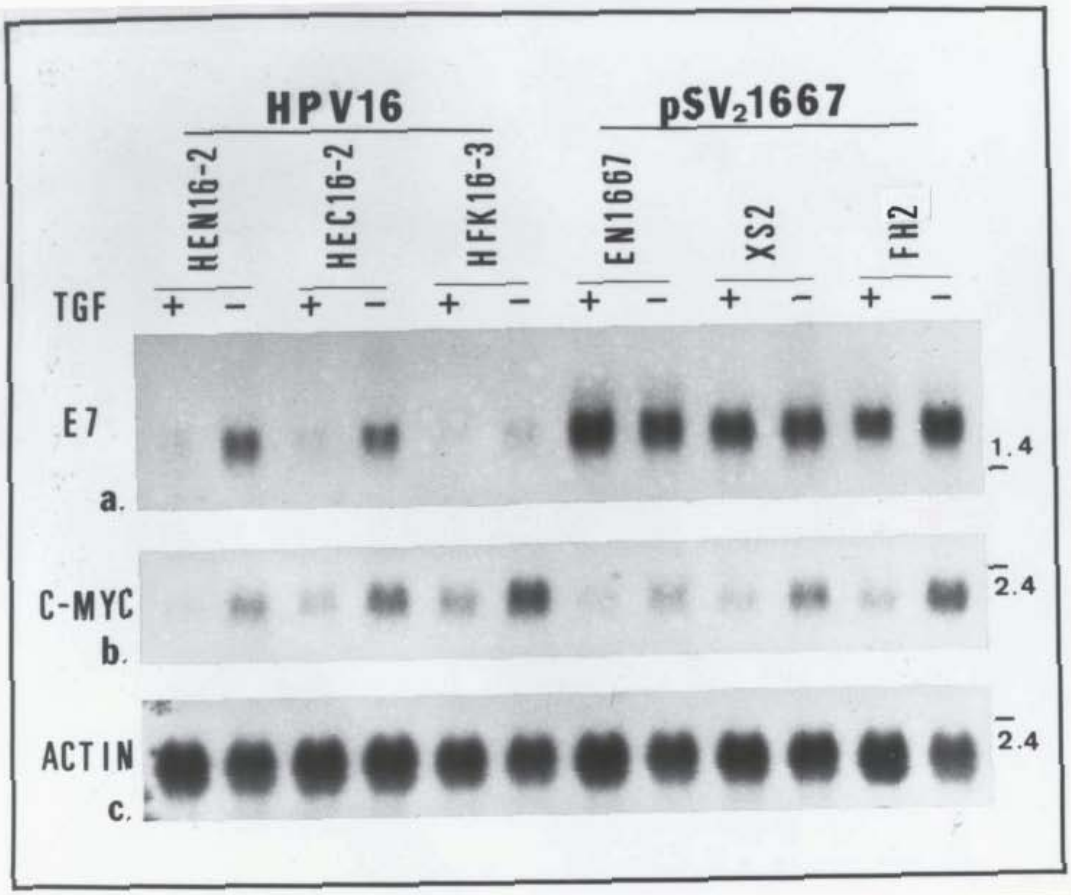
Samples treated or untreated with TGF-β are indicated by "+" or "-" on the top of the figure.

Molecular weight in kb was indicated on the right side of the figure

a: riboprobe for HPV16 E7 ORF.

b: probe for c-*myc*.

c: probe for γ-actin, as internal control.


HPV16
pSV2 1667
HEN16-2
HEC16-2
HFK16-3
EN1667
XS2
FH2
TGF + - + - + - + - + - + -
E7
a.
1.4
C-MYC
b.
2.4
ACTIN
c.
2.4

These results suggested that the HPV16 LCR was functional in controlling the E7 ORF in all the immortalized cells (Figure 3.11a). Since TGF-β repression to viral E7 may be mediated by cellular or/and viral factors (Pietenpol *et al.*, 1990; Pietenpol *et al.*, 1991), this result also indicated the functions of these factors must also have been retained in the immortalized cells. Expression of γ-actin was used as an internal control for Northern blot to monitor the quality of RNA transfer and detection (Figure 3.11c).

3.2.5 Expression of viral and cellular genes possibly involved in cell growth and cervical oncogenesis

The differences in the pathological features between the *in vivo* lesions from the immortalized HEN and HKC may be caused by other viral and/or cellular genes. One of the viral genes that potentially could contribute directly to the cellular phenotype is HPV16 E5. Although it has not been shown that HPV16 E5 is involved in immortalization or transformation of human cells, E5 does transform murine cells (Leechanachai *et al.*, 1992; Pim *et al.*, 1992; Cohen *et al.*, 1993; Straight *et al.*, 1993). Since the HPV16 E5 protein may interfere with the down-regulation of certain tyrosine kinase receptors for protein/peptide growth factors, a higher level of E5 expression could potentially lead to more active proliferation of the immortalized cells (Finbow *et al.*, 1991; Straight *et al.*, 1993; Bouvard *et al.*, 1994a). To address this consideration, expression of E5 in the immortalized cells cultured *in vitro* was examined by Northern blot (Figure 3.12). While an HPV16 E5-specific probe detected a 2.3 kb band in the HEC16-2 and HFK16-3, HEN16-2 did not produce, or produced undetectable, E5 mRNA (Figure 3.12a). Expression of γ-actin was used again as

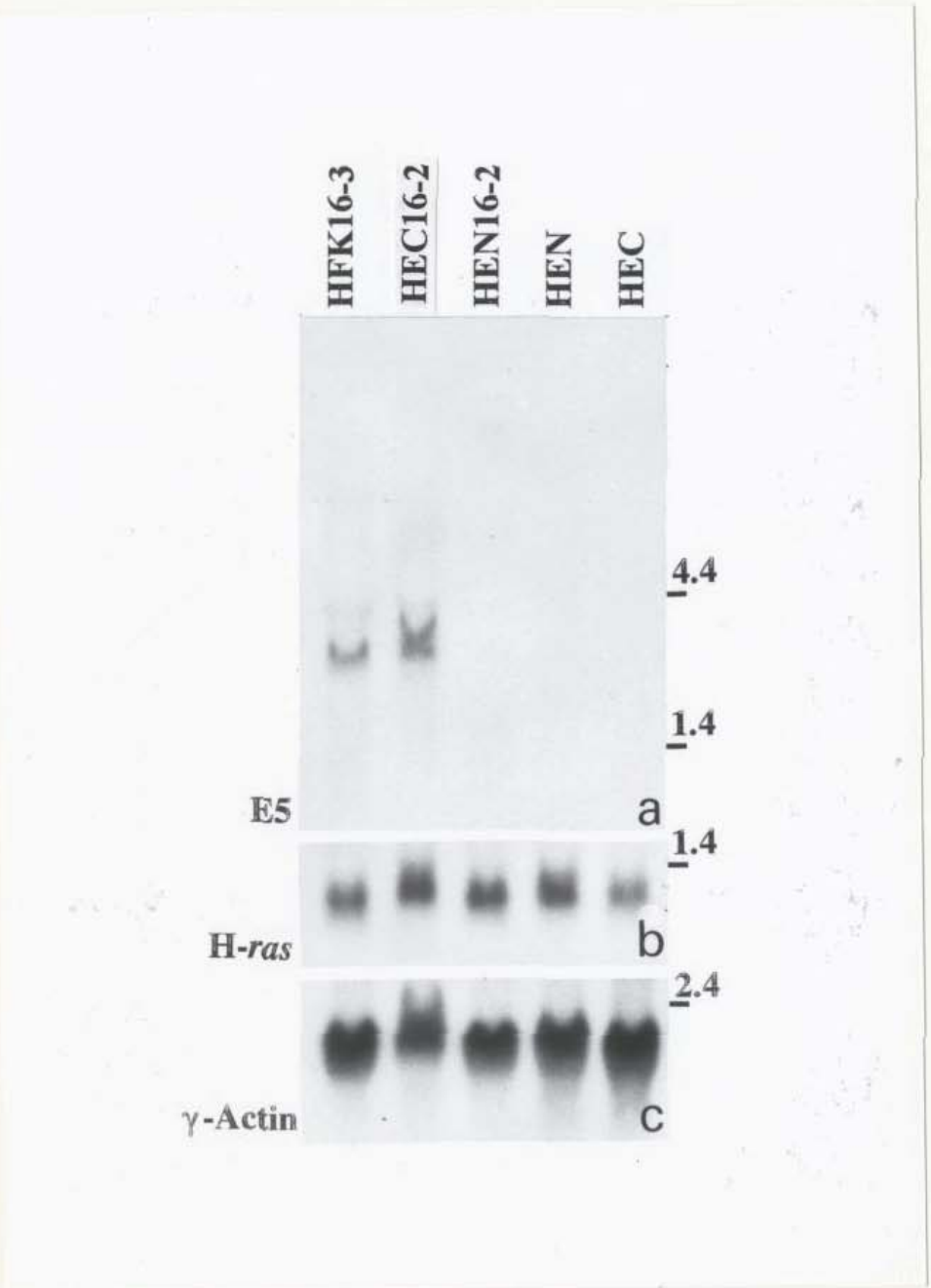
Figure 3.12 Northern blot analysis of *in vitro* expression of HPV16 E5, c-H-*ras* and γ-actin in HEN16-2, HEC16-2 and HFK16-3

Molecular weight was indicated on the right side of the figure.

a: probe for HPV16 E5.

b: probe for H-*ras*.

c: probe for γ-actin, as internal control.


HFK16-3
HEC16-2
HEN16-2
HEN
HEC
4.4
1.4
E5
a
1.4
H-ras
b
2.4
γ-Actin
c

an internal control for the sample loading in the Northern blot (Figure 3.12c).

Another possibility concerning the increased dysplastic phenotypes of the HEN16-2 *in vivo* lesion, was that cellular oncogenes were involved. The immortalized cells might differentially overexpress certain protooncogene(s) due to DNA recombination events that might have occurred during or after immortalization. Indeed, clinical studies indicated that overexpression of c-*myc* and H-*ras* protooncogenes was associated with oncogenesis of cervical neoplasia, and cases were reported in which HPV DNA integration occurred near the c-*myc* locus (Riou *et al.*, 1987; Crook *et al.*, 1989b; Crook *et al.*, 1989a; Lazo *et al.*, 1989; Crook *et al.*, 1990; Sagae *et al.*, 1990; Pim and Banks, 1991). To exclude the possibility that these two protooncogenes were up-regulated, expression of c-*myc* and H-*ras* was examined in the immortalized cells cultured *in vitro* by Northern blot assays. The blots used for probing HPV16 E7 and E5 were stripped, and rehybridized with probes specific for c-*myc* and H-*ras*, respectively. Results showed that neither c-*myc* (Figure 3.11b), nor H-*ras* (Figure 3.12b), was expressed with significant variations among the immortalized cells.

Thus, it was unlikely that HPV16 E5, c-*myc* or H-*ras* contributed significantly to the distinct *in vivo* phenotypes of the immortalized HEN.

3.2.6 Growth properties of the immortalized cells

One fundamental difference between normal and malignant cells is that malignant cells possess uncontrollable growth potential. In fact, increased growth constitutes the pathological basis for malignant diseases. Since oncogenesis is a multistep process, cells undergoing oncogenesis usually display different growth potentials depending on the oncogenic stage.

This biological behaviour of cells undergoing oncogenesis often can be reflected by several experimental criteria, such as optimal regeneration time, anchorage-independent growth and tumorigenicity in immuno-deficient animals. Since the immortalized HEN and HKC lesions possessed distinct dysplastic features, they may also have different growth potentials.

The doubling time of the immortalized cells was examined in *in vitro* monolayer culture. After plating 1 X 10^7 cells into 60-mm diameter plates, the cells were recovered and counted at two day intervals. Results showed that while HEN16-2 had an average doubling time of 25±2 hours, HEC16-2 and HFK16-3 had an average doubling time 31±4 and 24±3 hours respectively. This indicated that at the stage of immortalization, the *in vivo* dysplastic morphologies of the immortalized cells did not correlate with their *in vitro* growth.

The immortalized cells were further tested for their potential for anchorage-independent growth in soft agar essays. While SiHa cell, a cell line derived from a cervical carcinoma, formed colonies in soft agar, all the immortalized cells failed to do so in the same conditions (data not shown). For tumorigenicity, the cells were injected subcutaneously into nude mice. SiHa cells formed progressively growing tumours from the injected cell mass, while the cell masses of the immortalized cells regressed, and no tumour appeared over a period of 3-6 months (data not shown). These results showed that the immortalized cells had acquired only limited oncogenic potentials.

3.2.7 HPV16 E7 and CK expression in the tumour from fully transformed HEN

Although HEN16-2 was not tumorigenic in athymic animals, HEN16-2 treated *in vitro* with the carcinogen NMU produced tumours when injected into nude mice (Pater, M.M.

and Pater, A., unpublished observation), while NMU failed to immortalize or transform normal human cells (Munoz and Bosch, 1992; Garrett *et al.*, 1993). One primary tumour from NMU-treated HEN16-2 was examined for morphology, HPV16 E7 expression and CK expression, to determine whether and which changes were associated with the subsequently acquired tumorigenic phenotype. The tumour formed by the NMU-treated HEN16-2 displayed invasive growth and showed moderate squamous differentiation (Figure 3.13, H&E panel). *In situ* hybridization assays showed that the HPV16 E7 was expressed extensively in the tumour, even in areas with morphological terminal squamous differentiation (Figure 3.13, E7 panel, arrowhead). Similar to the *in vivo* implants formed by its parental HEN16-2, CK19 was expressed throughout the tumour epithelium and CK10 was hardly detectible in the superficial layers. However, the weakly expressed CK13 became more compartmentalized in the upper layers than did the premalignant lesion. In contrast, CK18 became undetectable in the tumour. These results suggested that, while the pattern of extensive expression of HPV16 E7 was retained in the fully transformed HEN tumour, or perhaps required for tumour development, the patterns of CK expression were not necessarily conserved during the progression of HPV16-related oncogenesis.

3.3 Discussion

Tissue-specificity and differentiation-dependency are inherent nature of HPV16 reproduction. These strict requirements for HPV16 vegetative replication have greatly hindered research on the virology and oncogenic mechanisms of HPV16, since no practical

Figure 3.13 RNA *in situ* hybridization and indirect immunofluorescence assays for the expression of HPV16 E7 and CKs in the tumour formed by NMU-transformed HEN16-2

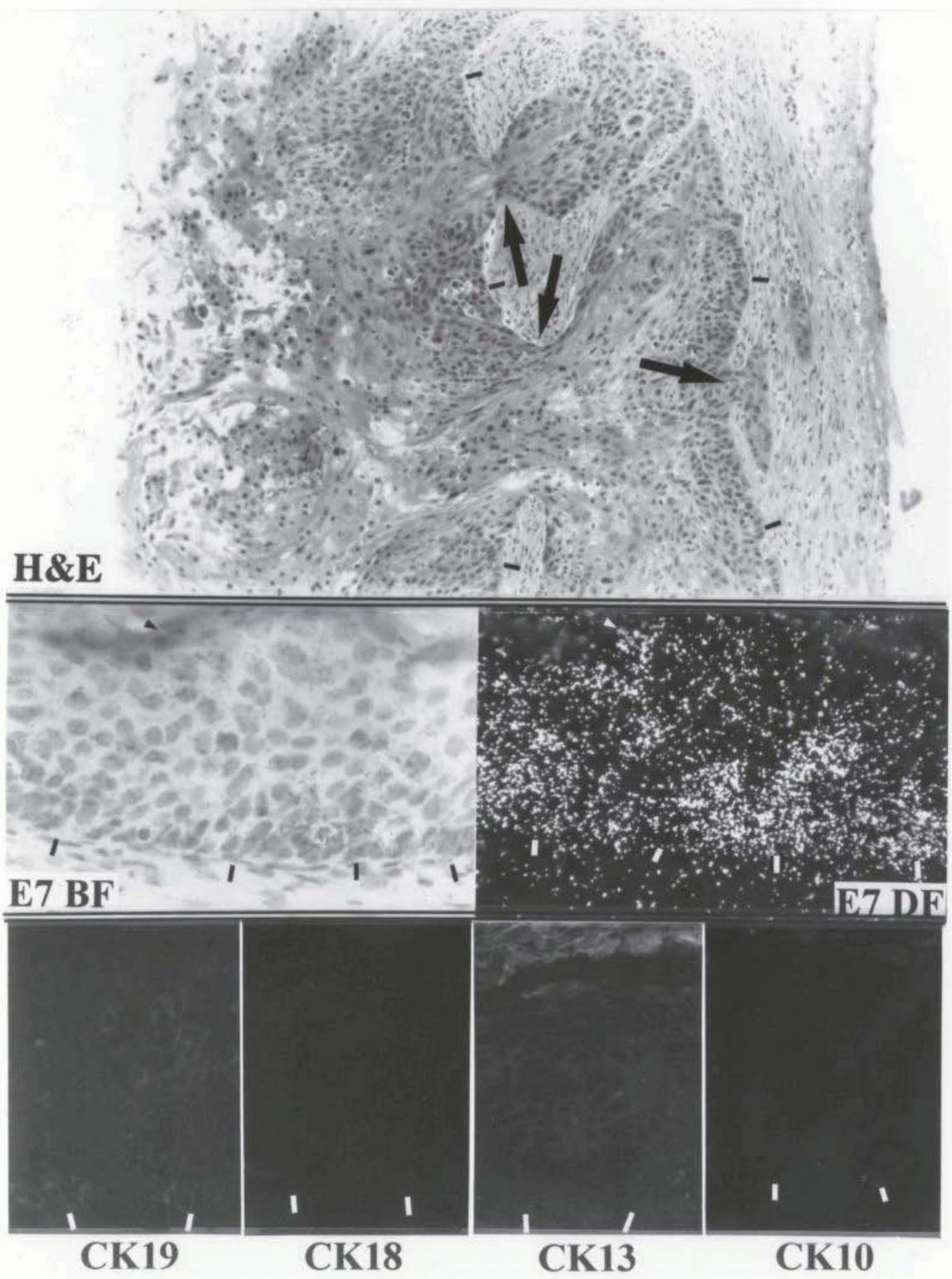
H&E: arrows indicate local tumour invasions into mouse stroma.

E7BF and E7DF: arrowheads indicate the superficial cells expressing viral E7 oncogene.

Panels in the bottom row: weak expression of CK19 throughout the lesion, and of CK13 in the upper half of the lesion.

Magnifications were 150 X in the H&E panel and 250 X in panels E7BF, E7DF, CK19, CK18, CK13 and CK10.

Basal membrane was indicated by bars.


H&E
E7 BF
E7 DF
CK19
CK18
CK13
CK10

experimental system supporting HPV vegetative infection has been available. Indeed, only very recently, some success was made to achieve vegetative infection in *in vitro* condition using a special cell line that contains episomal HPV31b genomes (Bedell *et al.*, 1991; Meyers *et al.*, 1992; Hummel *et al.*, 1992; De Geest *et al.*, 1993). Although mature virions were shown to be produced in this system, reinfection using the virions produced has not been reported. Therefore, studies on HPV16-mediated oncogenesis have relied on potential HPV target cells cultured *in vivo*, a condition that fails to allow programmed squamous differentiation and HPV16 vegetative infection.

In vitro monolayer cultured cells have limitations for HPV research. Indeed, gene expression of keratinocytes in monolayer culture is aberrant, perhaps due to the fact that the current *in vitro* culture regimen does not duplicate the environment of natural tissue (Turyk *et al.*, 1989). Since cells are grown on a two-dimensional plate in *in vitro* monolayer culture, the physical relationship among cells cannot be correlated with their cellular physiological or differentiation status. As a result of the lack of this correlation, and in consideration of possible aberrant gene expression and differentiation, determining the physiological and differentiation status of the cells cultured in *in vitro* monolayer is often difficult. This is particularly the case for interpreting a heterogenous expression pattern of genes related to differentiation. For example, CK13 is heterogeneously expressed in human genital epithelial cells cultured in *in vitro* monolayer (Tsutsumi *et al.*, 1992). Since the relationship between the expressing cells and non-expressing cells cannot be determined, confidence is lacking that this is due to ongoing differentiation, or aberrant expression. This dilemma can be avoided

by using the epithelia reconstruction systems, since the physical relationship between cells in the reconstructed tissues can always provide a reliable reference for making a confident judgement. For example, an upper layer expression of CK13 in the reconstructed SSE can be regarded as a reflection of ongoing normal squamous differentiation, while a CK13 expression in other epithelial compartments is a sign of aberrant differentiation.

3.3.1 Cervical squamous metaplasia and endocervical SCE

The *in vivo* implantation system enabled the current study to reconstruct well-differentiated SSE from *in vitro* cultured HFK and HEC. These reconstructed epithelia were morphologically very similar to their respective origin tissues from which the cultured cells were derived. CK expression patterns in these *in vivo* implants confirmed that these reconstructed SSE resemble their natural counterparts. Most importantly, CK18, which is detectible in monolayer HKC by antibody CY90 (Tsutsumi *et al.*, 1992), became negative in the *in vivo* HKC implants, as in native SSE. This result suggested that the aberrant gene expression in monolayer-cultured HKC was corrected in the *in vivo* condition. The subtle differences in the CK expression between the reconstructed HFK and HEC *in vivo* implants indicated that the differences between the native SSE may mainly result from the inherent nature of the respective epithelial cells, rather than from the anatomical micro-environments. Furthermore, the successful revelation of these subtle differences also attested to the reliability of this *in vivo* system for faithful reconstruction of squamous epithelia.

The observation that the *in vitro* cultured endocervical epithelial cells were reconstructed into metaplastic SSE, rather than SCE, was intriguing. However, it was not

surprising, in consideration of the morphological transition of HEN in monolayer culture (Turyk *et al.*, 1989). The metaplastic nature of the HEN implants was substantiated by the CK expression pattern. One of most prominent features for squamous metaplasia is the co-expression of CK18 and CK13, since the former is not detectible in native and mature metaplastic SSE, while the latter is not expressed in endocervical SCE. These two CKs were both fairly strongly expressed in the HEN implant, as in the naturally occurring immature metaplasia. It has been shown that in naturally occurring metaplasia CK19 expression becomes mostly compartmentalized in the bottom layers and CK10 protein emerges in the upper layers as metaplasia matures (Smedts *et al.*, 1990; Smedts *et al.*, 1993b). In the normal HEN *in vivo* implant, CK19 was expressed throughout the implant, while CK10 was not detectible. Thus, the combination of CK expression patterns suggested that the metaplastic state of the HEN *in vivo* implant may represent immature metaplasia.

The current study indicated that the progenitor cells of metaplastic SSE were present in the epithelial culture from the endocervix. Since the endocervices used to prepare HEN culture were distal to the TZ, it was unlikely that the HEN culture contained cells from the TZ. If the cell population in HEN culture was derived from endocervical SCE, then the metaplastic process that the progenitor cells of metaplastic SSE underwent must have occurred during either *in vitro* cultivation or *in vivo* reconstruction. The results from the current study argued for the former possibility. Indeed, the transition in the morphology of the *in vitro* HEN culture from pleomorphic to keratinocyte-like cells, which was not observed in HEC and HFK culture, may be associated with the metaplastic process. In addition, if the metaplastic process occurred only during *in vivo* epithelia reconstruction, it would be

expected that at least some cells with columnar morphologies should have existed in the reconstructed epithelium. The result from the current study, that no such cells were observed, even in the HEN implant that had been placed *in vivo* for a short period of time, was consistent with the view that the metaplastic changes in HEN occurred when the cells were cultivated in *in vitro* monolayer. Thus, it is an interesting possibility that the *in vitro* culture condition in KGM may initiate the progenitor cells of metaplastic SSE to undergo a process identical, or related to, the naturally occurring squamous metaplasia by a default mechanism. Furthermore, since the *in vitro* cultivated HEN produced immature rather than mature metaplasia, the process of metaplasia in *in intro* culture may be time dependent. This is consistent with the finding made with the naturally occurring metaplasia that metaplasia can be observed at various stages. This possibility implies that modification of the *in vitro* culture conditions may lead to better maintenance of glandular phenotypes and finally lead to successful reconstruction of SCE.

The exact endocervical cell type giving rise to the metaplastic HEN cannot be determined from the current study. The cells potentially derived from the stroma were effectively controlled by limited digestion of the biopsy tissues. In addition, KGM with low concentration of calcium is not suitable for the growth of fibroblasts, and preferentially selects epithelial cells. Thus, the precursors of HEN in the current study may be constitutively present in the endocervical SCE. This idea is consistent with the results of an extension study (Tsutsumi *et al.*, 1993). It showed that the proliferating cells in the HEN culture had properties of the mucus-secreting SCE cells, which are the major cell type in endocervical SCE. The current study also failed to address the relationship between the progenitor cells

for the metaplastic SSE and those for endocervical SCE. Again, based on the finding that the metaplastic cells contain mucus elements, the reserve cells and their derivative metaplastic cells can be regarded as being derived from de-differentiated and transdifferentiated endocervical mucus-secreting SCE cells. Consistent with this possible mechanism is the observation that some mucus-secreting cells in SCE are found to undergo DNA replication and cell division (Hiersche and Nagl, 1980). This observation was used to suggest that SCE is regenerated by replication of some of the differentiated functional SCE cells. The current study did not exclude the possibility that metaplastic SSE was derived from a unique category of bi-potent reserve cells in endocervical SCE. Under normal *in vivo* conditions, these bi-potent reserve cells regenerate the endocervical SCE. In a changed environment, such as in *in vitro* culture, the reserve cells may change their differentiation program, shifting toward the one required for SSE. In all the possibilities, metaplasia must inevitably involve a process of transdifferentiation from one cellular differentiation commitment to the other. The possible molecular mechanism involved in transdifferentiation and its impact on HPV16 activity will be discussed in Chapter 6. Although the questions concerning these progenitor cells remain unsolved, the finding of the current study, that the process of metaplasia occurred *in vitro* culture, will be of help to address the nature of the precursors of SCE and metaplastic SSE in future studies.

3.3.2 HPV16-mediated oncogenesis and genital epithelial cell phenotypes

Similar to their normal counterparts, the HPV16-immortalized cells cultured in *in vitro* monolayer did not show significant differences in morphology and CK expression. However,

when tested *in vivo*, significant differences emerged.

The immortalized HEN and HKC retained many basic features of their parental cells. Similar to normal HEN, the HPV16-immortalized HEN formed a multilayered lesion failing to show mature squamous differentiation. Consistent with the SCE origin of HEN, the HPV16-immortalized HEN *in vivo* lesion expressed the simple epithelial CK18. In addition, expression of CK19 and CK13, which were expressed in the normal HEN implant, was also retained in the immortalized HEN *in vivo* lesion. In contrast, the *in vivo* lesions from the HPV16-immortalized HKC displayed features typical of native SSE, such as compartmentalized cell morphology and mature squamous differentiation. Consistent with their morphology, differentiation marker CK10 was expressed in the correct epithelial compartment in the HKC *in vivo* lesions. Studies by others have also reported that the spontaneously immortalized keratinocytes (Boukamp *et al.*, 1988; Ryle *et al.*, 1989; Boukamp *et al.*, 1990b; Fusenig *et al.*, 1990; Breitkreutz *et al.*, 1991), or those immortalized by HPV16 (Waggoner *et al.*, 1990; Woodworth *et al.*, 1990b; Blanton *et al.*, 1991; Durst *et al.*, 1991; Merrick *et al.*, 1992) retained their potential for programmed squamous differentiation.

However, the immortalized cells also developed phenotypes that were distinct from those of their parental counterparts. The cells throughout the entire HEN16-2 *in vivo* lesion showed obvious dysplastic changes. In addition, CK18 and CK13 became more extensively expressed in the HEN16-2 *in vivo* lesion than in the normal HEN implant. In contrast to a more immature phenotype suggested by the extensively expressed CK18, the HEN16-2 *in vivo* lesion developed a positive, albeit weak, expression of CK10, which is normally present only in mature metaplastic SSE (Smedts *et al.*, 1992a; Smedts *et al.*, 1993b). The aberrant

co-expression of CK18 and CK10 suggested that the differentiation status in the immortalized HEN lesion was disorganized. This impression gained support from the expression pattern of the CK1 mRNA in the HEN16-2 lesion, which was distributed throughout the epithelium (Figure 3.9b), instead of being compartmentalized in the upper layer cells as did in the immortalized HKC *in vivo* lesions (Figure 3.9d and e) (Knapp and Franke, 1989; Durst *et al.*, 1991). Significantly, the immortalized HKC lesions did not show obvious dysplastic changes, in comparison with the immortalized HEN. However, disruption in CK expression was also observed in the immortalized HKC lesions. Expression of CK19 in the HFK16-3 lesion was no longer compartmentalized in the basal layer, as did it in the normal HFK implant, but also occurred in the suprabasal cells. In addition, CK13, which is the first and the second major CK expressed in the differentiating HEC and HFK, respectively, had a reduced expression in both the HKC and HFK lesions. Reduced expression of CK13 has also been reported in cervical premalignant lesions (Smedts *et al.*, 1990; Malecha and Miettinen, 1991).

The CK expression data obtained from the tumour formed by the NMU-treated HEN16-2 provided more insight into the relationship between CK expression and HPV16-mediated oncogenesis. CK18 was not expressed in this tumour, in contrast to the reconstructed *in vivo* epithelium and lesion from its normal and immortalized parental cells. This result suggested that the expression of the simple epithelial CKs may in fact reflect the differentiation status of the epithelia, rather than directly correlating with the severity of malignancy. However, since malignant severity of tumours does correlate with the maturity of differentiation, CKs such as CK18 may still be relevant markers for reflecting the severity of malignancies. The results indicated that the patterns of CK expression in HPV16-induced

SIL were greatly dependent on the types of the target tissues, although changes in CK expression may occur during the process of HPV16-mediated oncogenesis, possibly as a result of secondary genetic events.

3.3.3 HPV16-mediated oncogenesis and tissue-specific HPV16 E7 expression

The current study showed that the HPV16 E7 gene was differentially expressed in the immortalized HEN and HKC *in vivo* lesions, a difference not attributable to any significant difference in the status of viral DNA. Southern blot analysis showed that all three cell lines contained integrated viral DNA. In addition, the viral DNA sequences containing LCR, the early coding region and the early polyadenylation signal were apparently intact in all the cell lines. Consistent with DNA data, functional Northern blot RNA assays confirmed that the expression of E7 was still negatively responsive to TGF-β-mediated repression in all the cell types, indicating that the expression of viral oncogenes remained under control of the HPV16 LCR.

The *in vivo* expression of HPV16 E7 in the immortalized HKC lesions was compartmentalized strictly in the basal cells. In contrast, the expression of E7 was sustained throughout the HEN lesion, despite the similar viral DNA status in HEN16-2 to that in the immortalized HKC. Significantly, E7 expression in the W12 *in vivo* lesion also showed a compartmentalized pattern similar, but not identical, to that in the HKC lesions. Because W12 cells contain episomal HPV16 DNA (Stanley *et al.*, 1989; Sterling *et al.*, 1990), this result suggested that the restricted expression of the HPV16 E7 oncogene in the HEC lesions may be largely irrelevant to the status of viral DNA.

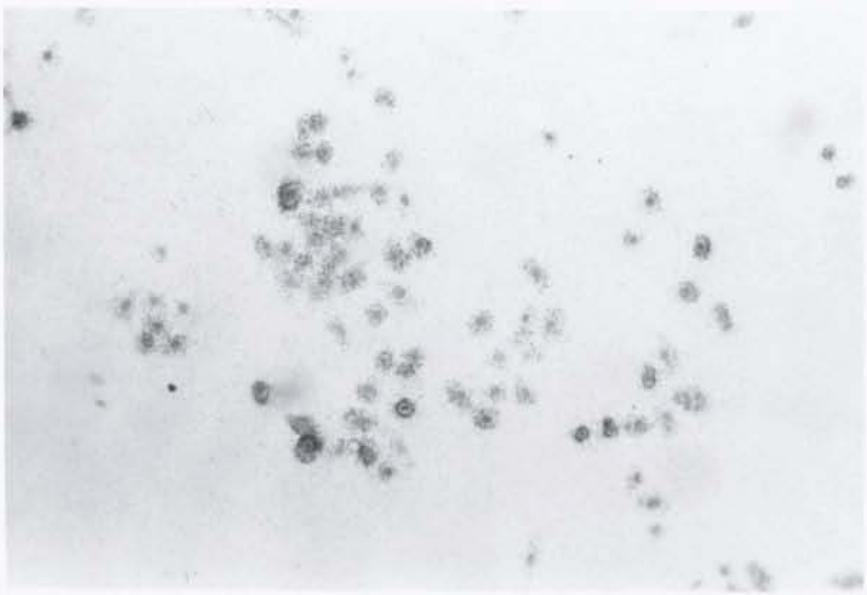
The failure of E7 expression in the upper layer cells of the HKC lesions was due to suppression of expression. This conclusion was based on the finding that CK1 in the HKC *in vivo* lesions was clearly expressed in the suprabasal cells that failed to express E7, while it was not expressed in the basal cells that showed E7 expression. Durst *et al.* (1991) also reported a similar phenomenon in their HPV16-immortalized HFK. Since most, if not all, W12 cells cultured in *in vitro* monolayer expressed E7 (Figure 3.14), the repression of E7 may only occur in the *in vivo* condition, or in conditions allowing programmed squamous differentiation. This view is consistent with the finding that although HEN16-2 and HEC16-2 expressed a similar level of E7 *in vitro*, the lesions formed by these two type cells expressed E7 in drastically distinct patterns *in vivo*. Indeed, although HEC16-2 expressed more abundant E7 *in vitro* than HFK16-3, both cells expressed E7 in a similar pattern *in vivo*. Thus, the differential E7 expression between the HEN and HKC *in vivo* lesions could be due to tissue specificity. This view is consistent also with other observations. The cancerous *in vivo* lesion from CaSki also expressed E7 in a pattern similar to the immortalized HEN lesion. Although the exact origin of CaSki cells is unknown, the pattern of CK expression in the CaSki *in vivo* lesion was consistent with that for cervical SCE (Sun *et al.*, 1993; Gorodeski *et al.*, 1994). Exclusive basal cell expression of the HPV16 E7 gene has not been reported in naturally occurring low grade preneoplastic lesions containing either integrated or episomal HPV16 DNA. However, it has been reported that carcinomas from native SSE, such as the vulva and penis, expressed HPV16 early genes in the basal layers (Higgins *et al.*, 1992c).

Figure 3.14 RNA *in situ* hybridization assays of the expression of HPV16 E7 in W12 cells cultured in monolayer

All the W12 cells in *in vitro* monolayer expressed HPV16 E7, in contrast to the compartmentalized expression of E7 in the *in vivo* lesion.

The magnification was 300 X.

Brightfield illumination.



3.3.4 HPV16-mediated oncogenesis and the target tissues of HPV16 infection

Being recognized as the natural target for HPV16 infection, human keratinocytes from the foreskin and ectocervix, and epithelial cells from the cervical TZ, have been used for studying HPV16-mediated oncogenesis (Woodworth *et al.*, 1989; Schlegel *et al.*, 1988; Barbosa and Schlegel, 1989; Munger *et al.*, 1989a; Sedman *et al.*, 1991; Halbert *et al.*, 1991). Consistent with the results from the current study, all the reported immortalized cells contained integrated viral oncogenes and actively expressed them. HPV16-immortalized HKC has been regarded as representing an early premalignant stage in the multistep process of HPV16-mediated oncogenesis, based on the fact that these cell lines are non-tumorigenic and usually lack anchorage-independent growth. Previous studies have also tested the immortalized or transformed HKC in various epithelia reconstruction systems (McCance *et al.*, 1988; Hudson *et al.*, 1990; Waggoner *et al.*, 1990; Woodworth *et al.*, 1990b; Durst *et al.*, 1991; Dollard *et al.*, 1992). Also consistent with the current study, lesions from some of these cells presented morphologies of low grade dysplasia. Durst *et al.* (1991) examined the expression of the HPV16 early genes in the reconstructed lesions from immortalized HFK, and obtained data in agreement with those from the current study. CK expression in the HPV-immortalized HKC was not systematically studied under conditions allowing programmed squamous differentiation, although it has been shown that the immortalized HKC retain the potential for squamous differentiation (McCance *et al.*, 1988; Waggoner *et al.*, 1990; Woodworth *et al.*, 1990b; Hudson *et al.*, 1990; Dollard *et al.*, 1992).

In contrast to HKC, endocervical epithelial cells have not been fully appreciated for

their potential significance in HPV16-mediated oncogenesis. This is probably because HPV infections mostly cause SSE lesions, and the possible HPV infections in tissues other than SSE were often neglected. Epithelial cells from the TZ, which are metaplastic in nature, have been used in *in vitro* immortalization assays, and immortalized epithelial cells from the TZ have been established (Waggoner *et al.*, 1990; Woodworth *et al.*, 1990b). Interestingly, when reconstructed into epithelia using the *in vivo* system as described by Barrandon *et al.* (1988), the immortalized TZ cells formed lesions similar to those from immortalized HKC (Waggoner *et al.*, 1990; Woodworth *et al.*, 1990b). This is possibly because the cells in the TZ are predominantly mature metaplastic cells, which are phenotypically similar to those of native SSE. Indeed, the normal TZ cells were reconstructed into well-differentiated SSE *in vivo* (Woodworth *et al.*, 1990b), which was morphologically similar to that from HFK and HEC (Sun *et al.*, 1992). Alternatively, technical differences in epithelia reconstruction may also contribute to the different maturity of squamous differentiation in the reconstructed epithelia from HEN and TZ cells. Treatment of cells with high concentrations of calcium, which is required in the method of Brandon *et al.* (1988) and was used by Woodworth *et al.* (1990b), may have enhanced squamous differentiation. It has been well established that calcium is one important mediator for squamous differentiation (Bikle and Pillai, 1993).

The current study revealed that HEN and HKC possessed differential pathological characteristics upon being immortalized by HPV16. The most striking *in vivo* feature for the HPV16-immortalized HEN was the high grade dysplastic morphology, which contrasted with the low grade dysplastic lesions from the immortalized HKC. This pathological difference can be substantiated by the pattern of CK expression and the pattern of HPV16 E7 expression in

the HEN and HKC lesions. The highly dysplastic HEN lesion extensively expressed the E7 oncogene and CK18, similar to naturally occurring HPV16-related high grade dysplasias (Smedts *et al.*, 1990; Durst *et al.*, 1992; Stoler *et al.*, 1992). The current study also showed that the pathological features of the immortalized HEN cannot be attributable to differential expression of the viral E5 gene since E5 expression in the immortalized HEN was almost undetectable. Consistently, there is no evidence that E5 of HPV is directly involved in oncogenesis, although E5 is potentially mitogenic due to its inhibiting effect on the down-regulation of tyrosine kinase receptors (Leechanachai *et al.*, 1992; Conrad *et al.*, 1993). In addition, overexpression of H-*ras* and c-*myc*, which has been found in some high grade CINs (Riou *et al.*, 1987; Sagae *et al.*, 1990; Riou *et al.*, 1992), was unlikely to be responsible for the high grade dysplastic changes in the immortalized HEN lesion. Thus, the difference in the dysplastic severity only be attributed to the differential expression of the HPV16 E7 oncogene in the immortalized HKC and HEN. The retained extensive expression of E7 in the tumour formed by the NMU-treated HEN16-2 was consistent with this view.

Results from the current study also indicated that the immortalized HEN may represent an early, or premalignant, stage of HPV16-mediated oncogenesis, despite the high dysplastic morphology. Indeed, the immortalized HEN was non-tumorigenic in nude mice, failed to grow in anchorage-independent conditions, and did not show more active growth than the immortalized HKC *in vitro*. Furthermore, like the immortalized HKC, the immortalized HEN can be subsequently transformed by treatment with chemical carcinogens such as NMU and cisplatin and (Pater, M.M., Pater, A. and Jin, G., unpublished observations). This indicated that other cellular events are required for the full transformation

of the HPV16-immortalized HEN, despite the extensive expression of HPV viral oncogenes in conditions allowing differentiation. Thus, these results indicated that the phenotypes of malignant morphology and tumorigenic growth were separable for HPV16-mediated oncogenesis. This idea is consistent with the pathological definition for CIN III/CIS, which is malignant in cytological morphology but is not in its growth property.

In all aspects, the results from the current study suggested that the cell or tissue types of HPV16 infection may be an important factor for the expression patterns of HPV16 oncogenes, for the expression patterns of CKs, and for the pathological features of the HPV16-mediated squamous lesions.

3.4 Summary

Morphology, expression of characteristic CKs, and expression of HPV16 E7 oncogene were comparatively studied in *in vivo* lesions reconstructed from three types of normal and HPV16-immortalized human genital epithelial cells. In addition, expression of CKs and HPV16 E7 was monitored in *in vivo* lesions from normal, immortalized and fully transformed HEN. In *in vivo* conditions, the normal HEN, HPV16-immortalized HEN and NMU-transformed HPV16-immortalized HEN respectively formed artificial lesions resembling immature metaplasia, high grade dysplasia, and full malignancy, which are three important states of cervical epithelia involved in cervical oncogenesis.

