MOLECULAR MECHANISMS OF GLUCOCORTICOID-DEPENDENT ONCOGENESIS AND EXPRESSION OF HUMAN PAPILLOMAVIRUS TYPE 16 DNA

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MOLECULAR MECHANISMS OF GLUCOCORTICOID-DEPENDENT ONCOGENESIS AND EXPRESSION OF HUMAN PAPILLOMAVIRUS TYPE 16 DNA

by

© Rakesh Mittal, M.D.

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ABSTRACT

Human papillomaviruses have been implicated as a causative agent in the etiology of many human cancers, especially cervical carcinomas. Our laboratory had previously shown that the presence of the steroid hormones, dexamethasone and progesterone, markedly enhances the transformation of primary rodent cells by HPV type 16 DNA in cooperation with the EJ-ras oncogene. This enhancement could have been direct, through a previously known glucocorticoid response element (GRE) located at nt position 7640 in the transcriptional regulatory region of the HPV 16 genome. Two additional GRE-like sequences were also found at nt positions 7385 and 7474. Alternatively, indirect mechanisms could be conceived through hormone-mediated expression of other cellular transcription factors which in turn modulate HPV gene expression. To address the role of the GRE located at nt position 7640, site-directed mutational analysis was performed. In transformation assays in cultured rodent cells and in transient CAT assays with the human cervical carcinoma cell line, HeLa, I found that loss-of-function mutations of this GRE retained the response to dexamethasone, indicating involvement of other factors. However, converting this GRE into the consensus sequence resulted in an increased frequency of transformation and a greatly increased expression in transient assays, indicating the role of this GRE also. Retention of a hormone response for the loss-of-function mutations led me to examine the role of the other two GRE-like sequences in transformation and gene expression. A series of single, double and triple mutants, containing different combinations of mutations in the three GREs, were tested in

transformation and transient gene expression assays. The results showed that all three elements are individually functional and are required to observe any hormone effect in both assays. The two newly identified GREs were further characterised using synthetic oligonucleotides. Both GRE sequences were studied for their ability to respond to dexamethasone in transient CAT assays. In addition, specific DNA-protein interactions were examined using several in vitro DNA-protein interaction assays. Results have shown that both GRE sequences respond to dexamethasone and bind specifically to a protein of 97 kDa, the molecular size of the native glucocorticoid receptor.

As the GRE at nt position 7640 is a composite GRE with an overlapping AP-1 motif, that interacts with the cellular c-jun and c-fos transcription factors, the role of these cellular oncogenes in glucocorticoid-dependent expression of HPV 16 genes was also examined. The results demonstrated that c-jun conferred a positive response of dexamethasone-induced expression of viral genes, whereas, presence of c-fos inhibited this response. Interestingly, the composite GRE was responsive to dexamethasone only in the presence of c-jun, indicating the special significance of the composite GRE in HPV gene regulation.

To examine if the GREs are functional in the principle host tissue for HPV 16 infection, primary human ectocervical cells were cultured and used to examine the effects of hormones on HPV 16 gene transcription. Viral RNA was examined using in situ hybridization, after transfecting either wild type or mutated HPV genomes into these cells. Viral transcription was observed for the wild type HPV genome only in

the presence of these hormones and could be blocked by RU486, an anti-progestin, in a dose-dependent fashion. Constructs with all single or double GRE mutations also responded to both hormones, whereas, a triple mutated construct, with all three GREs disrupted did not support any detectable induction of viral transcription. Thus, steroid hormones appear to be essential for episomal expression of HPV genes in primary human cervical cells. This result also emphasizes the role of hormones in early stages of HPV infection where the majority of HPV DNA is found episomally.

This thesis is dedicated to all the wonderful people I have known and will know.

Publications

1. Mittal R., Tsutsumi, K., Pater, A. and Pater M.M. (1993) Human papillomavirus type 16 gene expression in human cervical keratinocytes: Role of progesterone and glucocorticoids. Obstetrics and Gynaecology 81, 5-12.

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Abstracts

1. Mittal, R., Pater A., and Pater, M.M. A mutational analysis of the HPV 16 glucocorticoid response element (GRE) to study its role in glucocorticoid dependent transformation. 10th international papillomavirus workshop, Seattle, USA. (July 1991)

2. Mittal, R., Pater A. and Pater M.M. Three glucocorticoid responsive elements (GREs) are essential and sufficient for glucocorticoid mediated transformation by HPV type 16. 42nd annual meeting of the Canadian Society of Microbiologists, St. Johns, Newfoundland, Canada. (June 1992)

3. Mittal, R., Tsutsumi, K., Pater, A. and Pater, M.M. Steroid hormones are essential for expression of human papillomavirus type 16 DNA in primary human cervical keratinocytes. 11th international papillomavirus workshop, Edinburgh, Scotland. (Sept. 1992).

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α-MEM	=	Alpha modification of Eagle's medium
A	=	Adenine
AR	=	Androgen receptor
bp	=	Base pairs
BPV-1	=	Bovine papillomavirus type-1
BRK	=	Baby rat kidney
BRL	=	Bethesda Research Laboratories
BSA	=	Bovine serum albumin
C	=	Cytosine
CAT	=	Chloramphenicol acetyltransferase
cGRE	=	Composite glucocorticoid response element
CIN	=	Cervical intraepithelial neoplasia
CIP	=	Calf intestinal phosphatase
CK	=	Cytokeratin
CK II	=	Casein kinase II
cpm	=	Counts per minute
DMEM	=	Dulbecco's modification of Eagle's medium
DNA	=	Deoxyribonucleic acid
DNaseI	=	Deoxyribonuclease I
DTT	=	Dithiothreitol

E1A	=	Early region 1A
E1B	=	Early region 1B
E2-RE	=	E2-response element
EC	=	Embryonal carcinoma
EDTA	=	Ethylenediaminetetraacetic acid
ER	=	Estrogen receptor
ERE	=	Estrogen response element
FCS	=	Foetal calf serum
G	=	Guanine
GR	=	Glucocorticoid receptor
GRE	=	Glucocorticoid response element
HEPES	=	H-(2-hydroxyethyl)-1-piperazinuthanesulfonic acid
HPV	=	Human papillomavirus
Kb	=	Kilobase pair
KBM	=	Keratinocyte basal media
KDa	=	Kilodaltons
KGM	=	Keratinocyte growth media
LCR	=	Long control region
LTR	=	Long terminal repeat
MMTV	=	Mouse mammary tumor virus
MR	=	Mineralocorticoid receptor
NCR	=	Non-coding region

NEN	=	New England Nuclear
NF-1	=	Nuclear factor 1
NFA	=	Nuclear factor 1 associated factor
nGRE	=	Negative glucocorticoid response element
nt	=	Nucleotide
ONPG	=	0-Nitrophenyl β-D-Galactopyranoside
ORF	=	Open reading frame
PBS	=	Phosphate buffered saline
PMSF	=	Phenylmethylsulfonyl fluoride
PR	=	Progesterone receptor
PRE	=	Progesterone response element
PVF	=	Papillomavirus factor
RARE	=	Retinoic acid response element
RNA	=	Ribonucleic acid
rNTP	=	Ribonucleotide triphosphates
rpm	=	Revolutions per minute
SDS	=	Sodium dodecyl sulfate
SV40	=	Simian vacuolating virus 40
T	=	Thymine
T-antigen	=	Tumor antigen
TE	=	Tris-EDTA
TLC	=	Thin layer chromatography

TPA	=	12-0-tetradecanoylphorbol-13-acetate
UV	=	Ultraviolet
VDRE	=	Vitamin D3 response element

Chapter 1

HUMAN PAPILLOMAVIRUSES: TRANSCRIPTIONAL REGULATION AND ROLE OF STEROID HORMONES

In 1907, G. Ciuffo while studying the benign lesion "human verruca vulgaris" (common warts), found that extracts prepared from these warts were infective even after passing them through bacterial filters, thus ruling out any bacterial or protozoal etiology (Ciuffo, 1907, cited from Shah and Howley, 1990). It was realised that the lesions were caused by a newly recognized submicroscopic particle that later came to be known as a virus. These infectious agents were called "papilloma" viruses, because of the papillomatous structure of the lesion in microscopy. The first papillomavirus was discovered by Shope in 1933, who produced warts in the skin of either wild or domestic rabbits by inoculating them with wart extracts from wild cottontail rabbits (Shope, 1933). Since then, papillomaviruses have been isolated from a large number of species, including humans. Most of them were found associated with benign lesions of the skin and only in rabbits was a progression to carcinoma observed (Rous and Beard, 1935). Later, through the initial work of Herald zur Hausen (zur Hausen et al., 1974, zur Hausen, 1976, 1977a), it became evident that some papillomaviruses are linked to human cancers, especially, cancer of the cervix (reviewed in Shah and Howley, 1990). Since 1974 many details of these virus have been elucidated.

1.1 Characterisation and classification.

Papillomaviruses belong to the family papovaviridae. Virus particles are about 55 nm in diameter and have an icosahedral symmetry with 72 capsomers (the basic building blocks of the virus shell). All have a closed circular double-stranded DNA genome that is enclosed in an icosahedral virion. Unlike other human viruses, such as adenoviruses, it has not been possible to type the papillomaviruses by serological methods. No antisera to distinguish the isolates of human papillomavirus are currently available. As a result, the virus has been "typed" by liquid DNA hybridization under controlled conditions of stringency and different types are distinguished, based on the degree of conservation of their DNA sequences as measured by hybridization kinetics (Pfister, 1984). Therefore, characterisation of HPVs as distinct types is based essentially on the lack of homology between their DNAs. Accordingly, HPVs are considered as a different viral type, a subtype or a variant, depending on whether the homology is less than 50%, more than 50% or identical to another type with single nucleotide changes, respectively. Using such an analysis, more than 60 types of HPVs have been characterised (de Villiers, 1989), of which more than 20 are associated with lesions of the anogenital tract. These anogenital HPV types were further classified as either high risk (such as HPV types 16, 18, 31, 33, 35, 39, 52) or low risk (such as types 6 and 11), based on the likelihood of malignant progression of the lesions that they are associated with (de Villiers, 1989).

1.2 Genomic organization.

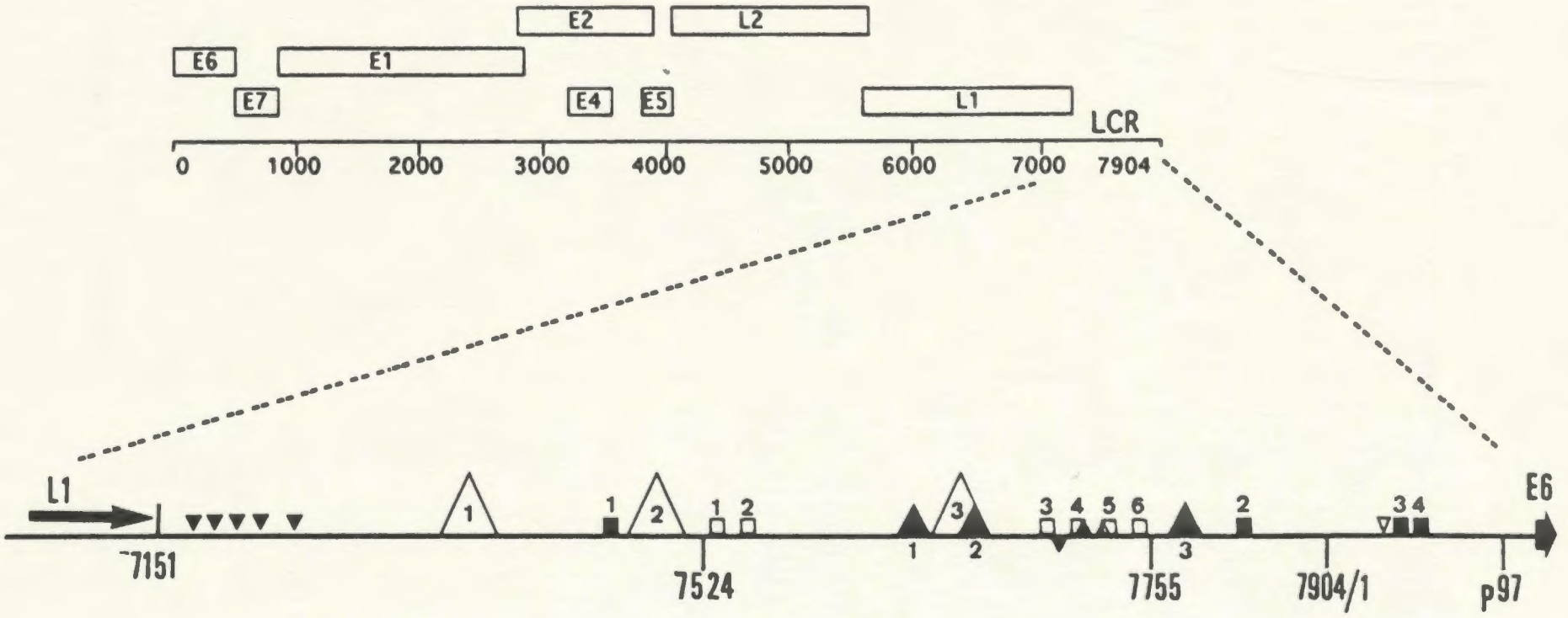
The advent of recombinant DNA technology has allowed cloning and sequencing of several human papillomaviruses. Their genomes are remarkably similar in size (about 8 kb) and show the same overall organization. All of the potential proteins synthesized by these viruses are encoded by only one of the two strands of the viral DNA. The other DNA strand is assumed to be non coding. The 8 kb genome is organized into three distinct regions and is based on the analogy with the bovine papillomavirus (fig. 1.1, reviewed in Giri and Danos, 1988).

1. An early region that encodes the viral proteins involved in viral DNA replication, transcription, and cellular transformation and are designated as E1, E2, E4, E5, E6 and E7.

2. A late region that encodes the viral capsid proteins, L1 and L2.

3. A non-coding DNA segment exists between the 5' end of the early region and the 3' end of the late region. This region is known by different names such as, long control region (LCR), non-coding region (NCR) or upstream regulatory region (URR) and contains many of the regulatory elements for transcription and replication. In this thesis I have used the term LCR for the regulatory region. Most ORFs are conserved and all are found at similar positions in all papillomaviruses on a single strand of DNA (reviewed in Broker and Botchan, 1986). As all the known genomes are organized similarly, it seems reasonable to extrapolate functional data from one virus to another.

Figure 1.1. Genomic organisation of human papillomavirus type 16 and its regulatory region. On top, the diagram of organization of the various early (E1, E2, E4, E5, E6, E7) and late (L1,L2) genes are shown as rectangular boxes. At the extreme right is a region, between the L1 ORF and E6 ORF, called the LCR (long control region). In the middle is an enlarged representation of the structural organization of the binding sites for various viral and cellular transcriptional factors that have been characterized and are listed on the bottom. This part of the figure is not to scale. The only characterized promoter for HPV transcription is shown as p97. The various symbols used for the different transcription factors is shown and number of binding sites for the same factors are in brackets.



△ = GLUCOCORTICOID RESPONSIVE ELEMENTS (3).

▲ = AP-1 BINDING MOTIFS (3).

▼ = PVF/TEF-II BINDING MOTIFS (6).

▲ = E-ELEMENT/TEF-I BINDING MOTIF (1).

▽ = SP-1 BINDING MOTIF (1).

△ = NF-1 ASSOCIATED FACTOR/NFA (1).

□ = NF-1 BINDING MOTIFS (6). #4 NF-1 IS CK ELEMENT.

■ = E2-RESPONSIVE ELEMENTS (4).

The best characterised papillomavirus is the bovine papillomavirus type 1 (BPV-1) and it has served as a prototype for studying the molecular biology of other papillomaviruses. However, in this brief introduction I will discuss mainly the human papillomaviruses and discuss BPV only where necessary. In the following sections, each of the regions of the genomic organization are briefly discussed.

1.2.1 Regulatory region

The long coding region (LCR) contains the regulatory region and contains an origin of DNA replication, a promoter for mRNA synthesis, transcriptional enhancer sequences and the 5' exons for mRNAs (reviewed in Lazo, 1988). Its size varies from 454 (HPV-8) to 979 (HPV-1) nucleotides and its sequence is much more variable than that of the viral ORFs.

The 5' extremity includes a domain which is GT-rich in genital papillomaviruses and AT-rich in the other viruses and probably includes an origin of replication (Waldeck et al., 1984). The 3'end of LCR is the most conserved region and contains 2 repeats of an ACC(N6)GGT palindromic sequence and promoter elements for RNA polymerase II (TATA and CAAT boxes). Located between these two regions of the LCR are the binding sites for several cell-specific factors and ubiquitous transcription factors (discussed later in section 1.5). Differences in the organization of some of these elements have been correlated with changes in virulence and oncogenic potential of some papillomaviruses (Rando et al., 1986). In addition, the LCR appears to be one of the major determining factors for

immortalisation and/or transformation of primary human (Romanczuk et al., 1991) and rodent cells (Rosen and Auborn, 1991).

1.2.2. E6 and E7 oncoproteins

Several lines of investigation have conclusively shown that E6 and E7 are the two major transforming oncogenes of human papillomaviruses (reviewed in Howley et al., 1991 and Vousden, 1990; discussed in detail in chapter 2). Here I will discuss only the structure-function analysis of the two oncoproteins and the relevance to their observed biological properties.

E6 is an unphosphorylated protein present in the nuclear and non nuclear membrane fractions of the cell (Grossman et al., 1989). One prominent feature of the protein is the presence of four repeats of the Cys-X-X-Cys motif, found in several nucleic acid binding transcription factors. Such repeats are involved in tetrahedral coordination of zinc and forms the so called zinc fingers (reviewed in Evans and Hollenberg, 1988). E6 protein, like other zinc finger proteins, was shown to be competent for zinc binding (Grossman and Laimins, 1989; Barbosa et al., 1989), DNA binding (Grossman et al., 1989; Imai et al., 1989; Mallon et al., 1987) and transcriptional activation (Desaintes et al., 1992; Lamberti et al., 1990; Gius et al., 1988). Recent studies have revealed that HPV 16 and 18 E6 protein, like SV40 large T antigen (LT) and adenovirus E1B, can interact with the cellular p53 tumor suppressor protein (Werness et al., 1990). However, unlike the LT and E1B, E6 binds to and promotes the degradation of the wild type p53 protein (Scheffner et al.,

1990) and also the mutant form of p53 (Scheffner et al., 1992). This degradation occurs via the ubiquitin-dependent pathway (Scheffner et al., 1990) and is mediated through a 100 kDa cellular protein, called E6 associated protein (Huibregtse et al, 1991, 1993). In addition, E6 of both the high and low risk HPV types are capable of interacting with p53, but degradation occurs only by the high risk types (Crook et al., 1991c). To date, no mutational analysis data is available to confirm the importance of p53 binding for the oncogenic properties of the E6 oncoprotein.

HPV E7 is a phosphoprotein (Seedorf et al., 1985; Smotkin and Wettstein, 1986, 1987). Although E7 was initially detected in the cytoplasm (Smotkin and Wettstein, 1987), it was subsequently found to be localized in the nucleus in association with the nuclear matrix (Sato et al., 1989a; Greenfield et al., 1991). Sequence and functional analysis of the protein indicated that it is similar to the other DNA tumor virus oncoproteins, SV40 LT and adenovirus E1A (Phelps et al., 1988, 1992b; Vousden and Jat, 1989). E7 of the high risk and low risk HPV types bind to the pRB tumor suppressor, but with distinct affinities (Dyson et al., 1989; Munger et al., 1989b; Barbosa et al., 1991; Gage et al., 1990; Heck et al., 1992). Thus, the affinity with which E7 binds to pRB was correlated with the in vitro transformation potential of the respective virus types. Like SV40 large T and E1A, E7 also binds preferentially to only the underphosphorylated form of pRB (Imai et al., 1991; Dyson et al., 1992) that negatively regulates cellular proliferation (reviewed in Green, 1989). Recently, E7 has also been shown to interact with other cellular growth regulators, such as p107, p130 and cyclin A (Dyson et al., 1992; Tommasino et al., 1993). In the

region towards the carboxyl terminus of the pRB binding domain are two serine residues which form part of a consensus sequence phosphorylated by casein kinase (CK) II (Firzlaff et al., 1991; Barbosa et al, 1990). The carboxy-terminal half of E7, like that of E6, contains two repeats of the Cys-X-X-Cys motifs (Watanabe et al., 1992) and is tetrahedrally coordinated by zinc (Barbosa et al., 1989). E7 induces cellular DNA synthesis, apparently through the region which binds pRB (Sato et al., 1989b; Banks et al., 1990a, 1990b; Rawls et al., 1990). The transforming activity and probably the normal function of the protein is related to its ability to bind pRB (Edmonds and Vousden, 1989, Chesters et al., 1990., Watanabe et al., 1990., Jewers et al., 1992, Phelps et al., 1992b). In addition, it has been shown that the amino-terminal region of E7 is responsible for the transforming activity of the high risk HPV types (Munger et al., 1991; Pater et al., 1992; Takami et al., 1992). Similarly, dominant mutations in this region of low risk HPVs can render their E7 transformation competent (Munger et al., 1991; Sang and Barbosa, 1992b), which is probably related to the ability of this region to interact with pRB. Another property of E7, shared by E1A, is its transactivating activity (Phelps et al., 1988, 1992a; Storey et al., 1990b; Munger et al., 1991). Extensive mutational analysis of E7 has revealed the following

1. pRB binding is necessary (Edmonds and Vousden, 1989; Sang and Barbosa, 1992; Phelps et al., 1992b)) but not sufficient (Jewers et al., 1992) for transformation.

2. CK II phosphorylation might contribute to the transforming activity. Whereas, CKII phosphorylation didn't appear to be important for transformation of primary cells (Chesters et al., 1990; Storey et al, 1990), transformation of established lines was

severely affected (Barbosa et al., 1990).

3. Mutations in all three regions affected the transactivating activity (Edmonds and Vousden, 1989; Storey et al., 1990). However, contradicting results have also been presented (Phelps et al., 1992b).

4. The Cys-X-X-Cys motifs are important for both transformation and transactivation (Edmonds and vousden, 1989; Storey et al., 1990; Chesters et al., 1990; Jewers et al., 1992; McIntyre, et al., 1993).

1.2.3. E1 ORF and protein

The functional role of this protein is mainly derived from studies on bovine papillomaviruses (Groff and Lancaster, 1986; Ustav and Stenlund, 1991), and more recently, from studies on HPV types 11, 16 and 18 where it has been shown to play an important role in replication of the virus (Chiang et al., 1992a, 1992b; del Vicchio et al., 1992; Remm et al., 1992). The E1 protein is a nuclear phosphoprotein, binds ATP and non-specifically interacts with DNA (Sun et al., 1990; Santucci et al., 1990; Blitz and Laimins, 1991; Lusky and Fontane, 1991; Bream et al., 1993). ATP binding has been shown to be important for its biological activity. More recently, specific DNA binding of E1 to the viral origin of replication has been demonstrated (Wilson and Ludes-Meyer, 1991; Ustav et al., 1991; Yang et al., 1991b; Remm et al., 1992; Bream et al., 1993). The E1 protein also interacts with the papillomavirus E2 protein which is thought to be involved in facilitating the binding and targeting of the E1 protein to the origin of replication (Mohr et al., 1990; Blitz and Laimins, 1991; Yang

et al., 1991b; Lusky and Fontane, 1991; Bream et al., 1993). A role of the E1 protein in immortalization of primary human keratinocytes by HPV 16 DNA has recently been demonstrated by Romanczuck and Howley (1992) and could be due to its function in HPV replication.