The current series of experiments revealed the following. First, cervical squamous metaplasia and squamous cell carcinomas may be derived from endocervical SCE cells. Also, HEN, or the precursor cells of HEN, may undergo the metaplastic, or a related process, by

default in *in vitro* culture. Secondly, the HPV16-immortalized HEN expressed the E7 viral gene extensively in the *in vivo* condition, while the immortalized HKC expressed the E7 message in a restricted pattern in the basal cells. In association with the extensive expression of the E7 viral oncogene was the high grade dysplastic morphologies in the immortalized HEN lesion, which were substantiated by the CK expression pattern. Since the expression of the HPV16 E7 oncogene is essential for oncogenic initiation and maintenance, endocervical epithelial cells may potentially be more susceptible to HPV16-mediated oncogenesis, compared with keratinocytes. Third, the results indicated that cell origins may dictate the basic patterns of CK expression in the squamous lesions, although changes in CK expression may also occur during the process of oncogenesis. The results implied that cell origins may also be important for the expression patterns of the integrated HPV oncogenes.

The most logical explanation for the above observations is that HKC may possess a cellular function to repress the expression of the integrated HPV16 oncogenes, and that this function is nonexistent or deficient in the SCE-derived cells. Then the most relevant questions was, "What was the nature of this cellular function?" This question is addressed in Chapter 4.

CHAPTER 4

IN VITRO MODELS

FROM CULTURED HUMAN GENITAL EPITHELIAL CELLS

4.1 Introduction

The differences in E7 expression between the HKC and HEN *in vivo* lesions strongly suggested that HKC, but not HEN, may possess a cellular function that represses integrated HPV16 oncogene. Since these two type lesions were obviously in a different differentiation status, the expression of HPV16 oncogenes from integrated viral DNA may be regulated by unique mechanisms in metaplastic and native SSE. This difference between native SSE and metaplastic SSE could potentially constitute the foundation for their differential oncogenic susceptibilities to HPV16 infections.

Although the tissue-specific differential expression of HPV16 oncogenes suggested by the current study has not been revealed by other studies, repression of HPV16 oncogene expression in HPV16-mediated premalignant lesions and its significance in HPV16-mediated oncogenesis have been under intensive intellectual and experimental scrutiny (zur Hausen and de Villiers, 1994). In recognition of the importance of viral oncoproteins in the initiation and maintenance of carcinogenesis, much attention has been focused on the levels and patterns of the expression of HPV oncogenes during the process of HPV16-mediated oncogenesis. It has been expected that, in association with the progression from premalignancies to fully developed malignancies related to HPV16 infections, there might be a transition of viral oncogene expression from a repressed to a derepressed state. This notion was inspired by and

based on observations from experimental models, as well as clinical samples.

4.1.1 Transition of viral oncogene expression in association with lesion severity and viral DNA status

The cardinal evidence for the hypothetical transition of viral oncogene expression during oncogenesis progression was from studies reporting that there were differential patterns of HPV16 oncogene expression and differential statuses of viral DNA in premalignant and malignant lesions. Several studies showed that, cells expressing viral oncogenes in the HPV16-mediated high grade premalignant lesions and fully developed cervical cancers tend to distribute evenly throughout the lesion (Durst *et al.*, 1991; Stoler *et al.*, 1992; Durst *et al.*, 1992). In these lesions, viral DNA most often becomes integrated into the cellular genome and viral infections are usually non-vegetative. In contrast, HPV16-infected low grade premalignant lesions have been reported to express viral oncogenes only in the differentiating upper layer cells and barely in the basal proliferating cells (Durst *et al.*, 1991; Stoler *et al.*, 1992; Durst *et al.*, 1992; Bohm *et al.*, 1993). These lesions were reported to contain episomal viral DNA and to support virus vegetative infections. Thus, compared with the extensively expressed HPV16 oncogenes in the high grade lesions, viral oncogene expression in the low grade premalignant lesions may be regarded as being repressed in the bottom undifferentiated cells. Since it has been widely accepted that genital malignancies, especially those derived from uterine cervices, develop from premalignant lesions, the de-repression of viral oncogenes and integration of viral DNA would be logically postulated to be associated with, or even required for, the progression of oncogenesis.

Experimentally, the current and previous studies showed that the immortalized HKC lines represented low grade premalignant lesions, since these cells have limited growth potential and retain the potential for programmed squamous differentiation in *in vivo* conditions (Waggoner *et al.*, 1990; Woodworth *et al.*, 1990b; Durst *et al.*, 1991). In addition, expression of viral oncogenes in the experimental premalignant HKC lesions was found to be compartmentalized in the undifferentiated proliferating basal cells, in contrast to the extensive E7 expression in naturally occurring high grade dysplasia, even though viral DNA in both types of lesions is in integrated forms. These data from the comparative studies on natural and experimental lesions have important implications. They indicated that, although HPV16 DNA integration may have the potential to result in fundamental changes in patterns of HPV oncogene expression, viral DNA integration does not necessarily lead to extensive expression of viral oncogenes. The disparity in the expression of viral oncogenes between the natural and experimental low grade premalignant lesions has been reasonably interpreted as being due to differences in viral DNA status. Indeed, unlike the experimentally immortalized cells, viral DNA in the natural low grade lesions is episomal rather than integrated. Nevertheless, HPV16 oncogene expression may be regarded as being repressed in the low grade premalignant lesions containing either episomal or integrated viral DNA, when compared with that in the high grade lesions. Therefore, if it is true that the HPV16-mediated malignant lesions develop from premalignant HPV16 infections, one of the oncogenic requirements for this progression logically would involve the failure of a mechanism(s) that represses the expression of HPV16 oncogenes.

The mechanism for repressing HPV16 oncogenes in the premalignant lesions with

either episomal or integrated viral DNA is not clear. However, as discussed in the following sections, several mechanisms were believed to be involved in the repression of HPV16 oncogene expression.

4.1.2 Repression of HPV oncogenes in the tumor-normal cell hybrids

As introduced in Section 1.3.5.3.4.2, studies with a cell hybrid model system showed that a function in normal cells may repress expression of HPV18 oncogenes (Rosl *et al.*, 1988; Bosch *et al.*, 1991; Rosl *et al.*, 1991). Upon fusing HeLa cells, which contain HPV18, with normal keratinocytes or fibroblasts, hybrid cells and their segregates can be selected for the non-tumorigenic and tumorigenic phenotypes. When tested *in vivo*, the non-tumorigenic hybrids halted growth after two days, which was preceded by repression of viral oncogene at transcription level. In contrast, the parental HeLa cells and the tumorigenic segregates displayed persistent expression of viral oncogenes, which was associated with continuous cell proliferation. Furthermore, although the tumorigenic cells and the non-tumorigenic segregates both expressed the viral oncogenes in *in vitro* monolayer culture, the nucleic acid demethylation agent 5-AZ repressed viral oncogene expression in the non-tumorigenic hybrid, but not in the parental cells or the tumorigenic segregates (Rosl *et al.*, 1988). Therefore, a dominant cellular mechanism in the normal cells was thought to be activated in *in vivo* condition by default, and to repress HPV18 oncogene via a trans-acting factor. It is further speculated that the cellular mechanism responsible for the *in vivo* repression of HPV18 oncogene expression may be susceptible to DNA hypermethylation, since long time *in vitro* cultivation may result in aberrant DNA methylation and suppression of genes not required for

in vitro growth (Antequera *et al.*, 1990).

4.1.3 Repression of HPV16 oncogenes by cytokines

One feature of the repression of the integrated HPV oncogenes revealed by the studies discussed above is that the repression was most prominent in *in vivo* condition. The most obvious difference between *in vivo* implantation and *in vitro* monolayer culture is that the cells in *in vivo* condition are subject to various cytokines that are involved in inflammation, immunological reactions and tissue repair. Thus, it is possible, and has been suggested, that the repression of HPV oncogene expression be mediated by, and even dependent on, these cytokines (zur Hausen, 1994).

The possible involvement of cytokines in repression of HPV oncogene expression is consistent with the finding, as also introduced in Section 1.3.5.3.4.2, that some cytokines indeed repress HPV16 expression in the immortalized cells. These cytokines include peptide growth factors, such as TGF-β (Woodworth *et al.*, 1990a; Gruppuso *et al.*, 1991; Braun *et al.*, 1992) and EGF/TGF-α (Yasumoto *et al.*, 1991), which are involved in differentiation and tissue repair. Cytokines involved in immunological functions, such as interferons, TNF-α, interleukin-6 and leukoregulin have also been shown to repress HPV oncogene expression (Woodworth *et al.*, 1992; Khan *et al.*, 1993b; Agarwal *et al.*, 1994; Kyo *et al.*, 1994). In addition, other diffusible agents, such as retinoic acid (RA) and steroid hormones, may also contribute to the modulation of HPV oncogene expression in *in vivo* condition. Indeed, RA was shown to repress HPV16 oncogene expression in some, but not in all, immortalized cell lines (Agarwal *et al.*, 1991; Bartsch *et al.*, 1992; Pirisi *et al.*, 1992; Agarwal *et al.*, 1993;

Khan *et al.*, 1993a; Merrick *et al.*, 1993; Agarwal *et al.*, 1994).

4.1.4 Repression of HPV16 oncogenes by viral DNA methylation

While it has been reported that DNA hypermethylation seems negatively to regulate a cellular mechanism that represses HPV18 oncogene expression (Rosl *et al.*, 1988), hypermethylation of viral DNA was also reported to repress HPV16 and HPV18 oncogene expression from episomal and integrated viral genome in monolayer culture (Rosl *et al.*, 1993; List *et al.*, 1994). In transient expression assays, in which most of the transfected viral DNA should be episomal, artificial methylation in the multiple CpG sites in the HPV16 and HPV18 LCRs extinguishes viral gene expression from the viral promoters. In a recent study it was shown that the HPV16 LCR contains two CpG sites that are sensitive to methylation for binding with an uncharacterized cellular factor, methylation-sensitive papillomavirus transcription factor (MSPF) (List *et al.*, 1994). Methylation at these two sites sabotages MSPF binding and results in the concurrent extinction of HPV16 oncogene expression. Interestingly, the studies provided evidence that viral DNA methylation may be involved in affecting the expression of integrated viral oncogenes at a level of chromatin structure (Rosl *et al.*, 1993). CaSki cells, which contain approximately 500 copies of integrated HPV16 DNA (Yee *et al.*, 1985), express a low quantity of viral messages in *in vitro* monolayer culture, which was believed not to be proportional to the dosage of viral genomes (Smotkin and Wettstein, 1986). This repressed expression of viral oncogenes in CaSki cells was attributable to viral DNA hypermethylation. DNA methylation may have promoted heterochromatinization of the DNA region containing the integrated viral DNA, since

nucleosome protection assays showed that the majority of viral DNA in CaSki cells is methylated and is incorporated into transcriptionally inactive chromatin (Rosl *et al.*, 1993).

4.1.5 Repression of HPV16 oncogenes and control elements for gene expression

Since the mechanisms controlling gene expression are very complex and random viral DNA integration may juxtapose viral genes in the vicinity of various cellular elements, suppression of integrated HPV oncogenes may occur at multiple levels.

As introduced in Section 1.3.4.2.8, most viral control elements are clustered in the LCR of HPV16, which contains the P97 major promoter and a complex enhancer. It has been shown that various *cis* modules in this enhancer modulate transcription from the P97 promoter via viral, as well as cellular, *trans*-acting factors (Butz and Hoppe-Seyler, 1993; Bernard and Apt, 1994). Transcripts initiated from the P97 promoter are potentially capable of encoding the E6 and E7 oncogenes and other downstream viral genes (Taniguchi and Yasumoto, 1990; Rohlfs *et al.*, 1991; Vormwald Dogan *et al.*, 1992). Thus, the control elements in the LCR may contribute to the programs of viral gene expression in the tissue-specific, differentiation-dependent viral life cycles. It has been expected that cellular factors specific for squamous differentiation should mediate the specificities of HPV infection, although no such factors have been found for HPV16 (Fuchs, 1993; Bernard and Apt, 1994). Since the HPV LCRs contain numerous binding sites for ubiquitous transcription factors, a delicate abundance balance of these factors and the complex interaction between them (Miner and Yamamoto, 1991) may play major roles in HPV gene expression. In addition, and as introduced in Section 4.1.4, epigenetic modification, such as methylation, at specific sites in

the HPV LCR also potentially regulates viral gene expression (List *et al.*, 1994; Rosl *et al.*, 1993). Despite the great efforts, the *modi operandi* of these control elements in relation to the specificity of cell type and differentiation remain largely elusive.

Although the HPV LCR may remain functional in the integrated viral genomes, as shown in the current study, cellular elements juxtaposed to the viral genome may modulate viral oncogene expression. First of all, viral gene expression may be initiated from cellular promoters (Vormwald Dogan *et al.*, 1992), and cellular enhancers may modulate viral promoters for viral gene transcription (von Knebel Doeberitz *et al.*, 1991). A juxtaposed cellular polyadenylation signal was also used for the transcription of viral oncogenes in the cervical cancer cell line CaSki (Smits *et al.*, 1991). In addition, control elements in cellular genomes may also affect viral oncogene transcription from integrated viral DNA at higher levels, such as chromatin structure. It has also been well documented that some cellular genes are clustered and their expression is controlled by DNA sequences called locus control regions. The regulation of gene expression by locus control regions is characterizcd by its effect on a group of genes located in a certain genomic locus in a tissue- and/or development stage-dependent pattern (Dillon and Grosveld, 1993). Expression of the genes in the β-globin locus is one good example for such control (Engel, 1993; Jane *et al.*, 1995). Furthermore, gene expression is regulated at the level of nucleosome assembly. Since promoters are active only in the correct chromatin configuration, modulation of gene expression must be accompanied with nucleosome modification and changes in chromatin structure (Workman and Buchman, 1993; Becker, 1994). Because viral DNA integration is a largely random event in relation to cellular DNA, integration of viral genome into different cellular DNA domains

may result in differential expression of viral oncogenes.

4.1.6 Objective

The results described in Chapter 3 showed that, while the expression of the integrated HPV16 E7 was repressed in the HKC *in vivo* lesions, it was not in the HEN *in vivo* lesion. Because the differentiation status in the HEN and HKC implants was fundamentally different, and HPV gene expression is closely associated with differentiation, the mechanism for the repression of HPV16 oncogene expression is possibly deficient or even totally unavailable in metaplastic SSE. One approach to elucidate the mechanism behind this phenomenon is to analyze the individual elements possibly involved in this repression mechanism comparatively in HKC and HEN.

Considering that the expression of HPV16 oncogenes, especially when integrated, is potentially modulated by numerous mechanisms and at multiple levels, the experiments in this chapter addressed three fundamental questions. The first question was, "Is the E7 repression mediated by *in vivo* factors, such as cytokines?" The second, "Is the E7 repression dependent on the HPV control elements?" And the third, "Under what condition can the E7 repression be disrupted?"

4.2 Results

The *in vivo* nude mouse implantation model used in Chapter 3 is undoubtedly the most relevant system to study the phenotype of cultured epithelial cells. However, it is not suitable for addressing the questions asked in this chapter for the following reasons. First, the *in vivo*

system would inevitably expose the subject cells to uncontrollable and undesirable *in vivo* factors. For example, the tissue trauma caused by surgical procedures may induce the release of many cytokines involved in tissue repair. In addition, residual immune functions in the athymic animals may release immunological cytokines. These cytokines would inadvertently contribute to the repression of HPV16 oncogene expression. Second, the *in vivo* system does not allow any systematic and controllable modifications of the epithelia reconstruction conditions. Indeed, on one hand, experimental animals may not tolerate the experimental chemicals. Further, the relatively unstable and uncontrollable level of the test chemical in the animal may also adversely affect the experimental observation.

To suit the special requirements for the objective of the current study, an *in vitro* epithelia construction system, the organotypic (raft) culture system, was used (refer to Figure 2.2). Raft culture was initially designed for constructing artificial skin, and was later adapted for HPV research (Bell *et al.*, 1983; McCance *et al.*, 1988; Wilson *et al.*, 1992). In the raft system, *in vitro* cultured epithelial cells are reconstructed into epithelial tissue on a fibroblast-containing collagen matrix that supports the growth of the epithelial cells above the media-air interface (Boukamp *et al.*, 1990a). This creates a polarized condition that mimics the growth conditions for epithelial tissues. Thus, the epithelial cells in the raft system are only exposed to a single type of exogenous cell, the fibroblast cells. Moreover, the raft system has the advantage of allowing the modification of the epithelial reconstruction conditions.

4.2.1 HPV16 oncogene repression independent of *in vivo* conditions

To test whether *in vivo* factors were involved in the repression of HPV16 oncogene

expression, the HPV16-immortalized HKC and HEN were reconstructed into epithelia using raft system, and the reconstructed epithelia were examined for E7 expression.

4.2.1.1 Morphology of the *in vitro* reconstructed epithelia

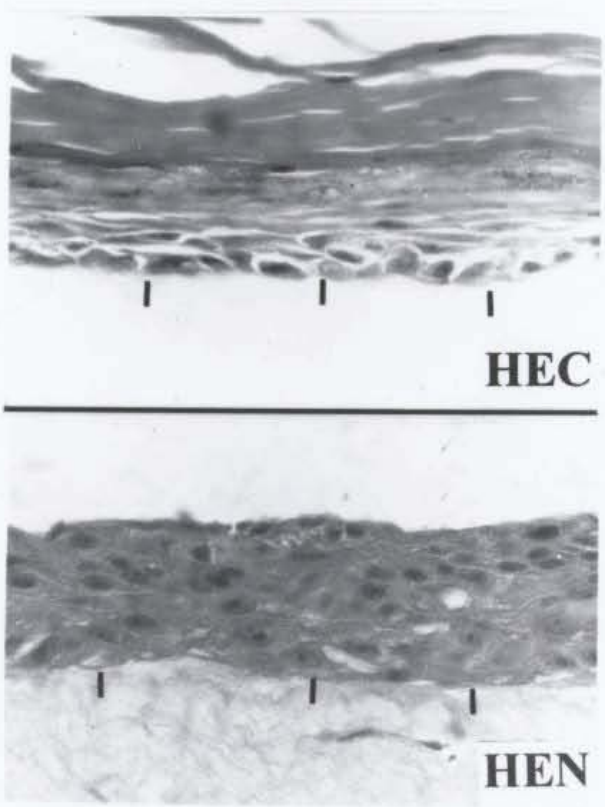
As a first step, normal HEN and HEC were reconstructed into epithelia rafts as experimental controls. Both the normal HEC and HEN formed stratified epithelia (Figure 4.1). Similar to that in the *in vivo* system, the normal HEC *in vitro* raft displayed a phenotype of well-differentiated SSE. The cells in different epithelial compartments showed distinctive morphologies. The basal layer consisted of cubical or oval cells with scarce cytoplasm. Starting from the suprabasal layers, the cells became increasingly elongated, and nuclei in the upper layer cells were condensed. Squamous differentiation was obvious, as indicated by the existence of typical inter-cellular bridges. Under the same condition, the stratified epithelium reconstructed *in vitro* from normal HEN was also squamous in phenotypes. However, the cells composing the epithelium did not show prominent cellular compartmentalization as seen in the HEC *in vitro* raft. Most cells in the HEN raft were oval in shape and horizontally arranged. The nuclei were generally uniform in size and shape throughout the epithelium, and did not show atypical features. Only few cells near the epithelial surface showed elongated cellular contour. These morphological features were consistent with those for metaplastic SSE. Although the morphologies of the normal HEN *in vitro* raft were largely in agreement with those in the HEN *in vivo* implant, there were some subtle differences. Most conspicuously, the cells in the HEN *in vitro* raft showed more elongated cellular contour than those in the *in vivo* implant. This may reflect the fact that the current *in vitro* condition for

Figure 4.1 Morphology of the *in vitro* raft epithelia reconstructed from HEC and HEN

The magnifications were 250 X.

H&E staining.

The intersection between the raft matrix and the epithelia was indicated by black bars.


HEC
HEN

epithelia reconstruction (Figure 4.1) was similar, but not identical, to that *in vivo* (Figure 3.3). This interpretation was substantiated by the patterns of CK expression in the normal HEC *in vitro* raft (see Section 4.2.3.2), and a similar view has also been suggested by other studies (Boukamp *et al.*, 1990a).

Under the same *in vitro* condition, HEN16-2, HEC16-2 and HFK16-3, the cell lines of HPV16 genomic DNA-immortalized HEN, HEC and HFK, formed lesions (Figure 4.2) comparable to those reconstructed *in vivo* (Figure 3.5). HEN16-2 formed a raft lesion morphologically similar to carcinoma *in situ*. The entire lesion consisted of immature cells with high nucleus/cytoplasm ratios. Inter-cellular bridges were observed in some cells. The interconnection between the cells appeared to be weak and the raft often showed breaks and detachments. The nuclei were atypical and hyper-chromatic. No programmed terminal squamous differentiation was observed. In addition, some of the superficial cells displayed a necrotic-like morphology (Figure 4.2, HEN16-2 H&E panel, indicated by the black arrow). These cells had small concentrated nuclei and very rich eosinophilic cytoplasm. Nuclei were not observed in some of these cells. These necrotic-like cells constituted a substantial fraction of the cells in some areas of the HEN16-2 raft lesion. In contrast, HEC16-2 and HFK16-3 formed well-differentiated SSE. Similar to their *in vivo* counterparts (Figure 3.5), the basal layer was composed of cubical cells and the cells in the suprabasal layers were horizontally elongated. The cells throughout entire HKC raft lesions rarely displayed typical dysplastic changes. Although the HFK16-3 and HEC16-2 rafts were processed in the same condition as the HEN16-2 raft, no typical necrotic-like cells and gross breaks were observed in the raft samples of these two HKC cell lines.

Figure 4.2 Morphology of and HPV16 E7 expression in the *in vitro* raft lesions reconstructed from HEN16-2, HEC16-2 and HFK16-3

Top row: HEN16-2; The black arrow in the HEN16-2 H&E panel indicates a necrotic-like cell.

Middle row: HEC16-2;

Bottom row: HFK16-3.

The white arrows and white arrowheads in the HEC16-2 and HFK16-3 E7DF panels indicate the E7-expressing cells located in the parabasal layer and in the basal layer, respectively.

The magnifications were 250 X.

Basal membrane was indicated by bars.

E7DF
E7BF
H&E
HEN16-2
HEC16-2
HFK16-3

4.2.1.2 Expression of HPV16 E7 oncogene in *in vitro* reconstructed lesions

The raft lesions from the immortalized cells were examined for the expression of HPV16 E7 oncogene by *in situ* hybridization assays. The same probe used for the *in vivo* analysis was used. As shown in Figure 4.2, the patterns of E7 expression in the raft lesions displayed were very similar features to those in their *in vivo* counterparts (Figure 3.8). In the immortalized HKC raft lesions, the expression of the viral E7 oncogene was compartmentalized in the bottom layers. In contrast, the same message was expressed in nearly all the dysplastic cells in the HEN16-2 raft lesion, except the cells with necrotic morphology. Despite being compartmentalized, the E7 expression in the immortalized HKC raft lesions also showed features distinct from those in their *in vivo* counterparts. Most of the parabasal cells in the immortalized HKC raft lesions also expressed E7 (Figure 4.2), in contrast to the *in vivo* implant lesions, in which E7 expression was strictly limited to the basal cells (Figure 3.8). Again, this may be explained by the possibility that the current regimen for the *in vitro* epithelia reconstruction supported a program of squamous differentiation very similar, but not identical, to the one in the *in vivo* condition.

Nevertheless, the compartmentalized expression of the E7 oncogene in the immortalized HKC raft lesions indicated that the cellular function responsible for the repression of integrated HPV16 oncogene expression was functional in the *in vitro* epithelia reconstruction system. Because this system contained only the test epithelial cells, a single type of fibroblasts and a defined medium, these results argued against the possible involvement of unique *in vivo* factors in the repression of HPV16 oncogenes.

4.2.2 Repression of HPV16 oncogene independent of HPV16 control elements

The second question concerned the possible involvement of HPV16 gene control elements in viral oncogene repression. The current study pursued a straightforward yes or no answer for this question. To this end, new cell lines were established from HEN, HEC and HFK by *in vitro* transfection of a plasmid that contained the HPV16 E6 and E7 genes controlled by heterologous gene control elements from SV40. These cell lines were reconstructed into epithelia *in vitro* and E7 expression in the reconstructed tissues was examined by *in situ* hybridization assays. The rationale for this experiment was that, if the E7 repression was mediated by an HPV16-specific mechanism, substitution of a heterologous promoter for the homologous HPV promoter would result in patterns of viral oncogene expression different from those seen in the HPV16 genomic DNA-immortalized HKC and HEN lesions.

The species- and tissue-specificities of SV40 infection are fundamentally different from those of HPV16. Logically, the functional mode of the gene control elements of SV40 should also be different from that of HPV16, as suggested by Chong's study (1991). Thus, the $pSV_2$1667 plasmid, which contained HPV16 E6 and E7 ORFs under the control of the SV40 early promoter (Figure 2.3), was transfected into primary HEN, HEC and HFK to establish new immortalized cell lines. In this plasmid, the polyadenylation signal was provided by the SV40 early polyadenylation sequence. In addition, the SV40 small t intron in the parental vector was retained to provide a splicing acceptor site for the possible splicing requirement for the HPV16 E6 and E7 mRNA (Belaguli *et al.*, 1992; Belaguli *et al.*, 1995).

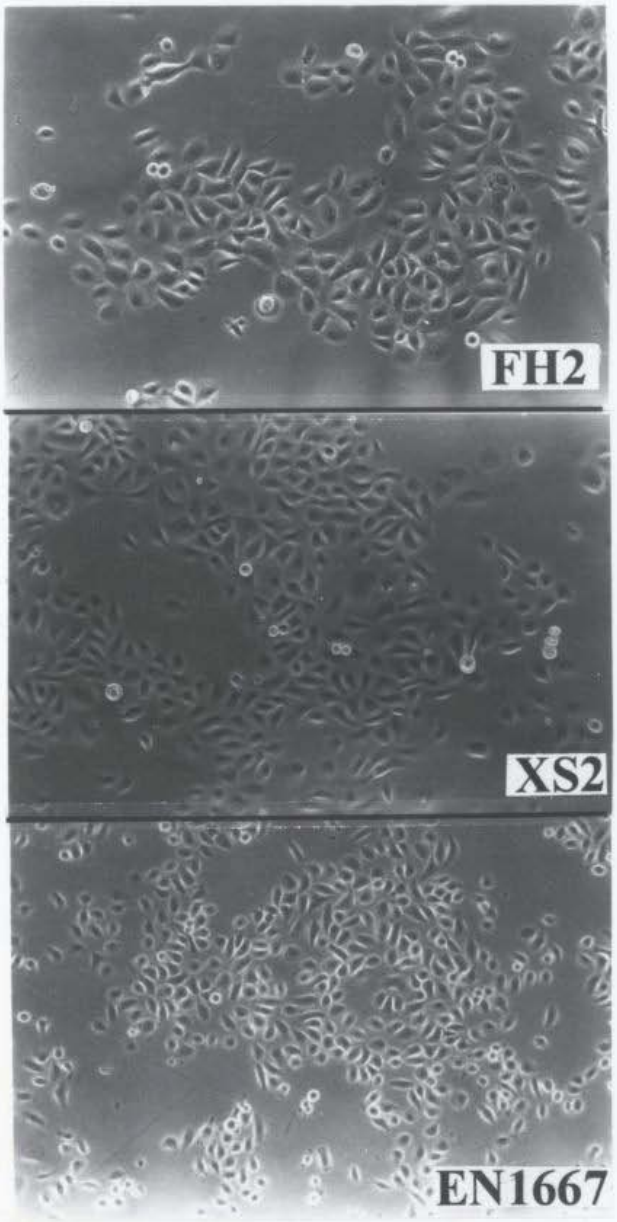
4.2.2.1 Immortalization of human genital epithelial cells by HPV16 E6-E7 expressed from a heterologous promoter

Normal primary epithelial cells from endocervix, ectocervix and foreskin were transfected by lipofection with pSV_21667, pSV_2CAT and pSV_21606 plasmids. The latter two constructs were used as negative controls. Their structure was similar to that of pSV_21667, except that the genes controlled by the SV40 promoter were for the chloramphenicol acetyl transferase (CAT) and the HPV16 E6 oncoprotein, respectively. The pSV_21667-transfected cells continued to proliferate when the pSV_2CAT- and pSV_21606-transfected cells stopped proliferation after 4-6 passages. At 11-13 passages, the pSV_21667-transfected cells showed a drastic slowdown in proliferation. This may represent the well-established phenomenon "crisis" (McDougall, 1994). The cells in crisis were passed to maintain sub-confluency, and a significant number of cells failed to reattach to the dishes. When the cell number in the culture did not increase significantly, they were maintained in fresh media without further passing, until new proliferating clones emerged. The initial growth of the proliferating clones was unstable and a substantial fraction of cells was lost during passing. Finally, one cell line from each cell type was established, and designated EN1667, XS2 and FH2 for the pSV_21667-immortalized HEN, HEC and HFK, respectively. When cell growth became stable, they were passed upon confluency with the ratio of 1:3, and maintained in continuous culture for more than six months to confirm their immortality. Similar to those immortalized by HPV16 genomic DNA, the pSV_21667-immortalized genital epithelial cells in *in vitro* monolayer culture showed no morphological features distinct to any cell types (Figure 4.3).

Figure 4.3 Morphology of the pSV$_2$1667-immortalized human genital epithelial cells in monolayer culture

The magnifications were 300 X.

Phase contrast micrography.


FH2
XS2
EN1667

4.2.2.2 Status and expression of transfected HPV16 E6-E7 oncogenes in $pSV_2$1667-immortalized cells

To examine the status of the transfected HPV16 E6-E7 oncogenes in the $pSV_2$1667-immortalized cells, DNA from these cells was analyzed by Southern blot with a DNA probe for HPV16 (Figure 4.4). To distinguish integrated and episomal forms of the transfected plasmid, DNA was digested with two restriction endonucleases, HindIII and XbaI. The two enzymes cut at single sites 6 nt apart in the plasmid. Thus, if the plasmid DNA was in an episomal form, the two enzymes would produce the same hybridization pattern. In contrast to the pattern predicted for the episomal plasmid, DNA from all the three cell lines showed different patterns for HindIII and XbaI digestion, suggesting that the transfected DNA was integrated into cellular genome. Furthermore, both XbaI and HindIII produced single bands for FH2, suggesting that $pSV_2$1667 was integrated into a single site in the cellular genome. In contrast, HindIII and XbaI produced multiple bands for XS2 DNA, suggesting multiple sites of integration and/or different rearrangements or deletions. In addition, EN1667 DNA showed two bands for HindIII and one band for XbaI, consistent with a pattern for at least two integration sites.

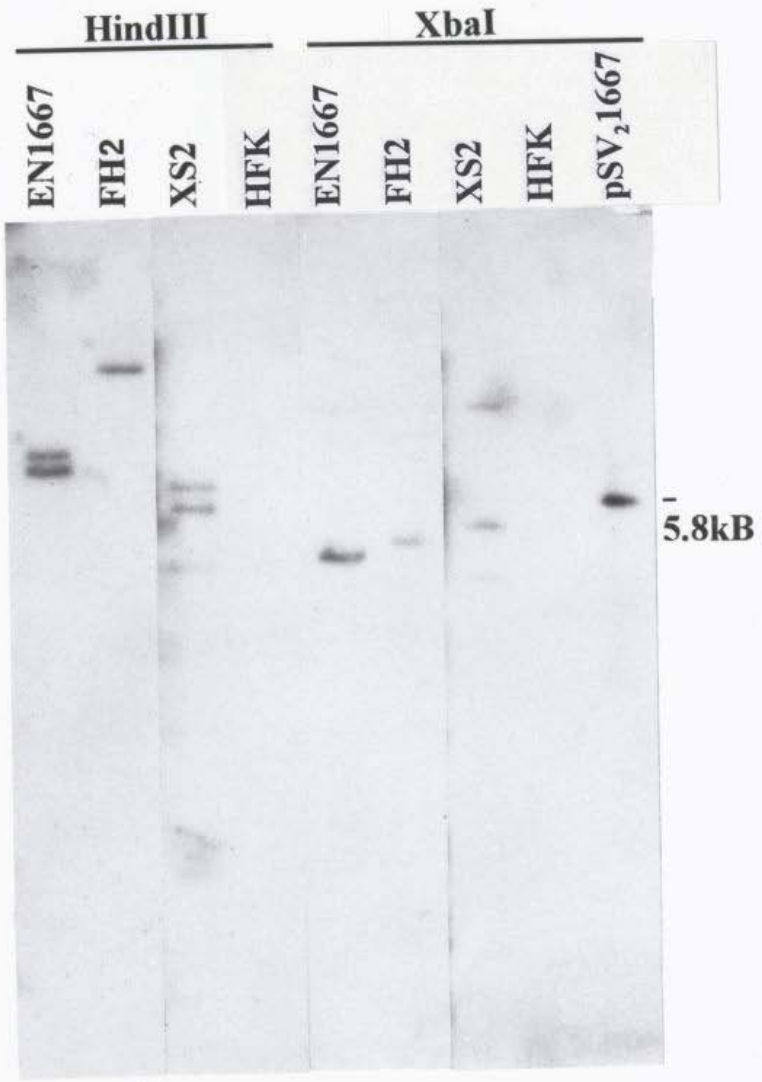
In vitro expression of the transfected viral E7 oncogene in the $pSV_2$1667-immortalized cells was examined by Northern blot (Figure 3.11). The E7-specific riboprobe used for *in situ* hybridization assays was used. The result showed that the E7 gene was expressed at a similar level among all the $pSV_2$1667-immortalized cell lines, while the $pSV_2$1667-immortalized cells expressed E7 at a higher level than the HPV16-immortalized cells (Figure 3.11a). This

Figure 4.4 Southern blot detection of the HPV16 E6-E7 sequence in EN1667, XS2 and FH2

The restriction endonucleases used are labelled on the top.

The molecular weight marker was indicated on the right side of the figure.

HFK was the DNA from the parental normal foreskin keratinocytes, used as negative controls.


HindIII
XbaI
EN1667
FH2
XS2
HFK
EN1667
FH2
XS2
HFK
pSV2 1667
5.8kB

indicated that the SV40 early promoter was more active than the HPV16 promoter in HEN and HKC cultured in *in vitro* monolayer condition.

To examine whether the heterologous SV40 early control elements behaved differently from those of HPV16, the $pSV_2$1667-immortalized cells were treated with TGF-β in monolayer culture, and the expression of HPV E7 was examined with Northern blot analysis to test the response of the SV40 promoter to TGF-β. Unlike the HPV16-immortalized cells, which showed a negative response to TGF-β for E7 expression, EN1667 and XS2 showed no response of E7 expression to TGF-β (Figure 3.11a). To exclude the possibility that the unresponsiveness of E7 expression to TGF-β in the $pSV_2$1667-immortalized cells was due to a deficient signal pathway for TGF-β, expression of c-*myc* in response to TGF-β was examined. It has been shown by others that TGF-β specifically represses c-*myc* expression (Woodworth *et al.*, 1990a; Gruppuso *et al.*, 1991; Braun *et al.*, 1992), and expression of HPV E7 abrogated the c-*myc* repression by TGF-β (Pietenpol *et al.*, 1990; Pietenpol *et al.*, 1991). Reprobing the RNA blot for E7 detection with a c-*myc*-specific probe clearly showed that the expression of c-*myc* was repressed by TGF-β in the HPV16-immortalized cells, as well as the $pSV_2$1667-immortalized cells (Figure 3.11b). Therefore, the signal transduction pathway for TGF-β was apparently functional in all the cell lines. These results indicated that the SV40 control elements, which were integrated into cellular genome, featured regulatory property distinct from that of the control elements of HPV16, as expected. Interestingly, E7 expression in FH2 was moderately repressed by TGF-β. This was possibly caused by modulation from a cellular TGF-β responsive element, in vicinity of which the $pSV_2$1667 was integrated.

4.2.2.3 HPV16 E7 expression from integrated $pSV_2$1667 in *in vitro* reconstructed lesions

The results described in Section 4.2.1.2 revealed that the cellular mechanism responsible for the repression of the integrated HPV16 oncogenes was functional in lesions reconstructed *in vitro*. To examine the involvement of the HPV16 LCR in the repression of HPV16 oncogene expression, the $pSV_2$1667-immortalized cells were reconstructed with the raft system, and the rafts were examined for E7 expression by *in situ* hybridization assays.

The three $pSV_2$1667-immortalized cell lines all formed multilayered lesions in the raft system. FH2 and XS2 raft lesions showed morphologies very similar to those of their HPV16-immortalized counterparts (Figure 4.5). The epithelia showed conspicuous cellular compartmentalization. The basal layers consisted of cubical cells with scarce cytoplasm. The elongated cells in the suprabasal layer were horizontally arranged, rich in cytoplasm, and abundant in intercellular bridges. Similar to their HPV16-immortalized counterparts, few typical dysplastic cells were found in those lesions formed by the $pSV_2$1667-immortalized HKC. In contrast, the multilayered lesion from EN1667 did not show obvious morphological compartmentalization (Figure 4.5). The lesion was occupied with cubical cells with similar size and contour. Most cells had round or oval nuclei with moderate to high nucleus/cytoplasm ratios. A few cells scattered throughout the lesion showed large irregular hyperchromatic nuclei that are consistent with the features for atypia. Although intercellular bridges were observed between the cells, they were not as prominent as in the $pSV_2$1667-immortalized HKC rafts. The cells in the EN1667 raft lesion appeared to detach easily from each other, resulting in fragmented appearance, which was not observed in the FH2 and XS2

Figure 4.5 Morphology and HPV16 E7 expression in the *in vitro* raft lesions reconstructed from EN1667, XS2 and FH2

Top row: EN1667. The black arrow in the H&E panel indicates a necrotic-like cell.

Middle row: XS2

Bottom row: FH2

The magnifications were 250 X.

The boundary between the reconstructed lesion and the collagen matrix is marked by bars.

H&E
E7 BF
E7 DF
EN1667
XS2
FH2

raft lesions. No mature squamous differentiation was observed in the EN1667 raft lesion. Some cells also showed necrotic-like morphology (Figure 4.5, EN1667 H&E panel, indicated by the black arrow). In all these aspects, the EN1667 raft lesion showed more features of metaplasia than dysplasia. This pathological picturein the EN1667 raft lesion was similar to that for atypical squamous metaplasia, or AIM (see Section 5.2.2.3 and refer to Figure 1.3B)(Fu and Reagan, 1989b).

To determine the pattern of the SV40 promoter-controlled expression of HPV16 E7, the raft lesions formed by these $pSV_2$1667-immortalized cells were examined by *in situ* hybridization assays with the same E7-specific probe used in previous experiments. Unexpectedly, E7 expression in the raft lesions from the $pSV_2$1667-immortalized HKC was compartmentalized in the bottom layer cells (Figure 4.5), similar to the pattern of E7 expression in the HPV16-immortalized HKC raft lesions (Figure 4.2). In contrast, E7 expression in the EN1667 raft lesion was detected almost uniformly throughout the metaplastic epithelium, similar to that in the HPV16-immortalized HEN lesions.

Although the cells immortalized by $pSV_2$1667 and HPV16 genomic DNA all exhibited compartmentalized expression of E7 oncogene, some subtle differences in the patterns of E7 expression were observed between the $pSV_2$1667- and HPV16-immortalized HKC raft lesions. While the E7 message was detected in all the basal cells and most parabasal cells in the HPV16-immortalized HKC raft lesions, E7 expression in the $pSV_2$1667-immortalized HKC raft lesions was mostly limited to basal cells and the expression level varied among the basal cells. In addition, a few E7-expressing cells also existed in the upper layer of the XS2 raft lesion, a phenomenon not observed in the HPV16-immortalized HKC raft lesions. These

differences between the HPV16- and $pSV_2$1667-immortalized HKC may result from the different characteristics of the SV40 and HPV16 promoters.

Nevertheless, these data strongly suggested that the cellular function responsible for the repression of the integrated HPV oncogenes was not dependent on the HPV16 promoter.

4.2.3 Disruption of HPV16 E7 repression

Another approach to reveal the nature of HPV16 oncogene repression was to test the conditions under which this function can be disrupted. As introduced in Section 4.1.3, a cellular function that represses HPV18 in *in vivo* condition is susceptible to DNA hypermethylation (Rosl *et al.*, 1988). Also as introduced in Section 4.1.5, viral oncogene expression from episomal HPV18 and integrated HPV16 DNA is subject to negative modulation by DNA hypermethylation of viral elements (Rosl *et al.*, 1993; List *et al.*, 1994; Gallego *et al.*, 1994). Although contradictory among these different studies on the exact role of DNA methylation in HPV expression, these data did suggest that DNA methylation be involved in repression of HPV16 oncogenes. This issue was pursued in the current study by testing the role of DNA methylation in regulation of HPV16 oncogene expression under conditions allowing programmed squamous differentiation.