1.2.4. E2 ORF and transregulatory protein

This coding region is apparently composed of three domains, the highly conserved amino-terminus and carboxy-terminus domains with a variable section overlapping with the E4 ORF (Fig. 1.1). The C-terminal region possesses a sequence specific DNA binding domain whereas the N-terminus contains a transcriptional activation domain (reviewed in Ham et al., 1991). E2 ORF encodes a trans acting factor which modulates viral gene expression from early region promoters through a specific cis acting sequence in the LCR, called the E2-response element (E2-RE) (Androphy et al., 1987; Li et al., 1989). At least four such elements are found in the LCRs of all the papillomaviruses sequenced so far. The full length E2 protein functions as a transcriptional activator (Spalholz et al., 1985; Phelps and Howley, 1987; Hirochika et al., 1987, 1988; Giri and Yaniv, 1988) or repressor (Chin et al, 1988; Bernard et al., 1989; Romanczuk et al., 1990), whereas, one or two alternatively spiced proteins containing the carboxy-terminal domain of the E2 ORF are transcriptional repressors (Lambert et al., 1987; Cripe et al., 1987; Chin et al., 1988; Chiang et al., 1991). The importance of this dual regulatory mechanism is discussed later in this chapter in section 1.5.1. Recently, another important role of E2 in HPV DNA replication has been demonstrated (Chiang et al., 1992a, 1992b; del Vicchio et al., 1992; Remm et

al., 1992). The amino-terminal region of the E2 protein is required for HPV DNA Replication and was dependent on the presence of E2-REs (Chiang et al., 1992b). In addition, replication by E2 protein was significantly enhanced in the presence of the E1 gene product (Remm et al., 1992). The alternatively spliced forms of E2 that contains only the carboxy-terminal region were found to be repressors of HPV replication (Chiang et al., 1992b). Thus, the alternatively spliced forms of E2 that function as transcriptional repressors are also repressors of replication. The molecular mechanisms of E2-dependent replication is thought to involve binding of E2 near the origin of replication, as a result of which changes occur in the local chromatin structure (Moskaluk and Bastia, 1988). The E2 protein was also shown to possess DNA bending properties that have been observed in other regulatory proteins involved in DNA replication and transcription (Bedrosian and Bastia, 1990).

1.2.5 E4 ORF and protein

This ORF overlaps E2 in all the papillomavirus genomes sequenced so far. It is an acidic phosphorylated protein and found in the form of several species with distinct cellular localizations (Grand et al., 1989; Rogel-Gaillard et al., 1992) . It constitutes about 20% of the total protein of HPV-1 infected warts and is principally found in the cytoplasmic compartment of the cell, in association with large inclusion bodies (Doorbar et al., 1986; Breitburd et al., 1987; Neary et al., 1987; Doorbar et al., 1991). It is speculated that E4 may have a role in either virion assembly or maturation (Doorbar et al., 1986, 1991). Recently, HPV 16 E4 gene product has been shown to

induce collapse of the cytokeratin matrix (Doorbar et al., 1991). However, no confirmed role of E4 has yet been deduced.

1.2.6. E5 ORF and protein

This small ORF, encoding a protein of about 7 kDa, has been described in some, but not all, papillomaviruses. It is localized in the variable region of the genome between or overlapping the E2 and L2 ORFs (fig. 1.1) and encodes a very hydrophobic protein (reviewed in Banks and Matlashewski, 1993). Attention has been focused on this ORF because it is one of the two transforming genes of BPV-1. Recent studies have implicated a role of HPV E5 in transformation and is discussed in a greater detail in chapter 2.

1.2.7. L1 and L2 ORFs and structural proteins

L1 ORF is highly conserved among the papillomaviruses and encodes the major capsid protein. The L1 protein has a molecular weight of 54-kDa and forms the 72 pentameric capsomers of the virion shell (reviewed in Broker, 1987)

L2 ORF encodes minor capsid protein of about 76 kDa that is less conserved and quite variable among HPVs. The localisation of L2 in the virion shell and its role in capsid assembly is not known. Both L1 and L2 are required for the formation of viral particles (Zhou et al., 1991). Nevertheless, in another report, overexpresssion of L1 alone is sufficient for particle formation and it is suggested that L2 might have a role in facilitating L1 in capsid assembly (Kirnbauer et al., 1992; Rose et al., 1993).

Neither of these two proteins are expressed in oncogenically transformed cells.

1.3 HPV life cycle

A major difficulty in studying HPVs has been the inability to cultivate a productive in vitro host cell culture system. Viral DNA replication, capsid protein synthesis and assembly of progeny virions takes place in the superficial highly differentiated keratinocytes (Stoler et al., 1989; Durst et al., 1992). Expression of HPV genes is related to the differentiated state of the cell (Stoler et al., 1989; Beyer-Finkler et al., 1990; Iftner et al., 1992), with low levels of viral transcription and replication in undifferentiated basal and parabasal cells. It is presumed that viral infection occurs in the basal epithelial cells, probably due to mechanical wounding. Recombinant DNA technology has made it possible to gain some insights into the regulation of viral gene expression and cellular factors involved in this process (Bernard, 1990). However, the controls that regulate late gene expression and productive infection remain largely unknown. There have been numerous attempts to reproduce the differentiation program of normal epithelial cells to allow productive infection. Initial studies done by Kreider et al (1987, 1990) have described a system in which HPV types 11 and 1 could be propagated in human tissue xenografts implanted in nude mice. More recently, two studies have utilized a modified in vitro collagen raft culture system (organotypic culture) to successfully demonstrate production of HPV 11 and 31 virions (Dollard et al., 1992; Meyers et al., 1992). Another study, using a

different approach and the W12 cell line containing episomal forms of HPV 16 DNA, has shown virion production (Sterling et al., 1990). These studies can be useful to examine the role of the co-factors, such as hormones, that are necessary for productive infection. However, the amount of virus recovered would not be sufficient to allow genetic analysis of the virus and/or mutant forms of the virus. Another problem is that the virus particles formed could not be used to infect cells in culture.

1.4 RNA transcription

Transcription initiates in most cases from one major promoter located just immediately upstream to the E6 ORF along a single strand of DNA and results in the production of complex and many different overlapping mRNAs (Rotenberg et al., 1989a, 1989b; Smotkin et al., 1989; Doorbar et al., 1990; Palermo-Dilts et al., 1990; Rohlfs et al., 1991; Sherman et al., 1992). An important difference between the high risk viruses (such as types 16, 18, 31 and 33) and the low risk viruses (such as types 6 and 11) is the presence of additional splice sites within transcripts spanning the E6 ORF that leads to the formation of truncated forms of the E6 protein, called E6*I and E6*II (Schneider-Gaticke and Schwarz., 1986; Smotkin and Wettstein., 1986; Doorbar et al., 1990; Rohlfs et al., 1991). The E6*I mRNA may allow a more efficient translation of the E7 ORF, as the E7 ORF would be more 5' on the mRNA for efficient translation initiation (Sedman et al., 1991). This difference in

production of truncated forms of E6 mRNA has been attributed to a alternate splicing within the E6 coding region, that is active only in the high risk HPV types and not in the high risk types (Smotkin et al., 1989). Another difference in viral gene transcription between the two HPV types is the presence in the low risk of E2 encoding mRNAs. In cancers, the E1 and E2 ORFs are usually disrupted due to integration of the viral genome into the host DNA, resulting in the abrogation of E2 mRNA synthesis (Baker et al., 1987; Le and Defendi, 1988; Matsukara et al., 1986). It has been suggested that altered expression of viral genes leads to progression of cervical lesions to increasing dysplasia (Shirasawa et al., 1988; Durst et al., 1992; Romanczuk and Howley, 1992; Stoler et al., 1992). Differences in the distribution of viral mRNAs was thereby correlated with the severity of the disease.

1.5 Regulation of HPV type 16 gene expression.

An important level in the regulation of expression of cellular and viral genes is of transcriptional initiation. In most cases, this is determined by the type of cis acting elements and their organization in a given promoter. Virus or host encoded trans acting factors stimulate transcription of these promoters. Thus, regulation of transcription is governed by the combined actions of various sequence specific DNA binding proteins (for reviews see: Dynan and Tjian, 1985; Ptashne, 1988; and Mitchell and Tjian, 1989). Some of these sequence-specific motifs are located proximal to the transcriptional start site and are generally ubiquitously expressed. By contrast, other site-specific DNA binding proteins, which regulate spacial and temporal patterns of

gene expression, bind to regulatory elements either at remote positions (enhancers) or dispersed between the sites for the ubiquitous promoter elements (Muller and Schaffner, 1990). Viruses, in particular DNA tumor viruses (for example SV40 and adenovirus), have served as useful models to study transcriptional regulation. Enhancers were first described in studies done with SV40 tumor virus (Banerji et al., 1981). Since my study involves transcriptional regulation of HPV type 16 DNA by steroid hormones, I will briefly review the literature on regulation of human papillomavirus gene expression and regulation of transcription by steroid hormones.

As mentioned earlier, one striking feature of all human papillomaviruses is their preferential tropism for epithelial tissues. HPVs are strictly epitheliotropic viruses and are dependent on differentiating keratinocytes for their replication. These viruses require factors that can only be provided by a differentiating cell of the permissive tissue. Hence, HPVs have been a good model to study epitheliotropism. The oncogenic nature of these viruses has provided a further reason to understand the mechanisms of viral gene expression, viral and cellular host factors which regulate programmed expression of the early and late genes and the genetic elements that are necessary to direct viral gene expression. In recent years, it has become increasingly clear that tissue tropism of many viruses is mediated through the complex interplay of viral and cellular host factors and is mediated through the regulatory regions of these viruses (reviewed in McKnight and Tjian, 1986). Thus the enhancer and promoter of the virus may be active in a particular cell type that contains an

appropriate supply of specific transcription factors (Mosthaff et al., 1985; Schirm et al., 1987; Thiesen et al., 1988; Dollard et al., 1993)).

All HPV genomes sequenced contain a non-coding region (LCR, or URR) located between the stop codon of L1 ORF and the first ATG codon of E6 ORF (fig. 1.1). Cloning these sequences upstream to enhancer-promoterless reporter genes have provided evidence that they contain sequences for both enhancer and promoter function (Marshall et al., 1989). Both, tissue-specific and constitutive enhancers, as well as, inducible enhancers have been found in many HPV types (Steinberg et al., 1989; Cripe et al., 1987; Gius et al., 1988; Hirochika et al., 1988; Gloss et al., 1987; Swift et al., 1987; Chin et al., 1989; Nakshatri et al., 1990). In this thesis I will stress more on the viral and cellular host factors responsible for HPV expression and will not delve too much into the structure of the LCR itself.

1.5.1 Role of virus-encoded gene products.

An enhancer sequence found in all papillomavirus types sequenced so far has been shown to be regulated in trans by the viral E2 ORF gene product and is important for early gene expression (reviewed in Ham et al., 1991). As discussed earlier, the action of the E2 gene product is pleotropic, affecting both transcription and replication. The E2 protein binds as a dimer (McBride et al., 1989) to a conserved palindromic motif, ACC(N6)GGT (Androphy et al., 1987; Hawley-Nelson et al., 1988). DNA binding is mediated by the carboxyl-terminal domain of the protein, whereas, the amino-terminal is responsible for transcriptional activation (Giri and

Yaniv, 1988). However, depending on the virus subtype studied and the context of its binding in the LCR, a given E2 gene product can be either a transcriptional activator (Thierry and Yaniv, 1987; Chin et al., 1988) or a repressor (Romanczuk et al., 1990). In genital HPVs, except HPV1a, the E2-binding motifs are arranged in a very characteristic manner. Two sites are situated very close to the ATG of the E6 ORF. A third site which is a perfect palindrome in HPV 6 and 11 and an imperfect one in HPV 16, 18 and 33 is situated about 100 nt further upstream. A fourth highly conserved E2 site is found about 400 nt further upstream (see fig. 1.1). The two sites near the promoter region of HPV 18 (Gius et al., 1988), HPV 16 (Phelps and Howley, 1987) and HPV 11 (Hirochika et al., 1988), do not appear to play a role in E2-dependent enhancer activity. However, recent evidence indicates that the full length E2 product actually represses HPV 16 and 18 transcription from these two E2 motifs in the context of their own promoter (Thierry and Yaniv, 1987; Bernard et al., 1989; Gloss and Bernard, 1990; Romanczuk et al., 1990; Dostani et al., 1991; Thierry and Howley, 1991; Sang and Barbosa, 1992a). It is hypothesised that this repression might be due to steric interference by E2 to the formation of a productive transcriptional initiation complex. Recently, Tan et al. (1993) have shown that one of the mechanisms of this repression is the displacement of another transcriptional activator, Sp1, from its binding site, which is one bp away from the E2 motif. Sp1 has been shown to be important for transcriptional stimulation of HPV 16 in epithelial cells (Gloss and Bernard, 1990). This arrangement of the Sp1 and E2 motifs is conservatively present in at least nine other genital HPVs, suggesting a

common regulatory mechanism (Tan et al., 1993). The E2 protein can also cooperate in a synergistic manner with other cellular factors (such as AP-1, glucocorticoid receptor and NF1), to activate transcription (reviewed in Ham et al., 1991; Gauthier et al., 1991; Monini et al., 1991). Transcriptional activation by E2 has also been attributed to its DNA-bending property (Bedrosian and Bastia, 1990).

The obvious question that arises is, "of what significance is E2-mediated repression for HPV-associated cancers?" The role of the other two upstream E2 motifs is also not clear. Indeed, the constitutive enhancers of HPV types 11, 16 and 18, that are cell specific and contain these E2 motifs, are also E2-independent (Cripe et al., 1987; Garcia-Carranca, 1988; Chin et al., 1989). One possible significance of this E2 repression comes from studies on the integration patterns of HPV DNA in cervical cancers and in vitro immortalized cells. In tumors and cervical carcinoma cell lines, integration of HPV DNA often disrupts the E1-E2 ORF (Baker, 1987; Durst et al., 1985; Schwarz et al., 1985; Choo et al., 1987). Negative regulation by E2 would be consistent with a model in which there is a derepressed regulation of HPV promoters after viral integration into the E2 ORF. This loss of E2 could then provide a selective growth advantage due to consequent deregulated expression of viral oncogenes and hence could represent an important factor in the progression of cervical lesions to a fully malignant condition (Sang and Barbosa, 1992a). Supporting this view are studies showing that mutations in either the E1 or E2 ORF increases the immortalizing efficiency of human keratinocytes by HPV 16 (Romanczuk and Howley, 1992). However, contradictory results have also been obtained which would

suggest otherwise (Lees et al., 1990; Storey et al., 1992). It is possible that both the transcriptional activating and repressing functions of E2 are stage-specific, depending on the differentiated state of the HPV-infected cell.

A possible role of another gene product, the E6 oncoprotein, in transcriptional regulation has been suggested for the HPV 16 and 18 LCRs (Gius et al., 1988; Lamberti et al., 1990; Sedman et al., 1991; Desaintes et al., 1992). Also, indirect evidence suggests a role of the E1 protein in transcriptional control (Romanczuk and Howley, 1992). Although, E6 protein can bind non-specifically to DNA and can also activate transcription from HPV and heterologous promoters, sufficient evidence is still lacking to implicate this direct mechanism in the controls of HPV gene regulation (Grossman et al., 1989; Imai et al., 1989; Lamberti et al., 1990; Desaintes et al., 1992).

1.5.2 Role of cellular factors in HPV transcription.

Although the E2 protein plays a key role in regulation of HPV gene expression, it is clear that numerous cellular factors are also intricately involved. One of the major questions to be addressed is the epithelial cell-specific nature of viral gene expression. Although no clear picture has yet emerged from many studies, the current data suggests that different HPVs have evolved distinct mechanisms to regulate their expression in the permissive host cell. The epitheliotropic nature of gene expression is partially brought about by the complex interaction of various cellular transcription factors binding to the viral enhancer-promoter region. So far, there is no single

factor that can be implicated in this cell-type specific expression. Binding sites for several nuclear proteins have been identified in the HPV 16 and 18 regulatory regions by mobilty shift, competition analysis and DNase footprinting (Garcia-Carranca et al., 1988; Gloss et al., 1989a, 1989b; Sibbet and Campo, 1990; Nakshatri et al., 1990). Among the known factors are the ubiquitously expressed NF1/CTF, oct-1, AP-1 and Sp1 transcription factors. Cis elements interacting with these factors have been found in the regulatory regions of HPV types 11, 16 and 18, distributed at different locations and in varying numbers in the LCR of these viruses. An important role of AP-1 in the tissue-specific enhancer activity of HPV 16 (Chan et al., 1990; Cripe et al., 1990) and HPV 18 regulatory regions has been suggested (Offord and Beard, 1990; Thierry et al., 1992). Binding of cellular jun-B factor to the AP-1 motifs in HPV 18 LCR has shown to be essential for enhancer function (Thierry et al., 1992). Another group has shown that a keratinocyte-specific transcriptional activator protein, KRF1, which binds specifically to the HPV 18 LCR, was found to act in concert with AP-1 to stimulate transcription (Mack and Laimins, 1991). In addition, oct-1 binds to a sequence overlapping this KRF1 motif, such that binding of either factor is mutually exclusive. This suggests that oct-1 may have a repressor function for HPV 18 gene expression and may partly be responsible for cell-type and differentiation-specific expression. Other studies have implicated the role of Sp1 factor as a major determinant of promoter activity for HPV 16 and 18 (Gloss and Bernard, 1990; Hoppe-Seyler and Butz, 1992, 1993).

Several nuclear factor 1 (NF1) binding sites are protected from DNase I in the

HPV 16 LCR and are also found in other sequenced HPVs (Gloss et al., 1989b; Nakshatri et al., 1990). In particular, an NF1 motif, located in at least six genital HPVs, is spaced exactly two bps away from another consensus element, termed NFA for nuclear factor associated factor (Chong et al., 1990). Both the NF1 and NFA motifs have been shown to be important for enhancer activity (Chong et al., 1991). In HPV 16 this NFA motif is similar to the octamer binding sites for oct-1 and oct-2 factors (Kemler et al., 1989). However, this sequence diverges in the low risk HPV types 6 and 11. Using mobilty shift and competetion analysis Chong et al. (1991) have shown that the HPV 16 NFA motif binds to oct-1 in addition to a novel factor, NFA. However, the HPV 11 motif exclusively binds to the NFA protein only. Similarly, Dent et al. (1991) have shown that the NFA motif binds to a novel octamer binding protein which is specifically expressed in cervical carcinoma cells and differs from the constitutively expressed oct-1. In contradiction to the study by Chong et al (1991), this same group (Morris et al., 1993a) has shown that the novel cervical tissue-specific octamer protein (presumably NFA and called cervical protein by this group), interacts only with the high risk HPV 16 and 18 NFA motifs and not with the corresponding sequence of HPV 6 and 11. Morris et al (1993a) have suggested that the constitutively expressed oct-1 behaves as a repressor of HPV 16 and 18 gene expression in non cervical cells by displacing the positively acting NF1 protein binding adjacent to the NFA motif. However, in the presence of the novel cervical protein (present only in cervical cells) and oct-1, the NFA motif acts positively. In light of the conserved arrangement of the NF1-NFA sequence and complex interactions of

at least three different factors at this site (oct-1, NFA and NF1), which appears to be different for HPV 16/18 and HPV 6/11, it is possible that the NFA motif may have an important role in tissue and species specificity and might explain some of the biological differences between these two virus groups. It is also possible that NFA/cervical protein is identical to an octamer factor studied by Royer et al. (1991), which shuttles between the nuclear and cytoplasmic compartments in a cell cycle-dependent manner. This could be a relevant instrument for HPV to couple its transcription to the cell cycle.

A keratinocyte-dependent enhancer element with a sequence, TTTGGCTT, was also found to be conserved in all genital HPV types (Cripe et al., 1987). An inverted form of this sequence is also found in the LCRs of several cytokeratin and involucrin genes (Blessing et al., 1987). It is tempting to speculate that this could be the epithelial cell-type specific element to explain epitheliotropism. However, further studies have found that this element binds a factor similar to the adenovirus NF1 site (Chong et al., 1991; Cripe et al., 1990) and only contributes to enhancer activity. Cell-type specificity was also attributed to another transcription factor called TEF-1. TEF-1 and its associated cell type specific co-activator was thought to activate HPV expression only in keratinocytes (Ishiji et al., 1992). Another factor called the papillomavirus enhancer-associated-factor (PVF), also was found to contribute to cell-type specificity (Chong et al., 1990). This PVF sequence was later shown to bind to the transcriptional enhancer factor, TEF-2 (Chong et al., 1991).

Another very important cis acting element discovered in several genital HPVs

are the steroid hormone receptor binding sites in the LCR, which confers hormone-induced expression of HPV genes (Chan et al., 1989; Gloss et al., 1987; Chong et al., 1990; Mittal et al., 1993a, 1993b). It is proposed that steroid hormones, progesterone and glucocorticoids, might play a very significant role in the HPV life cycle and HPV-mediated oncogenesis (Pater et al., 1988; zur Hausen, 1989a; Pater et al., 1990; Mittal et al., 1993a). Other physiological signals have also been shown to influence HPV gene expression. Examples include, EGF (Yasumoto et al., 1991), TGF-β (Woodworth et al., 1990b; Braun et al., 1990), retinoic acid (Pirisi et al., 1992; Bartsch et al., 1992; Khan et al., 1993a) and Leukoregulin and α and γ-interferon (Nawa et al., 1990; Woodworth et al., 1992b; Khan et al., 1993b). All of these factors repressed HPV gene expression in cell lines. Except for EGF, which seems to act through an EGF response element in the HPV 16 LCR, the mechanisms of action of the other factors are not known. Recently, it was demonstrated that a nuclear factor for interleukin 6 expression, called NF-IL6, binds to the HPV type 16 enhancer region and represses transcription (Kyo et al., 1993). The roles of any of these growth inhibitory factors in HPV life cycle is not clear.

In conclusion, results from these studies illustrate that ubiquitously expressed cellular factors might be the sole determinants for cell-type specific expression of HPVs. Some of these factors are associated with co-activators (Mack and Laimins, 1991; Ishiji et al., 1992; Chong et al., 1991), each binding with different relative affinities and/or post transcriptional modifications. Cell-type specific transcription can be brought about in two ways: First, by factors that are uniquely present in the cells

that supports promoter function. Secondly, due to induction by a factor that interacts with a DNA element that is normally occupied by a ubiquitous factor. A classic example would be the lymphoid B-cell specific oct-2 factor which binds to a site that normally is bound by the ubiquitous oct-1 factor (Kemler et al., 1991). For HPVs, it is more likely that neither of these two mechanisms exist. Instead, cell-type specificity might be due to a combination of synergistic and cooperative interactions between the ubiquitously expressed cellular factors that are expressed in varying amounts in particular cell types, and/or are differentially modified. This is supported by the observations that although HPV enhancer is inactive in fibroblasts, it binds to and gives identical footprints with extracts from different origins (Chong et al., 1991,; Cripe et al., 1990; Gloss et al., 1989a; Nakshatri et al., 1990). It is also possible that unknown co-activators may function differently in association with the same ubiquitous factors. This could explain the cell, tissue and species specific gene expression exhibited by different HPV types.