4.2.3.1 Decompartmentalized E7 expression by 5-AZ

Taking advantage of the capacity of the raft system to allow modification of the epithelia reconstruction conditions, 5-AZ was added to the culture media shortly before and during raft culture. E7 expression was examined in the 5-AZ-treated *in vitro* rafts by *in situ*

hybridization assays. An analogous experiment has been done by others, in which cells in monolayer culture were examined (Rosl *et al.*, 1988). In the current experiment, 5-AZ was tested with concentrations of 2 and 20 μM, since it has been shown that the cellular mechanism repressing HPV18 oncogene in monolayer culture is suppressed at these concentrations of 5-AZ (Rosl *et al.*, 1988).

As shown in Sections 4.2.1.2 and 4.2.2.3, both the HPV16- and $pSV_2$1667-immortalized HFK lines HFK16-3 and FH2 showed a restricted E7 expression in their respective raft lesions, although the HPV16-immortalized HFK expressed the E7 message in a wider compartment that included basal and parabasal cells. In the presence of 2 μM 5-AZ, the raft lesion formed by HFK16-3 retained a well-differentiated morphology (Figure 4.6), similar to that found without 5-AZ (Figure 4.2, HFK16-3, E7DF panel). The expression of the viral E7 oncogene was still compartmentalized in the basal cells and some parabasal cells, similar to the untreated cells. Unexpectedly, in the presence of 20 μM 5-AZ, the raft lesion showed almost no signs of mature squamous differentiation. The lesion consisted of large cells with big hyperchromatic nuclei of irregular shape. Significantly, expression of the viral E7 gene was no longer compartmentalized, but became detectible in cells throughout the lesion, except a few necrotic-like cells.

To determine if the decompartmentalized E7 expression in the HFK16-3 raft by 5-AZ was a general phenomenon for genital keratinocytes and HPV16-specific, the $pSV_2$1667-immortalized HFK line FH2 was also tested (Figure 4.7). Similar to that of HFK16-3, the morphology and the expression pattern of E7 in the FH2 raft lesion did not show significant changes in the presence of 2 μM 5-AZ. The cells in the raft retained compartmentalized

Figure 4.6 Morphology and HPV16 E7 expression in the *in vitro* raft lesions from HFK16-3 cells treated with 5-AZ

The cells were treated with either 2 μM (left column), or 20 μM (right column) 5-AZ, respectively.

The magnifications were 250 X.

The boundary between the reconstructed lesion and the collagen matrix is marked by bars.

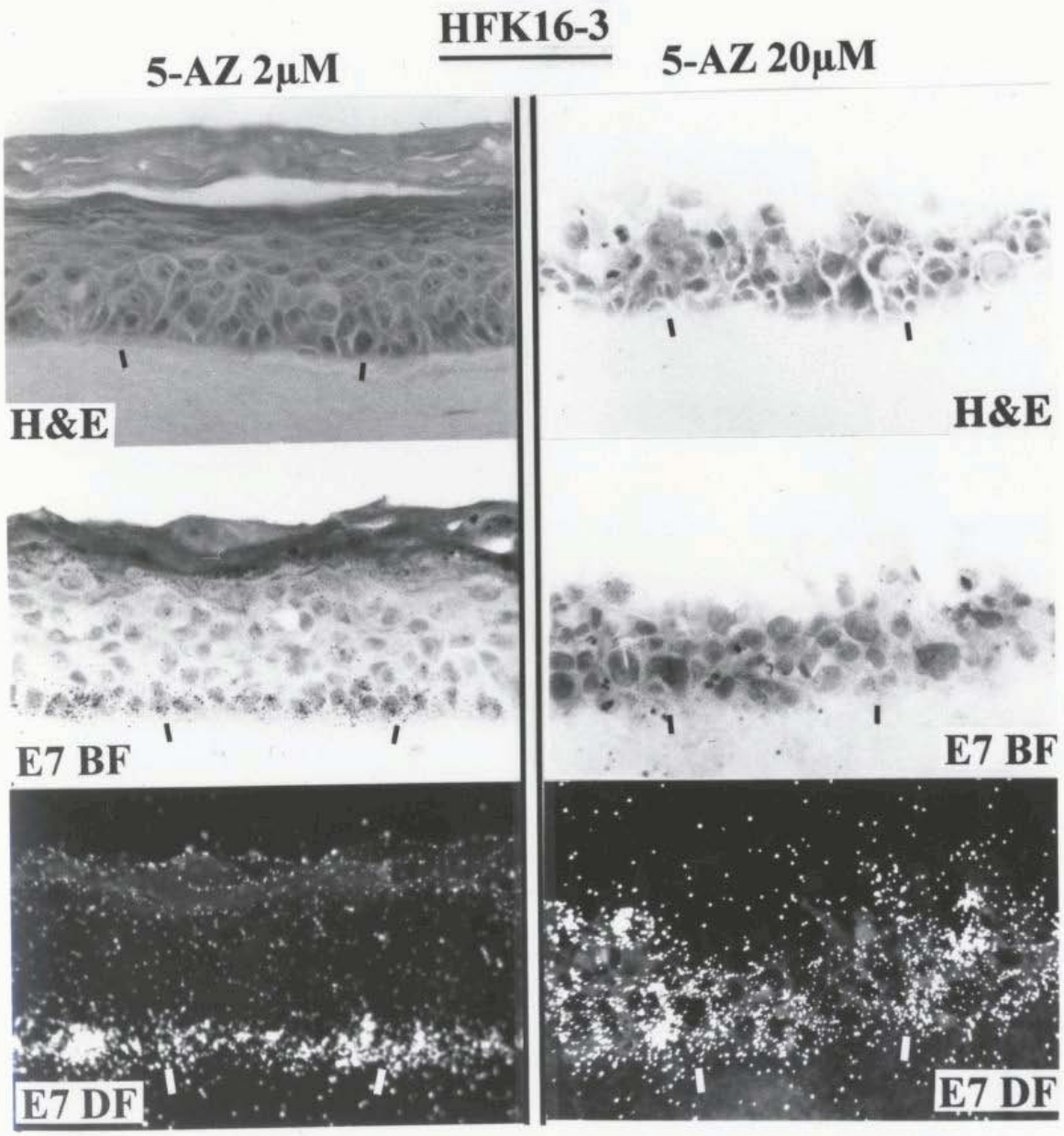
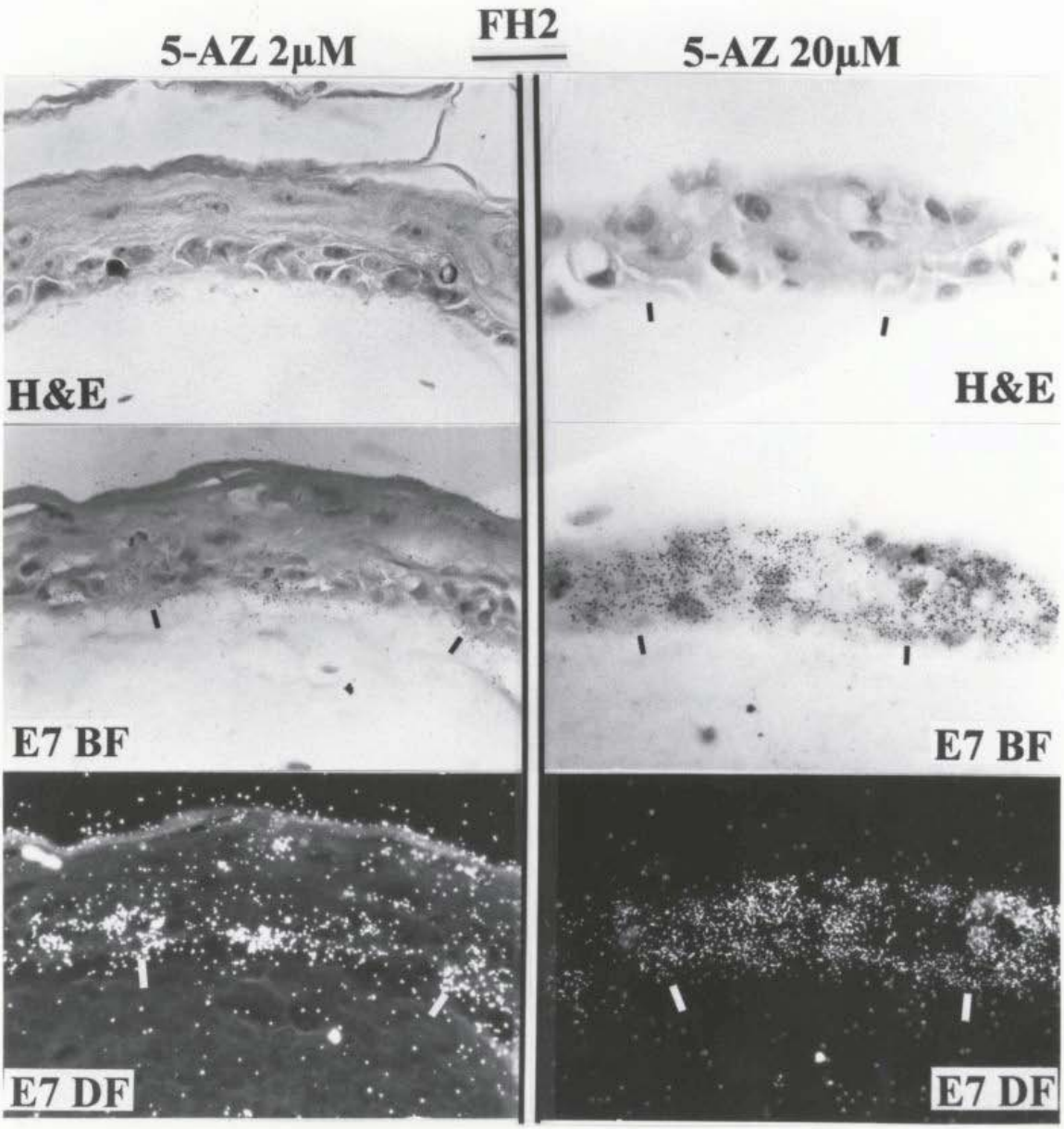

HFK16-3
5-AZ 2μM
5-AZ 20μM
H&E
H&E
E7 BF
E7 BF
E7 DF
E7 DF

Figure 4.7 Morphology and HPV16 E7 expression in the *in vitro* raft lesions from FH2 cells treated with 5-AZ

The cells were treated with either 2 μM (left column), or 20 μM (right column) 5-AZ, respectively.

The magnifications were 250 X.

The boundary between the reconstructed lesion and the collagen matrix is marked by bars.


FH2
5-AZ 2μM
5-AZ 20μM
H&E
H&E
E7 BF
E7 BF
E7 DF
E7 DF

morphologies and the E7 viral oncogene was mainly expressed in the basal cells. However, in the presence of 20 μM 5-AZ, the raft lesion failed to compartmentalize, and the expression of E7 became detectible in cells throughout the raft, as observed for HFK16-3.

Thus, these experiments showed that 5-AZ was able to disrupt the cellular function(s) that is responsible for the repression of the integrated viral oncogene controlled by either the HPV16 or SV40 control elements.

4.2.3.2 Selective disruption of cellular gene expression by 5-AZ

The association between the decompartmentalized E7 expression and disrupted morphological squamous differentiation in the HPV16 oncogene-immortalized HFK raft lesions in the presence of 5-AZ raised one question. Did 5-AZ cause disruption in the programmed squamous differentiation, which then resulted in decompartmentalized E7 expression, or *vice versa*? To address this question, normal HEC was also tested in the raft culture under the same conditions used for the immortalized HFKs, and the pattern of CK expression was examined in the rafts by indirect immunofluorescence assays (Figure 4.8).

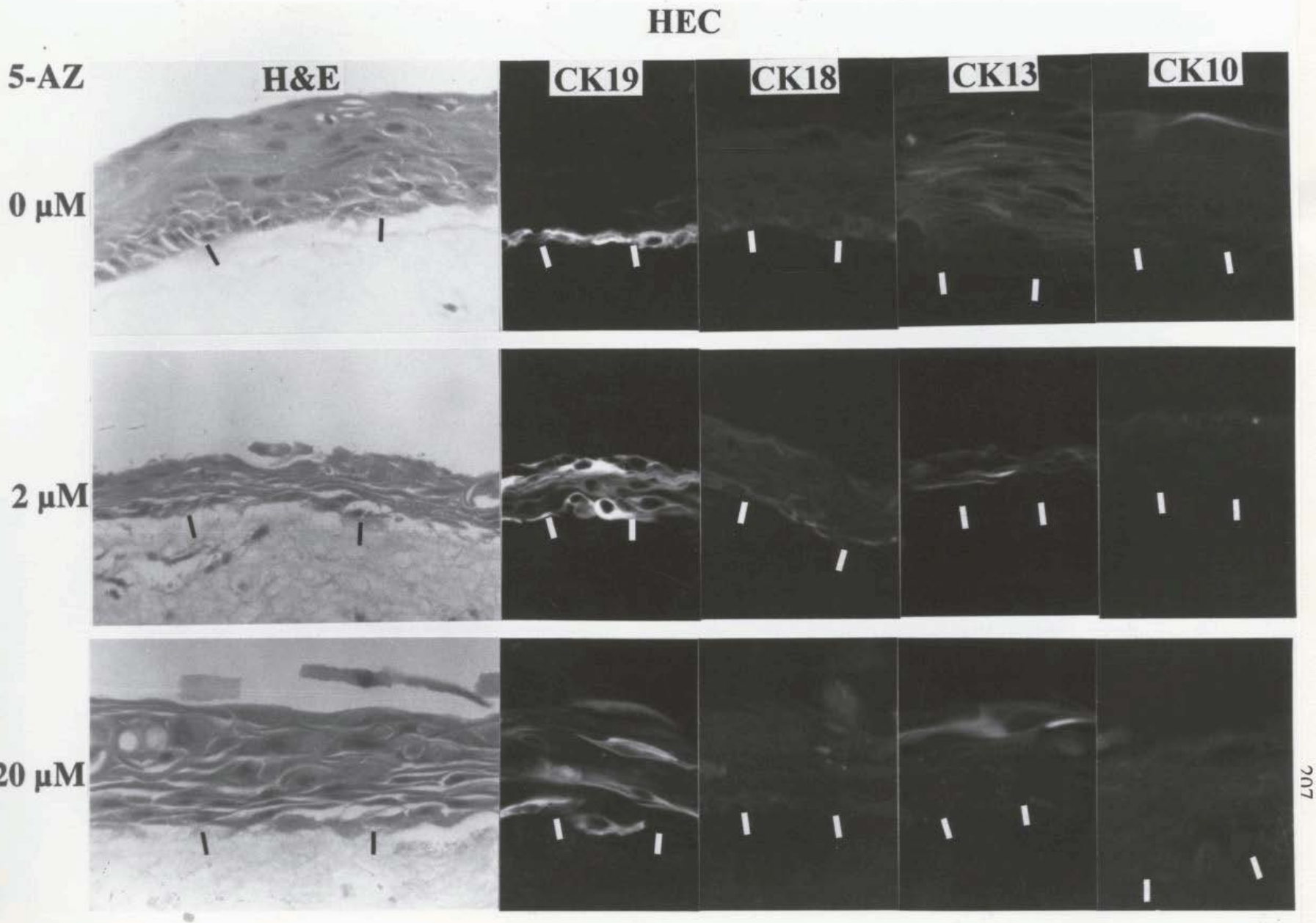
Similar to the pattern found *in vivo*, CK19 in the non-treated normal HEC raft was expressed uniformly in the basal cells and heterogeneously in suprabasal cells. However, CK18, which was completely undetectable in the normal HKC *in vivo* implants, was weakly expressed in the basal cells of the HEC raft. In addition, the two markers for terminal squamous differentiation, the expression of CK13 and CK10 was positive, but at a reduced level, in the upper layers of the HEC rafts, in comparison with that in the HEC *in vivo* implant. These results indicated that the epithelial homeostasis for mature squamous

Figure 4.8 Morphology and the CK expression in the *in vitro* raft epithelia from normal HEC treated with 5-AZ

Note the pattern change of CK19 expression in association with the various concentrations of 5-AZ.

The bars indicate the boundary between the epithelium and the collagen matrix.

The magnifications were 250 X.


HEC
5-AZ
H&E
CK19
CK18
CK13
CK10
0 µM
2 µM
20 µM

differentiation was not genuinely achieved in *in vitro* raft culture, although the raft system may closely mimic the *in vivo* conditions, as also suggested by others (Boukamp *et al.*, 1990a). This result is also consistent with the observations made in Sections 4.2.1.1 and 4.2.1.2.

In the presence of 2 μM 5-AZ, HEC formed an SSE with a reduced thickness (Figure 4.8). In the presence of 20 μM 5-AZ, the cells in the raft appeared to be proportionally increased in size, and the upper layer cells showed very rich cytoplasm. Even though the morphologies of the HEC rafts in both concentrations of 5-AZ were abnormal, morphological squamous differentiation appeared to be retained and no dysplastic changes were observed in the reconstructed epithelia. The expression patterns for CK18, 13 and 10 in the HEC rafts were largely unaffected by either 2 or 20 μM 5-AZ (Figure 4.8). However, 5-AZ modified CK19 expression in the HEC rafts concentration-dependently. With 2 μM 5-AZ, CK19 was expressed throughout the raft, whereas with 20 μM 5-AZ, CK19 expression became prominently heterogeneous among the cells, although the distribution of these CK19 positive cells remained throughout the raft.

The results indicated that although 5-AZ did not disrupt programmed squamous differentiation fundamentally, it did selectively affect the expression of genes associated with squamous differentiation.

4.3 Discussion

The organotypic (raft) culture system was used in the current study to reconstruct epithelia in an *in vitro* condition to address the questions that could not be answered by using

the *in vivo* system.

In the raft system, normal HEC and HEN formed epithelia resembling well-differentiated and metaplastic SSE, respectively. The results were completely consistent with the finding from the *in vivo* system. However, despite the morphological similarity, CK expression patterns in the normal HEC raft showed that the *in vitro* raft culture condition did not allow HEC to reach genuine epithelial homeostasis for mature squamous differentiation. For example, CK18, which neither is detectible in native SSE, nor was in the normal HKC *in vivo* implants, was weakly expressed in the basal layer of the normal HEC raft. Because HEC cultured in monolayer also weakly and heterogeneously expresses CK18 (Turyk *et al.*, 1989; Tsutsumi *et al.*, 1992), the CK18-expressing basal cells in the normal HEC raft may retain some characteristics of HEC cultured in *in vitro* monolayer. The upper layer expression of CK10 and CK13 in the normal HEC raft indicated that programmed squamous differentiation indeed occurred in raft culture. However, these two differentiation markers were stained considerably more weakly than in the *in vivo* implants. These results suggested that terminal differentiation in the normal HEC *in vitro* raft was delayed and/or not fully developed. This result is consistent with other reports. Boukamp *et al.* (1990a) also observed that the *in vitro* rafts formed by human keratinocytes from several anatomical sites fail to achieve full homeostasis for squamous differentiation. Indeed, attempts had been unsuccessful to propagate HPV using the raft system, until the very recent success by using a particular cell line harbouring HPV31b and supplementing the raft culture with TPA (Meyers *et al.*, 1992).

Despite such shortcomings, this system still provided important information on the cellular mechanism responsible for the repression of integrated HPV oncogenes.

4.3.1 Repression of integrated foreign genes in *in vitro* reconstructed epithelia

In contrast to normal HEC and HEN, and consistent with their behaviour *in vivo*, the HPV16-immortalized HKC and HEN were reconstructed into lesions resembling low grade and high grade dysplasia, respectively (Sun *et al.*, 1992; Sun *et al.*, 1993). The patterns of viral E7 expression in the rafts formed by the HPV16-immortalized cells were also consistent with those observed in the *in vivo* lesions. The E7 message was detected in cells throughout the HEN16-2 raft lesion. In contrast, the HPV16-immortalized HKC *in vitro* lesions again showed a clearly compartmentalized viral E7 expression, although the E7-expressing compartment was extended to parabasal cells. Nevertheless, this study suggested that the *in vivo* factors might not be responsible for the repression of the integrated HPV16 oncogene in differentiating HKC.

The reason for the extended E7 expression in the HPV16-immortalized HKC raft lesions remains unclear. Since the raft culture may support a differentiation program that was not genuinely identical to the *in vivo* one, as indicated by the patterns of CK expression in the normal HEC raft, the extended E7-expressing compartment can be logically attributed to a delayed squamous differentiation.

The repression function for integrated HPV16 oncogenes, which was observed in HKC under conditions allowing squamous differentiation, was not specific to the HPV promoter. This conclusion was drawn from the experiments examining the pattern of E7 expression in the cell lines containing integrated SV40-controlled HPV16 oncogenes.

First, the results showed that the activity of the SV40 promoter had at least two

features different from those of HPV16. In monolayer culture, the SV40 promoter appeared to be more active than the HPV16 promoter in both HEN and HKC. In addition, while the HPV16 promoter was negatively regulated by TGF-β, the SV40 promoter was not responsive to TGF-β. These results were good indications that the integrated HPV16 oncogenes in the $pSV_2$1667-immortalized cells were controlled by a heterogenous promoter that was fundamentally different in function from the homologous HPV16 promoter.

Secondly, the results showed that the $pSV_2$1667-immortalized HKC formed well-differentiated SSE with few dysplastic changes. This is consistent with the observation made by others, that the SV40-immortalized keratinocytes form SSE that is indistinguishable from that from normal keratinocytes (Lechner and Laimins, 1991). In contrast, the $pSV_2$1667-immortalized HEN formed a raft lesion that was similar to immature metaplastic SSE with some dysplastic changes. Most significantly, this experiment showed that, despite being controlled by a heterologous promoter, the expression of the HPV16 E7 gene in the $pSV_2$1667-immortalized HKC raft lesions was compartmentalized in the basal undifferentiated cells, while it was extensively expressed in the HEN raft lesion.

These findings had two important implications. First, they indicated that the cellular function(s) responsible for repressing the expression of the integrated HPV16 oncogenes in the immortalized HKC lesions may not as expected be specific to HPV16. Indeed, in consideration of the distinct tissue- and species-specificity of HPV16 and SV40 infections, the HPV16 and SV40 promoters may have a fundamental distinction in the mode of operation, as indicated by the experiments by Chong *et al.* (1991). The HPV16 promoter shows a strong specificity for epithelial tissue and has low activity in cells of mesenchymal

origins, such as human fibroblasts (Smits *et al.*, 1990; Chong *et al.*, 1991; Butz and Hoppe-Seyler, 1993). In contrast, it has not been shown that the SV40 early promoter possesses such specificity. The fact that the cellular function for E7 repression was observed to be active for these two different types of promoters raised the suspicion that this function may be largely independent of promoters, and may in fact affect a wide spectrum of promoters, including the SV40 promoter. However, it should be noted that because the $pSV_2$1667 construct contained the HPV16 E6/E7 genes, it can not be excluded that the compartmentalized expression of the E6/E7 was due to differential mRNA processing or stability in the course of squamous differentiation.

Secondly, the current study suggested that the function repressing the expression of integrated HPV16 oncogenes repression may be an inherent cellular mechanism in HKC but not in HEN. Indeed, since the current experiments used newly established cell lines, it was unlikely that the differential patterns in E7 expression between the immortalized HEN and HEC raft lesions were due to differential acquisition of cellular mutations. Because the HPV16 and SV40 promoters have different regulatory functions (Chong *et al.*, 1991), the promoter-independent, extensive expression of E7 in the HEN lesions suggested that the gene expression control in HEN was relaxed, and that the cellular environment in HEN was promiscuously permissive for promoter activities. The notion that the cells in metaplasia have relaxed control for gene expression is consistent with the fact that CKs such as CK13 and CK18, which are associated with distinct epithelial types and are exclusively expressed in SSE and SCE, respectively, are co-expressed in metaplastic SSE (Smedts *et al.*, 1993a). This nature of metaplastic SSE is also reflected in the transgenic mice studies. In transgenic mice,

expression of the transgenes may retain their original tissue- and differentiation-specificity. For example, transgenes under the control of the CK10 or αA crystallin promoter are expressed in the tissues and cells with the corresponding differentiation status (Griep *et al.*, 1993; Auewarakul *et al.*, 1994). However, in transgenic mice carrying HPV16 E6-E7 transgenes controlled by the promoter of the basal cell keratin CK14, the expression of transgenes becomes detectible in the cervix when estrogen was administrated to the animals to induce cervico-vagina metaplasia (Arbeit *et al.*, 1994; Arbeit *et al.*, 1995). Since the CK14 promoter is not directly activated by the estrogen receptor, the enhanced expression of HPV16 E6-E7 transgenes controlled by the CK14 promoter in the cervical cells possibly results from a promiscuously permissive cellular environment due to the estrogen-induced metaplasia. In addition, it is possible, but not proven, that other mechanisms, such as the post-transcriptional regulation, may also be unique in metaplastic SSE.

4.3.2 Repression of HPV expression, squamous differentiation, and DNA methylation

The expanded compartment of the E7-expressing cells in the HPV16-immortalized HKC *in vitro* lesions with delayed squamous differentiation suggested that the HPV repression mechanism may be closely associated with squamous differentiation. This is consistent with the inverse relationship between E7 expression and CK1 expression in the HPV16-immortalized HKC *in vivo* lesions (see Section 3.2.3). This view was further supported by the unexpected results from the 5-AZ-treated HKC raft culture.

5-AZ was designed as a chemotherapeutic agent for cancers (Jones, 1985). Although 5-AZ has been known to prevent DNA methylation by inhibiting enzymes that methylate the

cytidine residues in CpGs in eukaryotic DNA, the exact effect of 5-AZ on cell biology and gene expression is not clear. Equally unclear is the function of DNA methylation for gene expression. However, DNA methylation is clearly important for mammals, since knock-out mice with a disrupted DNA methylase were embryonically lethal (Li *et al.*, 1992). Substantial evidence implicates the involvement of DNA methylation at the CpGs in the regulation of gene expression (Razin and Cedar, 1991). Indeed, the CpGs in genomic DNA are unevenly distributed and they are more often clustered in the control regions of the housekeeping genes. These clustered CpGs are also referred to as CpG islands. The CpG islands in an actively expressed genomic locus are usually hypomethylated, whereas hypermethylated CpG islands are found in the inactive loci. Gene regulation by DNA methylation has been attributed to the methylation of specific sites in gene control elements, as seen in gene imprinting. The methylated CpG may interfere directly with the binding of certain transcription factors to their cognate DNA sequences (Prendergast *et al.*, 1991). Alternatively, the methylated CpG islands may be bound with CpG-binding proteins (MeCPs) (Meehan *et al.*, 1989). MeCPs are a family of proteins that bind to methylated DNA with various affinities. The binding activities of MeCPs to methylated CpG sites require specific sequences surrounding the methylated CpG islands (Zhang *et al*, 1989), and MeCP bound to CpG sites may prevent the formation of transcriptional complexes (Antequera *et al.*, 1990; Boyes and Bird, 1991; Boyes and Bird, 1992). In addition, DNA methylation may promote nucleosome assembly into heterochromatin, which is believed to be inactive for gene transcription (Keshet *et al.*, 1986). One feature of the gene control at the chromatin level is that it may exert control over genes in a region of the genome, referred to as the position effect. MeCPs may contribute to

heterochromatinization by stabilizing the nucleoprotein complex (Antequera *et al.*, 1990; Antequera *et al.*, 1989) and serving as phasing elements for nucleosome assembly via the methylated CpG (Meehan *et al.*, 1989). Thus, DNA methylation may function in gene regulation in a direct specific manner, and/or regulate expression of genes located in certain genomic domains at a more global level. Since MeCPs include different classes of proteins that show distinct binding specificity and affinity for methylated CpGs, the global effect of methylation on gene expression may also possess some selectivity (Jones, 1985).

DNA methylation patterns are tissue- and organ-specific, and are involved in cell differentiation. Studies on myogenesis provided the best studied example (Weintraub, 1993). DNA in some nonmuscle cells cultured *in vitro* for a long period is hypermethylated in the genomic locus containing the myo-D gene, one of the master genes for muscle differentiation. Treatment of certain fibroblast cell lines with 5-AZ converts some cells into clones with myogenic phenotypes. This transdifferentiation is associated with specific demethylation at the myo-D locus and concurrent activated expression of myo-D. In addition, myo-D expression is not the only requirement for myogenic differentiation, since cells of different lineages show differential susceptibilities to myo-D-induced myogenic differentiation. Keratinocytes are resistant to myogenic transdifferentiation by myo-D transfection alone and can be induced to transdifferentiate into myogenic cells by treatment of the myo-D transfected keratinocytes with 5-AZ (Boukamp *et al.*, 1992; Boukamp and Fusenig, 1993). In addition, cellular dedifferentiation and transdifferentiation events that are involved in tissue regeneration have also been found associated with changes in the general patterns of DNA methylation (Casimir *et al.*, 1988). Based on the ample evidence implicating DNA methylation

in cell differentiation, the current consensus regards DNA methylation as a passive mechanism for controlling cell differentiation. This passive control may enforce the active differentiation controls implemented by the complex active feedback circuits constituted by transcription factors (Blau, 1992). Such a passive control mediated by DNA-methylation may function to stabilize the patterns of gene expression established by active controls and thus prevent "leaky" expression of genes that are not required for or are inappropriate to the status of cellular differentiation.

DNA methylation has also been connected with viral infection and viral gene expression. The best studied example is EBV (Klein, 1993; Klein, 1994). The B-cells latently infected with EBV, including the *in vitro* immortalized B cells, contain episomal viral DNA that is highly methylated and does not support virus vegetative replication (Masucci *et al.*, 1989). Treatment of these cells with 5-AZ converts the program of viral gene expression from the one for latent infection to the other for vegetative infection. In association with this transition is the demethylation of the CpG sites in the control elements of the EBV genome. Since 5-AZ affects the differentiation status of a wide spectrum of cells, 5-AZ may also promote the transition from latent to vegetative infections via modulating the differentiation status of the latently infected B cells. As introduced in Section 4.1.4, DNA hypermethylation has also been reported to be involved in the expression of HPV18 and 16 oncogenes (Rosl *et al.*, 1993; List *et al.*, 1994). DNA methylation at multiple CpG sites in the LCR of episomal HPV18 DNA results in MeCP binding at these sites, which is accompanied by the extinguishment of viral oncogene expression. For HPV16, hypermethylation at two sites in the LCR down-regulates viral gene expression by blocking the binding of the transactivating

factor MSPF. In addition, the major part of the integrated HPV16 DNA in CaSki cells is methylated and incorporated into transcriptionally inactive chromatin.

The current experiments showed that 5-AZ disrupted the function for E7 repression in the immortalized HFK *in vitro* lesions. The molecular and biochemical mechanism for this activity is not clear. However, since the only known biochemical effect of 5-AZ is DNA demethylation, it is possible that the mechanism for E7 repression is related to DNA hypermethylation. In contradiction with the result from the cell hybrids system (Rosl *et al.*, 1988; Rosl *et al.*, 1991), 5-AZ in the current study decompartmentalized, or derepressed E7 expression, instead of resulting in repression of the viral E7 oncogene. The difference between the current and other studies could be due to the fact that the previous study used cells cultivated for a long period *in vitro*, and the experiment was carried out in monolayer culture. The data from the current study is consistent with those from the very recent studies suggesting the involvement of DNA methylation in repressing the expression of HPV16 and HPV18 oncogenes (Rosl *et al.*, 1993; List *et al.*, 1994; Gallego *et al.*, 1994). The current study was more relevant to understanding the mechanism for the involvement of DNA methylation in HPV16-mediated oncogenesis than the previous studies, in that the cells were examined in a condition allowing differentiation. The finding that 5-AZ derepressed the integrated viral genes controlled by HPV16 as well as SV40 promoters suggested the involvement of DNA methylation in the repression of integrated foreign genes. It also reinforced the notion that the repression of integrated HPV16 oncogenes in the immortalized HKC occurred as one part of a global regulation, rather than a specific one for the HPV16 promoter.

The parallel study of 5-AZ effect on the differentiation of normal HEC in raft culture provided additional information on the relationship between the function for the repression of integrated oncogenes and squamous differentiation. The results showed that 5-AZ did not cause a fundamental disruption in programmed squamous differentiation, since the expression of CK18, 13 and 10 was largely undisturbed by 5-AZ. However, the expression of CK19 was altered by 5-AZ in a concentration-dependent manner, consistent with the finding that E7 derepression by 5-AZ occurred most prominently in the rafts treated with the higher 5-AZ concentration. Thus, it seemed that the repression of integrated foreign genes in the HKC lesions was dependent on the level or/and extent of DNA methylation. This is consistent with the previous well-established finding that gene repression by DNA methylation in general is related to the density of methylated CpG islands in the promoters (Boyes and Bird, 1992).

Thus, 5-AZ is hypothesized to effect through a gene repression mechanism that is dependent on DNA methylation and is required for repressing gene expression selectively rather than specifically during squamous differentiation. Such a mechanism has the potential to play an important role in squamous differentiation. During the progression of squamous differentiation, the majority of cellular genes that are not involved in squamous differentiation should be gradually silenced, with the concurrent activation of genes related to squamous differentiation, including those for the CKs (Fuchs, 1990). The mechanism for the squamous differentiation-associated gene repression is open to speculation. Repression of gene expression can of course be achieved by an active mechanism mediated by up-regulation of *trans*-acting repressors or down-regulation of *trans*-acting activators. *Cis*-acting DNA elements have been described in the control region of CK genes, but their mechanisms of

action have not been clarified for repression (Leask *et al.*, 1990). In consideration of the harsh external environment to which native SSE is exposed, the mechanism for gene repression associated with squamous differentiation should function stably or even irreversibly. One rationale for this reasoning is that, as the differentiating cells become closer to the epithelial surface, they have more chance to contact external mutagens and mitogens. If cellular genes are not stably suppressed, the external mitogens and mutagens are able to reactivate the genes controlling cell proliferation and lead to cell replication. This would pose a neoplastic threat to the organism. DNA methylation-mediated gene repression seems to be an ideal avenue to arrive at the stability requirement for gene suppression. Indeed, gene suppression by DNA methylation is stable and heritable. Thus, it is possible that DNA methylation is involved in squamous differentiation and functions at the global level to repress the expression of genes that are no longer essential for cell functions. This notion is consistent with the finding that a long period of *in vitro* cultivation may result in hypermethylation of cellular DNA, and the inactive genes of cells cultured *in vitro* become hypermethylated.

This hypothetical scenario fully explains the promoter-independent, 5-AZ-susceptible features of the E7 repression function in the immortalized HKC *in vitro* lesions. Since the cellular genes involved in squamous differentiation may occupy only a small portion of the cellular genome, viral DNA integration into these domains should be relatively rare. Thus, it is logical that viral DNA integration in most cases occurs in cellular genomic loci that are suppressed during programmed squamous differentiation. As a result, when the cells begin their journey for terminal squamous differentiation, the integrated E7 gene that was under control of either the homologous HPV16 promoter or the heterologous SV40 promoter

would be repressed by the mechanism mediated by DNA methylation, along with the flanking cellular genes.

This hypothetical scenario can also explain the 5-AZ effects on the repression of E7, and on the expression of CKs, in the immortalized and normal HKC rafts. In the presence of 5-AZ, the DNA methylation-mediated mechanism for gene repression may be disrupted. However, because squamous differentiation remains directed and maintained by the active control mechanism, which is mediated by a dynamic balance of trans-acting factors. Although gene repression in the process of squamous differentiation may be "leaky" without the passive control, this may not pose a major problem for the normal HEC, since the normal cells contain neither mitogenic viral oncogenes nor growth-promoting cellular mutations. Thus, the program for squamous differentiation may proceed, as seen for the 5-AZ-treated normal HEC raft. However, without passive control, a differentiation program directed only by active control becomes susceptible to disruption. The effect of 5-AZ on the differentiation and E7 expression of the immortalized HFK *in vitro* lesions may reflect this possibility. The "leaky" expression of the viral oncogenes, which are mitogenic in nature, may be sufficient to disturb the differentiation program, leading to the atypical cellular phenotypes, in association with extended expression of the viral E7 oncogene.

4.4 Summary

The experiments in this chapter were designed to reveal the nature of the differential repression of integrated HPV16 oncogenes in the immortalized HEN and HKC. Using an *in vitro* raft system, the current study showed that the HKC-inherent, differentiation-associated

function for E7 repression was functional in the *in vitro* condition, was independent of HPV16 promoters, and was most likely susceptible to DNA demethylation. Since DNA demethylation not only decompartmentalized E7 expression in the immortalized HKC *in vitro* lesions, but also selectively disrupted the patterns of CK expression in the normal HKC raft, the cellular function responsible for repressing integrated foreign genes may also be involved in a gene repression mechanism that is mediated by DNA methylation, functions at a global level, and is associated with terminal squamous differentiation. In contrast to the cells in the HKC lesions, those in the HEN lesion may not only lack this repression function, but also have relaxed gene expression control. The difference between HEN and HKC in their controlling expression of integrated foreign genes may be the basis for their differential oncogenic susceptibility to HPV16 infection.

CHAPTER 5

NATURAL HPV16 INFECTIONS IN NATIVE AND METAPLASTIC SSE

5.1 Introduction

The *in vivo* and *in vitro* experimental models described in the previous two chapters provided provocative information on HEC and HEN concerning their differential expression of integrated foreign genes in conditions allowing differentiation. These results suggested that HEC possess a differentiation-associated mechanism that represses the expression of integrated exogenous genes. This mechanism seems to function with some selectivity but not with specificity. In contrast, HEN may transdifferentiate into a metaplasia-related state, which appears to be promiscuously permissive for the expression of integrated foreign genes. These findings could be relevant to the mechanisms underlying the differential oncogenic susceptibility of native SSE and metaplastic SSE to HPV16 infection.

5.1.1 Golden test of experimental data: verification in natural lesions

However, these experimental data should be verified by the golden test of experimental medicine, to confirm the relevance of experimental data to natural diseases by analysing the lesions *in situ*. Such a necessity arises mainly from the concern regarding the validity of the modelling systems. The modelling systems in the current studies were designed to mimic natural conditions as closely as possibly. However, they differed from the natural HPV16 infections in several important aspects. Therefore, the experimental systems may have

reflected only certain facets of the HPV16-mediated oncogenesis, or may not even have had relevance to the *in vivo* conditions at all. The most obvious difference of the modelling systems from the *in situ* lesions is that the experimental systems in the current studies used *in vitro* cultured epithelial cells, which in many physiological aspects may no longer be entirely equivalent to those in tissues. In addition, HPV16 oncogenes were introduced into the cultured cells by transfection, which is different from natural HPV entry for establishing viral infections. Since viral oncogenesis is a result of the interaction between cellular and viral activities, these differences between the experimental conditions and natural infections may create an artificial environment that may have limited relevance to the oncogenic process mediated by natural HPV infections. Such concern is consistent with the fact, that the natural equivalent of the experimental premalignant lesions reconstructed from the HPV16-immortalized HKC has not been found.

Examining natural HPV16 infections was also imperative for verifying the predictions made on the basis of the new findings from the experimental studies. Based on the experimental data, it is reasonable to predict that the expression of HPV16 oncogenes in lesions containing episomal viral DNA may also be distinct in native and metaplastic SSE. This is because HPV gene expression is dependent on squamous differentiation and the differentiation status of native SSE differs greatly from that of immature metaplastic SSE. This prediction gained support from the study on the cell line W12, the only available line containing episomal HPV16 DNA (Sterling *et al.*, 1990). The W12 *in vivo* lesion was similar to those of the HPV16-immortalized HKC in that they all displayed low grade dysplastic changes, in contrast to the high grade dysplastic changes in the HPV16-immortalized HEN

lesions (Figure 3.8). Significantly, in contrast to the extensive expression of the HPV16 E7 oncogene in the immortalized HEN lesions, the expression of E7 was restricted in the bottom cells in both the W12 and the immortalized HKC lesions, even though the W12 lesion expressed E7 in a wider epithelial compartment than the one from the immortalized HKC (Figure 3.7 and 3.8). The similarly but distinctly restricted expression of E7 in the W12 and HKC *in vivo* lesions were attributable to the different statuses of viral DNA in W12 and the immortalized HKC. While the former contains episomal HPV16 (Stanley *et al.*, 1989; Sterling *et al.*, 1990), viral DNA in the later was integrated. Therefore, these findings are consistent with the above prediction that the expression of the HPV16 oncogenes may be more dependent on the origin and/or differentiation of the infected tissues, rather than on the status of viral DNA. However, due to the lack of suitable cell lines and the concern over the validity of experimental conditions, this prediction must also be tested in natural HPV16 infections.

As introduced in Section 1.3.4.3, previous *in situ* hybridization studies on natural HPV16 infections have established a consensus on the patterns of the viral DNA amplification and late gene expression. However, an important virological question, the expression pattern of viral oncogenes in the low grade premalignant lesions undergoing vegetative virus replication, remains controversial (Durst *et al.*, 1991; Park *et al.*, 1991; Auvinen *et al.*, 1992; Stoler *et al.*, 1992; Bohm *et al.*, 1993). In addition, information from previous studies on natural HPV infections cannot explain the findings from the current experimental studies. Moreover, the relationship between the expression of HPV16 oncogenes and different types of target SSE tissues had not been vigorously probed.