1.5.3 Role of silencers in HPV LCR and negative regulation by cellular factors.

In recent years, silencer elements have also been discovered in the HPV LCR. Transcriptional silencers, defined by Brand et al. (1985), are cis acting elements which operate at a distance in an orientation independent manner to suppress transcription. Such cis acting negative elements have been described in HPV 6 (Wu and Mounts, 1988), HPV 8 (Reh and Pfister, 1990) and HPV 18 LCRs (Bauknecht et al., 1992). The HPV 18 silencer was shown to interact with a previously characterised YY1

repressor protein (Shi et al., 1991) and was shown to downregulate both constitutive and phorbol ester-induced expression of HPV genes at the level of transcriptional initiation (Bauknecht et al., 1992). Interestingly, the YY1 core element is also present in the anogenital HPV types 11, 16, 31 and 33. However, the relevance of these silencers in HPV life cycle and their biological significance is not understood. Possibly, the presence of both silencers and activators allows these viruses to be regulated both positively and negatively, thus allowing a fine tuning of transcriptional control.

Another interesting control mechanism with some biological significance is the involvement of a putative suppressor gene on chromosome 11, which is found deleted in many cervical cancers (Atkin and Baker, 1988). Experimental studies have shown that chromosome 11 harbours a gene which suppresses expression of HPV 16 and 18 genes (Smits et al., 1990; Rosl et al., 1988). In addition, cell fusion and microinjection studies have shown that chromosome 11 is able to suppress tumorigenicity of HPV transformed cells (Rosl et al., 1991; Koi et al., 1989; Saxon et al., 1986). This was correlated with a concurrent decrease in the expression of HPV oncogenes. HPV 16 enhancer-promoter was also shown to be more active in fibroblasts deleted of chromosome 11 (del-11 cells) and could be easily transformed compared to normal diploid fibroblasts which were not permissive for HPV 16 gene expression (Smits et al., 1988 and 1990). The region responsible for this downregulation was delineated to nt 59-112 in the HPV 16 promoter (Smits et al., 1990). Additional studies provided evidence that deletion of chromosome 11 induces

or activates a cellular homolog of SV40 small t-antigen (Smits et al., 1992a). SV40 small t-antigen interacts with and modulates the activity of the protein phosphatase 2A subunit, PP2A, (Yang et al., 1991a). In del-11 cells, the steady state levels of protein phosphatase 2A subunit are increased (Smits et al 1992b), indicating that HPV promoter region could have been influenced by a PP2A activity present in del-11 cells. Recently, quantitative differences were observed for proteins binding the TATAAA box region of HPV 16 promoter in del-11 and diploid cell extracts (Smits et al., 1993). This led to the speculative assumption that either TATA binding proteins or a modification of factors binding this region are involved in the chromosome 11-mediated repression of HPV 16 genes. The likely involvement of chromosome 11 in HPV gene regulation supports the intracellular surveillance hypothesis (CIF theory) of zur Hausen (1991) for cervical carcinogenesis (discussed in chapter 2). Accordingly, it is postulated that a cellular interfering factor (CIF), putatively residing on chromosome 11, suppresses HPV expression in normal cells and it is the inactivation of the CIF genes that leads to unregulated increased expression of HPV oncogenes and development of cervical carcinomas. Although quite attractive, more research is required to substantiate the role of chromosome 11 in HPV gene expression.

1.6. Transcriptional regulation by steroid hormones

Steroid hormones are a group of naturally occuring compounds which act to

coordinate complex biological events involved in development, differentiation and also responses to a number of physiological stimuli (reviewed in Evans, 1988). Three classes of steroids have been identified based on their biological functions. These are the adrenal steroids (cortisol and aldosterone), sex steroids (estrogen, progesterone and testesterone) and calcitriol (vitamin D_3 metabolite). Through the initial work by Jensen and Gorski, it was realized that steroid hormones work through specific intracellular receptors (Jensen, 1968; Gorski, 1968). At present, these intracellular molecules comprise a superfamily of ligand-dependent transcription factors (reviewed in Fuller, 1991). Recombinant DNA technology has played an important role in understanding the molecular mechanisms of steroid hormone action and has allowed cloning and identification of the various hormone receptors and their DNA recognition sequences. A clear picture has emerged about how steroid hormones perform their functions. It is thought that the free pool of glucocorticoids, which are lipid soluble, enters the cells by diffusion (Furu et al., 1987) and bind with high affinity to specific cytoplasmic receptors called the glucocorticoid receptor (GR, Yamamoto and Alberts, 1976). Prior to hormone binding, the GR is found in an inactive state in a multiprotein complex that appears to contain a single molecule of GR associated with several heat shock proteins, including hsp 90, hsp 70, and hsp 56 (reviewed in Pratt et al., 1992a; and Smith and Toft, 1993). Upon hormone binding, hsp 90 dissociates from the GR followed by activation or transformation of the hormone-receptor complex. It is thought that the heat shock proteins may be involved in the proper folding and stabilization of the receptor molecule (Pratt et al.,

1992b), as well as, for high affinity steroid binding to the receptor protein (Bresnick et al., 1989; Ohara-Nemato et al., 1990). Following activation, the receptor is translocated into the nuclear compartment of the cell, attains a high affinty for DNA (Howard and Distilhorst, 1988) and dimerizes either prior to or concurrent with DNA binding (Tsai et al., 1988; Wrange et al., 1989).

1.6.1 Structural and functional analysis of the glucocorticoid receptor.

Molecular cloning and sequencing of cDNAs have greatly facilitated our understanding of ligand-dependent intracellular receptors for steroid hormones and have allowed us to group these receptors in one superfamily (reviewed in Evans, 1988). All of them consist of three major domains: a conserved DNA binding domain, joined to a carboxy-terminal hormone binding domain and a non conserved amino-terminal domain (Muller and Renkawitz, 1991). The exact locations and functions associated with these domains have mainly been derived from the study of the GR. I will also discuss other steroid hormone receptors only where necessary.

The DNA binding domain comprises the central region of the GR receptor molecule, is highly conserved and contains eight conserved cysteine residues which tetrahedrally coordinate two zinc atoms (Freedmann et al., 1988). This forms two zinc fingers important for the structural integrity and sequence-specific DNA-binding property of this domain (Hard et al., 1990; also reviewed in Schwabe and Rhodes, 1991). A transactivation function and a hormone-independent nuclear localization signal has also been identified in this region (Hollenberg and Evans, 1988; Picard and

Yamamoto, 1987; Guiochon-Mantel et al., 1989). Swapping experiments have provided evidence that the first zinc finger and few amino acids between the cysteines of the second zinc finger confer target gene specificity (reviewed in Freedman, 1992).

The hormone binding domain lies at the carboxy-terminal end of the GR and has a complex structural and functional organization. In addition to binding the ligand, this region also contains a dimerization signal which is similar to those for the estrogen (ER) and progesterone (PR) receptors (Kumar and Chambon, 1988; Guiochon-Mantel et al., 1989; Fawell et al., 1990). It also contains sequences for interaction with the hsp 90 (Denis et al., 1988; Pratt et al., 1988), a ligand-dependent nuclear localization signal for the GR and PR (Picard and Yamamoto, 1987; Guiochon-Mantel et al., 1989) and a transcriptional transactivation function which is common for the GR, ER and PR (Hollenberg and Evans, 1988; Webster et al., 1988; Dobson et al., 1989).

The N-terminal domain of the hormone receptor superfamily is the most highly variable region, both in length and amino acid sequence (reviewed in Beato, 1989). This region of the receptor consists of amino acids which are negatively charged and are important for the transcriptional transactivation function of the GR (Giguere et al., 1986), PR (Tora et al., 1988), ER (Tora et al., 1989) and the androgen receptor (AR, Simental et al., 1991).

1.6.2 Hormone response elements (HRE).

Gene transfer techniques and DNA-Protein interaction studies have identified

consensus sequence elements which are the binding sites for various steroid receptors (reviewed in Beato et al., 1989). Response elements have been found in a variety of hormone responsive genes and are sufficiently homologous to derive a consensus sequence (see table 1.1). In most cases these sequences have a palindromic structure with two unequal halves seperated by three non-conserved nucleotides. The HREs can be superficially divided into three subgroups: the GRE/PRE, the ERE and the TRE subgroups (table 3.1). One exception to the palindromic rule is the retinoic acid response element (RARE) present in the promoter region of the retinoic acid receptor gene. This element contains a direct repeat of the sequence (G/A)GTTCA seperated by 5 nucleotides (de The et al., 1990). Orientation and spacing between the two half sites of HREs have been shown to be important for specificity of response to the ligand and also for the receptor molecule (Umesono et al., 1991; Naar et al., 1991). In addition, nucleotides important for receptor interaction to the DNA have been mapped for several steroid hormone receptors. Studies have shown that the functional consensus GRE sequence is $5'G^{-6}G^{-5}T^{-4}A^{-3}C^{-2}A^{-1}NNNT^{+1}G^{+2}T^{+3}T^{+4}C^{+5}T^{+6}$ 3' (Beato et al., 1989), where N is a degenerate nucleotide and the numbers represent positions of the basepairs in the HRE palindrome. The three bp spacing is very important for DNA binding specificity. Interestingly, this sequence can bind to the PR, AR and mineralocorticoid (MR) receptors as efficiently as the GR (reviewed in Beato, 1989). Mutagenesis experiments with the GREs have demonstrated that changes at nucleotides at position G^{+2}, T^{+3}, C^{+5}, C^{-2}, and A^{-3} are not tolerated for either DNA binding or

Table 1.1. Consensus DNA sequence elements for various steroid hormone receptor binding sites. The core consensus sequences for the various members of the steroid hormone receptor superfamily are overlined. All the hormone response elements are palindromes, except for the RARE which contains a direct repeat of the sequence (A/G)GTTCA. The response elements are for receptors that bind glucocorticoids (GRE), progesterone (PRE), mineralocorticoids (MRE), androgens (ARE), estrogen (ERE), thyroid hormone (TRE), vitamin D (VDRE) and retinoic acid (RARE). n represents either A, G, C or T.

SEQUENCE	NAME
AGAACAnnnTGTTCT	(GRE, MRE, PRE or ARE)
AGGTCAnnnTGACCT	(ERE)
AGGTCA---TGACCT	(TRE)
TGGTGA-n-TCACCG	(VDRE)
AGTTCAnnnnnAGTTCA	(RARE)

glucocorticoid inducibility (Noordeen et al., 1990), whereas, changes in other bases to a certain degree are. Most of these results were further confirmed from methylation interference and DNase protection studies that showed that GR makes contacts at the guanines at positions +2, +5, -2 and -5 of the palindromic sequence (Scheidereit et al., 1984 and 1986). Similarly, the T's at position +3 and +4 appear to be very important for target site discrimination (Truss et al., 1990; Cairns et al., 1991), because changing the T's at these positions to A^{+3} and C^{+4} converts it into an estrogen response element (Klock et al., 1987). The palindromic nature of many steroid hormone response elements suggests that hormone receptors bind to DNA as dimers (Perlmann et al., 1990).