5.1.2 Specific aims

The objectives of the experiments in this chapter were to examine the patterns of HPV16 infections in native and metaplastic SSE and, thereby, to test the relevance of the experimental data in natural HPV16 infections.

5.2 Results

5.2.1 Rationale and design of experiments

Most of the archival pathological blocks were obtained from Department of Pathology, the Sir Mortimer B. Davis-Jewish General Hospital, Montreal, by courtesy of Dr. Ferenczy. The rest were from the Departments of Pathology, General Hospital and Grace Hospital in St.John's, by the courtesies of Drs. Haegert and Pirai, respectively. For samples from Montreal, the clinical and pathological diagnoses were blinded until the study was completed.

HPV16 infections in native SSE and metaplastic SSE were represented by VIN and CIN, respectively. The VINs were chosen as the representatives for the HPV16 infections in native SSE because VINs, unlike ectocervical CINs, had fewer chances of being mixed with the CINs originated from metaplastic SSE, such as those in endocervix or TZ. Furthermore, if this were to happen, VINs can be more easily distinguished from metaplastic SSE-derived CINs by histological features than the native SSE-derived CINs. To ascertain that the CINs representing HPV16 infections in metaplastic SSE were indeed derived from metaplastic SSE, the lesions with unambiguous underlying cervical glands, the landmark for an endocervical origin, were examined (Saito *et al.*, 1987; Fu and Reagan, 1989b). In addition, particular

attention was given to the CIN lesions located immediately adjacent to SC junction, or the SCE-proximal lesions. This was mainly based on the consideration for the progressively dynamic nature of cervical metaplasia. As discussed in Section 3.1.1, metaplastic SSE may exist in various statuses of transdifferentiation, reflected in the forms of stages. Immature metaplasia is often SCE-proximal and may have more phenotypes similar to those of SCE than the SCE-distal metaplasia. In contrast, mature metaplasia is usually SCE-distal and may be indistinguishable from native SSE phenotypically. Since HPV16 replication is dependent on squamous differentiation, differences in differentiation states in metaplastic SSE may affect the pattern of HPV16 infection. Also because cervical metaplasia develops from SCE into SSE, the metaplastic SSE immediately adjacent to SC junction not only should be relatively immature in squamous differentiation, but also may have minimal variations in differentiation status.

In this study, serial sections were examined for the mRNA of the HPV16 E7 oncogene and L1 structural gene, and HPV16 DNA, by *in situ* hybridization assays. For detecting viral E7 and L1 mRNAs, the sense strand-specific antisense riboprobes labelled with ^{35}S were prepared from HPV16 subgenomic fragments cloned in BlueScript SK+II vector by *in vitro* transcription. The E7 probe in this study was the same one used in experimental studies described in Chapter 3 and 4. Both the E7 and L1 probes were verified by sequencing. The signals for RNA *in situ* hybridization were detected by autoradiography. As introduced in Section 3.2.3, positive signals were represented as white dots in darkfield micrography, while they were black in brightfield micrography.

For viral DNA detection, the full length HPV16 genomic DNA was labelled with

biotin as probes. The sections used for DNA *in situ* hybridization assays were heat-treated to denature DNA duplex, and in some cases were digested with RNase to eliminate possible cross-hybridization between the DNA probe and RNA sequences. The hybridized probes were detected immuno-chemically and the signals were presented as deep blue colorimetric deposits. This detection system allows a clearer distinction for the signal subcellular distribution. Furthermore, this detection system also allows identification of the status of viral DNA (Cooper *et al.*, 1991b). Cells with integrated viral DNA show a pattern of punctate staining in the nuclei, while cells with episomal viral DNA display diffuse nuclear staining (Cooper *et al.*, 1991a; Cooper *et al.*, 1991b; Troncone *et al.*, 1992). Exemplary staining patterns for integrated and episomal HPV16 DNA are shown in Figure 5.1. Status of viral DNA can also be extrapolated on the basis of the signal distribution in the epithelial compartments. The cells in lesions with integrated viral DNA most often contain a similar amount of viral DNA due to clonal expansion, while viral DNA was amplified in a subpopulation of cells in lesions containing episomal viral DNA and supporting viral DNA amplification (Figure 5.1). Thus, a pattern of positive diffuse staining in a subpopulation of cells located in a certain epithelial compartment would suggest that these cells supported the amplification of episomal viral DNA. A typical picture for lesions with episomal viral DNA is represented by a vegetative infection, in which the positive viral DNA staining should occur in individual cells in the upper layer differentiating cells.

Based on the combined information on the expression of viral genes and amplification of viral DNA, it was possible to assess the state of HPV16 infection. Viral structural proteins and viral DNA are the basic prerequisites for virion assembly. Viral early proteins are required

Figure 5.1 Characteristic staining patterns of viral DNA in lesions with integrated and episomal HPV16

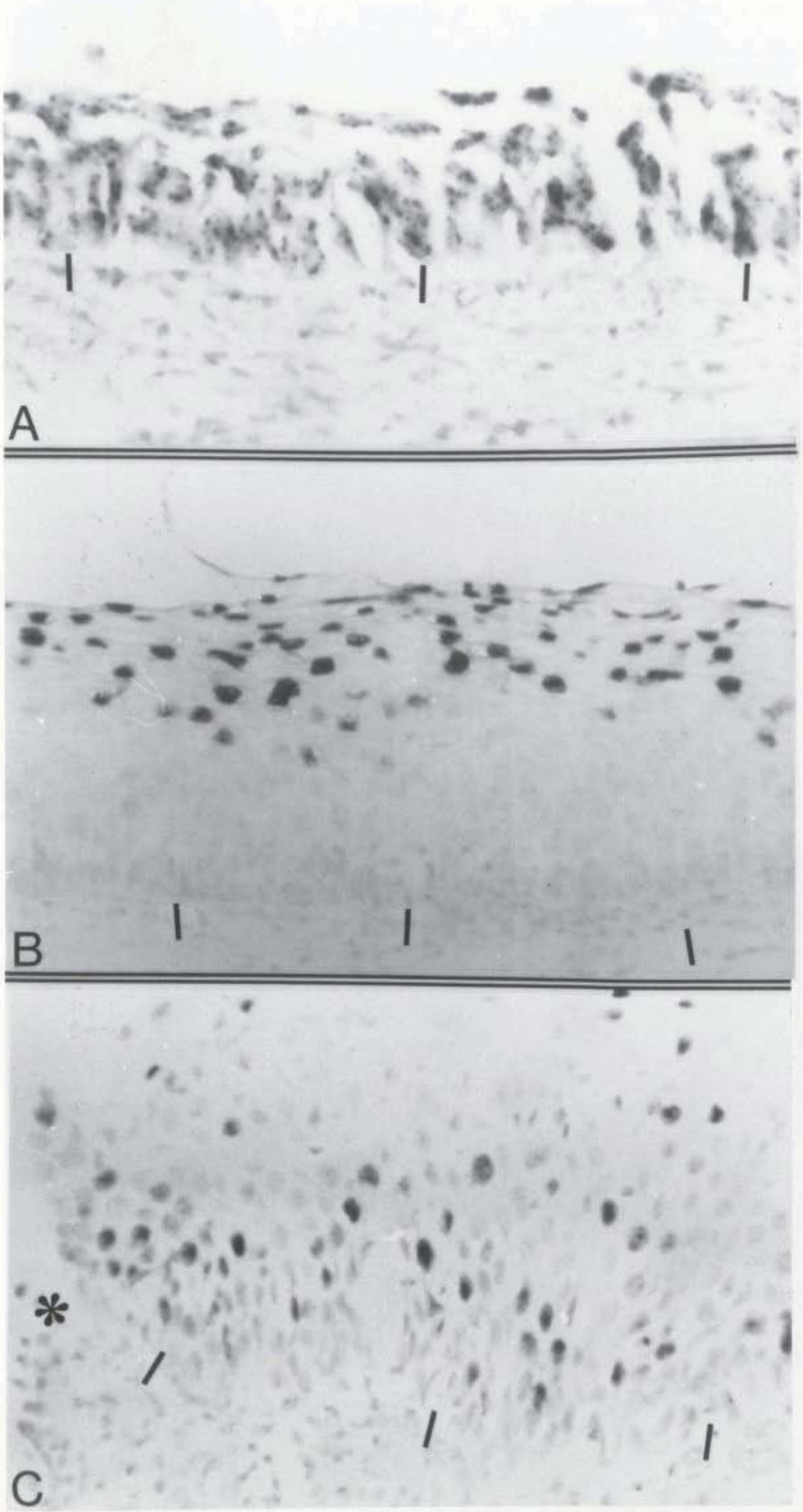
Viral DNA status in the lesion can be confidently judged according to the staining patterns in the nuclei and the distribution pattern of the positive cells (Cooper *et al.*, 1991a; Cooper *et al.*, 1991b). Lesions with integrated viral DNA, if the copy numbers of the integrated viral DNA are sufficient for *in situ* detection, will show a punctate staining pattern, and most cells in the lesion will show the same staining pattern, as exemplified in panel A. For lesions with episomal viral DNA, the DNA staining is diffuse throughout the nuclei, and only certain subpopulation of the cells in the lesion are positive, as shown in the B and C panels.

A: DNA *in situ* hybridization assay of the *in vivo* lesion from CaSki. CaSki cells, which contain approximately 500 copies of HPV16 (Yee *et al.*, 1985), exhibited a pattern of punctate nuclear staining, in all the cells in the *in vivo* lesion.

B: DNA *in situ* hybridization assay for a natural HPV16-infected CIN distal from SCE. HPV16 DNA was detected mostly in the upper layers of the lesion, and the staining pattern was nuclear and diffuse.

C: DNA *in situ* hybridization assay for a natural HPV16-infected CIN proximal to SCE. Although the staining pattern was nuclear and diffuse, similar to that in the B panel, substantial number of the viral DNA-amplifying cells was located in the bottom layers.

The snowflake indicates SC junction.


A
B
C
*

to prepare the cellular environment for the expression of viral late genes and amplification of viral DNA. Thus, E7 expression, DNA amplification and L1 expression should all be detectible in lesions undergoing vegetative infections. Absence of either DNA amplification or L1 expression would indicate that the lesion cannot produce virions and was in a state of non-vegetative infection. In lesions with non-vegetative infections as a result of viral DNA integration, viral DNA staining should either be negative due to the low copy numbers of integrated viral DNA, or present a punctate pattern usually due to limited numbers of integration sites (Cooper *et al.*, 1991b; Nuovo *et al.*, 1991a). The viral L1 gene expression is usually undetectable in lesions with integrated viral DNA, due to disrupted integrity of viral genome (Higgins *et al.*, 1992b).

In cases for which sufficient samples were available, HPV16 infection was confirmed by PCR amplification of the E6 sequences, and attempts were made to determine the differentiation status by examining the patterns of CK expression. One of the major practical hindrances for the current study was the amount of sample material available. Due to the benign nature of the lesions examined in the current study, the biopsy tissue pieces were usually very small. The results in this Chapter was summarized in Table 5.1.

5.2.2 HPV16 infections in native SSE

Based on the expression pattern of the viral L1 structural gene, HPV16 infections in VINs can be divided into categories of either vegetative or non-vegetative infections, as follows

Table 5.1 Summary of results on HPV16 infection in native SSE and metaplastic SSE

"+": positive

"-": negative

"(+)": weak positive

NA: not available

ND: not done

Lesions F8, F9, F11, F13, F15, F17 and F18 were physiological or pathological conditions that are known not related to HPV16 infections and were tested as blinded negative controls.

*: Ethidium bromide staining

§: Southern blot

No	PCR		In situ			Diagnosis
	EB*	S-blot§	DNA	E7	L1	
V4	+	+	+	+	-	VIN
V17	+	+	(+)	+	+	VIN
V22	+	+	+	+	+	VIN
V25	+	+	(+)	+	-	VIN
V26	+	+	+	+	+	VIN
V27	+	+	+	+	+	VIN
V31	+	+	+	+	+	VIN
V38	+	+	-	NA	NA	VIN
V39	+	+	+	+	+	VIN
V41	+	+	+	+	+	VIN
V43	+	+	+	+	+	VIN
C7	+	ND	-	+	-	CIS
C21A1	+	ND	+	+	+	CIN
C21A3	+	ND	-	+	-	CIS
C48A1	+	ND	+	+	+	CIN
C48A1-b	+	ND	+	+	+	CIN
C48A2	+	ND	-	+	-	CIN-CIS
H1	NA	NA	NA	-	-	CIN
H2	NA	NA	NA	-	-	CIN
H6A1	NA	NA	NA	+	-	CIN-CIS
H10A1	NA	NA	+	+	+	CIN
H10A2	NA	NA	-	+	-	CIS
H11E	NA	NA	+	+	+	CIN
H11M	NA	NA	+	+	+	CIN
H13	NA	NA	+	+	+	CIN
H15	NA	NA	-	+	-	CIS
F1	N/A	NA	+	+	+	VIN
F8	NA	NA	-	-	-	Vulval Paget's
F9	NA	NA	-	-	-	Vulval epithelial hyperplasia
F10	NA	NA	NA	-	-	Cervical hyper/parakeratosis
F11	NA	NA	-	-	-	Vulval Paget's
F12	NA	NA	-	-	-	Cervical condyloma
F13	NA	NA	-	-	-	Vulval condyloma
F14	NA	NA	-	-	-	VaIN
F15	NA	NA	-	-	-	Vulval condyloma
F16	NA	NA	-	-	-	CIN
F17	NA	NA	-	-	-	Normal endocervix
F18	NA	NA	-	-	-	Vulval condyloma
M1	NA	NA	-	+	-	CIS
M3G1	NA	NA	-	+	-	CIS
M3G3	NA	NA	+	-	-	CIN
M3G4	NA	NA	+	+	-	AIM
M4G1	NA	NA	+	+	-	CIN
M4G2	NA	NA	+	+	-	AIM
M4G2b	NA	NA	+	+	-	CIN

5.2.2.1 VINs with vegetative virus replication

The F1 VIN lesion represented a low grade VIN (Figure 5.2). DNA *in situ* hybridization showed that the cells showing positive HPV16 DNA staining were mostly localized in the upper layers near the surface of the lesion (indicated by a black arrow). The nuclear staining was in a diffuse pattern, and was positive only for a subpopulation of the upper layer cells, consistent with the pattern predicted for the amplification of the viral episomal DNA. The HPV16 L1 gene was expressed also in cells near the surface of the lesion. The expression of L1 appeared to be activated very abruptly, and high levels of expression were observed in individual cells or clusters of cells. The positive staining for both viral DNA amplification and L1 expression suggested that HPV16 infection in this lesion was vegetative. In contrast to the signal distribution for viral DNA amplification and L1 expression, E7 expression in this lesion was mainly restricted within the bottom layers, spanning approximately 2-3 cell layers. The E7 signal was mostly cytoplasmic. Its distribution in the bottom epithelial compartment was continuous, in contrast to the abruptly activated expression of the viral L1 gene in the upper layer cells.

VIN lesions may contain very rich stroma papillae, which protruded into the proliferated epithelium. This made E7 expression appear to be more abundant in these VINs. The V43 VIN lesion exemplified this feature well (Figure 5.3). The basal membrane on the tips of stroma papillae, as marked by the short white bars, was high above the lowest basal membrane surrounding the bases of stroma papillae, as indicated by long white bars (Figure 5.3, E7BF and DF panels). Careful examination consistently identified a compartmentalized

Figure 5.2 ***In situ*** **hybridization assays of F1 VIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

For abbreviations, see the lists.

The black arrow in the DNA panel indicates a nucleus with positive staining for HPV16 DNA. The arrows in the L1DF and L1BF panels indicate cell clusters positive for L1 expression.

The magnifications were 200 X.

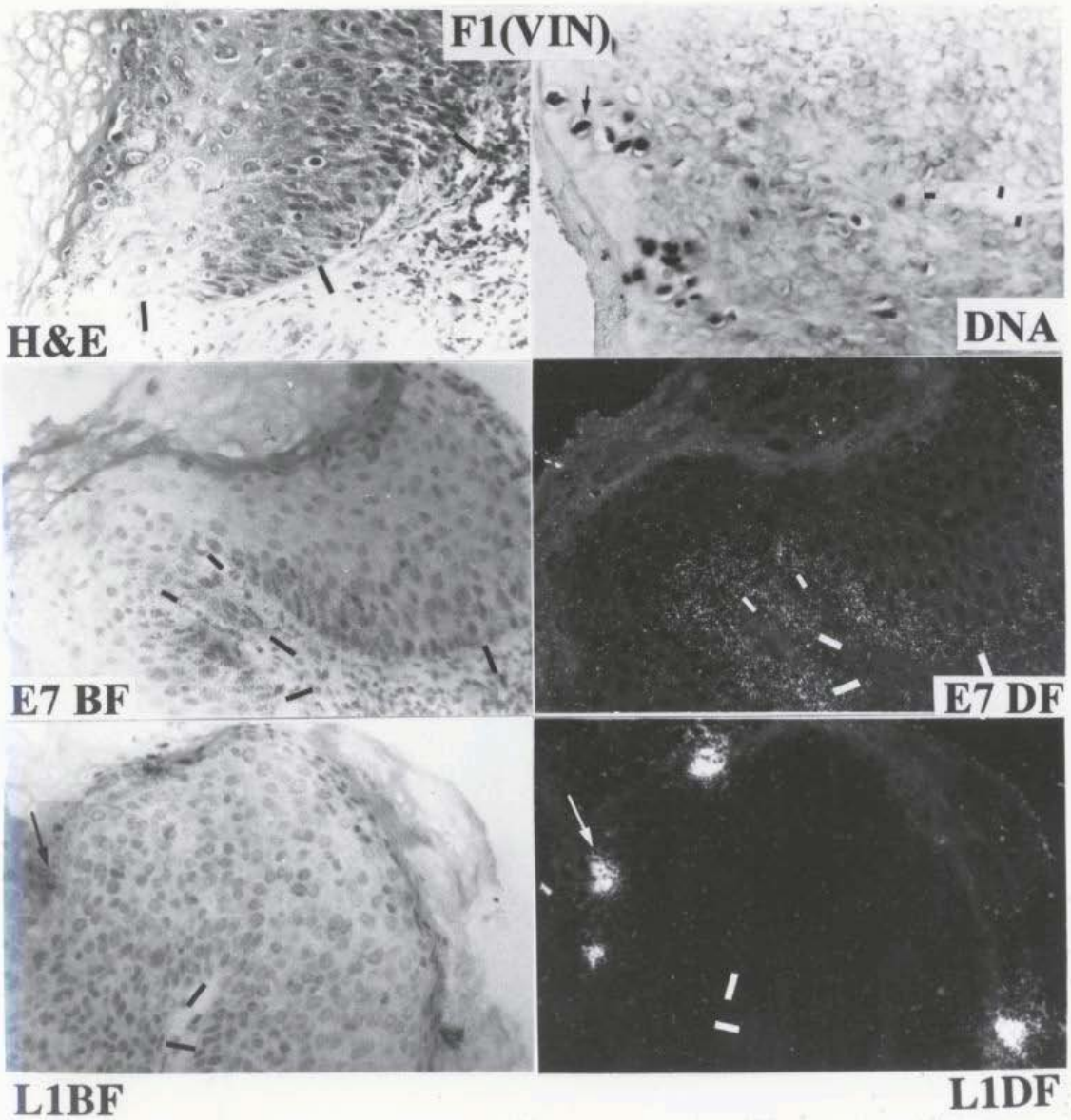

F1(VIN)
H&E
DNA
E7 BF
E7 DF
L1BF
L1DF

Figure 5.3 ***In situ*** **hybridization assays of V43 VIN lesion**

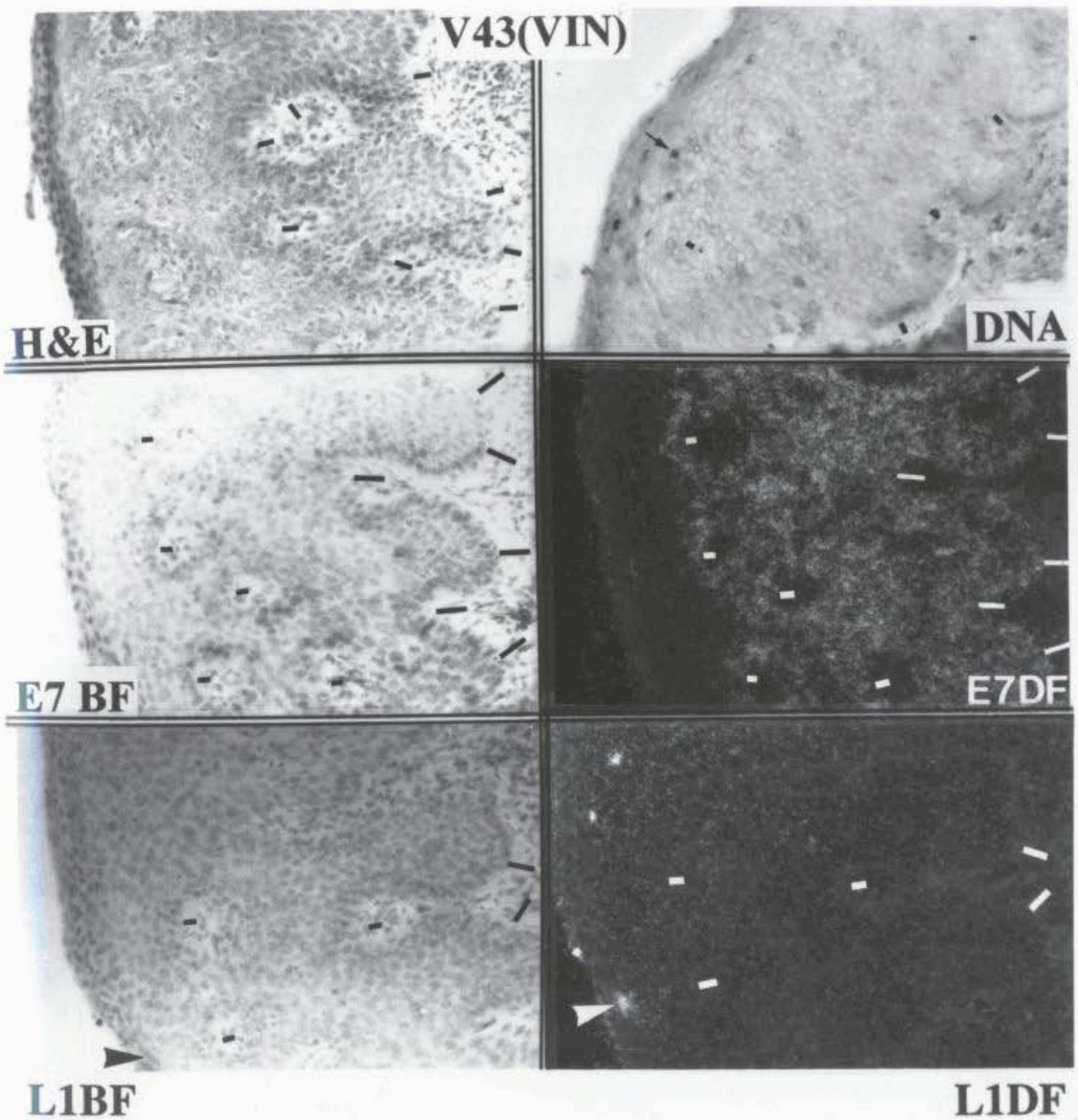
HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

For abbreviations, see the lists.

The black arrow in the DNA panel indicates a nucleus with positive staining for HPV16 DNA. The white arrowheads in the L1BF and L1DF panels indicate the cell positive for L1 expression.

The magnifications were 200 X.


V43(VIN)
H&E
DNA
E7 BF
E7DF
L1BF
L1DF

expression of E7 in the bottom 2-3 layers of cells closest to the basal membrane. In this lesion, viral DNA amplification and L1 expression occurred in the upper layer cells, suggesting that V43 represented vegetative infection.

In addition to consistent E7 expression in the bottom layer cells, cells in the upper layers occasionally showed positive E7 signals, as in the V27 VIN lesion (Figure 5.4, E7DF panel, indicated by the white arrow). Comparing the signals under darkfield and brightfield illuminations, it appeared that most, if not all, of the silver grains were in the nucleus. This was particularly clear in the F1 VIN lesion under higher magnification and brightfield illumination (Figure 5.5, indicated by arrows). These nuclear signals were seen in individual cells sporadically distributed in the lesion, or in small cluster of cells, and appeared to be completely resistant to DNase I digestion. Nevertheless, the presence of viral DNA amplification and L1 expression suggested that these lesions were HPV16 vegetative infections. The V41 VIN lesion was another example of HPV16 vegetative infection in native SSE (Figure 5.6).

5.2.2.2 VINs with non-vegetative virus replication

The two VIN cases observed with non-vegetative infection were exemplified by the V4 VIN lesion. While viral E7 expression and DNA amplification were detectible, the L1 probe detected no signals (Figure 5.7). This suggested that HPV16 infection in this lesion was non-vegetative. Since the individual cells positive for viral DNA showed a diffuse staining pattern, and were located mostly in the upper layers (Figure 5.7, DNA panel, indicated by the black arrow), this non-vegetative state was unlikely to be due to viral DNA integration.

Figure 5.4 ***In situ*** **hybridization assays of V27 VIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

For abbreviations, see the lists.

The black and white bars indicate the basal membrane.

The arrows in the E7 panels indicate the E7 nuclear signal in the upper layer cells of the epithelium.

The arrowheads in the L1 panels indicate the cell clusters positive for L1 expression.

The magnifications were 200 X.

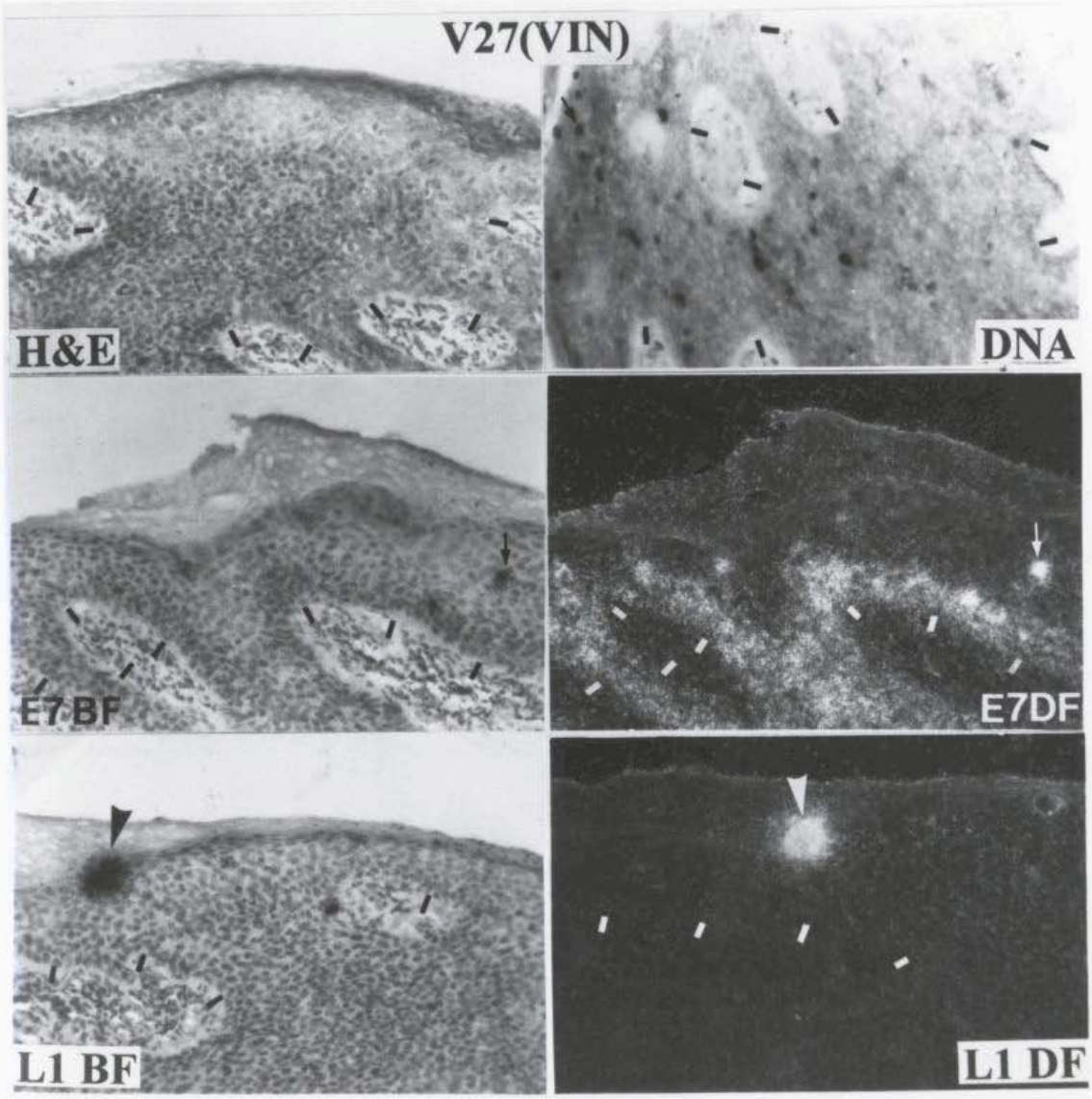

V27(VIN)
H&E
DNA
E7 BF
E7DF
L1 BF
L1 DF

Figure 5.5 ***In situ*** **hybridization assays of F1 VIN lesion pretreated with DNase I**

This section was treated with DNase I before the *in situ* hybridization assay with the E7-specific riboprobe.

The arrows in the E7DF and E7BF panels indicate the same cells.

The black and white bars indicate the basal membrane.

The magnifications were 280 X.

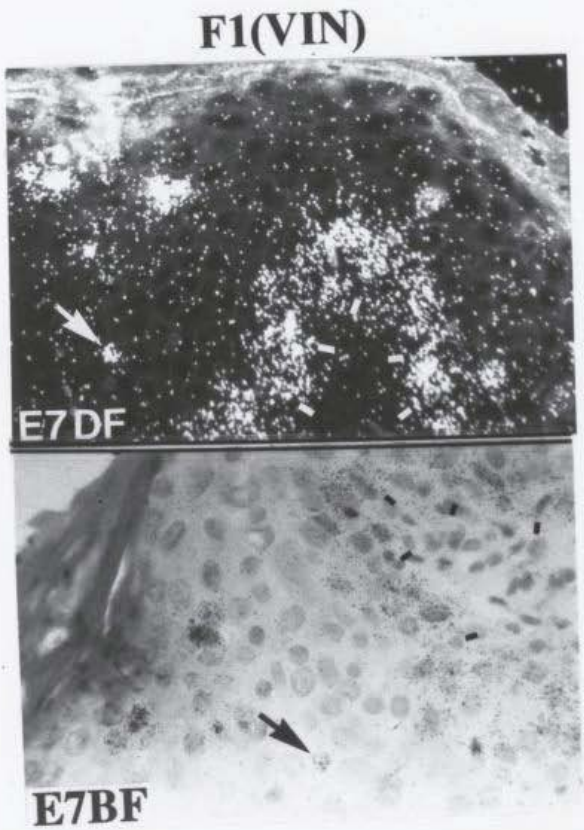

F1(VIN)
E7 DF
E7BF

Figure 5.6 ***In situ*** **hybridization assays of V41 VIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

The black arrow in the DNA panel indicates a nucleus with positive staining for HPV16 DNA.

The arrows in the L1BF and L1DF panels indicate the cell clusters positive for L1 expression.

The magnifications were 200 X.

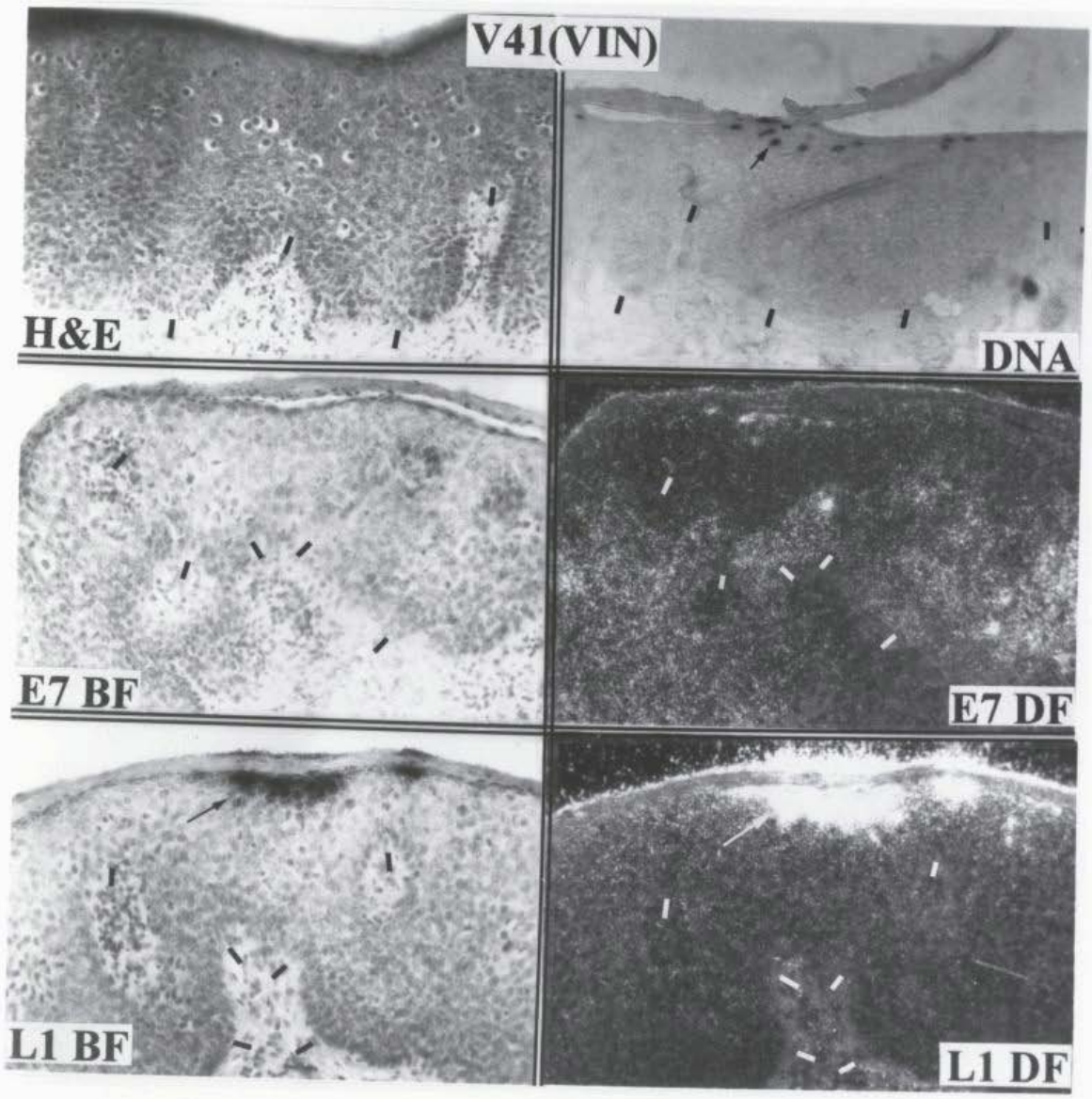

V41(VIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

Figure 5.7 ***In situ*** **hybridization assays of V4 VIN lesion**

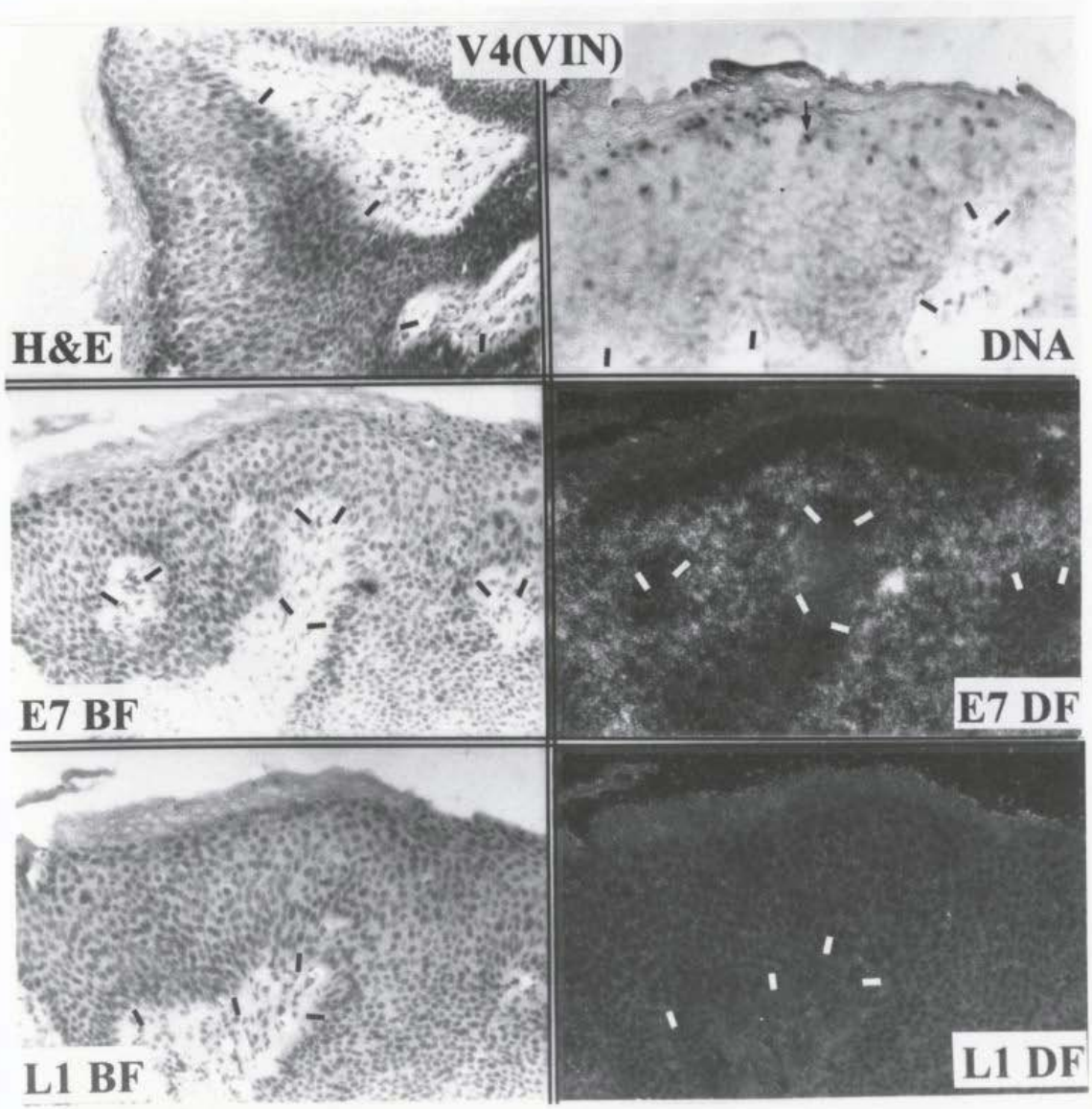
HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

The black arrow in the DNA panel indicates a nucleus with positive HPV16 DNA staining.

Note that the L1 probe detected no signal (L1DF panel), even though DNA amplification was positive (DNA panel).

The magnifications were 200 X.


V4(VIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

Similar to the VINs with HPV16 vegetative infections, the expression of E7 was also detected in the bottom layer cells in the VIN lesions with non-vegetative infections. This indicated that the compartmentalised E7 cytoplasmic expression was a general feature for both HPV16 vegetative and non-vegetative infections in the VINs.

For the convenience of distinguishing the non-vegetative state of HPV16 infections containing episomal viral DNA from those with integrated viral DNA, or INI, the infection state with episomal viral DNA and non-detectible L1 expression was referred to as transitional non-vegetative infection (TNI). This was based on the speculation that, since the TNIs contained episomal viral DNA, this state of non-vegetative infection was potentially able to be corrected under suitable conditions. In contrast, INI, the irreversible non-vegetative infections contain integrated viral genomes, and therefore theoretically cannot produce progeny viruses using the integrated viral genome as templates (Howley, 1990).

5.2.3 HPV16 infection in cervical metaplastic SSE

Similar to VINs, vegetative infection and TNI were also observed in CINs. Besides the variations in the state of infections, CINs also showed variations in the status of viral DNA, of being either episomal or integrated. These variations were closely associated with the location of the lesions, in relationship to the cervical SCE, or SC junction.

5.2.3.1 Vegetative infection in CINs immediately adjacent to SC junction

The C21A CIN lesion represented a low grade CIN that was located immediately adjacent to SCE and had underlying cervical glands (Figure 5.8, DNA panel, SC junction

Figure 5.8 ***In situ*** **hybridization assays of the SCE-proximal C21A1 CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were detected.

A SC junction can be seen in the DNA panel, as indicated by a black snowflake.

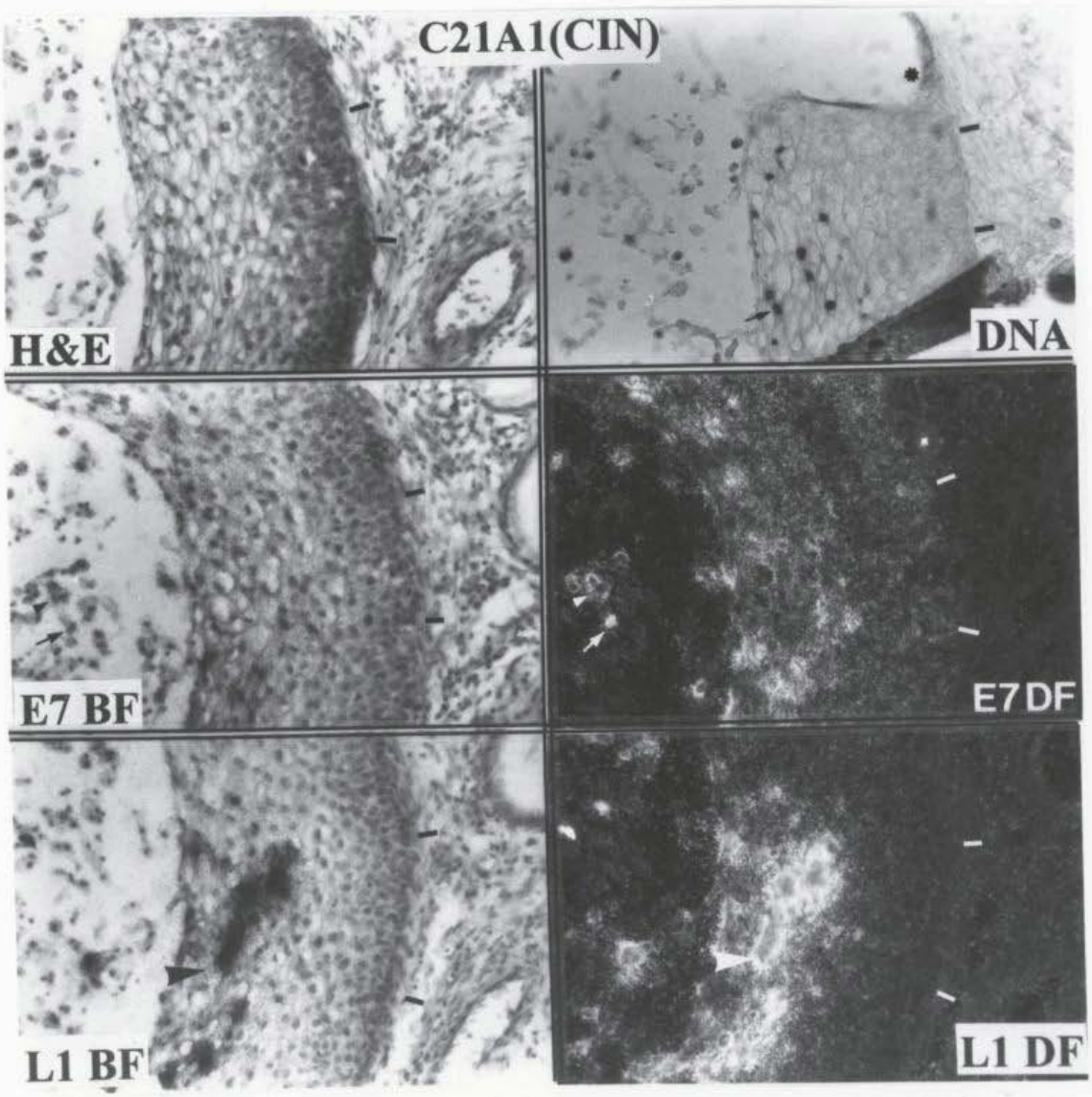
The black arrow in the DNA panel indicates a nucleus with positive staining for HPV16 DNA.

In the E7DF panel, the white arrowhead and white arrow indicate E7 cytoplasmic and nuclear signals in the defoliated cells, respectively.

The arrowheads in the L1DF panel indicate L1 signals.

The magnifications were 200 X.

The black and white bars indicate the basal membrane.


C21A1(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

indicated by the black snowflake). This lesion showed typical squamous differentiation (Figure 5.8, H&E panel), and thus morphologically resembled mature metaplastic SSE. Similar to VINs, viral DNA amplification (Figure 5.8, DNA panel, indicated by the black arrow) and L1 expression (Figure 5.8, L1DF panel, indicated by the white arrowhead) were localized in the upper layer cells. These patterns suggested that this lesion represented a vegetative infection. In this lesion, viral L1 expression was very active, and some cells defoliated from the lesion surface also expressed L1.

In sharp contrast to the VINs, the C21A CIN lesion expressed E7 throughout the epithelium, even in the cells defoliated from the surface of the lesion (Figure 5.8, E7BF and DF panels, indicated by arrow and arrowheads). The predominant E7 signals were cytoplasmic, although some cells in the upper layers also showed nuclear E7 signals. This can be clearly demonstrated in the defoliated cells, most of which displayed cytoplasmic E7 signals and a few cells showed nuclear signals. Although E7 expression in the upper layers appeared to be stronger than that in the bottom layers, this was possibly due to the appearance of nuclear signals in the upper layers. Indeed, when compared with the upper layer regions that did not show nuclear signals, E7 cytoplasmic signals in the bottom layers had a similar strength to that in the upper layers.

The C48A1 CIN lesion was another example of HPV16 vegetative infection in metaplastic SSE bordering SCE (Figure 5.9, SC junction indicated by the snowflakes). Although this lesion also represented a low grade dysplasia, metaplasia was not as mature as that in C21A. Similar to C21A1 (Figure 5.8), viral DNA amplification and L1 expression were detected in the upper layer cells, consistent with the expected pattern for vegetative virus

Figure 5.9 ***In situ*** **hybridization assays of the SCE-proximal C48A1 CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

SC junction was indicated by snowflakes in the E7 and L1 panels.

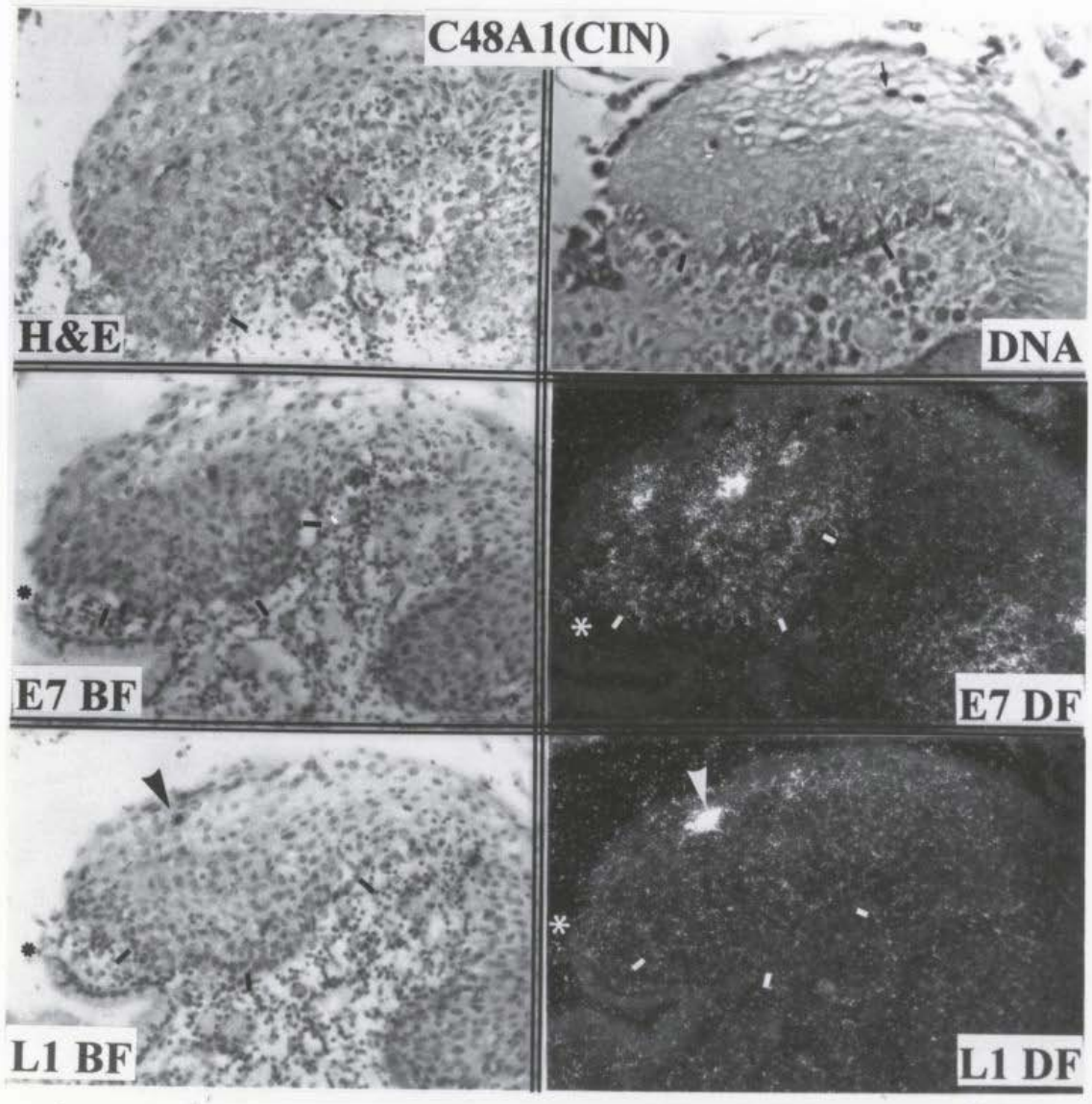
The black and white bars indicate the basal membrane.

The black arrow in the DNA panel indicates a nucleus with positive staining for HPV16 DNA.

The arrowheads in the L1 panels indicate positive L1 signals.

Note the sharp boundary between the E7-negative SCE and the E7-positive SSE, due to the well-preserved SCE in this case.

The magnifications were 200 X.


C48A1(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

replication. However, unlike C21A1 (Figure 5.8), HPV16 L1 in C48A1 was not expressed as actively, and only a few cells had amplified viral DNA. Consistent with the result from C21A1, the E7 cytoplasmic signal was distributed throughout the lesion, with a similar intensity in the bottom as the upper layers (Figure 5.9, E7DF panel). This suggested that the non-compartmentalized E7 expression in the metaplastic SSE was unlikely to be correlated with the activity of viral DNA amplification, and was not associated with the level of L1 expression. In the C48A1 CIN lesion, it can be more clearly seen that the expression of viral E7 and L1 genes only occurred in the metaplastic SSE, but not in SCE.

The H11E and H13 CIN lesions represented two more examples of HPV16 vegetative infections in metaplastic SSE immediately adjacent to SC junction (Figure 5.10 and 5.11). Consistently, both CINs showed extensively expressed E7 cytoplasmic signals in the metaplastic SSE, but not in the SCE (Figure 5.10 and 5.11, E7 panels, SC junction indicated by the white snowflakes).

5.2.3.2 Non-vegetative infection in CINs adjacent to SC junction

As among VINs, lesions positive for DNA amplification but negative for L1 expression were also found. Those TNIs at SC junction had particular features.

The M4G2b CIN lesion was located immediately adjacent to SC junction (Figure 5.12), and pathologically was consistent with a low grade dysplasia. The morphology of M4G2b (Figure 5.12, H&E panel) did not show significant differences from the CIN lesions supporting vegetative replication (Figure 5.8-5.11, H&E panels). Similar to CINs with vegetative infection, HPV16 E7 was expressed throughout the lesion. However, while the

Figure 5.10 ***In situ*** **hybridization assays of the SCE-proximal H11E CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

SC junction was indicated by the snowflake in the E7DF panel.

The black and white bars indicate the basal membrane.

The magnifications were 200 X.

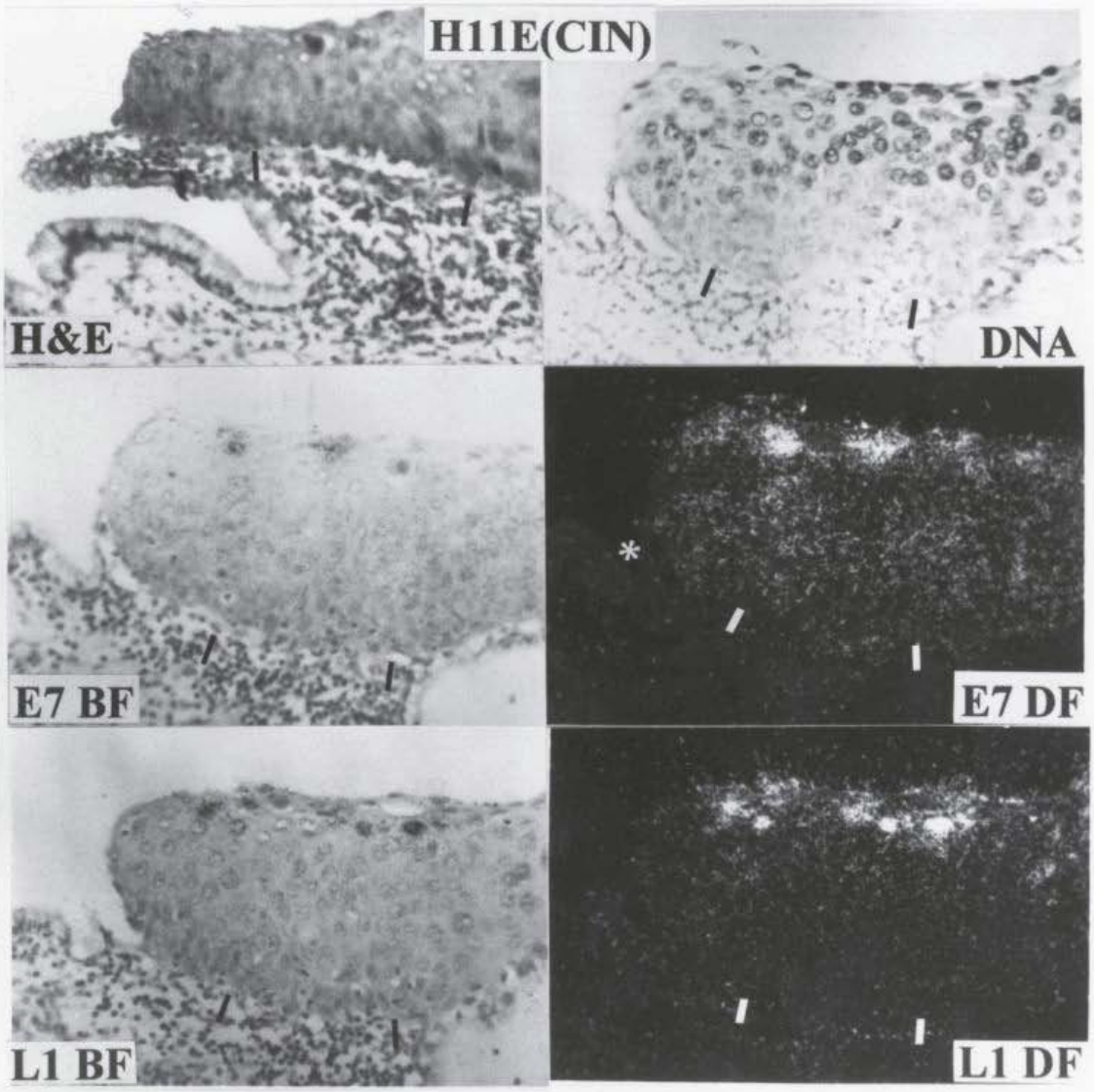

H11E(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

Figure 5.11 ***In situ*** **hybridization assays of the SCE-proximal H13 CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

SC junction was indicated by snowflakes in the E7 and L1 panels.

The black arrow in the DNA panel indicates a nucleus with positive staining for HPV16 DNA.

The black and white bars indicate the basal membrane.

The magnifications were 200 X.

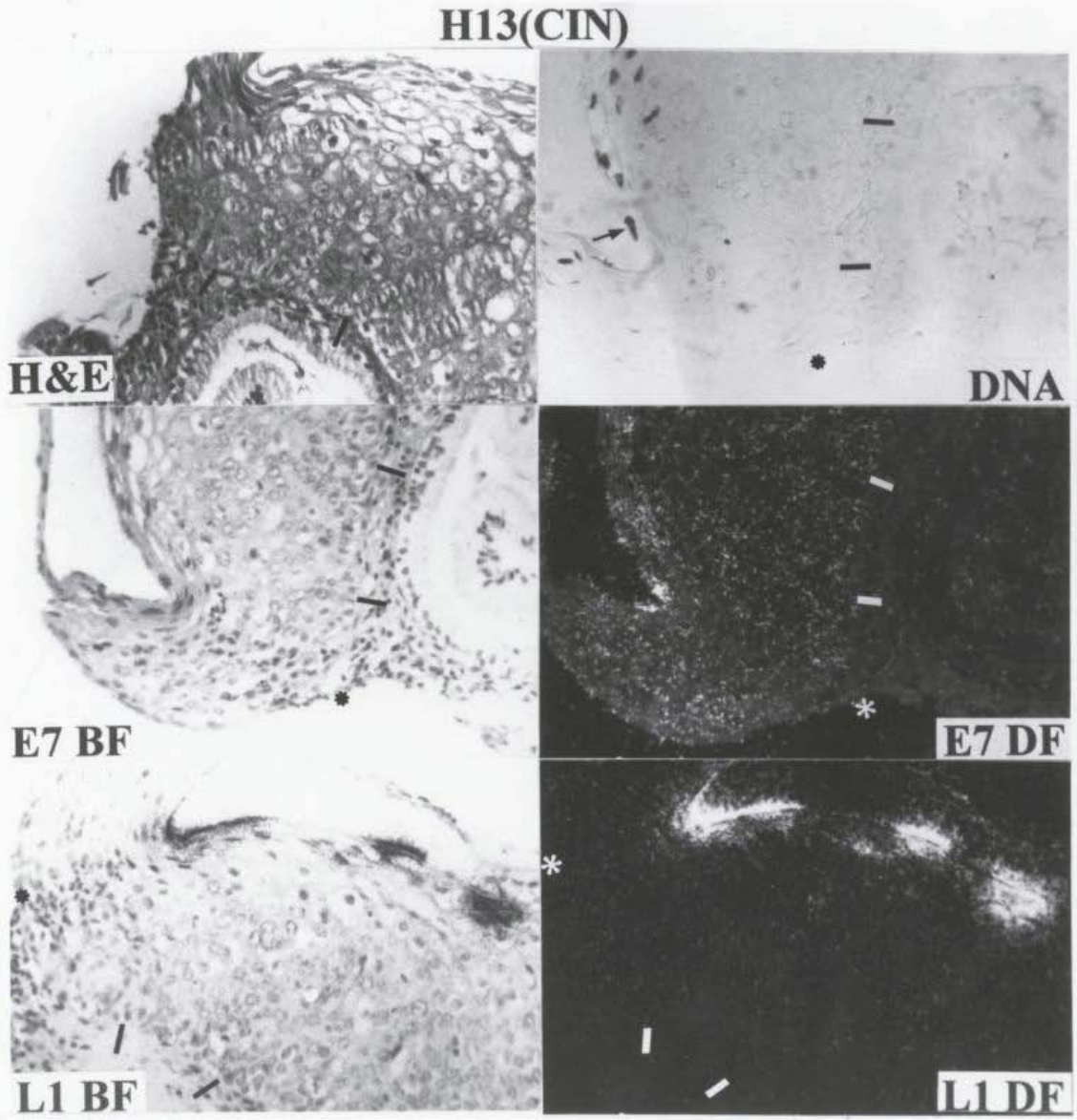

H13(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

Figure 5.12 ***In situ*** **hybridization assays of the SCE-proximal M4G2b CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

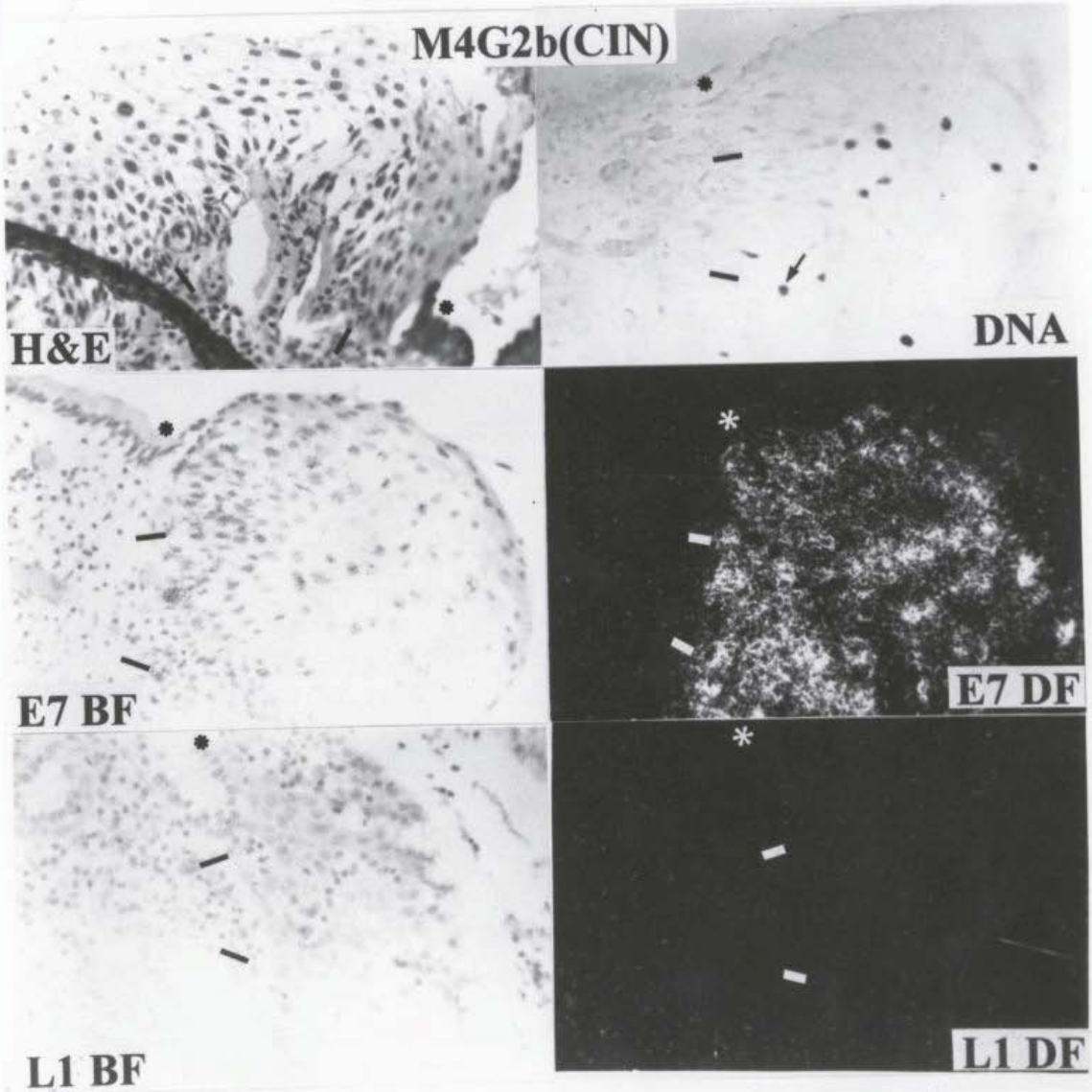
SC junction was indicated by snowflakes.

The black and white bars indicate the basal membrane.

The black arrow in the DNA panel indicates a nucleus with positive staining for HPV16 DNA, which was located quite close to the basal membrane.

Note the highly heterogenous signal strength of E7 expression (E7 panel). Also, note the sharp boundary between the E7-negative SCE and the E7-positive SSE.

The magnifications were 200 X.


M4G2b(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

extensive E7 cytoplasmic signals in the lesions with vegetative infections were generally homogeneous, the intensity of the E7 signals in M4G2b was very heterogeneous among the cells. The E7 signal in M4G2b was predominantly cytoplasmic, and was not restricted to any particular epithelial compartment. Despite a strong positive signal for E7 mRNA, L1 mRNA was not detectible, suggesting a non-vegetative infection. DNA *in situ* hybridization showed that only a subpopulation of cells in the lesion was positive for viral DNA amplification, and the cells positive for DNA staining did not show a punctate nuclear staining pattern that would have suggested viral DNA integration (Cooper *et al.*, 1991b). Therefore, this state of non-vegetative infection was unlikely to have been due to viral DNA integration, rather representing a TNI. Interestingly, the cells amplifying viral DNA in M4G2b tended not to be limited to the epithelial compartment near the lesion surface (Figure 5.12, DNA panel), as do the CINs with vegetative infections (Figure 5.8, DNA panel). Thus, the TNI in this lesion was accompanied with an aberrant compartmental distribution of viral DNA-amplifying cells.

This association between TNI and aberrant viral DNA amplification in metaplastic SSE was manifested more clearly in the M4G1 CIN lesion (Figure 5.13). While the E7-expressing cells in M4G1 were distributed throughout the lesion, the signal intensity varied greatly. As in the M4G2b lesion shown in Figure 5.12, the heterogeneity in E7 expression did not show any particular pattern of compartmentalization. In contrast to the positive E7 expression, L1 mRNA was not detectible, as for M4G2b in Figure 5.12. Again, this state of non-vegetative infection was unlikely due to viral DNA integration, since viral DNA was stained in a subpopulation of cells and the staining pattern was consistent with that for episomal viral DNA. In agreement with the finding in the CIN with non-vegetative infection

Figure 5.13 ***In situ*** **hybridization assays of the SCE-proximal M4G1 CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were detected.

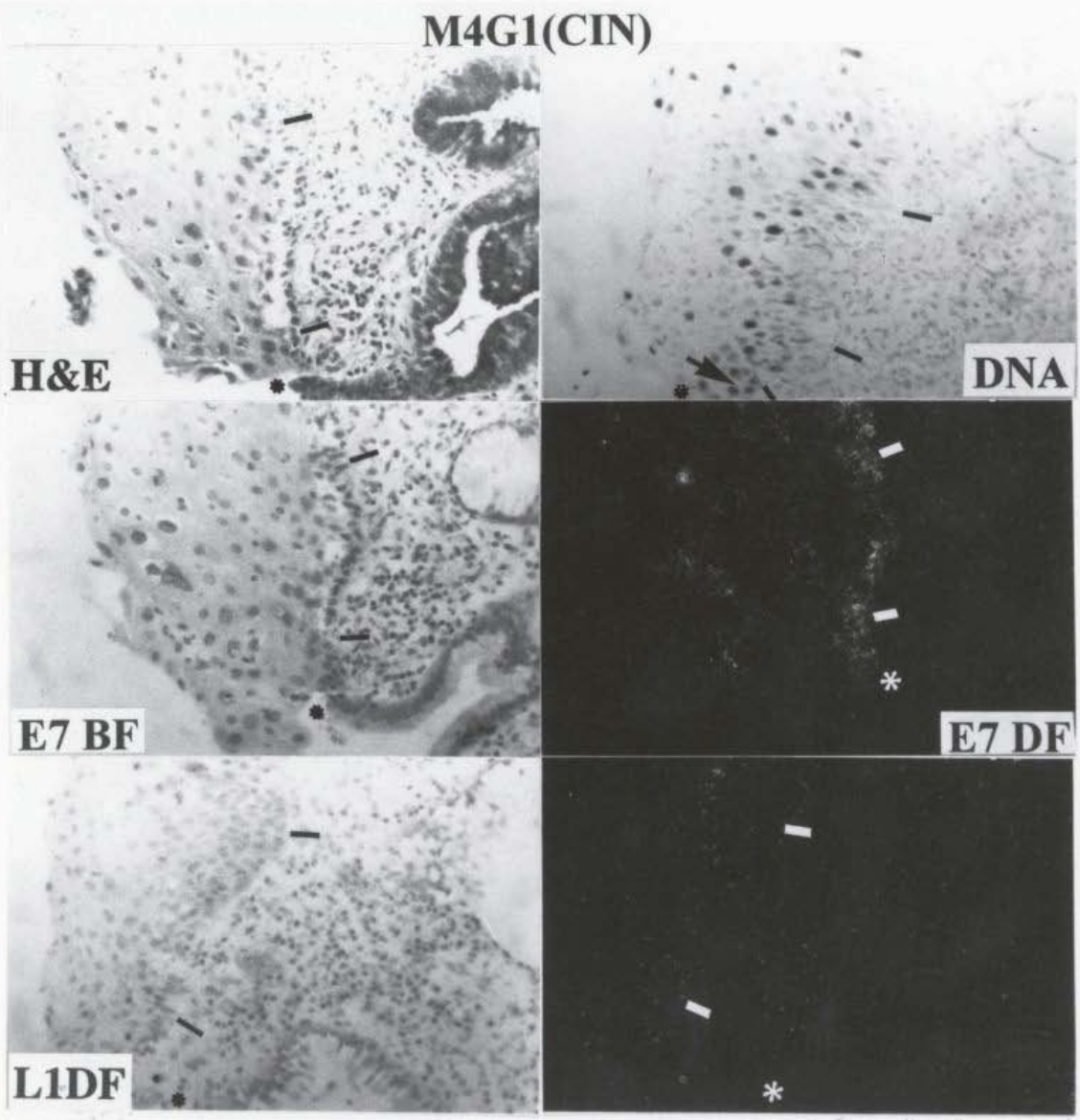
SC junction was indicated by snowflakes.

The black and white bars indicate the basal membrane.

The black arrow in the DNA panel indicates a viral DNA-positive cell, which was located at the basal membrane and was immediately adjacent to SC junction. An enlarged picture for DNA staining was shown in Figure 5.1C, which showed SC junction more clearly. Note that the SCE-proximal end of the lesion featured viral DNA-amplifying cells compartmentalized in low half, while the viral DNA-amplifying cells at the SCE-distal end tended to distribute also in the upper layer cells (DNA panel).

The L1 probe failed to detect any signal and the E7 probe did not detect obvious nuclear signal in any compartment of the lesion, in spite of the presence of viral DNA-amplifying cells in the low half of the lesion.

The magnifications were 200 X.


M4G1(CIN)
H&E
DNA
E7 BF
E7 DF
L1DF

(Figure 5.12), and in contrast to that in the CINs with vegetative infections (Figure 5.8-5.11), most viral DNA-amplifying cells in the M4G1 were not located in the upper layer compartment (Figure 5.13, DNA panel). Instead, the cells amplifying viral DNA were mostly localized within the lower half of the lesion at the end proximal to SC junction (Figure 5.13, DNA panel, indicated by the black snowflake). Interestingly, viral DNA amplification seemed to occur even in cells very close to the basal membrane (Figure 5.13, DNA panel, the black arrow). However, at the SC junction-distal region where metaplasia is usually more mature than the SC junction-proximal end, viral DNA amplifying cells appeared to tend to distribute evenly in the lesion. Thus, this type of non-vegetative infection, TNI with aberrant viral DNA amplification, was a general feature of HPV16 infection in metaplastic SSE close to SC junction.

5.2.3.3 Non-vegetative infection in atypical immature metaplasia (AIM)

Atypical immature metaplasia, or AIM, is recognized as cervical SSE lesions with limited atypical changes and prominent phenotypes of immature metaplasia (Fu and Reagan, 1989a). AIM has a close relationship with SCE and immature metaplasia. Fu and Reagan (1989a) suspected that AIM is caused by HPV infection in immature metaplasia. The M4G2 lesion provided a good example for HPV16 infection in AIM (Figure 5.14, H&E panel). The SCE-proximal end of the stratified lesion was composed of cells with similar sizes and cellular contours, and showed almost no mature squamous differentiation. The homogeneity of cell population is one feature of AIM, which is different from the heterogeneous cell population in CIN lesions. The hyperchromicly stained nuclei and higher than normal nucleus/cytoplasm

Figure 5.14 ***In situ*** **hybridization assays of the M4G2 AIM lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

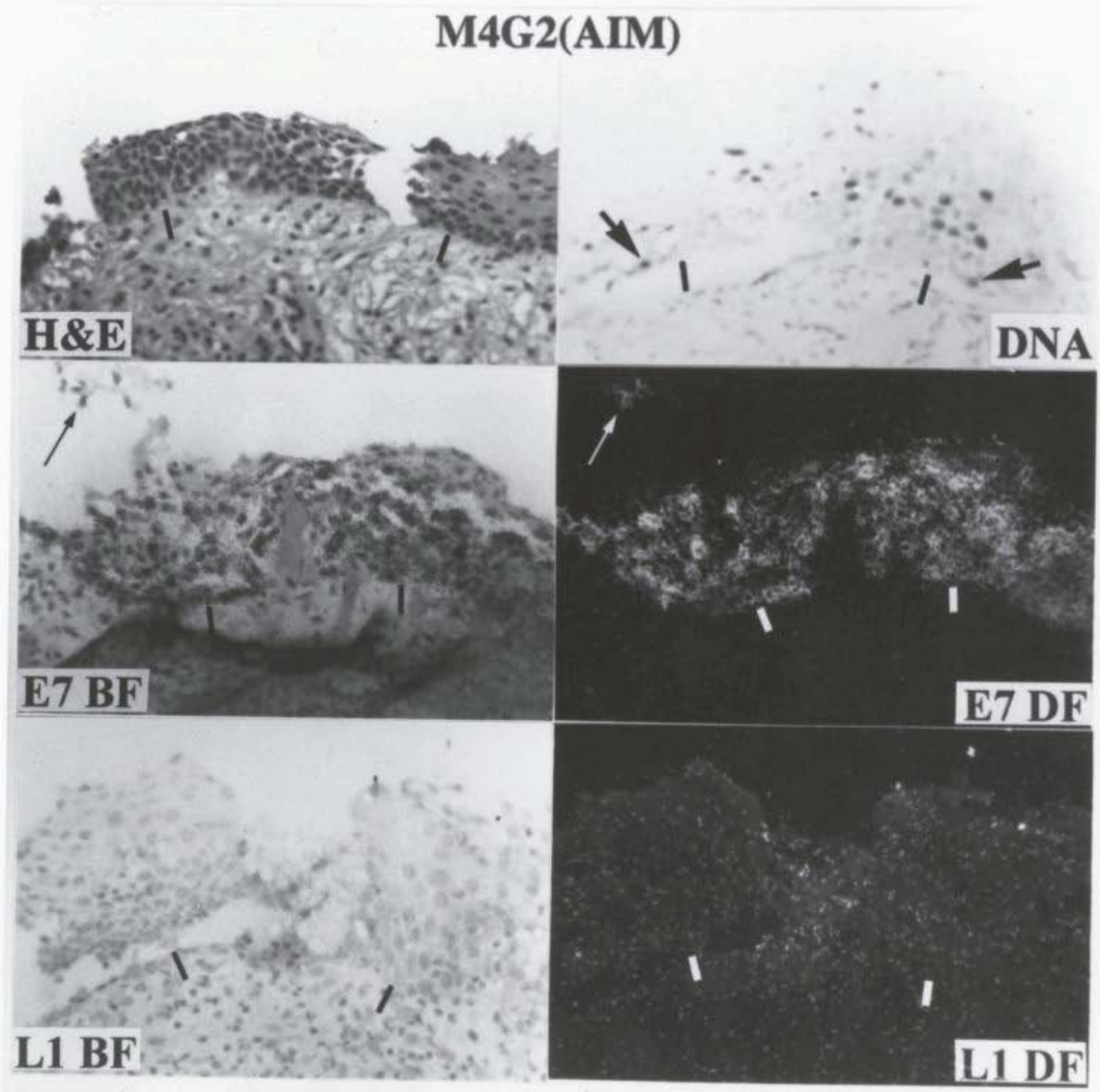
The black arrows in the DNA panel indicate the viral DNA-positive cells at, or very close to, the basal membrane.

The arrows in the E7 panels indicates the detached metaplastic cells expressing E7.

Note that although most of the viral DNA-amplifying cells were located in the low half of the lesion (DNA panel), the E7 signal was distributed uniformly and heterogeneously throughout the lesion, and no obvious E7 nuclear signal was found in the low half of the lesion (E7 panels).

The L1 probe failed to detect any signal.

The magnifications were 200 X.


M4G2(AIM)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

ratios were indicative of their atypical nature. However, the nuclei did not show the drastic irregularity seen in CIN lesions. This type of lesion was similar to the reconstructed lesion from the $pSV_2$1667-immortalized HEN, EN1667 (Figure 4.5). The right end of the M4G2 lesion showed an increased compartmentalization in cellular morphology, indicating a relatively mature metaplastic phenotype (Figure 5.14). Another feature of immature metaplasia was that the epithelium was very fragile, as indicated by the easy fragmentation in the epithelium and detachment of the epithelium from the basal membrane. This lesion was no exception. It can be clearly seen that parts of the lesion were fragmented and detached (Figure 5.14, H&E panel). This was reminiscent of the experimental atypical metaplasia reconstructed from the $pSV_2$1667-immortalized HEN (Figure 4.5, EN1667 H&E panel).

While the expression of the HPV16 E7 oncogene and viral DNA amplification were detected in the M4G2 lesion, viral L1 mRNA was not detectible (Figure 5.14, L1DF panel), consistent with the pattern for a TNI. Similar to the pattern found in the HPV16-infected CINs at SC junctions (Figure 5.7-5.12), the HPV16 E7 gene was expressed throughout the lesion, including the cells detached from the lesion (Figure 5.14, E7DF panel, indicated by the white arrow). Also similar to the TNI in CINs at SC junction (Figure 5.12 and 5.13), the intensity of the E7 signal was highly heterogeneous among the cells (Figure 5.14). Also, the E7 signal was almost exclusively cytoplasmic. Significantly, the cells amplifying viral DNA in this lesion were also distributed mostly in the low half of the lesion, and even in the cells at, or very close to the basal membrane (Figure 5.14, DNA panel, indicated by black arrows). Thus, the TNI in AIM was also associated with aberrant viral DNA amplification, similar to the TNIs in metaplastic SSE at SC junctions.

The pathological features of the M3G4 lesion were also consistent with those for an AIM (Figure 5.15, H&E panel). The pattern of HPV16 infection in this lesion suggested that the state of HPV16 infection was a TNI. Similar to the M4G2 AIM lesion (Figure 5.14), the features of the M3G4 lesion included the extensive heterogeneous E7 expression in the entire lesion (Figure 5.15, E7DF panel), viral DNA-amplifying cells in the basal layer (Figure 5.15, DNA panel), and non-detectible L1 expression in the lesion (Figure 5.15, L1DF panel).

Since these AIM lesions contained a large amount of viral DNA, as suggested by the positive staining for DNA *in situ* hybridization, the negative staining for L1 expression attested to the RNA specificity of the L1 probe (L1DF panel in Figure 5.14 and 5.15). This was also the case for the E7 probe, since the E7 signal appeared almost exclusively cytoplasmic in such aberrant TNIs, and the occasional occurrence of E7 nuclear signals seemed not to correlate with the location of the viral DNA-amplifying cells (Figure 5.14, E7DF panel).

Thus, TNIs with irregularly distributed viral DNA-amplifying cells occurred in the AIM lesions.

5.2.3.4 Vegetative infection and TNI in CINs distal from SC junction

SCE-distal CINs with vegetative infections may also show extensive cytoplasmic HPV16 E7 expression. As an example, the H11M CIN lesion showed patterns of viral gene expression and DNA amplification (Figure 5.16), similar to the HPV16 infections proximal to SC junction. The cytoplasmic E7 signal was almost evenly distributed throughout the lesion, with viral DNA amplification and L1 expression in the upper layers. The C38A2 CIN

Figure 5.15 ***In situ*** **hybridization assays of the M3G4 AIM lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

The black arrows in the DNA panel indicate viral DNA-positive cells at, or very close to, the basal membrane.

The magnifications were 200 X.