1.6.3 Mechanism of transactivation by steroid hormones and their receptors.

The MMTV DNA has served as a paradigm for the study of glucocorticoid regulated expression of genes transcribed by RNA polymerase II (reviewed in Truss et al., 1992). In the general model of steroid hormone-activated gene expression, the steroid hormone receptor is first converted from a non DNA-binding complex in the cytoplasm to a DNA-binding protein in the nucleus. This activated receptor then binds in a sequence-specific manner to hormone-regulated genes to induce transcription. The MMTV promoter contains 4 copies of the hexanucleotide, TGTTCT, each constituting one half of the palindromic receptor binding site. Each of these motifs can bind and also respond to the GR, PR, AR and MR in cells expressing these receptors (reviewed in Truss et al., 1992). Deletion and insertion

mutagenesis has shown that a strong cooperativity exists between the upstream distal and the three downstream proximal motifs (Chalepakis et al., 1988). Two types of mechanisms have been described for transactivation of hormone responsive genes by steroid hormones and their respective receptors. The first mechanism involves other cellular transcription factors while the second involves the role of chromatin structure in steroid hormone regulation.

1.6.3.1 Role of transcription factors in transcriptional activation by steroid hormones.

Development of in vitro reconstituted cell free transcription systems combined with other in vitro DNA-binding assays and in vivo studies have greatly enhanced our understanding of the molecular mechanism of steroid hormone induction. There are two different mechanisms by which hormone receptors might influence transcription: (1) modulating the formation of a stable preinitiation complex at the promoter or (2) cooperating with other enhancer and/or promoter specific transcription factors.

(1) Enhancement of an assembly of a stable pre-initiation complex at the promoter region.

Transcription of protein encoding genes begins with the formation of an initiation complex (reviewed in Zawel and Reinberg, 1993). In vitro reconstituted systems have revealed that assembly of proteins at the transcriptional initiation site

occurs in a defined order. The TATA box binding factor (TFIID) first binds to the TATA element present upstream of the start site. Next, the general transcription factors, TFIIA and TFIIB, associate with the TFIID-DNA complex and lay the foundation for the subsequent binding of RNA polymerase II, TFIIF, TFIIE, TFIIH and TFIIJ (reviewed in Zawel and Reinberg, 1993). As a result, a transcriptionally active complex is formed which is sufficient for basal levels of transcription. However, for higher induced levels, other DNA-binding specific transcription factors are required (reviewed in Mitchell and Tjian, 1989). Naturally occcuring genes contain a variety of cis acting elements that are binding sites for specific DNA-binding transcription factors, also called activators. Basically, transactivators are comprised of at least two domains: one for DNA binding and another for transcriptional activation. These activation domains are believed to enhance transcription by promoting or stabilizing the formation of an initiation complex by interacting with the general transcription factors (reviewed in Ptashne and Gann, 1990). Many studies have implicated TFIID and TFIIB as potential targets for transactivators (reviewed in Greenblatt, 1991; Lin et al., 1991). However recent studies have shown direct physical interactions between activation domain of these activators with the general transcription factors, TFIID, TFIIB and TFIIH (reviewed in Zawell and Reinberg, 1992). In addition, other proteins, which are termed adaptor, coactivator, mediator, and/or bridging proteins, may mediate protein-protein interactions between activation domains and the general transcription factors (reviewed in Lewin, 1990).

Most of the studies to examine the mechanisms by which hormone receptors interact with the general transcription factors came from in vitro reconstitution experiments. In such a system the concentration of the receptor, ligand, general transcription factors and target genes are amenable to easy manipulation. Using such a strategy it was shown that the progesterone receptor is able to confer induction of correctly-initiated transcripts of a template DNA in the presence of RNA polymerase II and nucleotides (Klein-Hitpass et al., 1990; Bagachi et al., 1990a). Competition analysis and kinetic studies have shown that the steroid receptor enhances the formation of a rapid start complex by the RNA polymerase II (Klein-Hitpass et al., 1990; Bagachi et al., 1990b). This appears to be by enhancing the assembly of a committed complex of transcription factors at the TATA box. In a similar fashion the glucocorticoid receptor (Tsai et al., 1990) and the estrogen receptor (Elliston et al., 1990) were shown to stimulate RNA synthesis in this in vitro system.

The understanding of the mechanisms of hormone stimulation was highlighted in studies of the ovalbumin promoter and induction of in vitro transcription by COUP-TF, a member of the steroid hormone receptor superfamily. It was shown that a second factor, S300-II, was required for in vitro transcription of the ovalbumin promoter (Sagami et al., 1986). This factor itself did not bind DNA, but functioned indirectly by stabilizing the binding of COUP-TF to its recognition sequence (Tsai et al., 1987). Thus S300-II exhibited properties of an adaptor molecule. Subsequent cloning of this factor established its identity as the general transcription factor, TFIIB (Ha et al., 1991; Malik et al., 1991). These results revealed that TFIIB, not only

functions as a passive target for activators, but also, actively stabilizes the binding of upstream factors to their respective regulatory elements. More interestingly, these data suggested that TFIIB could be a general target for the steroid receptor superfamily of proteins.

As mentioned earlier, steroid hormone receptors have multiple activation domains localized in various regions of the molecule. In a recent report it was shown that the action of at least three members of the steroid receptor superfamily, COUP-TF, estrogen and progesterone receptors was mediated by a direct interaction of these receptor molecules with TFIIB (Ing et al., 1992). It was proposed that TFIID and the steroid receptor initially and continously associate and dissociate with their respective binding elements. However, in the presence of TFIIB, both factors are likely to bind more stably to their recognition elements and thus assist in forming a more stable initiation complex to initiate transcription in a productive manner. This interaction of the steroid receptor with TFIIB might be a critical step since the association of TFIIB with the transcription initiation complex is known to be rate-limiting (Lin and Green, 1991). More recently, another target of hormone receptor mediated transactivation was established by demonstrating a functional interaction of estrogen receptor with TFIID (Bron et al., 1993).

(2) Cooperation with other site-specific DNA-binding transcription factors.

Several reports suggest that steroid receptors act by cooperating with other cellular transcription factors. Cooperativity has been observed for a variety of heterologous

binding sites, including NF1, Sp1 and oct-1 factors (Schule et al., 1988a, 1988b; Strahle et al., 1988). A downstream NF1 site was shown to be important for glucocorticoid and progesterone induction of the MMTV promoter (Kalff et al., 1990) in cell free transcription systems. Mutational analysis (Miksicek et al., 1987; Buetti et al., 1989) combined with gene transfer experiments (Bruggemeier et al., 1990) have also established an essential role of the NF1 motif in hormone induction of the MMTV promoter. However, quite unexpectedly, it was revealed that purified hormone receptor did not cooperate, but rather competes with NF1 for binding (Bruggemeier et al., 1990). This is because the binding sites for both factors overlap with each other by several base pairs. This hormone effect and the observed requirement of the NF1 motif for optimal steroid response led to some classical studies regarding the involvement of chromatin structure which is discussed in the following section.

Two degenerate octamer binding motifs (oct-1) were also shown to be involved in mediating hormonal induction of the MMTV promoter (Bruggemeier et al., 1991). Mutations at this site significantly reduced hormone induction of this promoter (Toohey et al., 1990). In contrast to the NF1, which competes with the receptor for binding, the binding of oct-1 to its recognition sequence is strongly enhanced in the presence of the GR or PR (Bruggemeier et al., 1991). Therefore, oct-1 in this system acts not only to cooperate with DNA binding, but also in functional synergism.

1.6.3.2 Role of chromatin structure in hormonal regulation.

The MMTV paradigm has again served as a useful model to explore the role of chromatin structure in hormone-mediated regulation. As mentioned earlier, competetive binding was observed in vitro at the MMTV promoter between the hormone receptor and NF1. However, in vivo data suggested that glucocorticoid treatment induces NF1 binding to the MMTV promoter (Cordingley et al., 1987). In the absence of hormones no binding was observed at the NF1 site. Beato's laboratory has explored the possibility that glucocorticoids alter the chromatin structure of the DNA such that it allows NF1 binding. Two preliminary studies had indicated that the MMTV LTR becomes hypersensitive to DNase1 digestion after hormone treatment in the region which contains the HREs (Zaret and Yamamoto, 1984). In addition, this region is also organized into three well positioned nucleosomes (Richard-Foy and Hager, 1987), one of which covers the regulatory region containing the hormone response elements. Increased hypersensitivity to DNase I digestion suggested that the hormone has induced a structural change in the DNA by displacing or removing the nucleosome covering the HREs. In vitro nucleosome reconstitution experiments have shown that the MMTV HRE region has an inherent ability to adopt a preferred conformation with the DNA double helix and positions itself in a very precise manner on the surface of the histone octamer (Perlmann and Wrange, 1988; Pina et al., 1990). Detailed analysis of this preferred path made it clear that two of the four HREs present in the MMTV LTR have their major groves facing outward, whereas the NF1 motif faces inwards (Pina et al., 1990). In agreement with this it was also shown that the glucocorticoid receptor can bind to

the MMTV promoter when it is organized into nucleosomes (Perlmann and Wrange, 1988). The current model of transactivation would then be that receptor binding displaces a nucleosome which then uncovers the NF1 binding site and allows formation of a stable transcriptional initiation complex. In the absence of hormones, the promoter is silent due to the masking of NF1 binding site by the nucleosome. In this respect, hormone receptor acts as a modulator of transcription by serving as an entry point for other promoter specific and basal transcriptional factors.

1.6.4. Positive and negative regulation of steroid hormones through overlapping factor binding sites.

Positive or negative regulation of expression of genes by glucocorticoid hormones has been identified at sequences where the GRE overlaps the binding sites for non-receptor transcription factors that negatively or positively regulate these genes. Such a mechanism would involve steric inhibition of binding for positive or negative factors at these overlapping motifs (reviewed in Ponta et al., 1992). In another situation, no competitive inhibition is involved, rather the steroid hormone receptor interacts with other transcription factors and binds as a complex to these overlapping motifs. Such an element with overlapping factor-binding sites has been referred to as "composite GREs" (cGRE) and was first described by Diamond et al. (1990) for the rat proliferin gene.

Negative and/or positive regulation by steroid hormone receptors, in particular GR, has been described for several genes (reviewed in Wahli and Martinez, 1991).

The prolactin gene is both positively and negatively regulated by estrogen and glucocorticoids, respectively. Estrogen induction is through an ERE (Adler et al., 1988) and requires cooperative interaction with the pituitary specific "pit-1" factor (Day et al., 1990). Glucocorticoids on the other hand inhibit prolactin gene expression, apparently through a composite GRE which has binding sites of some unknown cellular factor (Sakai et al., 1988). Such negatively regulating GREs were called "nGRE" (n for negative). Similar nGREs were also reported for the pro-piomelanocortin gene (Drouin et al., 1989) and choroinic gonadotropin α-subunit gene (Akerblom et al., 1988) which apparently act by sterically interfering with the binding of a positively acting cAMP response element binding (CREB) protein (Nakai et al., 1991; Akerblom et al., 1988). This inhibition was mediated by the DNA-binding domain of the GR indicating that DNA binding of GR is required (Akerblom et al., 1988; Oro et al., 1988). GR also negatively regulates genes by binding to GREs overlapping with other regulatory elements, e.g. AP-1 in the case of α-fetoprotein gene (Guertin et al., 1988; Zhang et al, 1991) or the TATAA box element in the case of human osteocalcin gene (Stromstedt et al., 1991). The human osteocalcin gene is also positively regulated by vitamin D and retinoic acid. This occurs through a vitamin D response element (VDRE), that is also a target of a negatively acting AP-1 protein (Schule et al., 1990; Owen et al., 1990). In this case, induction by these hormone receptors is due to competitive inhibition of a negatively acting protein.