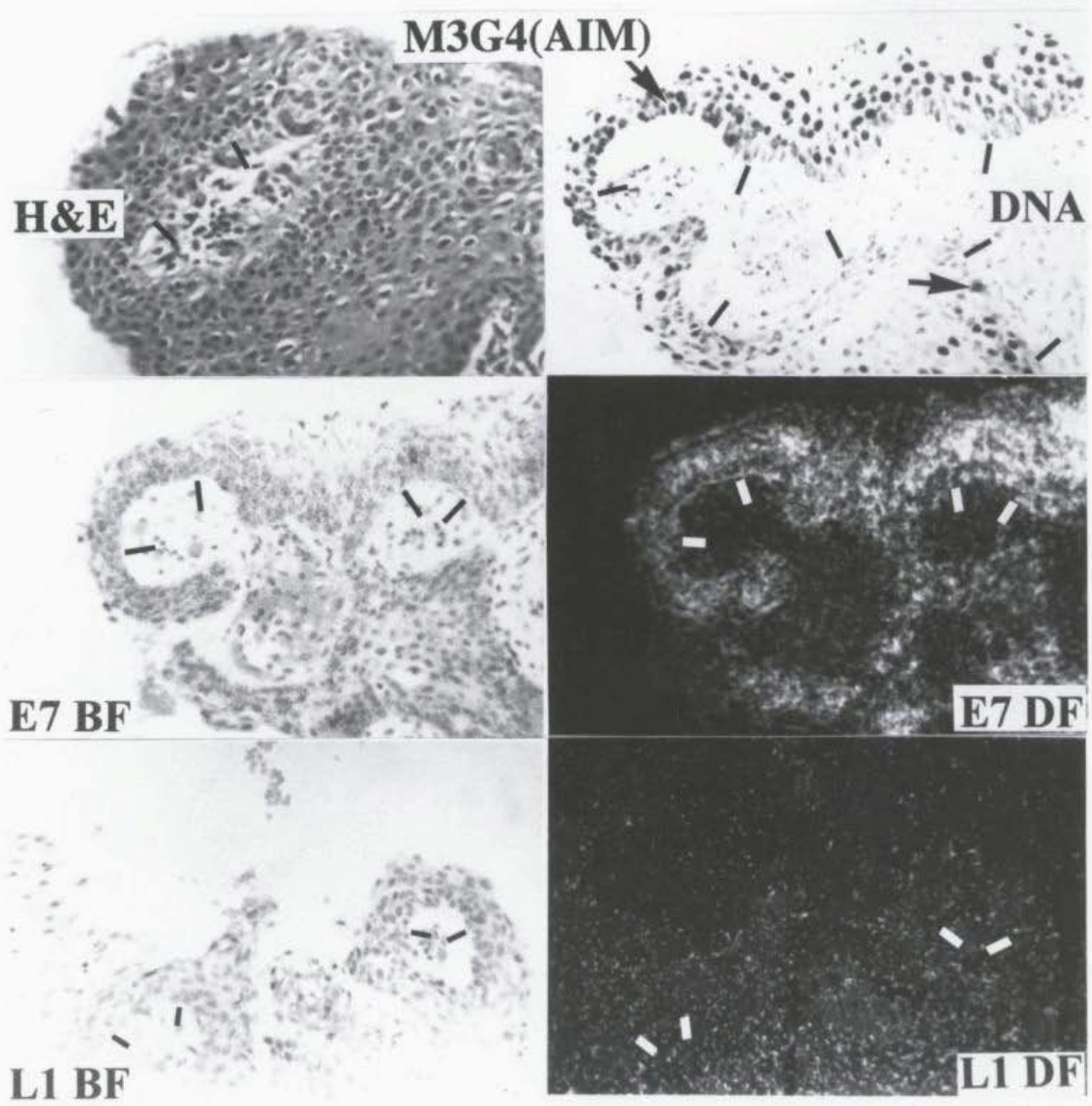
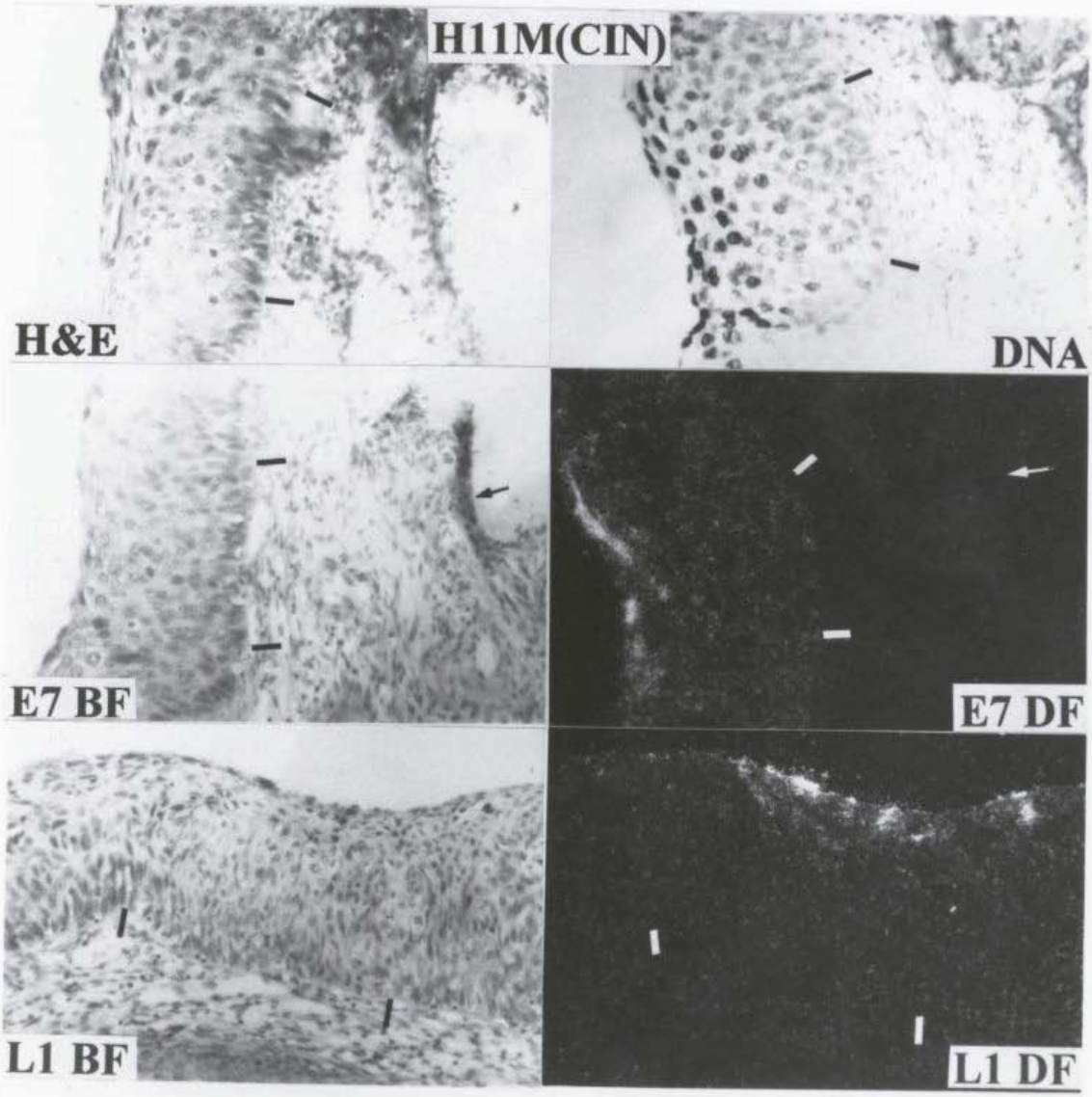

M3G4(AIM)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

Figure 5.16 ***In situ*** **hybridization assays of the SCE-distal H11M CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

The magnifications were 200 X.


H11M(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

lesion was another example of a CIN located distal from SC junction (Figure 5.17). Consistently, the viral DNA amplification and L1 expression occurred in the upper layers of the lesion, while the E7 cytoplasmic signal was distributed almost evenly throughout the lesion, and the nuclear signal in some upper layer cells.

However, subtle variations in E7 expression were observed in the C48A1-b, F2F3 and M3G3 CIN lesions, all of which were located distally from SC junctions. In C48A1-b, the E7 cytoplasmic signal showed an abrupt reduction in the upper half of the lesion. Viral DNA amplification and L1

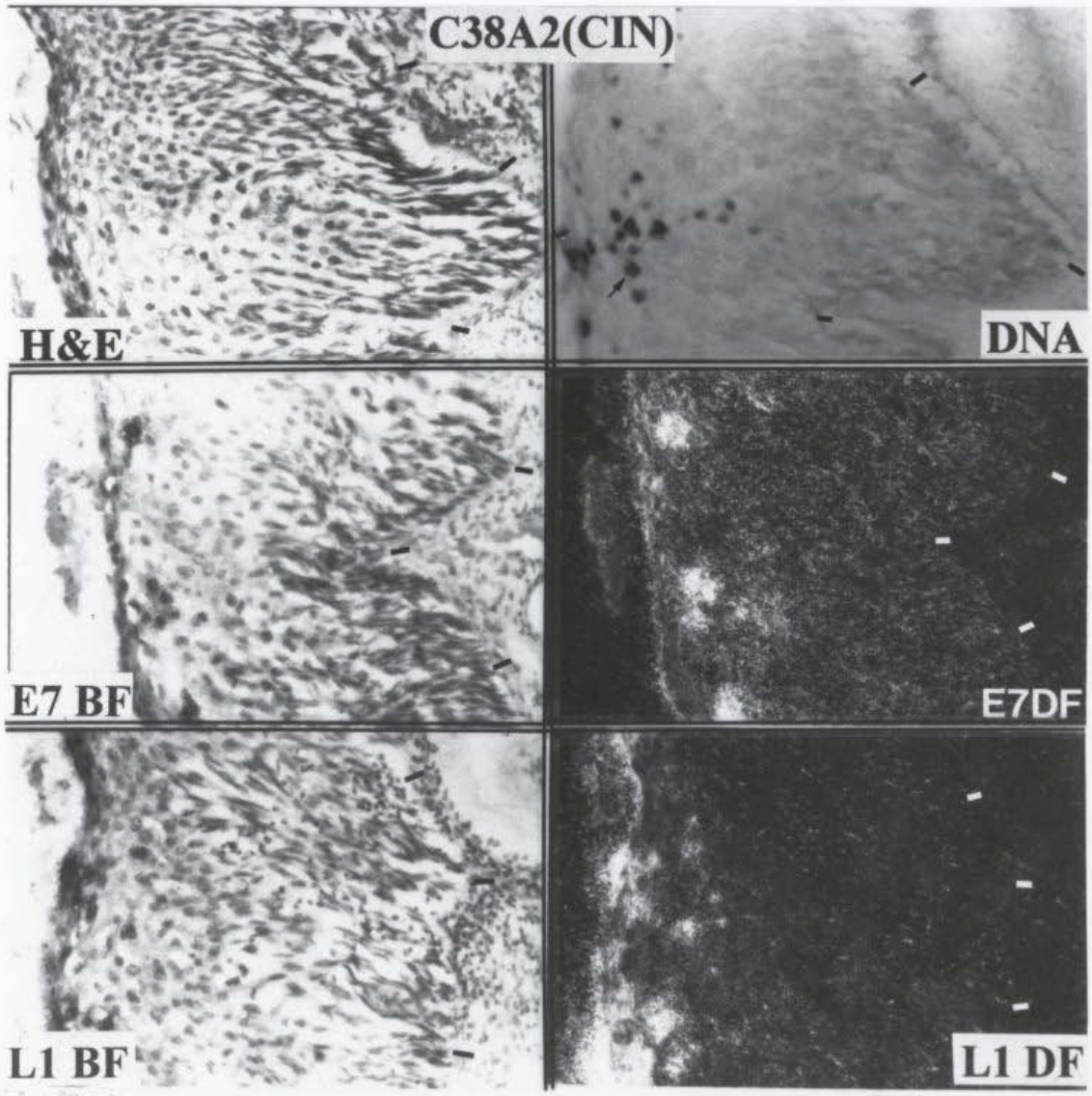
Figure 5.17 ***In situ*** **hybridization assays of the SCE-distal C38A2 CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

The black arrows in the DNA panel indicate a viral DNA-positive cell.

The magnifications were 200 X.


C38A2(CIN)
H&E
DNA
E7 BF
E7DF
L1 BF
L1 DF

expression occurred in the upper layers, although at a relatively low level of activity (Figure 5.18). The pattern of E7 expression in C48A1-b bore some similarity to that in HPV16-mediated VINs (Figure 5.1-5.6), although the epithelial compartment expressing E7 was relatively wider, extending to the middle of the C48A1-b lesion (Figure 5.18, E7DF panel). In contrast to C48A1b, the F2F3 CIN lesion showed an enhanced cytoplasmic E7 expression in the upper half of the lesion, although E7 was also expressed in the bottom layers at a relatively low level (Figure 5.19, E7DF panel). The underlying cervical gland showed no E7 signal (Figure 5.19, E7DF panel, indicated by the white arrow). In this lesion, individual cells with active viral DNA amplification and L1 expression were located in the upper layers. Since viral DNA amplification and L1 expression occurred in the expected epithelial compartments, the C48A1b and F2F3 lesions supported vegetative infections. The M3G3 CIN lesion provided another example of the variable HPV16 activities in metaplastic SSE distal from SCE. Although the M3G3 CIN lesion showed active viral DNA amplification (Figure 5.20), E7 signals were not detectible in the bottom layers. In the upper layer cells, the E7 and L1 probes detected only ambiguous, if any, signals. None of the lesions located distally from SC junctions showed patterns of HPV16 activities consistent with the aberrant TNIs seen in the lesions bordering SCE.

These data suggested that HPV16 lesions distal from SC junction may have more variations in viral activities than those occurring at SC junction.

5.2.3.5 CINs with INI

HPV16 DNA was not detectible in some CIN lesions positive for the HPV16 E7

Figure 5.18 ***In situ*** **hybridization assays of the SCE-distal C48A1-b CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

The black arrow in the DNA panel indicates a nucleus positive for viral DNA staining.

The arrows in the L1 panels indicate positive L1 signals.

Note in this particular sample that the E7 cytoplasmic signal was mostly distributed in bottom half of the lesion.

The magnifications were 200 X.

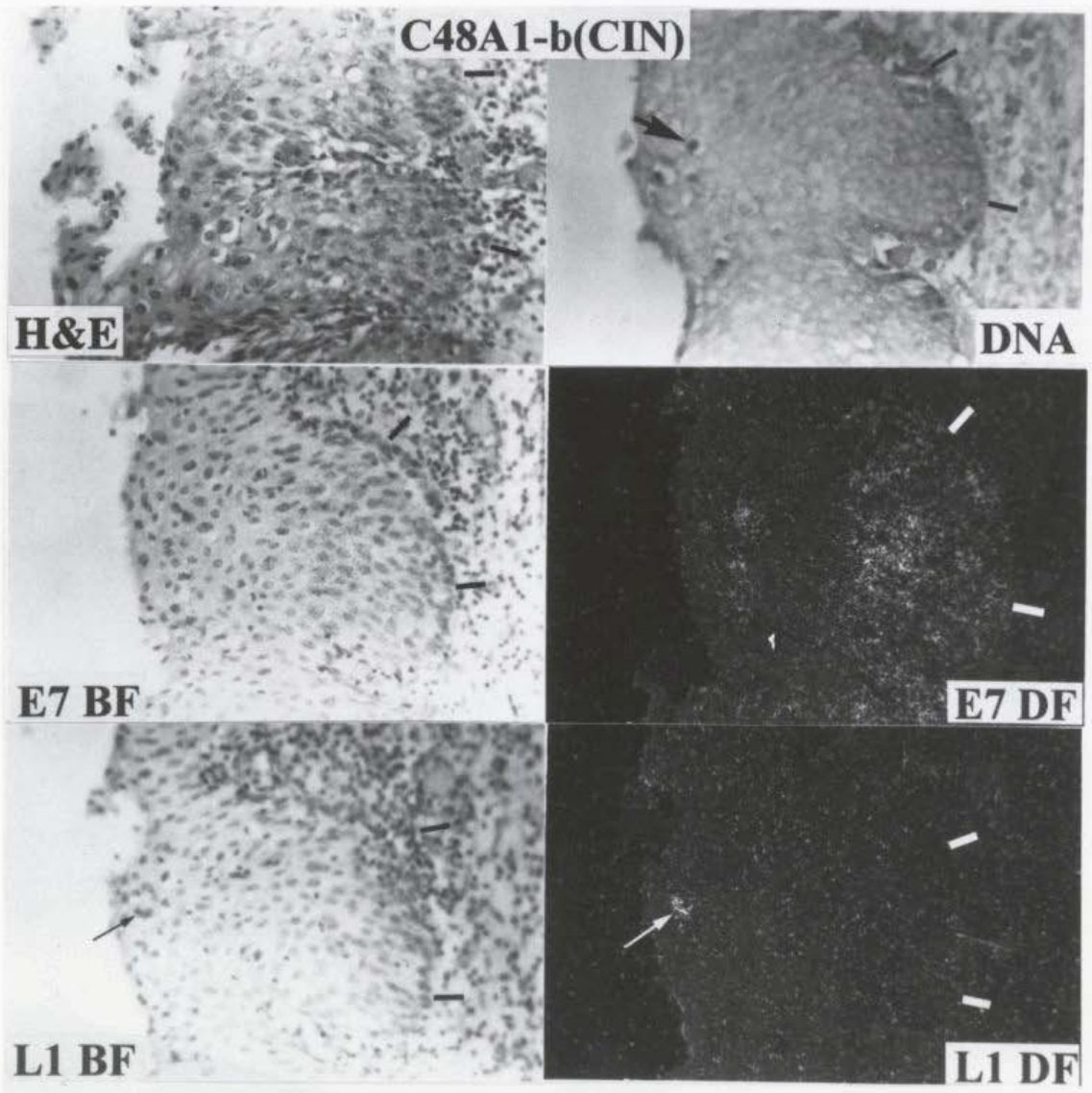

C48A1-b(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

Figure 5.19 ***In situ*** **hybridization assays of the SCE-distal F2F3 CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

Viral DNA amplification was active in this lesion (DNA panel).

The arrows in the E7 panels indicate the E7-negative cervical gland underlying the lesion.

The magnifications were 200 X.

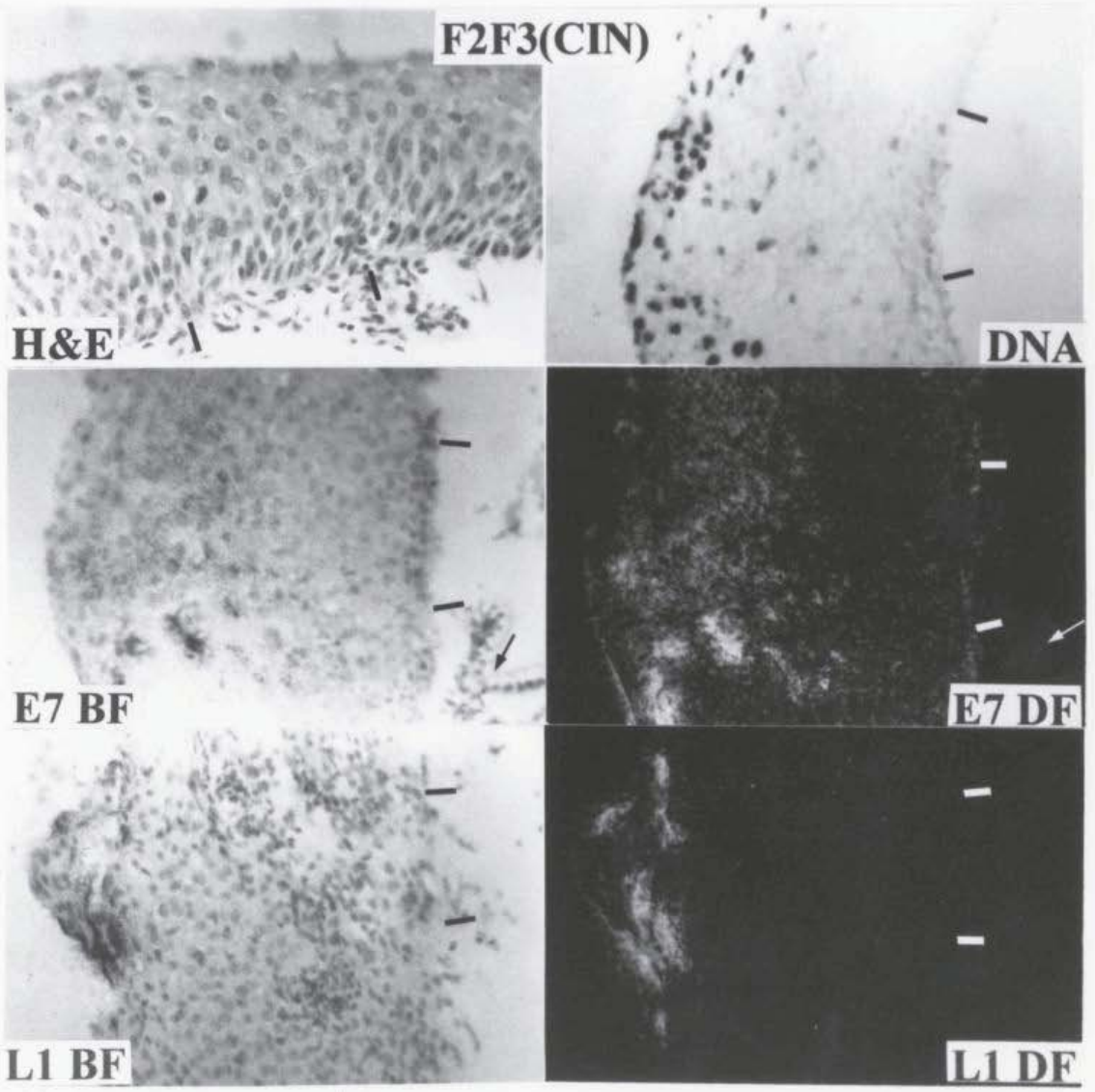

F2F3(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

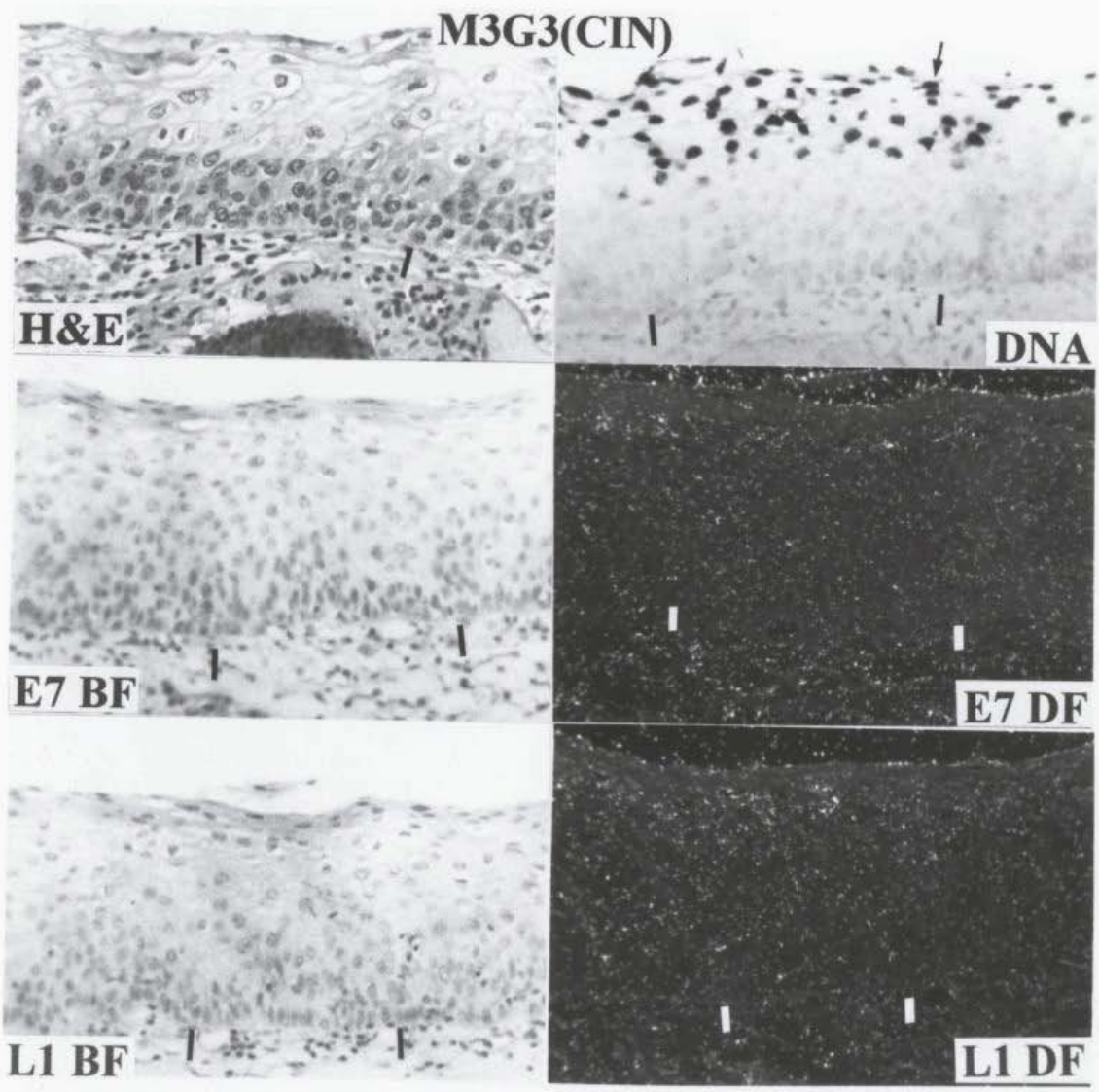
Figure 5.20 ***In situ*** **hybridization assays of the SCE-distal M3G3 CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

Viral DNA amplification was high in this lesion (DNA panel), while the E7 and L1 probes detected almost no signal (E7 and L1 panels).

The magnifications were 200 X.


M3G3(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

mRNA, in contrast to the finding that all the VINs expressing E7 oncogene showed positive signals for viral DNA amplification (see Section 5.2.1). These CIN lesions were observed in both the SCE-proximal and -distal regions of metaplastic SSE. The failure to detect viral DNA amplification in these lesions by the conventional DNA *in situ* hybridization may be due to the limited amounts of integrated HPV16 DNA and no or few copies of episomal HPV16 DNA (Nuovo *et al.*, 1991b). The assumption that these viral DNA-negative lesions contained integrated viral DNA is consistent with the finding that these lesions showed no detectible L1 expression. Similar observations have also been made by others (Nuovo *et al.*, 1991b; Stoler *et al.*, 1992; Cooper *et al.*, 1991b). Thus, these lesions may represent INIs.

The H10A2 CIN lesion was an example of a CIN lesion with integrated HPV16 DNA. This lesion was located within the endocervix and the pathological profile of H10A2 was consistent with that of a CIS (Figure 5.21, H&E panel). There were no signs of viral DNA amplification (Figure 5.21, DNA panel) and L1 expression (Figure 5.21, L1DF panel) in H10A2, although expression of the viral E7 oncogene was detectible (Figure 5.21, E7DF panel). The E7 signals were predominantly cytoplasmic, and similar to other CIN lesions, were distributed throughout the lesion. However, some subtle differences in the E7 expression pattern existed between H10A2 and the CIN lesions that were positive for viral DNA amplification. When compared with CINs supporting vegetative infections, H10A2 was characterized by the absence of nuclear E7 signals in the lesion (Figure 5.21, E7DF panel). In comparison with that in CIN lesions with TNI, the E7 cytoplasmic expression tended to be more homogeneous in H10A2. These features all suggested that the state of HPV16 infection in H10A2 was an INI (Durst *et al.*, 1991).

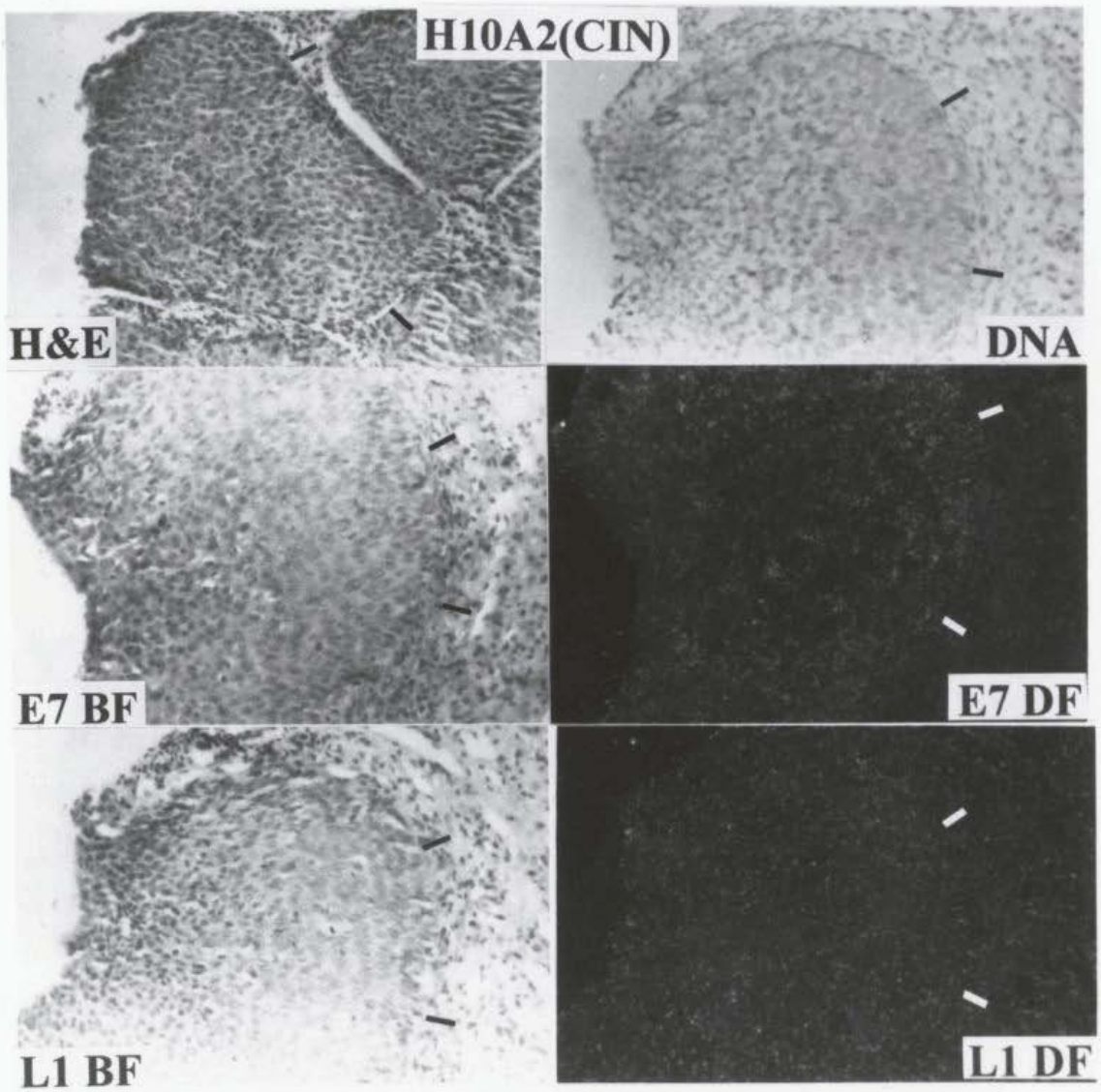
Figure 5.21 ***In situ*** **hybridization assays of the SCE-proximal H10A2 CIS lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

By comparing with background signals, it can be clearly seen that the HPV16 E7 signals were distributed quite evenly throughout the lesion (E7DF panel), while viral DNA and L1 expression were negative (DNA panel and L1DF panel, respectively).

The magnifications were 200 X.


H10A2(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

In addition, some CINs with integrated HPV16 DNA showed a gradual transition in their E7-expressing compartments. C48A2 was negative for both viral DNA amplification and L1 expression, consistent with the pattern observed for an INI (Figure 5.22, DNA panel and L1DF). HPV16 E7 was expressed throughout the epithelium on the left end, which was proximal to SC junction (Figure 5.22, E7DF panel). The expression of E7 at the SC junction-distal region in C48A2 became gradually compartmentalized to the lower layers (Figure 5.22, E7DF panel).

This gradual transition in E7 expression was also observed in another possible INI H6A1, which was negative for L1 expression (data not shown). Similar to C48A2, E7 expression in the H6A1 CIN lesion was detectible throughout the lesion at the left end, which was proximal to SC junction (Figure 5.23, E7DF panel). As the lesion extended distally, E7 expression was gradually limited to the lower half of the lesion, then to the bottom 2-3 layers, and finally to basal cells. The boundary between the infected and non-infected regions can be clearly seen (Figure 5.23, E7DF panel, indicated by arrows). This pattern of transitional E7 expression in C48A2 was accompanied by decreasing dysplastic severity and increasing maturity of squamous differentiation (Figure 5.23, E7BF panel). No typical nuclear E7 signal was observed in both the lesion C48A2 and H6A1. Thus, the SCE-proximal regions in CINs with INI resembled the experimental lesions from the immortalized HEN, in that E7 was extensively expressed (refer to Figure 3.5*b* and 3.8*b*). In contrast, the SCE-distal regions analogous to the experimental lesions from the immortalized HKC, in that E7 expression was restricted in the bottom layers (refer to Figure 3.5*c* and 3.5*d*, Figure 3.8*d* and 3.8*f*, and their legends).

Figure 5.22 ***In situ*** **hybridization assays of the SCE-proximal C48A2 CIN lesion**

HPV16 DNA, E7 mRNA and L1 mRNA were examined.

The black and white bars indicate the basal membrane.

Viral DNA amplification and L1 expression were negative (DNA and L1 panels).

While E7 signals were distributed throughout the lesion at the left, SCE-proximal end of the lesion, it became restricted at the right, SCE-distal end.

The magnifications were 200 X.

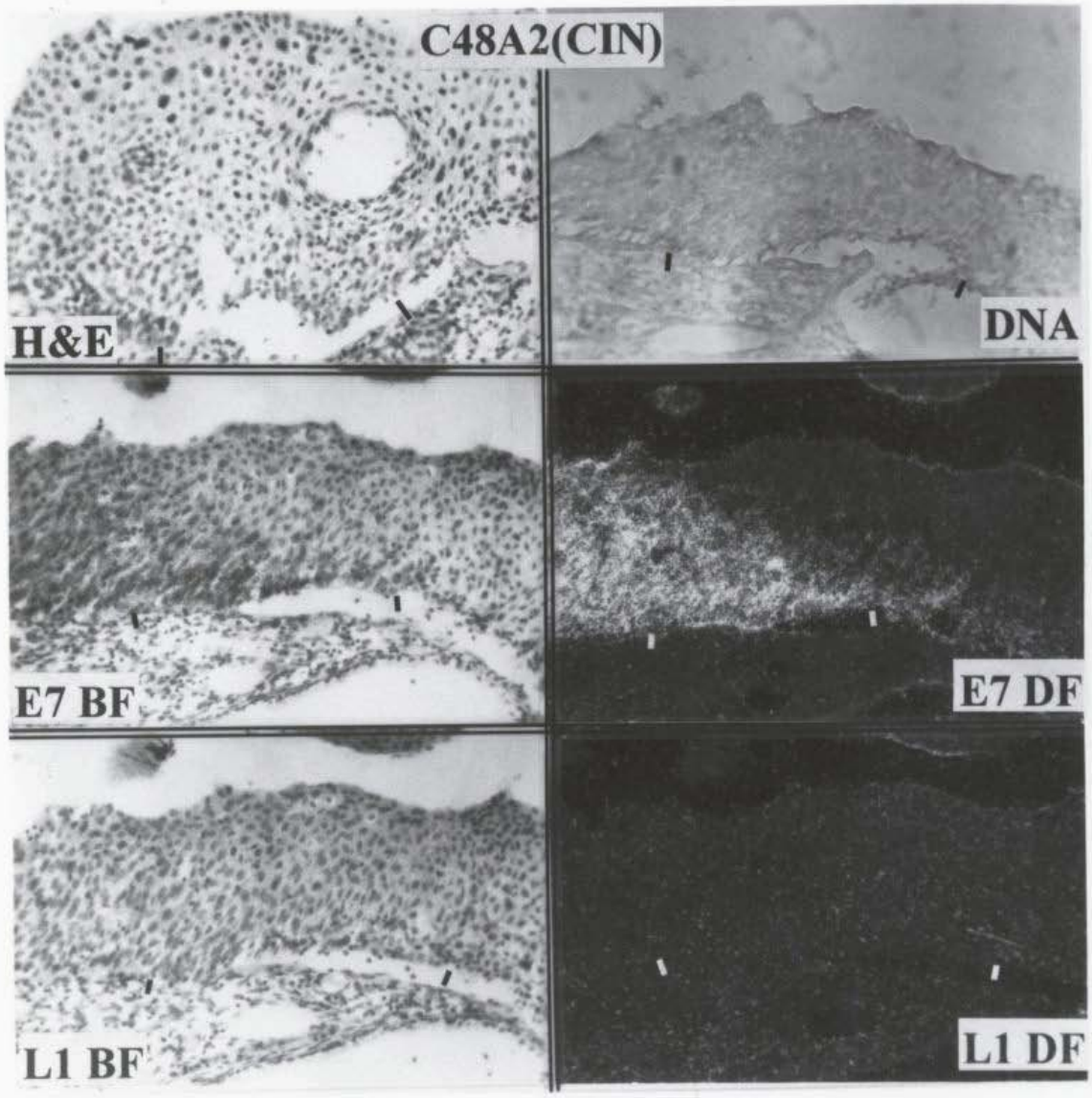

C48A2(CIN)
H&E
DNA
E7 BF
E7 DF
L1 BF
L1 DF

Figure 5.23 ***In situ*** **hybridization assays of the SCE-proximal H6A1 CIN lesion**

The black and big white bars indicate the basal membrane.

The slender white rods indicate the surface of the lesion (E7DF panel).

E7 signals were distributed throughout the lesion at the left, SCE-proximal end, and it became more restricted as the lesion extended towards the right, SCE-distal end. The very right end of the lesion was negative for E7 expression, and the arrows indicate the boundary of this transition. The small arrowheads indicate a fold in the section, which caused high nonspecific background locally.

The magnifications were 200 X.

H6A1(CIN)
E7 DF
E7 BF

5.2.4 Confirmation of HPV16 DNA sequences in VINs and CINs

To reexamine whether the lesions that showed positive signals for HPV16 by *in situ* hybridization assays harboured *HPV16, DNA* was extracted from paraffin-embedded blocks and was examined by PCR, when tissue was available (Figure 5.24, and Table 5.1). Using a pair of primers that flanked a 323 bp sequence in the HPV16 E6 ORF, PCR amplified DNA sequences with the expected size from the lesions that were positive for HPV16 E7 mRNA by *in situ* hybridization assays. The amplified sequences were then confirmed by Southern blot analysis under stringent condition. The results showed that the *in situ* hybridization protocol used in this study was reliable for detecting HPV16 infections.

5.2.5 CK expression in natural HPV16 infected lesions

In an attempt to substantiate the relationship between squamous differentiation and HPV16 gene expression, the protein expression patterns for CK10, CK13, CK18 and CK19 were examined in the HPV16 infected lesions by indirect immunofluorescence assays. However, partially due to the limited amount of tissues in the archival blocks, no conclusion could be drawn from these experiment (data not shown).

5.3 Discussion

The studies in this chapter examined natural HPV16 infections occurring in VINs and CINs. The experimental design of the current study was distinguished in several aspects from

Figure 5.24 PCR amplification of HPV16 E6 sequence from clinical samples

Black bars indicate a 600 bp molecular marker.

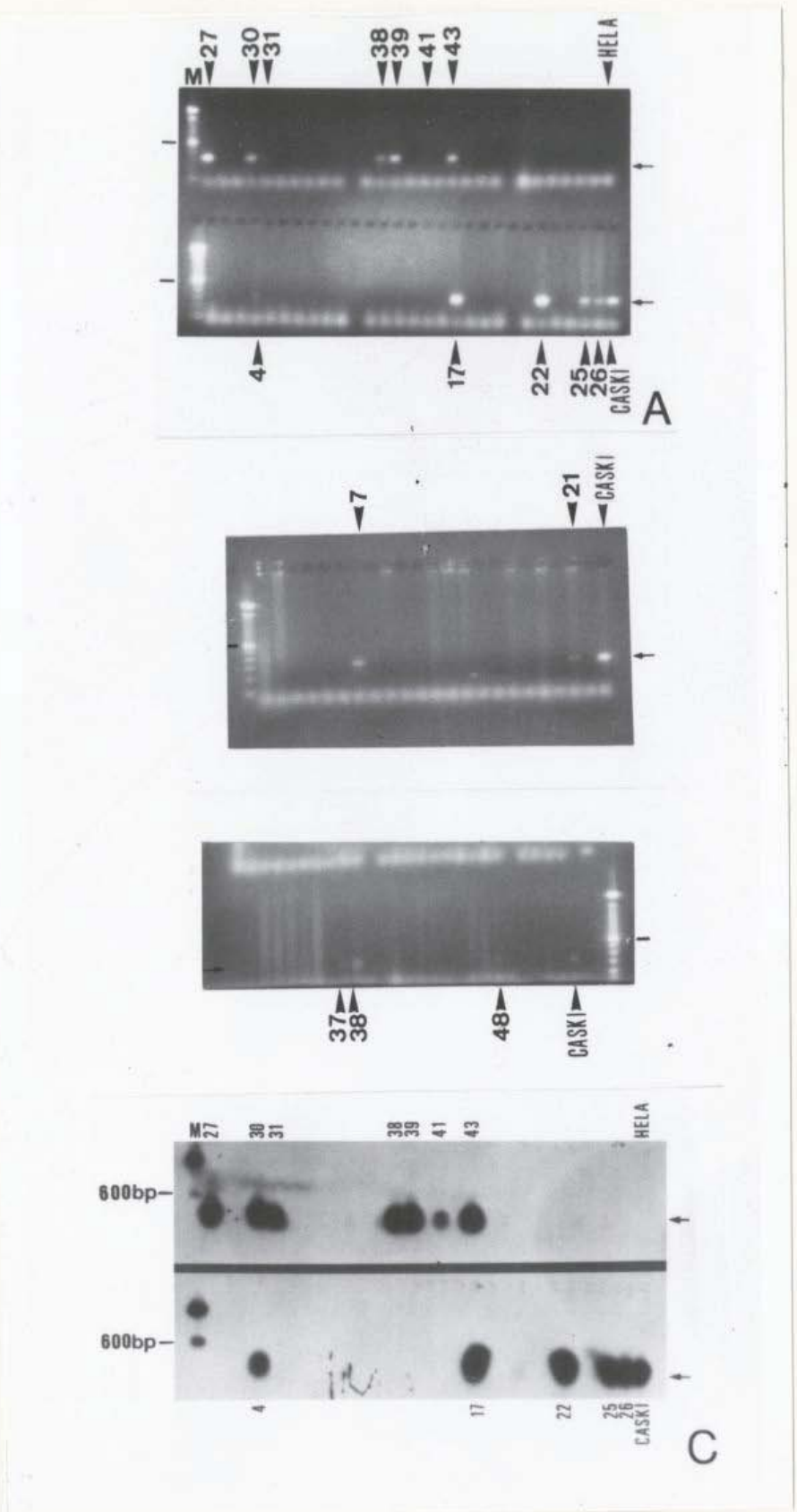
Black arrows indicate amplified products of expected size of 323 bp.

CaSki and HeLa cells were used as positive and negative controls, respectively.

A: PCR-amplified products from the "V" series biopsies, upon being separated on an agarose gel and stained with ethidium bromide.

B: PCR-amplified products from the "C" series biopsies, upon being separated on an agarose gel and stained with ethidium bromide.

C: Southern blot analysis of the PCR-amplified samples in panel A with [^{32}P]-labelled HPV16 under stringent condition.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similar studies reported by others (Crum *et al.*, 1988; Durst *et al.*, 1991; Park *et al.*, 1991; Auvinen *et al.*, 1992; Durst *et al.*, 1992; Higgins *et al.*, 1992b; Stoler *et al.*, 1992; Bohm *et al.*, 1993; Jochmus *et al.*, 1993).

First, great care was taken to select suitable premalignant lesions to represent infections in the two different types of HPV16 target tissues, the native and metaplastic SSE. The current study chose VINs to represent HPV16 infection in native SSE, instead of ectocervical CINs. The reason was that the epithelial origins of CINs were not always clearly identifiable, due to the complex and dynamic nature of the histo-anatomical structure in the cervix. Under certain circumstances, a lesion originated from epithelium in the TZ, which is covered by metaplastic SSE, may be inadvertently miscategorized as originating from the ectocervix, which is lined with native SSE. If the biopsy tissue is large enough to provide sufficient histological information, this miscategorization can usually be realized and corrected. For example, the presence of an underlying cervical gland would indicate the endocervical origin of the lesion. However, the subject lesions for the current study were premalignant. Therefore, the biopsy tissues were generally small, and often could not provide the histological information required for confidently confirming their epithelial origins. This problem was circumvented by choosing VINs to represent HPV16 infections in native SSE, since lesions from vulvas should have no chance of being miscategorized as originating from cervical metaplastic SSE. This precaution was proven to be fully warranted, since two lesions categorized as VINs, and another two as CINs, were actually CINs and VINs, respectively. These mistakes were realized based on the expression patterns of HPV16 E7 oncogene in the lesion, and confirmed by histopathology reexamination (Dr. Ferenczy, personal

communication).

To assure that the lesions representing HPV16 infections in metaplastic SSE were genuinely from metaplasia, the epithelial origins of the CIN lesions were confirmed by the existence of underlying cervical glands. In addition, based on the results from the experimental studies described in Chapters 3 and Chapter 4, the current study recognized the dynamic, transitional nature of metaplasia and its potential to affect HPV16 activity. The histo-anatomical relationship of the CIN lesions to SC junctions was used as a reference for the maturity of metaplasia. This was based on the consideration that, although CK expression may provide some information on the progression of normal metaplasia, their expression might be disrupted during the HPV-induced oncogenic process, as indicated by studies on natural lesions (Smedts *et al.*, 1992b; Smedts *et al.*, 1990; Smedts *et al.*, 1993a) and the current study on experimental lesions (Chapter 3). In addition, limited amounts of biopsy tissue also prevented the collection of informative data on CK expression in most cases.

Another feature of the current study was that particular attention was given to the lesions immediately adjacent to SC junction. The selection of these samples was based on the consideration that these lesions should represent HPV16 infections in more immature metaplasia, because metaplasia may be associated with the exposure of SCE to the vaginal environment and develops from cervical SCE to SSE (Fu and Reagan, 1989b; Jenson and Lancaster, 1990). Thus, the SCE-distal region of a metaplasia must occur prior to the SCE-proximal region of the same metaplasia. Another reason for examining the SCE-proximal lesions was the speculation that HPV infection is initiated in the exposed cells at SC junction (Jenson and Lancaster, 1990; zur Hausen and de Villiers, 1994). Consistently, HPV infection

has been reported to be established in the cervical epithelium proximal to the endocervix and extended toward the ectocervix (Gupta *et al.*, 1989). Therefore, the variation in infection duration among the SCE-proximal lesions should also be less than that among the distal lesions.

In association with above efforts, attempts were also made to correlate the states of HPV16 infection and the statuses of viral DNA with the locations of the lesions. The states of HPV16 infection were extrapolated by examining the three milestones in the HPV16 life cycle, early gene expression, viral DNA amplification and the expression of viral structural genes. For a productive infection, the viral early gene E7 must be expressed first to establish the necessary cellular environment. Then, the viral DNA must be amplified as progeny viral genomes. Finally, the viral L1 structural gene must be expressed to package the progeny genomes into virions. This pattern of programmed sequential viral activities is the common regimen for all viruses to reproduce, and this law has been recognized for, and applied to guide HPV research (Kreider *et al.*, 1987; Beyer Finkler *et al.*, 1990; Stoler *et al.*, 1990; Dollard *et al.*, 1992).

The combined efforts produced results that contributed to successfully revealing several distinct features of HPV16 infections in native, mature metaplastic and immature metaplastic SSE.

5.3.1 E7 expression from integrated viral DNA

In the current study, some CINs showed a pattern of HPV16 activities suggesting that viral DNA was integrated into cellular genomes. Consistent with other reports, these CINs

were negative for viral DNA amplification and L1 expression. Also, most of these CINs displayed morphological features of high grade dysplasia and expressed the viral E7 oncogene extensively in the lesions (Figure 5.21).

Significantly, this study also revealed that the expression of the HPV16 E7 oncogene in the lesions containing integrated viral DNA (Figure 5.22 and 5.23) may be associated with squamous differentiation and dysplastic severity. This observation was most clearly demonstrated in the lesion H6A1 (Figure 5.23). At the SCE-proximal end, the lesion showed dysplastic changes consistent with those for high grade dysplasia, which included the lack of mature squamous differentiation and extensive expression of HPV16 E7. This picture showed a strong resemblance to the one present in the experimental lesions from the immortalized HEN (Section 3.2.2.1 and 3.2.3, and Figure 3.5 and 3.8). In contrast, the SCE-distal region of H6A1 showed mature squamous differentiation with almost no dysplastic change, which was accompanied with a pattern of E7 expression limited to the basal cells. This picture was reminiscent of the one found in the experimental *in vivo* lesions reconstructed from the immortalized HKC. Interestingly, a gradual, continuous transition existed between the two regions of the lesions, which clearly showed the association among squamous differentiation, E7 expression and dysplastic severity. Thus, the results from this study suggested that, the metaplastic lesions may gradually acquire the differentiation-associated gene repression function, as metaplasia progresses and acquires mature squamous differentiation. Thus, maturing metaplastic transdifferentiation results in the restricted E7 expression and decreased dysplastic severity of the lesions. This notion is consistent with the observation that the mature metaplastic SSE eventually becomes morphologically indistinguishable from native

SSE under physiological conditions (Carmichael and Jeaffreson, 1941; Fu and Reagan, 1989a), and achieves the profile of CK expression very similar to that in ectocervical native SSE (Smedts *et al.*, 1990). It is also in agreement with the results from experiments with the epithelial cells from the TZ, which may represent mature metaplastic cells. TZ cells form well-differentiated SSE similar to native SSE, and the HPV16-immortalized TZ cells form low grade dysplastic lesions similar to those from the immortalized HFK, upon being reconstructed into epithelia *in vivo* (Woodworth *et al.*, 1990b).

VIN with INI was not observed in the current study. All of the VINs examined showed viral DNA amplification, indicating that they all contained episomal viral DNA. Although it cannot be absolutely excluded the possibility that episomal and integrated viral DNA was coexistent in some lesions, it was unlikely that these VINs contained only integrated viral DNA. This was based on the fact that all the VINs showed differentiation-dependent viral DNA amplification in a subpopulation of cells localized in the superficial layers, an indication for viral episomal DNA (Cooper *et al.*, 1991b). In addition, the pattern of E7 expression in the VINs was also not consistent with that expected for integrated viral DNA. The VINs examined in the current study expressed E7 mRNA in the bottom layers of the lesions. This pattern was analogous to that in the reconstructed W12 *in vivo* lesion, which contains viral episomal DNA, and was distinct from the pattern for the reconstructed HPV16-immortalized HKC *in vivo* lesions, which contained integrated viral DNA. In addition, the HPV16 E7 expression in the well-differentiated penile SCC, which was believed to contain integrated viral DNA, was also found to be restricted in the basal cells (Higgins *et al.*, 1992b; Higgins *et al.*, 1992c). Therefore, all the results supported that the VINs examined in this

study contained episomal viral DNA.

5.3.2 E7 expression in lesions containing episomal HPV16 DNA

The current study showed that the E7-specific riboprobe detected cytoplasmic and nuclear signals in the lesions containing episomal HPV16 DNA. The cytoplasmic and nuclear signals had several distinct features.

5.3.2.1 E7 cytoplasmic signals

The E7 cytoplasmic signals should represent the mRNA for the expression of the E7 oncogene. The current study demonstrated a clearly differential pattern for the expression of the HPV16 E7 oncogene in native and metaplastic SSE. While the expression of HPV16 E7 in native SSE was compartmentalized in the bottom layers, expression was distributed throughout the metaplastic lesions. The extensive E7 expression was most convincingly shown by the obvious E7 cytoplasmic signal in the defoliated cells of the metaplastic lesions. The significance of this difference in HPV16-mediated oncogenesis will be discussed in Chapter 6.

The compartmentalized expression of E7 in the HPV16-induced VINs was in contrast to the pattern of E7 expression reported by other studies, in which the E7- or E6-E7-specific probes detected signals in the upper layer differentiating cells of CINs and VINs (Crum *et al.*, 1988; Park *et al.*, 1991; Auvinen *et al.*, 1992; Higgins *et al.*, 1992b; Bohm *et al.*, 1993; Jochmus *et al.*, 1993). However, the pattern of E7 expression in VINs revealed by the current study was consistent with that found in the HPV6 vegetative infections occurring in the

external genital region (Iftner *et al.*, 1992). Similar to the current study, the expression of the viral E7 gene in the HPV6-induced low grade dysplastic lesions was reported to be restricted in the undifferentiated bottom 2-3 cell layers. The extensive E7 expression in the CINs examined in the current study is also different from the reported by other studies (Crum *et al.*, 1988; Durst *et al.*, 1991; Auvinen *et al.*, 1992; Durst *et al.*, 1992; Stoler *et al.*, 1992; Bohm *et al.*, 1993). Previous studies showed that the expression of HPV16 early genes, including E7, are activated in the upper layer differentiating cells, similar to that in VINs. The disparities between the results from the current study and those by others will be discussed in Section 5.3.4.

The extensive E7 expression was a consistent feature for HPV16 infections in metaplastic SSE. Also, while all the metaplastic lesions at SC junction showed a similar intensity of the E7 cytoplasmic signals throughout the lesions, variation in the relative strength of the E7 signals was observed in some metaplastic lesions distal from SC junction. The difference between the lesions adjacent to and distal from SC junction may be a reflection of different properties of metaplastic SSE at these two locations. Considering the possibilities that metaplasia develops from SCE (Fu and Reagan, 1989b) and that HPV infection may preferentially initiate in the exposed basal-like cells at SC junction (Gupta *et al.*, 1989), the HPV16 lesions at SC junction should have more immature metaplastic phenotypes and a shorter infection history than those distal from SC junction. In addition, the maturity of squamous metaplasia and the duration of HPV16 infection should be more uniform among the SCE-proximal lesions than the SCE-distal lesions. The shorter infection history would dictate less accumulated cellular mutations and less local immuno-pressure in the lesions at

SC junction than those distal from SC junction. The uniformity in all the above aspects may explain the consistence in the pattern of E7 expression in the SCE-proximal lesions. In contrast, the SC junction-distal CINs may represent "aged" HPV16 infections. The aged infections may differ from the infections at SC junction in many aspects, as a result of progressing transdifferentiation, accumulation of cellular mutations, and increased local immunity against the infected cells.

First, the maturing metaplastic SSE gradually and progressively establishes the program for squamous differentiation, and acquires the squamous differentiation-associated functions identical or similar to those inherent in native SSE. This view is consistent with the suppressed E7 expression found in the upper layer of the C48A1b CIN lesion (Figure 5.18), which was located distally from SC junction. The mechanism responsible for this repression may be analogous to the one in native SSE, and it may become available as a result of the more mature squamous differentiation in the C48A1b lesion.

In addition, the longer history of HPV16 infection in these aged lesions may be accompanied with an accumulation of cellular mutations. This may be the case particularly for the HPV16 infections in metaplastic SSE, since the expression of HPV16 oncogenes, which induces mitosis and mutations, was more extensive in metaplastic lesions than in those of native SSE. The mutations introduced and accumulated as a result of the expression of HPV16 oncogenes may cause aberrations in the programs for epithelial differentiation, resulting in altered patterns of E7 expression in the epithelium. The pattern of E7 expression seen in the F2F3 CIN lesion (Figure 5.19) may represent an example for such a possibility.

Furthermore, aged lesions may be subject to more immunological pressure from the

locally infiltrated immunity effector cells. These effector cells may release immunological cytokines that have been shown to mediate the repression of HPV16 gene expression (Woodworth *et al.*, 1992; Khan *et al.*, 1993b; Agarwal *et al.*, 1994; Kyo *et al.*, 1994; Majewski *et al.*, 1994; De Marco *et al.*, 1995). Consistent with this scenario, the M3G3 CIN lesion (Figure 5.20) did not express a significant amount of the viral E7 mRNA, despite the existence of a large amount of amplified viral DNA. Therefore, M3G3 possibly represented HPV16-induced lesions undergoing recovery.

Thus, these exceptional cases found in SC junction-distal metaplastic lesions indicated the importance of studying viral gene expression in the context of epithelial differentiation and infection history.

5.3.2.2 E7 nuclear signals

In contrast to the differential distribution pattern of the E7 cytoplasmic signals in the native and metaplastic lesions, the E7 nuclear signal was mostly observed in the upper layers and rarely in the bottom layers in the native as well as the metaplastic lesions. The nature of the nuclear signal was not clear. Nuclear signals detected by probes for HPV16 early genes have also been reported by other studies (Stoler and Broker, 1986; Crum *et al.*, 1988; Higgins *et al.*, 1992b; Bohm *et al.*, 1993). These nuclear signals were suggested to be the result of cross-hybridization between the probes and DNA sequences (Higgins *et al.*, 1992b), or to represent introns for viral transcripts downstream of the E7 ORF (Bohm *et al.*, 1993). The results from the current study were in agreement with the latter interpretation. First, the E7

nuclear signal was not always associated with viral DNA amplification. In the SCE-proximal metaplastic TNIs, the epithelial compartment amplifying viral DNA was the bottom half of the lesion (Figure 5.12 and 5.13, DNA panels). If the E7 nuclear signal had been derived from cross-hybridization between the riboprobe and amplified viral DNA, the E7 nuclear signal would have been restricted in this epithelial compartment. However, nuclear signals were largely absent in these lesions. Second, the activity of viral DNA amplification was not always proportionally correlated with the occurrence of the E7 nuclear signals in the vegetative CIN lesions. An example is the H13 CIN lesion, which displayed active viral DNA amplification in the upper layers and had very few cells showing typical E7 nuclear signals (Figure 5.11, DNA panel). Third, the E7 nuclear signal appeared to be persistent in the sample pretreated with DNase before hybridization assays (Figure 5.5).

E7 nuclear signals appeared to be correlated with the states of HPV16 infection. Typical E7 nuclear signals were observed frequently in the lesions with vegetative infections, less prominently in those with TNIs, and rarely in the lesions with INIs. For example, the TNIs exemplified by the M4G1 CIN (Figure 5.13) and V4 VIN lesions (Figure 5.7) showed few E7 nuclear signals. The INIs exemplified by the H10A2 CIN lesion (Figure 5.21) displayed almost no E7 nuclear signals. Thus, E7 nuclear signals may be derived from mRNA species that are required for the late phase of virus reproduction and contain the E7 ORF sequences as introns.

The E7 ORF sequences may be transcribed as introns for the other early mRNAs downstream of the E7 ORF. A cDNA containing E6*** and E5 ORFs has been isolated from the W12 cells and low grade dysplasia biopsies (Doorbar *et al.*, 1990; Sherman *et al.*, 1992).

The E7 ORF was removed from this transcript by splicing at the nt 226 donor and nt 3357 acceptor. Since the W12 cells and the low grade premalignant lesions contain episomal viral DNA, this transcript may represent a physiological product related to viral reproduction. Alternatively, the E7 ORF sequence has the potential to be transcribed as introns for the viral late genes downstream of the early genes. Consistent with this possibility, promoters for late genes have been found in the 5'-end of the LCRs of cutaneous papillomaviruses including BPV, HPV1, CRPV, and HPV8 (Baker and Howley, 1987; Wettstein *et al.*, 1987; Palermo Dilts *et al.*, 1990; Stubenrauch *et al.*, 1992). Interestingly, these studies showed that the transcripts from these late promoters contain two short sequences from the LCR and the E4 ORF preceding the late ORFs. Most of the early gene ORFs, including the E7 ORF, are spliced out as introns. Since the viral structural genes and the early genes downstream of the E7 gene are expressed at very high level, some E7 introns might escape destruction and become detectible by the E7 probe, giving rise to strong nuclear signals in the upper layers. The location of the promoter for the HPV16 late genes has not been established. Therefore, the question remains open. Studies on HPV31b, which is closely related to HPV16 and shares significant genomic sequence and organization homology with HPV16, showed that the transcription of L1 can be initiated from the early promoter used also for the transcription of the E7 ORF (Hummel *et al.*, 1995; Pray and Laimins, 1995). Thus, the L1 transcripts initiated from this promoter should inevitably contain the E7 ORF, possibly as an intron. In addition, the regulation for gene expression of papillomavirus is complex. Regulation at the RNA level may occur not only at the level of specific promoter activation (Stubenrauch *et al.*, 1992; May *et al.*, 1994b; Stubenrauch and Pfister, 1994; Hummel *et al.*, 1995), but also at levels of

splicing (Hummel *et al.*, 1995), mRNA stability (Kennedy *et al.*, 1990; Kennedy *et al.*, 1991) and/or polyadenylation signal usage (Baker and Noe, 1989; Furth and Baker, 1991; Furth *et al.*, 1994). Thus, any fluctuation in these regulatory functions may affect the occurrence and pattern of the E7 ORF-containing intron. This may partially explain why the appearance of E7 nuclear signals was not always proportional to that of L1 signals in lesions examined by the current study.

5.3.3 HPV16 vegetative infections and TNIs in lesions at SC junction

The current study revealed that HPV16 infections may be either vegetative or non-vegetative, as judged by the expression of the HPV16 L1 structural gene. As discussed in Section 5.3.1, a non-vegetative infection with undetectable viral DNA amplification may be regarded as INIs containing a low amount of integrated viral DNA. In contrast, the non-vegetative lesions supporting the amplification of viral DNA in a subpopulation of the cells would represent TNI. TNIs were found in both native and metaplastic SSE. While TNI in VINs showed patterns of E7 expression and viral DNA amplification similar to those in VINs with vegetative infection, TNIs in SCE-proximal metaplastic SSE exhibited features in E7 expression and DNA amplification distinct from those in CINs with vegetative infections.

In the HPV16-induced CIN lesions at SC junction, the failure to express viral late genes was not the only feature that distinguished TNI from vegetative infections. The SCE-proximal TNIs expressed viral E7 oncogene more heterogeneously among the cells. In addition, the amplification of viral DNA in the SCE-proximal TNIs was also characteristic. In vegetative infections, HPV16 DNA amplification occurred in the upper layer cells, as also

reported by others (Crum *et al.*, 1988; Auvinen *et al.*, 1992; Durst *et al.*, 1992; Higgins *et al.*, 1992b; Stoler *et al.*, 1992; Bohm *et al.*, 1993). In contrast, the TNIs at SC junction featured viral DNA-amplifying cells distributed sporadically throughout the lesion, and/or compartmentalized in the low half of the lesions. Individual cells in the basal layer occasionally also showed viral DNA signals, although less frequently than in other epithelial compartments. Interestingly, a recent study on a single CIN case also reported that HPV16 DNA amplification occurred at the transition from the basal layer to the upper layers of the epithelium (Kube *et al.*, 1994). Since E7 expression, viral DNA amplification and L1 expression represent the most important events during viral life cycles, alterations in their patterns indicated that the cellular environment in the SCE-proximal lesions with TNI was profoundly dysregulated and the cells were deficient in epithelial homeostasis control.

The topographic distribution of the aberrant TNIs suggested an association between the infection states and the maturity of squamous metaplasia. While the AIM lesions, which showed no mature squamous differentiation, were all TNIs with aberrant viral DNA amplification, only some SCE-proximal lesions, which may display morphologically mature squamous differentiation, were aberrant TNIs. Consistent with this view, TNIs in the metaplastic SSE distal from SC junction and in native SSE did not show aberrant viral DNA amplification and heterogeneous E7 expression. In addition, the relationship between TNI and the status of squamous differentiation was corroborated by the distribution pattern of the viral DNA-amplifying cells in the TNIs. In the AIM lesions and the SCE-proximal end of CIN with TNIs, the majority of the viral DNA-amplifying cells were located within the lower half of the lesion. In contrast, the viral DNA-amplifying cells tended to distribute throughout the

epithelium at the SCE-proximal end of CIN with TNI. This was most clearly demonstrated in the M4G1CIN lesion (Figure 5.13, DNA panel, or Figure 5.1C).

The association between the maturity of metaplasia and TNI with dysregulated viral DNA amplification may derive from the inherent nature of metaplasia. The metaplastic cells, even in an inappropriate epithelial compartment, may transiently fulfill the cellular conditions required for viral DNA amplification, as an inevitable result of transdifferentiation. Consistently, immature metaplasia in physiological conditions extensively expresses CK13, which is associated with terminal squamous differentiation in mucous SSE and is not expressed in SCE (Smedts *et al.*, 1992b). CK13 first appears in the bottom layers of the metaplastic SSE, and becomes compartmentalized in the upper layer differentiating cells as metaplastic SSE matures (Gigi-leitner *et al.*, 1986; Levy *et al.*, 1988; Smedts *et al.*, 1993b). Thus, the redistribution of viral DNA amplifying cells in association with the progression of metaplasia was correlated with the process of the natural histogenesis of metaplasia.

5.3.4 Disparities between current and previous studies

The results from the current study were consistent with those from other groups on several issues, such as the squamous differentiation-dependent expression of HPV16 L1 gene and amplification of viral DNA in lesions with vegetative infections. The current study is also in agreement with others for the extensive expression of the HPV16 E7 oncogene, non-detectable L1 expression and undetectable viral DNA amplification in high grade dysplasias and cervical SCC. However, this study also provided results that were either different from, or not reported by, previous similar studies. These disparities may be explained by several

distinct features of the current study.

The most prominent difference between the current study and the others was the E7 expression pattern in the HPV16 vegetative infections. While the current study showed that the expression of the viral E7 oncogene was different in HPV16 infections in native and metaplastic SSE, previous studies did not reveal this difference. Most previous studies showed that HPV16 E7 was very weakly, or not expressed at all, in the bottom layers in all the low grade lesions. The expression of E7 was believed to be activated in the upper layer of the lesion in a squamous differentiation-dependent manner, similar to that of other viral genes. This disparity may be attributed to the design of the probes. Most of the previous studies used probes that included the 3'-end of the E7 ORF beyond nt 880 (Durst *et al.*, 1991; Park *et al.*, 1991; Auvinen *et al.*, 1992; Durst *et al.*, 1992; Higgins *et al.*, 1992b; Stoler *et al.*, 1992; Jochmus *et al.*, 1993; Kube *et al.*, 1994). In contrast, the probe used in the current study contained only the 5'-end of the E7 ORF spanning from nt 502 to nt 720.

A promoter inside the HPV16 E7 ORF was postulated recently. This E7 promoter was thought to be activated by squamous differentiation and to initiate transcription for other early genes downstream of E7, such as E1^E4 and E5 (Higgins *et al.*, 1992b). In addition, studies on HPV31b, which has the genome structure very similar to that of HPV16 (Goldsborough *et al.*, 1989), indicated that the mRNAs for E1^E4, L1, and possibly L2, can be initiated from the E7 promoter located at the 3'-end of the E7 ORF (Hummel *et al.*, 1992; Hummel *et al.*, 1995; Pray and Laimins, 1995). Similar E7 promoters have also been described in HPV6 and 11 (Chow *et al.*, 1987; Rotenberg *et al.*, 1989). Thus, the signals detected by the HPV16 E7 probes containing the 3'-end of the E7 ORF may actually derive

from hybridization of the probes to mRNAs originating from the E7 promoter. E1^E4 mRNA has been well established to be highly expressed in the upper layer cells (Crum *et al.*, 1988; Durst *et al.*, 1991; Park *et al.*, 1991; Durst *et al.*, 1992; Higgins *et al.*, 1992b; Stoler *et al.*, 1992; Bohm *et al.*, 1993). Therefore, the strong E1^E4 signal detected by the E1-overlapping probe used by others may mask the relatively weak E7-specific signal. Indeed, the transcription level of the E1^E4 mRNA was suggested to be 50 to 100 times more prevalent than the level of E7 mRNA (Iftner *et al.*, 1992). The importance of probe design was illustrated in studies investigating the pattern of E7 expression in HPV6 infections (Iftner *et al.*, 1992). With an E7-specific probe spanning most of the E7 ORF, E7 expression in the HPV6-induced genital condylomas was detected in the upper layers (Stoler *et al.*, 1989). However, a recent study using an E7-specific probe containing only the 5'-end of the E7 ORF found that E7 expression was compartmentalized in the bottom 2-4 cell layers (Iftner *et al.*, 1992). This pattern of HPV6 E7 expression in native SSE reported by Iftner *et al.* (1992) is very similar to the HPV16 E7 expression in the VINs observed in the current study.

Results from one previous study also argued against the view that the expression of viral E7 was activated in the upper layers of the HPV16 lesions (Higgins *et al.*, 1992b). Interestingly, this argument was based on *in situ* hybridization assays that examined HPV16 E6 and E7 oncogene expression using subgenomic probes spanning the entire E6 or E7 ORFs. The E6 probe detected signals distributed almost uniformly in the CIN and VIN lesions, although the E7 signal was distributed weakly in the bottom layers and very strongly in the upper layers. Based on the knowledge that HPV16 E6 and E7 genes use the same promoter, the report proposed that the expression of HPV16 E6-E7 oncogenes is not

activated in the upper layers and the signal detected by the E7 probe reflects the transcripts of the viral genes downstream of the E7 ORF. This study is consistent with the current one, in recognizing the expression of E7 mRNA in the bottom layers and arguing against an enhanced E7 expression in the upper layer cells. The reason for the extensive E6 expression in the HPV16-induced VINs in this study, in contrast to the compartmentalized E6 expression in HPV6 infection (Iftner *et al.*, 1992), is unclear. Possibly the probe design is the reason. A recent study with a refined E6 probe detected almost no E6 signal in HPV16-containing low grade CINs (Bohm *et al.*, 1993). Thus, the HPV16 E6 probe spanning the entire E6 ORF may not be truly E6-specific *per se*. Indeed, HPV gene expression is controlled by very complex splicing mechanisms. A recently described example is the control on the HPV31b E1^E4 and L1 expression (Hummel *et al.*, 1995; Pray and Laimins, 1995). Both mRNAs can be initiated from the HPV31b p97 promoter, and the transcripts are alternatively spliced to remove only part of the E6 ORF for encoding E1^E4 or L1. Similarly, the transcripts from the P97 promoter of HPV16, from which E6 and E7 are transcribed, are potentially capable of encoding most of the viral early genes, and the spliced transcripts for these ORFs retain a short 5'-sequence of the E6 ORF as an mRNA leader sequence (Doorbar *et al.*, 1990; Rohlfs *et al.*, 1991; Nasseri *et al.*, 1991; Vormwald Dogan *et al.*, 1992; Sherman *et al.*, 1992; Sherman and Alloul, 1992). Thus, an HPV16 probe spanning the entire E6 ORF may also detect these transcripts for the downstream early ORFs. Therefore, differences in probe designs may greatly affect the outcomes of *in situ* hybridization experiments.

Another explanation for the differences between many of the previous and the current studies may lie in the sample selection. Based on the available literature, no systematic study

has focused on the HPV16-induced lesions at SC junction. Most of the previous studies did not carefully identify the histological origin of the lesions. As shown clearly in the current study, CIN lesions distal from SC junction often displayed subtle variations in the patterns of E7 expression. Thus, the results obtained by examining lesions of the SCE-distal category, especially when the number of cases is limited, may reflect only one facet of HPV16 infection. In fact, the E7 expression patterns in the SCE-distal lesions observed in the current study bore some similarity to those reported by Crum *et al.* (1988) and Bohm *et al.* (1993). These studies examined HPV16 gene expression in two cases of VINs (Bohm *et al.*, 1993; Crum *et al.*, 1988) and two cases of CINs (Bohm *et al.*, 1993), using E7 probes similar to the current study. They showed that E7 expression in these cases was extremely weak, and the few E7 signals were mostly nuclear in only a few upper layer cells. The pattern of E7 expression described by these workers was very similar to that in the SCE-distal lesion M3G3 (Figure 5.20) examined in the current study. The importance of sample selection was also reflected in the patterns of viral DNA amplifications. Indeed, the distribution patterns of viral DNA-amplifying cells in the vegetative infections were consistent in the current and previous studies, although the current study revealed an aberrant viral DNA amplification associated with TNIs in metaplastic SSE proximal to SC junction.

Ambiguity in results may also stem from a combination of technical factors, such as the combination between the sensitivity of the *in situ* hybridization assays and the selection of infected tissues. The importance of the combined effects of these two factors may be represented by the CIN lesion F2F3 (Figure 5.19). F2F3 was located distally from SC junction, but was metaplastic in nature, as indicated by the underlying cervical gland. Viral

DNA amplification and L1 expression occurred only in the upper layers. While the E7 oncogene was expressed throughout the lesion, its expression level in the upper layers was enhanced. Because the cytoplasmic E7 signal in this case was exceptionally abundant in the upper layers, a low sensitivity in the *in situ* hybridization assays may result in revealing only the upper layer signals. This would lead to the impression that E7 was expressed only in the upper layer cells, a picture reported by several studies (Durst *et al.*, 1991; Park *et al.*, 1991; Auvinen *et al.*, 1992; Durst *et al.*, 1992; Stoler *et al.*, 1992; Jochmus *et al.*, 1993; Kube *et al.*, 1994).

5.4 Summary

The results from this series of studies on natural HPV16 infections were in agreement with those from the experimental studies reported in Chapter 3 and 4. The maturity of metaplasia was shown to be associated with the expression patterns of viral oncogenes from the integrated viral genomes in the CIN lesions. This was indicated by the extensive expression of E7 in the CIN lesions proximal to SC junction, and the repressed expression of E7 in the lesions distal from SC junction. Thus, the gene repression function that acted on the integrated viral genes may exist in the mature metaplasia but not in the immature metaplasia.

Also consistent with the experimental studies, the current study showed that viral activities were different in vegetative infections occurring in native and metaplastic SSE, as well as in immature and mature metaplastic SSE. The expression of HPV16 E7 was compartmentalized in the lower layers of the native SSE lesions, while E7 expression was extensive in the metaplastic SSE lesion, especially in the immature metaplastic SSE lesions.

The current study also showed that TNIs in immature metaplastic SSE displayed aberrant viral DNA amplification. This unique state of TNI in immature metaplasia was transient in nature, and tended to be corrected with the progression of metaplasia.

All these results indicated that the metaplastic SSE had distinct features for HPV16 activities, and these features were dynamic in relation to the maturity of the metaplastic transdifferentiation. The significance of these unique features of metaplastic SSE in relation to HPV-mediated oncogenesis will be discussed in Chapter 6.

CHAPTER 6

GENERAL DISCUSSION:

SQUAMOUS METAPLASIA AND HPV16-MEDIATED ONCOGENESIS

This project was undertaken to elucidate the virological and cellular mechanisms responsible for the high oncogenic susceptibility of metaplastic SSE to HPV16 infections. The results constituted the first systematic study of the relationships among human genital epithelial cells, squamous metaplasia, HPV16 infections and HPV16-mediated oncogenesis.

The current study featured two modelling systems with three types of human genital epithelial cells that are potential targets for HPV16 infections. Because HPV16 infection is tissue-specific and the oncogenesis by tumour viruses is closely associated with the dysregulated interactions between viral and cellular elements, the necessity of using the natural target cells of HPV16 infection to study HPV16-mediated oncogenesis is obvious. Therefore, epithelial cells from the human foreskin, ectocervix and endocervix were used in the current study to represent all the possible cell types susceptible to HPV16 infection in the genital area (Burghardt, 1986; Jenson and Lancaster, 1990).

By using newly established cell lines instead of tumour cell lines, the current study circumvented the following potential problems. Tumour cell lines are not suitable for studying the initial oncogenic events mediated by HPV16 due to several reasons. First, tumour cell lines were most often established from malignant tumours and they represent late, not early, stages of oncogenesis. Therefore, they may carry accumulated mutations that are often sufficient for the advanced stages of oncogenesis. Second, cultivation for a long period of

time *in vitro* may introduce selected phenotypes that interfere with experimental observations. Third, the differentiation status of the tumour line cells may be affected by the expression of cellular and viral oncogenes. Therefore, the exact origin of the tumour cell lines cannot be reliably ascertained. These problems were addressed in the current study using primary human genital epithelial cells and their newly established derivatives, which were ascertained for their origins.

Another problem-solving feature of the design of this study was that the experimentally manipulated epithelial cells were examined in epithelia reconstruction systems for their behaviour under the influence of HPV16 oncogenes. It has been well recognized that cells cultured in *in vitro* monolayer, especially epithelial cells, do not fully resemble those in the original tissues. In the conventional monolayer submersion culture, the epithelial cells are placed in a two-dimensional, unpolarized environment, instead of the polarized environment in a three-dimensional space, as in tissue. Thus, tissue homeostasis cannot be established in monolayer culture. Lack of homeostasis in monolayer culture inevitably results in altered expression of many genes responsible for cellular phenotypes, such as differentiation. As a result, the physiological and pathological phenotypes of cells in monolayer culture generally cannot be confidently correlated with those of their counterparts in tissue. The problems arising from the disparities between monolayer culture and natural tissues were minimized in the current study by limiting the culture time in monolayer and analysing the cells with *in vivo* and *in vitro* epithelia reconstruction systems. The two epithelia reconstruction systems have different characteristics. The *in vivo* system more closely mimics the natural condition. The shortcoming of the *in vivo* system for lacking reflexibility was complemented by the *in vitro*

system, which partially mimics natural condition but allows modification of the epithelial reconstruction conditions. The principles of these two systems were introduced in Chapter 3.

The current study on clinical natural HPV16 infections was also characterized by distinct features. As introduced in Section 5.1 and discussed in Section 5.3.1, the clinical samples were selected carefully to represent HPV16 infections in native SSE and metaplastic SSE. With the samples obtained from three pathology departments, the current study standardized the HPV16-induced metaplastic lesions for their status of differentiation by their relative position to SC junction. With these reliable tissues, the expression of the HPV16 E7 oncogene and the amplification of viral DNA were correlated with the state of the viral infection and status of the viral DNA.

The combination of these features enabled the current study to shed new light on several topics concerning HPV16 infection and the mechanism(s) of HPV16-mediated oncogenesis.

6.1 Distinct properties of target tissues for HPV16 infection and expression of HPV16 oncogenes

The current study indicated that HPV16 was competent for viral vegetative life cycles in both native SSE and metaplastic SSE. However, HPV16 activities in these two types of epithelia were distinct, due to the fundamentally different patterns of squamous differentiation in native and metaplastic SSE. These results are consistent with the hypothesis of this thesis that the HPV life cycles are closely predicated on the differentiation and homeostasis of native

and metaplastic SSE.

6.1.1 Stable homeostasis in native SSE and differentiation-associated gene repression

Native SSE is one of the biggest tissues in the human body. It covers most of the exposed anatomical regions including the vulva, vagina and ectocervix. Native SSE has a single, highly specialized function, to isolate and to protect the organism from the harsh external world.

Native SSE is characterized by its physical strength and impermeability, which are required by SSE to function as a mechanical barrier against physical, chemical and biological agents from outside. The external environment contains various agents that are potentially carcinogenic, but native SSE is quite resistant to malignant transformation. Consistent with this observation, native SSE is designed with a stratified structure, in which the epithelial cells undergo programmed differentiation (refer to Figure 1.3A and Section 1.3.3.3). Cells that possess proliferation potential and thus are susceptible to malignant transformation are located in the bottom layers. In contrast, cells having detached from the bottom compartment of the epithelium are permanently withdrawn from cycling. The potentials of other cellular activities in the upper layer cells are also highly restricted. Only genes involved in the SSE terminal function are expressed, to convert the cellular resource efficiently to produce differentiation-associated products, such as CKs. Therefore, the majority genes in the upper layer cells are inactive as squamous differentiation progresses (Fuchs, 1993). This pattern of programmed differentiation renders the upper layer cells virtually non-transformable. As a result, the transformation-susceptible basal cells are protected by the overlying

transformation-resistant, irreversibly differentiated cells from direct contact with external mutagenic agents. Considering the importance of squamous differentiation in preventing neoplastic transformation, the control mechanisms for squamous differentiation in native SSE should be dominant and stable. Indeed, studies of spontaneously immortalized epidermal human keratinocytes showed that the potential for squamous differentiation is stably retained even after the cells were malignantly transformed (Boukamp *et al.*, 1988; Fusenig *et al.*, 1990, Breitkreutz *et al.*, 1991).

The terminal nature of squamous differentiation suggests that the programmed gene expression during squamous differentiation have special requirements for the control mechanisms. This control mechanism(s) should allow the programmed switching of gene expression to occur, and meanwhile it should also render the inactivated genes permanently suppressed. Since an active control mechanism is susceptible to disruptions (Blau, 1992), a passive control should be involved in the gene repression during squamous differentiation. This hypothetical passive control mechanism would cooperate with, or supplement, the function of the active control mechanism. The current study showed in Chapter 4 that DNA methylation, a candidate passive control mechanism, may be involved in the repression of certain cellular genes during squamous differentiation. DNA methylation has not been reported to be involved in squamous differentiation. However, the inheritable nature of the DNA methylation-mediated gene repression can fulfil the stability requirement of squamous differentiation and the epithelial homeostasis control in native SSE (Boukamp *et al.*, 1988; Breitkreutz *et al.*, 1991; Fusenig *et al.*, 1990). The epigenetic nature of the DNA methylation-mediated gene repression predicts that this mechanism may function at a global level. This is

consistent with the finding of the current study using the HPV16 and SV40 promoters. Both promoters were subject to the differentiation-associated gene repression, and the repression of both promoters was equally sensitive to the 5-AZ DNA demethylation agent. The possible mechanisms for DNA methylation-mediated gene repression and the effects of 5-AZ have been discussed in Section 4.2.3.