Cooperative interactions at composite elements was demonstrated for the ER

and AP-1 for the chicken ovalbumin gene (Gaub et al., 1990) and between GR and AP-1 for the rat proliferin genes (Diamond et al., 1990). In both cases the overlapping binding sites were indeed bound by both AP-1 and the steroid receptors. However, in the case of the rat proliferin genes, AP-1 behaves as a selector of, either a positive or negative regulator by hormones (Diamond et al., 1990). Thus, the element was inactive in the absence of AP-1, and was either positively regulated (in the presence of c-jun homodimers) or negatively regulated (in the presence of c-jun/c-fos heterodimers) by glucocorticoids (Diamond et al., 1990). This phenomenon is interesting because it allows diversity and regulatory versatility of gene expression. However, regulation of such a kind remains a model and has been demonstrated only with cloned composite elements of the proliferin gene promoter. Whether or not such a regulation actually happens at the complex proliferin promoter is yet to be established. More recently, differential regulation between the GR and MR was demonstrated at the proliferin cGRE (Pearce and Yamamoto, 1993). Although both GR and MR interact with the same sequence, yet they behaved in opposite manners (Pearce and Yamamoto, 1993). It is also suggested that this mode of complex regulation might be relevant and important for many physiological signals governed by steroid hormone receptors, nonreceptor cellular factors and composite elements that bind with these factors (reviewed in Funder, 1993). It is also possible that different members of the AP-1 and steroid hormone receptor families interact with each other to result in different physiological responses of cGRE regulated genes (reviewed in Miner and Yamamoto, 1991; and Lamph, 1991). It is interesting to note

that HPV 16 regulatory region also possesses a cGRE at nt position 7640, which contains an AP-1 motif overlapping a non-canonical GRE (Chan et al., 1990). In this thesis (chapter 5) I have studied regulation of this cGRE by GR, c-jun and c-fos in the context of both, the full length HPV 16 enhancer region and also with an isolated sub-region of this enhancer.

CHAPTER 2

HUMAN PAPILLOMAVIRUSES AND THEIR ROLE IN TRANSFORMATION.

2.1 Introduction

Several decades after the first demonstration that cell free extracts from wart tissue transmitted HPV, the malignant potential of cottontail rabbit papillomavirus was observed in rabbits (Rous and Beard, 1935). Decades later, it was shown that cervical cancers contained genetic material that specifically hybridized with DNA isolated from human warts (zur Hausen et al., 1974) and it was hypothesized that cervical cancers are caused by papillomavirus infections (zur Hausen, 1976 and 1977). It was during this later period that papillomavirus-specific cytopathic changes were demonstrated in cervical lesions (Meisels and Fortin, 1976; Purola and Savia, 1977; Laverty et al., 1978) and the genetic heterogeneity of human papillomaviruses was recognized (Gissmann and zur Hausen, 1976). Specific HPV types 5 and 6 were then identified in skin cancers from patients with epidermodysplasia verucciformis and additional types were identified in a wide variety of benign and malignant human tumors of the skin, cervix, anogenital region, respiratory tract and oral cavity (reviewed in Shah and Howley, 1990). To date, over 60 types of HPVs have been identified (de Villiers, 1989), more than 20 of which are associated with anogenital lesions. In addition, more than 85% of cervical carcinomas contained HPV DNA, predominantly of the high risk HPV types (Lorincz et al., 1992).

2.2 Assay systems to study transformation in vitro

Most of the early observations about the tumorigenic nature of papillomaviruses were based on tumors occuring in domestic or laboratory animals, which were amenable to both epidemiological and experimental studies (reviewed in Pfister, 1984). In contrast to the animal models, however, in humans a logarithmic relation exists between cancer incidence and age, suggesting that not one but several random events must be required for the development of a tumor (Armitage and Doll, 1957; recently reviewed in Vogelstein and Kinzler, 1993). It became increasingly clear that most human malignancies could not be attributed to a single infectious agent.

One of the major limitations to human papillomavirus research was the inability to propagate the virus in culture. HPVs infect only epithelial tissues and the viral replication cycle is linked to the process of epithelial cell differentiation (Blanton et al., 1991). Mature virions are found only in the superficial highly differentiated keratinocytes (Stoler et al., 1989; Durst et al., 1992). Since this state of differentiation has not been achieved in tissue culture, propagation of HPVs has been, for the most part, frustrating. However, with the advent of recombinant DNA technology, it has been possible to clone and sequence viral DNA from infected tissues, enabling researchers to recognize multiple HPV types exhibiting distinct tissue tropisms associated with clinical lesions. Using such techniques, it has also been possible to dissect different sub-regions of the viral DNA and assess some of their biological properties when introduced into cultured cells by DNA transfections. Thus,

these techniques have allowed us to introduce cloned viral DNA into established or primary cells and look for cell biologic effects such as morphological transformation, anchorage-independent growth, cooperation with cellular oncogenes, life span, immortalization, differentiation and cell growth.

2.2.1 Transformation of established lines by HPVs.

It was shown earlier that DNA from tumors introduced into established mouse fibroblasts (NIH 3T3) could impart a malignant phenotype (Tabin et al., 1982; Reddy et al., 1982). Similar experiments have shown that the genomic DNA taken from a cervical adenocarcinoma induced focus formation in NIH 3T3 cells (Tsunokawa et al., 1986). It was revealed that the transforming sequences were derived from HPV 16 sequences present in the tumor DNA. Mouse NIH 3T3 and C127 cells and rat 3Y1 cells have been extensively used to study the oncogenic potential of several HPV types and have provided us with much information on the mechanisms of transformation. These established lines have been very useful to study morphologic transformation. Ordinarily, these cells are contact inhibited and grow as a flat monolayer. However, upon transformation, these cells become more refractile, lose their contact inhibition and form a focus of densely packed cells. Cells cloned from these foci display characteristics of transformed cells, including growth in low serum, higher saturation density, anchorage independence and tumorigenicity in nude mice.

The oncogenic potential of HPVs was directly demonstrated by transfecting a cloned dimer of HPV 16 into 3T3 cells (Yasumoto et al., 1986 and 1987). Foci

which displayed characteristics of a transformed phenotype appeared and were tumorigenic in nude mice. Employing a different strategy, HPV early region driven by strong heterologous promoters (usually SV40 early promoter or retroviral LTR) demonstrated transformation of 3T3 cells (Matlashewski et al., 1987) and anchorage-independent growth (Tanaka et al., 1989; Vousden et al., 1988; Yutsudo et al., 1988). Subsequently it was shown that HPV 16 and 18 E7 ORF is sufficient for morphologic transformation but required the E6 ORF for tumorigenicity in nude mice (Phelps et al., 1988; Tanaka et al., 1989; Bedell et al., 1987, 1989).

A variety of different HPV types were also tested in a similar fashion. Transformation of C127 cells by HPV types -1, 5, 16 and 18 were reported (Watts et al., 1984, 1987; Morgan et al., 1988). Differences were observed among different HPV types, correlating with the oncogenic potential of these viruses. The E6 and not E7 encoding ORF of HPV 8, which is associated most commonly with cutaneous carcinomas in EV patients, was able to transform C127 and Rat 1 cells only after G418 selection (Iftner et al., 1988). The E5a gene of HPV 6c can induce morphologic transformation and anchorage-independent growth of 3T3 and C127 cells (Chen and Mounts, 1990).

The rat 3Y1 cells have also been used to show that HPV 16 and 18 (Kanda et al., 1987; Noda et al., 1988; Watanabe and Yoshiike, 1988) and their respective E7 genes (Kanda et al., 1988b; Tanaka et al., 1989) are sufficient for transformation. HPV 1a could impart features of transformation in 3Y1 cells (Green et al., 1986). It was also recently demonstrated that the E6 genes of HPV types 5, 8, and 47

induced morphological transformation of 3Y1 cells more frequently than those of HPV types 14, 21, and 25 (Kiyono et al., 1992). Morphological changes induced by the E6 genes of several HPV types was also correlated with the risk of malignant transformation of lesions associated with these HPVs (Hiraiwa et al., 1993). One important consequence of using these transformation assays was the identification of regions required for oncogenicity (Watanabe et al., 1990). It also enabled investigators to dissect the structural and functional properties of the HPV transforming genes (discussed earlier in chapter 1).

2.2.2 Transformation of primary rodent epithelial cells by HPVs.

It was soon realized that morphologic transformation of established cells was not sufficient to convincingly conclude that HPV genes were the only determinants for oncogenicity, since established lines cannot be considered as normal cells. As mentioned before, in the multi-step model of tumorigenesis other cellular genetic events are important for transformation (reviewed in Vogelstein and Kinzler, 1993). Consequently, in vitro assays were developed to explain this multi-step process. In this process it is assumed that a tumorigenic cell evolves as a consequence of a series of gene activations/inactivations, each one of which contibutes to the overall phenotype of a malignant cell (Burnet, 1957; Foulds, 1958; Nowell, 1976). Studies of two virally encoded oncogenes, the middle (MT) and large T (LT) genes of polyomavirus (Rassoulzadegan et al., 1982), set the initial relationship between oncogenes and the multistep nature of oncogenesis. Neither of the oncogenes alone

was able to transform primary cells but the two were able to cooperate to elicit a fully malignant phenotype. This model was then extended to a number of cellular and viral oncogenes. Using this type of cooperation, oncogenes were grouped into two main classes: The establishment class of genes which are capable to establish primary cells in vitro and the transforming class which required the cooperation of an establishment gene in order to transform primary cells. Over the years, it has become a standard assay to assign an oncogenic property to a gene if it is able to cooperate with one of the transforming class of oncogenes (includes EJ-ras, c-fos, c-myc etc). Using such a cooperating assay, the early region of several oncogenic HPV types (16, 18, 33 and 35) driven by heterologous promoters were found to be able to cooperate with cellular oncogenes to transform primary baby rat or baby mouse kidney epithelial cells (Matlashewski et al., 1987; Storey et al., 1988; Phelps et al., 1988; Kanda et al., 1988a). Neither HPV DNA nor the oncogenes alone were sufficient for transformation. In this assay the early region of low risk HPV types 6 and 11 were either inactive or transformed at a very low frequency, only if expressed at sufficiently high levels (Storey et al., 1990a). These studies also showed that continous expression of the E7 gene is required for maintenance of the transformed phenotype and for continued cell proliferation (Crook et al., 1989b; Storey et al., 1991). The above mentioned studies were performed using strong heterologous promoters and the full length HPV 16 or 18 genomes were very inefficient in these assays. In a landmark experiment, the role of co-factors required for HPV transformation was stressed when it was shown that the full length HPV 16 genome

could cooperate with the EJ-ras or c-fos oncogenes to efficiently transform primary cells provided steroid hormones, dexamethasone or progesterone, were present in the media (Pater et al., 1988; Crook et al., 1988; Pater et al., 1990). Cooperating activity was greatly diminished without hormones. These studies were able to identify a very important co-factor essential for the transforming activity of HPV 16 DNA. It was suggested that oral contraceptive pills, which contained progesterone like derivatives, may be capable of stimulating the expression of HPV oncogenic proteins in infected individuals through the hormone response element present in the HPV regulatory region (Pater et al., 1988; Gloss et al., 1987). The hypothesis gained support from reports that oral contraceptives were associated with a high risk of developing invasive cervical cancers (Melamed and Flehinger, 1973; Stern et al., 1977; Swan and Brown, 1981; Vessey et al., 1983; Ebeling et al., 1987). Interestingly, the non-oncogenic HPV types 6 and 11 were unable to transform even in the presence of hormones even though HPV 11 LCR contained a hormone-responsive GRE (Pater et al., 1988; Crook et al., 1988; Gloss et al., 1987). However, as suggested by Pater et al. (1988), hormone-induced expression levels of HPV oncogenes are important for transformation. This was reflected in another study where duplication of the HPV 11 LCR, in the context of the whole genome, resulted in a glucocorticoid-dependent transformation of the non-oncogenic HPV 11 DNA (Rosen and Auborn, 1991).