6.1.2 Relaxed homeostasis in metaplastic SSE and persistent expression of HPV16 oncogene

The protective barrier on the surface of the endocervix is provided by the multifunctional SCE, which is physically less robust than SSE but able to perform other functions, such as mucus excretion. This choice is obviously based on the physio-anatomical requirement of the endocervix. Unlike vulva, vagina and ectocervix, which are exposed directly to the external environment and subject to stringent mechanical conditions, the endocervix is relatively sheltered, functioning as the passage for sperm migration. However, the anatomy of the cervix is dynamic rather than stationary. Physiological and pathological alterations in the anatomy of the cervix may expose certain regions of the cervical SCE, most often those at or near SC junctions, to the vaginal environment. Obviously, the exposed SCE is not physio-anatomically compatible to the new environment, and therefore this condition should be corrected. The option apparently taken by nature is to allow the exposed cervical SCE to transdifferentiate into SSE by default. This scenario was clearly shown by the current study experimentally. This view is also supported by previous morphological observations on metaplasia, by means of ultrastructure, nucleic acid incorporation autoradiography, *in toto*

organ *in vitro* culture, and analysing patterns of CK expression (Carmichael and Jeaffreson, 1941; Schurch *et al.*, 1978; Smedts *et al.*, 1993a).

Because metaplasia is a gradual and progressive process, the programmed transition of gene expression responsible for the transdifferentiation from SCE to SSE should also be changed gradually and progressively. This phenomenon of gradual and progressive metaplasia may reflect the possibility that the transdifferentiation requires dismantling the original differentiation control circuit for SCE and establishing the new one for SSE. In association with this gradual transition, therefore, there may be a window period in which the transcription regulators responsible for different differentiation phenotypes may be expressed with overlap. For example, transcription regulators responsible for the glandular phenotypes may be co-expressed with those for squamous phenotypes within a certain time frame during metaplasia. This would lead to a mixed phenotype that is partially glandular and partially squamous. Although no experimental data had been available thus far to substantiate this hypothesis at the level of molecular biology, transitional co-expression of CKs that are characteristically and exclusively expressed in SCE and SSE is the norm for immature metaplastic SSE (refer to Figure 3.1A) (Smedts *et al.*, 1993b). Section 3.2.1 of the current study on CK expression and morphology in the artificial metaplasia also addressed this issue.

Such a cellular environment may lead to deregulated expression of many genes, since the combination of transcription factors may have composite trans-acting activities, or crosstalk effects (Miner and Yamamoto, 1991). Indeed, the plethora of independent and combinational effects of transcription regulators may create a cellular environment that supports promiscuous gene expression. Furthermore, this state of relaxed gene control may

be aggravated by the absence of, or deficiency in, the passive control mechanism that is inherent in native SSE. Thus, the upper layer cells of the metaplastic SSE may show patterns of gene expression and DNA replication similar to those of the bottom layer cells. This scenario may explain why metaplastic SSE, especially immature metaplasia, more often displays relaxed homeostasis, as indicated by cycling cells in the upper layers of metaplasia (Schellhas and Heath, 1969; Averette *et al.*, 1970) and decompartmentalized CK expression in immature metaplasia (Smedts *et al.*, 1993a). Theoretically, foreign genes having invaded the cells as a result of virus infection may also be expressed in a dysregulated manner. This is consistent with the finding of the current study that both the HPV16 and SV40 promoters were active in the experimentally reconstructed immature metaplastic lesions from the immortalized HEN.

6.2 Different features of HPV16 infections in native and metaplastic SSE

E7 and E6 are the major viral genes involved in HPV16-mediated oncogenesis. The oncogenic potential of the E7 oncoprotein is attributed biochemically to its interference with at least two cell cycle regulators, pRb and p107. Thus, the E7 oncoprotein can be regarded biologically as a mitogen that acts on the central circuit for cell cycle control. The E6 oncoprotein abrogates the functions of the p53 tumour suppressor protein, which regulates genomic integrity control and apoptosis (Tommasino and Crawford, 1995). Although the E7 and E6 genes of HPV16 are the best biochemically and molecular biologically characterized viral oncogenes, a basic virological question has not been solved: "How is the expression of E6 and E7 regulated during vegetative infection and oncogenesis?" The current study

revealed distinct patterns of HPV16 E7 oncogene expression in native and metaplastic SSE. In fact, the results from the current studies on natural HPV16 infection suggested that HPV16 activities in these two types of SSE are different, possibly due to the fundamental differences in their differentiation programs.

6.2.1 HPV16 vegetative infection in native SSE

One of the major controversies surrounding the expression of HPV16 E7 oncogene is whether E7 is expressed in the bottom layer cells in the infected SSE. The current study revealed E7 expression patterns that were distinct in native and metaplastic SSE and have not been reported by other studies (Crum *et al.*, 1988; Durst *et al.*, 1991; Durst *et al.*, 1992; Higgins *et al.*, 1992b; Stoler *et al.*, 1992; Bohm *et al.*, 1993). The HPV16 E7 oncogene was highly expressed in the bottom layer cells of native SSE. E7 expression in the upper layers was suppressed. Since HPV16 replication was vegetative in these native SSE lesions, these results indicated that E7 expression in the upper differentiating cells is not required at a high level for HPV16 reproduction. A similar pattern for the E7 expression has been reported in the HPV6-infected native SSE (Iftner *et al.*, 1992; Oft *et al.*, 1993). Therefore, it may be a general phenomenon that the E7 gene is required to be expressed in the undifferentiated proliferating cells for HPV vegetative infection. Because the HPV16 E6 gene is controlled by the same promoter as E7, E6 expression most probably parallels that of E7. Consistently, E7 and E6 can be transcribed as a bicistronic message from the same promoter, and both ORFs were reported to be translatable from this mRNA (Altmann and Trachsel, 1993; Tan *et al.*, 1994).

The expression of the E7 gene in the bottom layer cells is consistent with the expected functions for a viral early protein and the biochemical properties of the E7 protein. Early proteins of viruses are expected to promote cell proliferation for increasing the pool size of the infected cells, and to prime the infected cells for later stages of viral life cycles (Fields and Knipe, 1990). The findings of the current study helped to address the long standing theoretical dilemma for interpreting the reported differentiation-dependent expression of the E7 oncogene in relationship to its biochemical properties and oncogenic potential. Indeed, it is difficult to reconcile the reported upper layer expression of the E6 and E7 oncoproteins with low grade dysplasias that are characterized by neoplastic proliferation of the bottom cells, rather than the upper layer cells. In addition, squamous differentiation and cell cycling are mutually exclusive in native SSE (Fuchs, 1990). Active expression of the mitogenic E7 oncoprotein in the upper layers would potentially interfere with the program of squamous differentiation (Merrick *et al.*, 1992; Blanton *et al.*, 1992), which is required by HPV for normal vegetative life cycle. Based on the above argument, the reported upper layer expression of HPV16 oncogenes does not logically fit in the picture for HPV vegetative infection.

It should be noted that although the current study revealed that the expression of the HPV16 E7 oncogene was repressed in the upper layers of the native SSE lesions, the HPV16 E7 oncogene cannot be excluded from being expressed at a reduced level in the upper layer cells. This is consistent with the suggestion that the upper layer-expression of the viral E7 oncogene may be required for reactivating the cellular genes involved in the cellular mechanisms for DNA replication (Merrick *et al.*, 1992; Demeter *et al.*, 1994). However, the

reactivation of cellular genes is not the only possible mechanism for providing the DNA replication proteins required for viral DNA amplification. The cellular proteins can be procured post-transcriptionally, such as by protein stabilization. In fact, a full reactivation of the cellular DNA replication mechanism may not be ideal for the viral DNA amplification, since replication of cellular DNA will inevitably compete with viral DNA amplification for the limited resources. In addition, abnormal replication of cellular DNA may also activate p53-independent control mechanisms that impose restraints on viral DNA amplification. The E1 and E2 proteins of papillomaviruses are involved in directing cellular DNA replication machineries to the viral replication origin (Frattini and Laimins, 1994; Kuo *et al.*, 1994), and are expressed in the SSE compartment partially overlapping the one for E7 expression (Stoler *et al.*, 1992; Bohm *et al.*, 1993). Therefore, E1 and E2 proteins are potentially capable of forming complexes with the cellular DNA replication proteins and protect these proteins from degradation following the permanent arrest of cell cycling. Indeed, counteracting cellular proteins is a strategy repeatedly and productively utilized by viral proteins, as exemplified by HPV oncoproteins.

Based on the findings of other studies, and in the light of the current one, the vegetative life cycle of HPV16 in native SSE may feature the expression of viral genes in three phases. The first phase is in the cells of the bottom 2-4 layers, in which the E7, and perhaps the E6, genes are expressed to promote cell proliferation. The second phase commences when the proliferating cells detach from the basal membrane and begin programmed terminal squamous differentiation. The differentiation program triggers a suppressed expression of the E6 and E7 genes and an activated expression of the downstream

early genes, such as E1 and E2, which regulate the expression of viral genes directly and promote DNA amplification. In the third phase, the viral L1 and L2 mRNAs are transcribed from the amplified viral DNA, leading to virion assembly. The pattern of phased expression of viral genes is also a feature for other DNA tumour viruses, such as SV40, EBV and adenovirus (Fields and Knipe, 1990).

The differentiation-dependent repression of E7 expression in native SSE may be mediated by both cellular and viral functions. One possible cellular mechanism is DNA methylation, which is involved in squamous differentiation, as shown in the current study. Consistent with this possibility, the expression of HPV16 oncogenes has been shown to be subject to the regulation of viral DNA methylation (Rosl *et al.*, 1993; List *et al.*, 1994). The viral mechanism for the repression of E7 expression may involve the viral E2 protein, which has been shown to be expressed in the upper layer differentiating cells (Durst *et al.*, 1991), and negatively transactivate the expression of the viral oncogenes via the HPV enhancer (Marshall *et al.*, 1989; Bernard *et al.*, 1989; Bedrosian and Bastia, 1990; Gloss and Bernard, 1990; Durst *et al.*, 1991). The E2C produced by alternative splicing may also suppress HPV16 E7 at the level of transcription initiation (Doorbar *et al.*, 1990; Sousa *et al.*, 1990; Chiang *et al.*, 1991; Sherman *et al.*, 1992). Another potential mechanism for the repressed expression of E7 in the upper layer cells is differentiation-dependent alternative splicing. The E6 and E7 ORFs could be transcribed in the upper layer cells, but be removed from the mRNA as introns. cDNAs consistent with such splicing events have been found (Doorbar *et al.*, 1990; Sherman *et al.*, 1992). This scenario is also consistent with a recent report that the differentiation-dependent alternative splicing is important for regulating the expression of the

HPV31b E1^E4 gene and late genes, in addition to promoter usage (Hummel *et al.*, 1995).

6.2.2 HPV16 vegetative infection and TNI in metaplastic SSE

The viral E7 oncogene was also expressed in the bottom layer in the HPV16-infected metaplastic SSE, as in native SSE. However, the expression of the viral E7 oncogene was not restricted to the bottom layers of the HPV16-infected metaplastic SSE, but was expressed at a similar level throughout the lesion.

This observation poses a dilemma to the understanding of HPV16 life cycles in metaplastic SSE. In theory, the phased expression of viral genes would benefit efficient expression of viral genes that are suited for specific functions during the virus vegetative life cycle. Successful switching between these phases of viral gene expression should be important for the progression of the virus replication program. If the upper layer repression of E7 in the native SSE lesions represented a switch for the expression of viral genes in the HPV16 replication program, such a switching scheme should be advantageous for the productive virus replication. A disruption in this switching scheme would result in inefficient virus reproduction, or in an extreme case, even non-vegetative infection. Thus, why could HPV16 vegetative replication still occur, if not more efficiently, in metaplastic SSE? An answer to this question could be rationalized from an evolutionary point of view.

All the HPVs are evolutionarily related. In fact, HPV16 probably evolved from HPVs that infected native SSE (Chan *et al.*, 1992). Infection in the genital epithelia may have provided an additional niche that benefited the contagious spreading of HPV16. The human behaviour related to reproduction would provide a greater and more reliable chance for the

virus to spread than other social contacts, since this behaviour is undoubtedly one of the most intimate and most conserved. In addition, the exposed basal-type cells at the SC junctions may indeed provide a unique opportunity for the HPV to establish a primary infection (see Section 6.5.1.1).

One challenge for HPV16 vegetative replication in the metaplastic SSE was probably that the metaplastic SSE, or certain stages of it, lacks the cellular function(s) required to down-regulate the expression of the E6 and E7 genes and/or to activate the expression of the downstream early genes in the upper layer cells. HPV16 may have developed a strategy that allows efficient progression of vegetative life cycles without the down-regulation of the E6 and E7 gene expression by, for example, modifying functions of individual virus-encoded products. Indeed, the increased pRb-binding affinity of the E7 oncoprotein and enhanced p53-destabilizing activity of the E6 oncoprotein, which are the biochemical foundations for HPV16-mediated oncogenesis, may represent one aspect of such adaptions. It is logical that the strategy used for adapting to the new cellular environment in metaplasia should render HPV16 capable of replicating in the metaplasmic SSE on one hand, while on the other hand it would preferably allow the virus to retain the original competency to replicate in native SSE, such as in penile SSE. The capability to replicate in both types of SSE should be advantageous for HPV16, since this allows HPV16 to infect both the female and male genital epithelia. From a lighthearted perspective, HPV16 perhaps learned that the best way to coevolve with humans is to couple the viral reproduction with that of humans.

The dynamic nature of the differentiation status in metaplasia dictates that HPV16 life cycles in metaplastic SSE should also be dynamic, since the program of HPV16 life cycles is

dependent on squamous differentiation. In conditions that the differentiation status of metaplastic SSE fulfils the requirement for the vegetative virus replication, a vegetative infection would result. However, at certain metaplastic stages, the differentiation status of the metaplastic SSE may not satisfy these requirements. HPV16 vegetative life cycles may be arrested at a certain phase, resulting in a non-vegetative infection. Since metaplasia is progressively transitional, the non-vegetative infection resultant from the incomplete squamous trans-differentiation of the host tissue, or TNI, could be specific to certain time windows during metaplasia. Therefore, TNI theoretically may convert into a vegetative replication program as transdifferentiation progresses and metaplastic transdifferentiation evolves into the phenotypic equivalent of native SSE. The current study demonstrated that the TNI program did exist in the HPV16-infected immature metaplastic SSE.

The current study showed that TNIs in immature metaplasmic SSE featured distinct patterns of viral E7 expression and DNA amplification. This phenomenon can be well explained by the possibly lax controls for gene expression and homeostasis in immature metaplastic SSE (refer to Section 6.1.2), and could be significant for HPV16-mediated cervical oncogenesis (see Section 6.3).

6.2.3 INIs in native and metaplastic SSE

The current study shed new light on the mechanism responsible for the repression of integrated viral E7 oncogene in native SSE (Durst *et al.*, 1991). The repression of the integrated HPV16 oncogene in native SSE was possibly mediated by a mechanism that was dependent on squamous differentiation. This native SSE-inherent mechanism may involve

DNA methylation, and may function at a global level with some selectivity, but no specificity. The current study revealed that this mechanism was deficient in immature metaplastic SSE, but was acquirable in mature metaplastic SSE. Therefore, the integrated viral oncogenes were expressed more extensively in immature metaplastic SSE than in mature metaplastic SSE or native SSE.

Although suppression of the integrated and episomal HPV16 oncogenes may both involve DNA methylation, the exact mechanisms could differ. Methylation, heterochromatinization, and suppressed expression of the integrated viral gene occur as one integral part of the cellular genome, and may depend on the nature of the cellular sequences flanking viral DNA, rather than of the viral DNA (Keshet *et al.*, 1986; Buschhausen *et al.*, 1987). Thus, repression of integrated HPV genes is not specific for the HPV. Consistently, integrated adenovirus DNA was also reported to be suppressed and extensively methylated (Doerfler, 1993). In contrast, DNA methylation-mediated suppression of episomal HPV genes can be attributed to DNA methylation at specific sites in the viral LCR (Rosl *et al.*, 1993; List *et al.*, 1994), which may result in specific interference of transcription initiation from the specific viral promoter (Boyes and Bird, 1991).

6.3 Oncogenic risks resulting from HPV16 infection in different SSE

The results of the current study indicated that the high susceptibility of metaplastic SSE to HPV16-mediated oncogenesis may reside in the transdifferentiation process required for metaplasia. The deregulated cellular environment in metaplasia may result in sustained expression of HPV16 oncogenes and incoordinate amplification of viral DNA. These irregular

viral activities, in conjunction with the deregulated cellular environment, may tend to induce cellular genomic mutations and viral DNA integration, the molecular events required for oncogenesis.

6.3.1 Increased cellular genomic mutations induced by extensively expressed viral oncoproteins

As introduced in Section 1.3.5.3.2, the oncogenic functions of E7 and E6 are attributable to their abilities to promote progression through the cell cycle, to interfere with senescence control, and to induce genomic mutations. Therefore, the expression of viral oncogenes is one important aspect for the oncogenic potential of HPV16 infection to the infected tissue.

In native SSE, only the basal cells are competent to proliferate. Not coincidentally, the current study showed that the HPV16 E7 oncogene was expressed only in the bottom cells of native SSE. Thus, viral oncoproteins are able to drive the bottom cells of native SSE into continuous cycling. Although the viral oncoprotein-driven cell proliferation in the bottom layer may induce cellular mutations, the mutations introduced during bottom cell proliferation should be limited in numbers. This is because the cells will be forced to commence the squamous differentiation program upon detaching from the basal membrane (Boukamp *et al.*, 1988; Fusenig *et al.*, 1990; Breitkreutz *et al.*, 1991). The differentiating upper layer cells are withdrawn from cycling, due to the dominant homeostasis control and suppressed viral oncogene expression. Therefore, no more mutations are introduced into the cellular genome. The limited number of cellular mutations introduced during basal proliferation is unlikely to

disrupt the cell cycle arrest of the upper layer cells, since important cellular control mechanisms are usually functionally redundant. In addition, the differentiation-associated passive gene repression mechanism may function at a global level to reinforce the cell arrest, by suppressing genes not involved in the differentiation program including those for cell replication. This repression mechanism can also effectively nullify the accumulated cellular genomic mutations. Therefore, the infected cells undergoing terminal differentiation no longer impose a neoplastic risk to the host, because these cells are destined to complete the differentiation program and slough off from the surface of the epithelium.

A different scenario may occur in HPV16 infections in the metaplastic SSE. As discussed in Section 6.1, the immature metaplastic SSE may have a relaxed epithelial homeostasis control, resulting from progressive readjustment of the expression of the genes that are required for metaplastic transdifferentiation. Relaxed homeostasis may render the cells more susceptible to signals that stimulate cells to replicate. This notion is consistent with the observation that replication of cellular DNA occurs not only in the basal cells, but also occurs sporadically in the upper layer cells of metaplastic SSE (Schurch *et al.*, 1978; Smedts *et al.*, 1993a). This is in strong contrast to the native SSE, in which DNA replication occurs only in the basal layer. Thus, HPV16 infection in metaplastic SSE, in which E6-E7 was extensively expressed, is potentially able to promote a wider spectrum of cells into cycling, including those that have left the basal membrane. Furthermore, since the metaplastic lesion may not have established the passive gene repression mechanism intrinsically functional in native SSE, mutant cellular genes could be continuously expressed in the upper layer metaplastic cells. Therefore, the upper layer cells of the infected metaplastic SSE proliferate

as actively and dyscoordinately as the bottom layer cells, allowing continuous accumulation of cellular genomic mutations. This condition could persist until the metaplastic transdifferentiation progresses to maturity. In mature metaplasia, the epithelial homeostasis becomes stably controlled, and the differentiation-associated passive gene repression mechanism is established. As a result, cell cycle arrest in the upper layer improves, and cellular genomic mutations accumulated during immature metaplasia are effectively nullified by the restricted gene expression in upper layer cells. The combination of these changes reduces the rate of mutation accumulation and breaks the aggregating cycles of mutation accumulation.

6.3.2 Increased viral DNA integration as a result of extensive and incoordinate DNA amplification

As discussed in Section 6.2.2, the relaxed homeostasis control and extensive viral oncogene expression may lead to the active incoordinate cellular DNA replication in metaplastic SSE. Because DNA recombination involves DNA strand separation (Lewin, 1994; Alberts, 1994) and strand separation is also one intermediate step required for DNA replication, persistently activated DNA replication driven by viral oncoproteins in metaplastic SSE may provide more DNA in the configuration required for HPV DNA integration. The current study showed that TNI in immature metaplasia was associated with incoordinate viral DNA amplification, as indicated by the dissociation between amplification of viral DNA and expression of the viral structure genes. The dissociation between these two events could aggregate the susceptibility of metaplastic SSE to viral DNA integration. It can be reasoned

that amplification of viral DNA without accompanying expression of viral structural proteins probably leaves a high level of viral DNA without the protection of viral capsomeres. The high dosage of exposed viral DNA in cells undergoing active, incoordinate, cellular DNA replication may impose a higher risk to the host cells for viral DNA integration. Furthermore, the current study showed that each *de novo* infection of HPV16 in metaplastic SSE may have to transverse a TNI window. Therefore, each episode of HPV16 infection in metaplastic SSE poses as high-risk for viral DNA integration.

In contrast, a different scenario occurs for HPV16 infections in native SSE, since squamous differentiation in native SSE is intrinsic, rather than acquired. In the early stage of HPV16 infection in native SSE, the intact homeostasis control and restricted viral oncogene expression should disfavour not only the accumulation of cellular mutations, as discussed in section 6.3.1, but also the integration of viral DNA. Therefore in native SSE, TNI with aberrant viral DNA amplification should occur less frequently, or occur only to the established HPV16 infections as a result of, or secondarily to, grossly altered squamous differentiation. This is consistent with the finding of the current study that no aberrant type of TNI was found in the VINs. Because squamous differentiation is stable in native SSE, this hypothesis implies that HPV16 infection alone may not be sufficient to sabotage the differentiation program, and thus cause HPV DNA integration and malignant progression in the native SSE lesions. Logically, other factors should be required to promote HPV-mediated oncogenesis in native SSE. Alternatively, other oncogenic mechanism(s) independent of HPV infection may be involved. These possibilities would explain not only why SSC occurs much less frequently in native SSE than metaplastic SSE, but also why most cervical carcinomas contain HPV

genome, in comparison of only 30 percent in vulval carcinomas (zur Hausen and de Villiers, 1994).

6.4 Factors modulating metaplasia as cofactors for HPV16-mediated cervical oncogenesis

HPV16 infection alone has been suggested to be insufficient for oncogenesis. This prediction is based on the finding that HPV-mediated oncogenesis has a long incubation time (Ponten *et al.*, 1995). The suspected cofactors involved in H

retinoids was found to induce squamous metaplasia through an unknown mechanism. Expression of retinoid receptors in the cervical epithelia is influenced by the estrus cycles of experimental animals. Interestingly, retinoid has been shown to modulate HPV16 expression in the *in vitro* culture of HPV-immortalized cells (Bartsch *et al.*, 1992; Pirisi *et al.*, 1992; Khan *et al.*, 1993a; Merrick *et al.*, 1993; Agarwal *et al.*, 1994; Creek *et al.*, 1994; Shindoh *et al.*, 1995). Since no RA responsive elements have been reported in the HPV16 genome, the regulation of HPV16 expression by retinoic acid may be indirect, via modulating the status of cell differentiation.

Another factor shown to modulate metaplasia is the estrogen steroid hormone (Nonogaki *et al.*, 1990). Similar to retinoids, estrogen functions by binding to specific cytoplasmic receptors, which bind specific DNA sites to trans-regulate gene expression. Estrogen has been known specifically to induce metaplasia in experimental animals (Firmin-Gomes-Teixeira and Anthonioz, 1989). Unlike retinoids and glucocorticoids, estrogen has not been shown to modulate HPV16 gene expression, except in SiHa cells, a cancer cell line containing integrated HPV16 DNA (Mitrani-Rosenbaum *et al.*, 1989). There is no well-established responsive element for the estrogen receptor in HPV16 genome (Mitrani-Rosenbaum *et al.*, 1989). Nevertheless, estrogen has been shown very recently to contribute to cervical carcinogenesis in a transgenic mice model (Arbeit *et al.*, 1993; Arbeit *et al.*, 1994; Arbeit *et al.*, 1995). The transgenic mice carried the HPV16 oncogenes under the control of the CK14 promoter. Low dose estrogen stimulation induced the expression of the HPV16 transgenes in the cervix, and cervical carcinomas occurred in the estrogen-treated transgenic mice. These results can be interpreted to indicate that the transdifferentiation of

the estrogen-induced metaplasia allows the expression of the HPV16 oncogenes, which then promotes transformation of the cervical cells. This is based on the fact that the CK14 promoter is not directly responsive to estrogen (Arbeit *et al.*, 1995), and the enhanced expression of the transgene may occur as a result of a globally relaxed control for gene expression during the process of estrogen-induced metaplasia.

6.5 A comprehensive hypothesis on HPV16-mediated cervical oncogenesis

The results from the current study allowed the formulation of a hypothesis for HPV16-mediated cervical oncogenesis, from the perspective of the histogenesis of metaplasia and HPV16 infection. This hypothesis differs from those by others (Shah and Howley, 1990; Howley, 1991; zur Hausen, 1994) in that it recognizes the differential HPV16 activities in native and metaplastic SSE. It emphasizes that a unique stage during HPV16 establishing vegetative infection in metaplastic SSE may impose a high-risk for HPV16-mediated oncogenesis.

6.5.1 HPV16 infection and oncogenesis in metaplastic SSE

The hypothetical natural history of HPV16 infection in metaplastic SSE, in relation to oncogenesis is summarized in Figure 6.1 (refer also to Figure 1.4). Infection of HPV16 in metaplastic SSE may be initiated by virus entry into cells in SCE, as suggested by the ubiquitous existence of HPV receptor(s) on the surface of different cell types (Muller *et al.*, 1995). Virus entry in SCE is not necessarily followed by expression of any viral genes, as a result of incompatible cellular environment in SCE. Synchronous replication with the cellular

Figure 6.1 Schematic presentation of a comprehensive hypothesis on the natural history of HPV16 infection and HPV16-mediated oncogenesis in cervical metaplastic SSE

The states of HPV16 infection in metaplastic SSE are dependent on the status of metaplastic transdifferentiation.

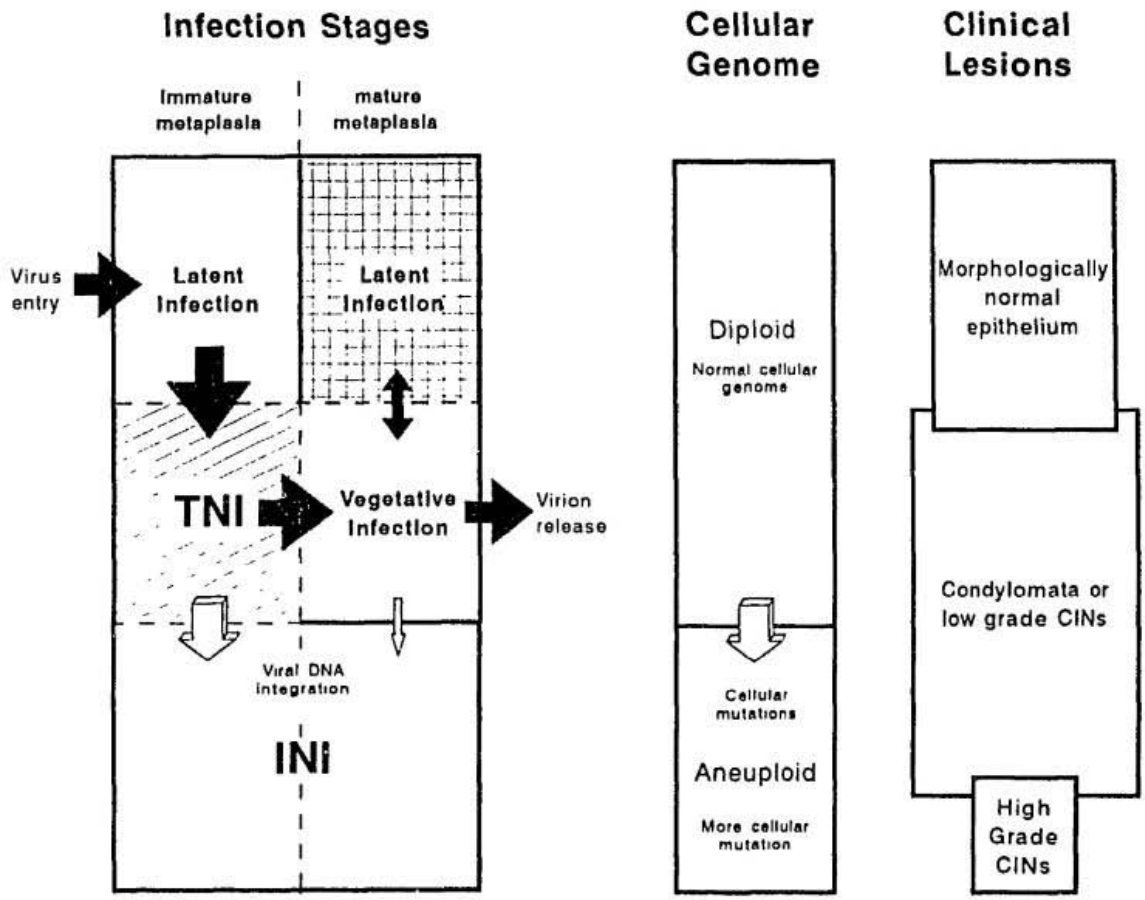
Virus entry in immature metaplastic SSE, and possibly SCE, is followed by a latent infection (Stippled square). Although virus infection may also be initiated at any stage during the process of metaplasia, early stages of metaplasia and SCE may provide an accessible gateway for virus entry.

As transdifferentiation proceeds, HPV16 infection becomes TNI (Striped square). Cells with HPV16 TNI are prone to viral DNA integration and cellular genomic mutations.

Vegetative infection (Open square) may derive from a TNI as a result of mature metaplastic transdifferentiation. Vegetative infection has a reduced risk for viral DNA integration and cellular genomic mutations, compared with TNI.

Vegetative infection may regress to, and develop from, a latent infection in mature metaplasia (checked area).

HPV16 infection and oncogenesis in native SSE are thought to be similar to that in the mature metaplasia (refer to Figure 1.4).


Infection Stages
Cellular Genome
Clinical Lesions
Immature metaplasia
mature metaplasia
Virus entry
Latent Infection
Latent Infection
TNI
Vegetative Infection
Virion release
Viral DNA integration
INI
Diploid
Normal cellular genome
Cellular mutations
Aneuploid
More cellular mutation
Morphologically normal epithelium
Condylomata or low grade CINs
High Grade CINs

DNA ensures that viral genomes persist in the SCE cells. This situation may represent one form of primary latent infection (Burghardt, 1986; Gupta *et al.*, 1989). Alternatively, HPV16 infection in metaplastic SSE may be initiated by virus entry into cells of early stage metaplasia. In this stage of immature metaplasia, the proliferating basal cells are protected only by a single layer of overlying dystrophic columnar cells, and may easily become exposed to the surface (Fu and Reagan, 1989b; Jensen *et al.*, 1992).

Metaplastic transdifferentiation of the latently infected SCE or the *de novo* entry of HPV16 in the immature metaplasia may be followed by immediate expression of viral early genes, in contrast to the latent infection after virus entry in SCE. This is consistent with the finding of the current study that HPV16 activities were found only in the stratified epithelia, and never in the simple epithelium. At this stage, the viral program for vegetative infection cannot be completed and HPV16 infection is non-vegetative, because the squamous phenotype acquired by metaplastic SSE is still insufficient to support viral late gene expression. The deregulated cellular environment intrinsic to metaplastic transdifferentiation supports only abnormal viral activities, including sustained expression of the viral oncogenes, incoordinate amplification of viral DNA and failed expression of the late genes. These dysregulated viral activities, in conjunction with deregulated cellular activities, lead to an increased incidence of cellular genomic mutations and viral DNA integration. Although these aberrant TNIs in immature metaplasia impose a high oncogenic risk, the episode of such high risk windows is transient and thus results in limited cellular mutations. Therefore, the lesions may manifest themselves clinically as benign or low grade dysplastic lesions.

Vegetative infection is achieved as metaplastic transdifferentiation of a TNI progresses

toward mature metaplasia. With the progressive improvement of squamous differentiation, viral structural proteins are expressed and HPV16 infection becomes vegetative. As a result of an improved epithelial homeostasis control, incoordinate viral activities are corrected to various extents. Viral DNA amplification and late gene expression become associated with each other, and occur in appropriate epithelial compartments. However, due to an underdeveloped squamous differentiation or an inherent deficiency in the squamous differentiation program of metaplastic SSE, vegetative infection in metaplastic SSE may still feature a sustained expression of viral oncogenes. Nevertheless, an improved epithelial homeostasis control in more mature metaplasia renders the extensively expressed viral oncoproteins less effective to promote cell division than in immature metaplasia. Thus, mutation accumulation is retarded and the incidence of viral DNA integration is reduced in this stage. Clinically most lesions of vegetative infection may manifest as low grade dysplasia.

The prolonged virus infection in mature metaplasia may provoke responses from the immuno-surveillance system. Immuno-cytokines released by the immunological effector cells down-regulate the expression of viral oncogenes, containing the infection in a latent state. Theoretically, the secondary latent infection in mature metaplasia is fundamentally different from the primary latent infection occurring in immature metaplasia. The latter are due to the incompatible cellular environment in the infected tissue, while the former need not be.

HPV16 infections become irreversibly non-vegetative when viral DNA is integrated. The majority of the viral DNA integration events should occur at the stage of TNI, although it may occur less frequently during vegetative infections. Any factor that prolongs the course of metaplastic transdifferentiation, or the time length of the aberrant TNI, may contribute to

viral DNA integration. The outcome of HPV16 INIs is also dependent on the course of metaplastic differentiation. In the case in which the differentiation-associated gene repression mechanism has not been established and homeostasis control is still lax, a sustained expression of viral oncogenes would result in mutation accumulation at a high rate. The accumulative mutations may halt the development of the metaplastic transdifferentiation and directly affect the phenotype of the infected cell. These phenotypic changes may result in the failure of virus vegetative replication, loss of the coexisting episomal viral DNA, and expression of high grade pathological features.

Although INI clearly represents an increased neoplastic threat, its oncogenic potential is still restrained by several cellular functions. One function is related to the histogenetic nature of metaplasia. As transdifferentiation progresses to mature squamous differentiation, the epithelial homeostasis control and differentiation-associated gene repression mechanism become improvingly functional. Both cellular functions not only restrict the expression of the integrated viral oncogenes, but they also suppress the neoplastic effects of activated, or overexpressed, cellular protooncogenes resulting from HPV16 mutagenic functions. The combination of these effects may arrest the viral oncoprotein-driven cell replication and could prevent the accumulation of mutations, resulting in lesion regression. The sites of viral DNA integration in the cellular genome could be critical for the differentiation-associated suppression of the integrated viral oncogenes. Expression of the viral oncogenes would not be suppressed, if the viral oncogenes are integrated into a genomic locus that is not subject to the differentiation-associated repression. However, in consideration of a relatively small proportion of genes thought to be actively expressed in the later stages of squamous

differentiation, the differentiation-associated gene repression mechanism would repress most of the integrated foreign genes. Another potential cellular function that can resolve INI is the alternative p53-independent apoptosis (Donehower *et al.*, 1992; Clarke *et al.*, 1993; Haupt *et al.*, 1995). Activation of this function by irregular cell activities may eliminate potentially malignant growth. As an ultimate means of suppressing malignant proliferation, the M2 phase of senescence control is activated when cell division reaches a finite limitation. Senescence controls would permanently arrest the growth of the abnormal cells, providing another opportunity for the immune system to eliminate the transformed cells.

The transition between the infection stages of HPV16 infection in metaplastic SSE is also associated with the differentiation status of the infected metaplastic SSE. Since the gene expression program of HPV infection is dependent on squamous differentiation, the transitions between infection stages can be regarded as being subject or secondary to the progression of metaplastic transdifferentiation. Because cervical squamous metaplasia is unidirectionally progressive from SCE to SSE, the transition from a primary latent infection (in immature metaplasia) to aberrant TNI and to vegetative infection should also be unidirectional. Phrased differently, a vegetative infection in metaplastic SSE cannot revert to a TNI or a primary latent infection, although it may be inter-convertible with the secondary latent infection in mature metaplasia. This irreversible transition of infection stages predicts that the potentially high oncogenic TNI window in immature metaplasia can only develop from a primary latent infection, or from a *de novo* infection in immature metaplasia. Transition from a vegetative infection in mature metaplasia to the aberrant TNI would be rare if not possible. This hypothesis implies that medical interference should focus on blocking the

pathways leading to the aberrant TNI for reducing the risk of HPV-mediated oncogenesis in metaplastic SSE. This hypothesis also predicts that therapeutical reagents that prevent metaplasia and promote squamous differentiation may be effective for reducing the risk of HPV-mediated oncogenesis in metaplastic SSE.

6.5.2 HPV16 infection and oncogenesis in native SSE

The hypothesis that was proposed according to previous information for guiding the current study (Figure 1.4) remains valid for HPV16-mediated oncogenesis in native SSE. The following supplements the above picture with the new findings of the current study.

After virus entry at the basal cells exposed by micro-traumas, HPV16 initiates the program for vegetative replications immediately, since programmed squamous differentiation is an inherent nature of native SSE. The viral oncogenes are expressed in the bottom cells to promote cell proliferation, while viral DNA amplification and expression of viral structural genes are sequentially activated following upward cell movement and squamous differentiation. Due to the strict homeostasis control and effective differentiation-associated gene repression function, cell cycle arrest in the upper layer cells is stable, preventing the accumulation of mutations. The differentiation program leads to the terminal death of the upper layer cells, including those with mutations. Under unknown conditions and through unknown mechanisms, HPV16 vegetative infection may become TNI and INI with a low frequency. Different from TNIs in metaplastic SSE, TNIs in native SSE may occur secondarily to vegetative infections. In addition, both TNI and INI in native SSE impose a less severe neoplastic threat to the host, due to stable homeostasis control, dominant

differentiation-associated passive gene repression mechanism, and restricted viral oncogene expression, as also discussed in Section 6.5.1 for mature metaplastic SSE. Therefore, oncogenesis in native SSE may involve other etiological factors, either in cooperation with, or independent of, HPV infections. The potential factors cooperating with HPV for oncogenesis would be those affecting squamous differentiation and thus promote aberrant TNI in native SSE. This scenario may explain not only the low incidence of carcinomas in native SSE, but also the low proportion of HPV16-related malignancies in carcinomas from native SSE.

6.6 Conclusions

The current study used experimental model systems and examined clinical samples to establish the relationship between squamous metaplasia, HPV16 infection and cervical cancer. The results from the *in vivo* experimental systems showed that metaplastic SSE and HPV16-mediated squamous cell carcinomas were derived from the cervical SCE. Further, the artificial *in vivo* lesions from the HPV16-immortalized HEN display dysplastic changes more atypical than, and distinct from, those from the HPV16-immortalized keratinocytes originated from native SSE. Significantly, the HPV16 E7 oncogene was extensively expressed in the former, while expression of E7 was restricted in the latter. The results from the *in vitro* experimental system indicated that the extensive E7 expression in the former and restricted E7 expression in the latter were not dependent on *in vivo* cytckines, the residual immune functions in nude mice, or the control domains of the HPV16 LCR. By modulating the conditions for tissue reconstruction, the current study showed that a gene repression function in HKC may be

responsible for the tissue-specific restriction of E7 expression. My results also suggested that this function is dependent on squamous differentiation and mediated by DNA methylation, and down-regulates gene expression selectively but not specifically at a global level.

The results from this research program revealed that the naturally occurring HPV infections were also tissue-specific and differentiation-dependent. The expression of the viral E7 oncogene was extensive in metaplastic SSE, whereas it was restricted in native SSE. In addition, my results showed that, non-vegetative infections in immature metaplastic SSE differed from vegetative infections. The former featured distinct patterns of viral DNA amplification and viral E7 oncogene expression. This aberrant type of TNI may represent an inevitable step for productive HPV16 infection in metaplastic SSE. The finding from the studies on natural infection also indicated that the metaplastic cell can acquire the native SSE-inherent gene repression function when metaplastic differentiation is maturely completed.

Based on these results of the current study and in combination with the results of others, the transdifferentiation process required for metaplasia is hypothesized to be the central knot that ties HPV16-mediated oncogenesis to the metaplastic SSE. Metaplastic transdifferentiation may result in a deregulated cellular environment that leads to a relaxed epithelial homeostasis control. In addition, metaplastic SSE may also lack the differentiation-associated, native SSE-inherent gene repression function, although metaplastic SSE may acquire this function under physiological conditions. In the context of the above cellular and tissue environments, the viral activities could be dysregulated and be prone to induce cellular genomic mutations and viral DNA integration, the molecular basis for HPV16-mediated oncogenesis. Thus, HPV-mediated malignancy is attributable to the biochemical properties

of viral E6 and E7 oncoproteins, may be related to the infection route provided by a particular histological structure in the cervix and, as suggested by the current study, could be associated with a unique differentiation program of the metaplastic SSE. The transient, potentially oncogenic TNI window for HPV16 in immature metaplasia, as compared with the vegetative infection of HPV16 in native SSE, may constitute the foundation of the differential susceptibility of metaplastic SSE and native SSE to HPV16-mediated oncogenesis.

CHAPTER 7

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