2.2.3. Transformation of primary human and rodent fibroblasts by HPV DNA.

Transfected HPV 16 DNA can extend the in vitro life span of primary human foreskin fibroblasts and rat embryo fibroblasts (Cerni et al., 1990; Matkaleshewski et al., 1988; Pirisi et al., 1987). The E6/E7 region of HPV 16 expressed from an SV40 promoter was sufficient for immortalization of primary human lung fibroblasts (Watanabe et al., 1989). The E6/E7 regions of HPV types 6, 16 and 18 could cooperate with activated EJ ras oncogene to fully transform primary rat embryo fibroblasts (Bedell et al., 1989; Chester and McCance 1989). These results are interesting, because although HPV naturally infects only epithelial cells, in vitro the viral oncoproteins are capable to immortalize primary fibroblasts. However, unlike epithelial cells, the full length HPV genomes were either inefficient or deficient in immortalizing human fibroblasts (see below). This differential activity could be a property of the LCR region which is the prime determinant of epithelial cell specificity (as discusssed in chapter 1). Studies by Romanczuk et al. (1991) and Rosen and Auborn (1991) for primary human and rodent epithelial cells also support this suggestion.

2.2.4 Transformation of primary human epidermal and cervical keratinocytes by HPVs.

Since HPV is an epitheliotropic human virus, it became essential to demonstrate the properties of the transforming genes in human keratinocytes. It was possible to obtain stable cell lines by transfecting HPV 16 and 18 DNA into primary foreskin keratinocytes. These cells were immortalised, contained HPV DNA and expressed

HPV-specific RNA, but were not tumorigenic in nude mice (Durst et al., 1987a; Pirisi et al., 1987 and 1988; Kaur and McDougall 1988, 1989). The E6 and E7 regions were found to be responsible and sufficient for this immortalization (Kaur et al., 1989; Hawley-Nelson, 1989; Munger et al., 1989a; Halbert et al., 1991) and altered differentiation (Barbosa and Schlegel, 1989; Hudson et al., 1990). Subsequently, much effort was put into characterising these cell lines, including their patterns of keratin gene expression and differentiation capability (Durst et al., 1987a, 1991; Pirisi et al., 1988; Kaur and McDougall, 1988, 1989; Tsutsumi et al., 1992). It was also observed that late passages of some of these lines became tumorigenic in nude mice (Kaur and McDougall, 1989; Hurlin et al., 1991), indicating that additional genetic events had happened. A similar observation was made for HPV-immortalised rat embryo fibroblasts (Inoue et al., 1991). The immortalizing efficiency of various HPV types were also correlated with the in vivo oncogenic potential. Thus, HPV types 16, 18, 31, and 33 were able to readily immortalize human epidermal cells, whereas, HPV types 1a, 5, 6b and 11 were inactive (Woodworth et al., 1988, 1989). However, some recent studies have provided evidence for a weak immortalizing /transforming activity for the low risk HPV types in human and rodent cells (Storey et al., 1990a; Rosen and Auborn, 1991; Halbert et al., 1992). In addition, E6 genes from the low risk viruses were able to complement the E7 of high risk viruses in immortalization of primary cells (Halbert et al., 1992). The two-step model of oncogenesis was also investigated and it was shown that subsequent transfection of activated ras oncogene rendered the cells transformed and tumorigenic (Rhim et al., 1989; DiPaolo et al.,

1989; Matlasheswski et al., 1988; Cerni et al., 1990).

In parallel experiments, HPV types 16, 18 and 33 DNA was able to immortalize human ectocervical and endocervical epithelial cells, the presumed progenitor cells of cervical carcinomas and targets for HPV infection (Pecoraro et al., 1989; Woodworth et al., 1990a; Tsutsumi et al., 1992; Gilles et al., 1993). HPV 6b DNA however did not display immortalizing activity. In another very interesting study it was observed that HPV 16-immortalized endocervical cells displayed a carcinoma in situ-like pathology after transplantation into nude mice, whereas, the immortalized ectocervical and foreskin keratinocytes displayed mild dysplasia (Sun et al., 1992). This study addressed the interesting question about the origins of squamous cervical carcinomas.

2.3 Mechanisms of transformation by HPVs.

In the analysis of many tumor viruses, two consistent features emerge for the mechanism of tumor induction by these viruses (reviewed in Green, 1970):

1. Viruses introduce at least one functioning gene into their respective host cells. This gene(s) may or may not become covalently linked to the host cell DNA.
2. Expression of this persisting gene is required to maintain the transformed state of this cell.

The genetic studies described in previous sections provided ample evidence that the E6 and E7 genes are the primary HPV genes involved in transformation of cultured cells. These genes are consistently retained and expressed in cervical

carcinomas (Lehn et al., 1985; Pater et al., 1986), cell lines derived from them (Yee et al., 1985; Pater and Pater, 1985) and in vitro HPV-immortalized and/or transformed cells. Much work has gone into understanding the mechanisms of transformation by HPVs and it would be far from true to say that we have been completely successful in arriving at a single mechanism. However, over the years several theories and speculations have been considered, some of which I have tried to recapitulate below.

2.3.1 zur Hausen's "CIF" theory.

As is currently understood, DNA tumor viruses behave as effectors of cellular transformation by stimulating host cell DNA synthesis. Such viruses would require host cell DNA replication for their DNA synthesis and seem to depend on a replicating cell for their propagation (Green, 1970). Induction of cellular DNA synthesis appears to depict an adaptive mechanism acquired to overcome this restriction, thus permitting viral gene expression and productive infection. Viruses defective in late functions would continue to initiate cellular DNA synthesis without viral replication, leading to unrestricted cell growth. According to the HPV-cancer hypothesis of zur Hausen, it is proposed that HPV encodes a transforming factor (E6/E7 genes), that is normally suppressed in cells by a cellular interfering factor referred to as "CIF" (zur Hausen, 1977b, 1989a). This theory assumes a balanced control of viral transforming factors. According to this model, CIF counteracts the transforming "effector" genes to suppress or disrupt the function of their gene

products. Mutational events affecting one CIF gene, mediated by initiators, such as mutagenic co-factors or arising spontaneously, could disturb the effector gene balance, leading to increased cellular DNA synthesis and cell growth. Inactivation of both alleles would then result in viral carcinogenesis (zur Hausen, 1986). The "CIF" theory would predict the following: tumor progression in a stepwise manner, long latency periods between infection and cancer, monoclonal origin of tumors, chromosomal abnormalities and aneuploidy of tumor cells, recessive nature of malignancy after fusion with normal cells, synergism between viral effectors and mutagenic co-factors and/or initiators and tumor promotion by growth stimulating events. Many of these predictions have been verified and several lines of evidence support the "CIF" theory.

Indeed, like the SV40 and adenovirus counterparts, HPV E7 gene has been shown to induce cellular proliferation, DNA synthesis and progression of the cell cycle (Sato et al., 1989b; Banks et al., 1990a, 1990b; Rawls et al., 1990). Fusion studies of an HPV-positive cervical cancer cell line and normal cells resulted in non tumorigenic hybrids despite normal levels of HPV E6/E7 gene expression (Bosch et al., 1990). Innoculation of these non tumorigenic hybrids or HPV immortalized cells into nude mice resulted in a remarkable decrease of HPV gene expression (Bosch et al., 1990, Durst et al., 1991). However malignant cells continued to transcribe their E6/E7 genes at higher levels. These results indicated that under in vivo situations the putative CIF gene was activated in non malignant cells, by unknown humoral mechanisms, resulting in supression of HPV expression. It is postulated that

inactivation of the CIF gene in malignant cells allows continous expression of HPV genes. In vitro 5-azacytidine treatment suppressed HPV expression only in the non-tumorigenic hybrids or immortalized cells and not in the malignant cells (Rosl and zur Hausen, 1988). This effect was interpreted as activation of CIF genes in the former by demethylation. In another study, the bacterial CAT gene driven by the HPV 18 regulatory region was stably transfected into cervical carcinoma cells and then fused with normal cells (Rosl et al., 1991). In the non malignant hybrids, CAT gene expression was again suppressed at the level of transcriptional initiation. Cycloheximide treatment abrogated this response, pointing out the existence of a suppressor protein molecule. Finally, it has been speculated that this putative CIF gene might be located on chromosome 11 (Rosl and zur Hausen, 1988). Evidence is available from somatic cell fusion and microinjection studies that chromosome 11 contains a tumor supressor activity (Stanbridge 1976, Saxon et al., 1986, Koi et al., 1989). Interestingly, human embryo fibroblasts with deletions in chromosome 11 are more susceptible to transformation by HPV 16 DNA (Smits et al., 1988). The role of genes located on chromosome 11 in transcriptional regulation of HPV has been discussed earlier in section 1.5.3.

2.3.2 HPV and the oncogene activation theory.

Amplification and increased expression of cellular oncogenes have frequently been observed in cervical carcinomas. In a majority of tumors c-myc amplifications and gene rearrangements have been described (Riou et al., 1985, 1987, 1990a, 1990b,

Ocadiz et al., 1987; Iwasaka et al., 1992). However, contraditory results are also available (Ikenberg et al., 1987). Increased levels of the c-myc oncogene have also been observed in cell lines derived from cervical carcinomas (Durst et al., 1987b; Couturier et al., 1991) and cells immortalized by the high risk HPVs (Crook et al., 1990; Sun et al., 1992). The potential role of ras has been recognized in experimental studies (discussed in section 2.2.2), but conflicting results have been obtained about the frequency of ras activation in cervical tumors and HPV-related cancers (Riou et al., 1988; Sagae et al., 1990; Anwar et al., 1992 and 1993; Pelisson et al., 1992). The early advocates of the oncogene hypothesis postulated that latent viruses and covert cancer genes pre-exist in normal cells and are "activated" to tumor viruses and cancer genes by mutations (Huebner and Todaro, 1969). Although the oncogene hypothesis gained momentum for cancer-causing retroviruses, it did not correlate well with the picture for DNA tumor viruses. In conclusion, oncogene activation as a sole mechanism for HPV transformation is conflicting and disputable.

2.3.3 Inactivation of tumor supressor genes. The "Howley" hypothesis.

Recent biochemical studies have suggested another mechanism by which the transforming proteins of HPVs might exert their effect on cellular proliferation. A class of cellular genes, variously named anti-oncogenes, tumor supressor genes or recessive oncogenes have been described (reviewed in Weinberg, 1991; Marshall, 1991). Inactivation of such genes, by deletions or mutations, has shown to be associated with a number of different human tumors (Lee et al., 1988). Two such

genes of interest are the retinoblastoma pRB and the p53 gene.

Substantial interest in the RB gene was generated when it was discovered that its protein exists within DNA tumor virus transformed cells in the form of complexes with various viral encoded oncoproteins. The SV40, human BK and human JC large T antigens (DeCaprio et al., 1988, Dyson et al., 1990), adenovirus E1A (Whyte et al., 1988) and HPV E7 oncoproteins (Munger et al., 1989b), have been shown to interact with the pRB protein. Regions mapped for pRB binding on E7 and other viral proteins were also essential for their transforming activity (Barbosa et al., 1990). In addition, in vivo or in vitro oncogenicity of various HPV types was clearly correlated with the efficiency with which E7 binds to pRB (Munger et al., 1989b, 1992; Gage et al., 1990, Heck et al., 1992; Sang and Barbosa, 1992b). What is the fuctional consequence of this interaction ? pRB has been shown to be a cell cycle regulator, switching betwen hyperphosphorylated and relatively hypophosphorylated forms during the cell cycle (Buchkovich et al., 1989). It is a negative regulator of growth (DeCaprio et al., 1989) and inhibits progression of the cell cycle into the S phase (Goodrich et al., 1991). HPV E7 gene product binds to the underphosphorylated form of pRB (Munger et al., 1992), thereby removing the constraints imposed on progression to the S-phase.

Another line of evidence has indicated that the cellular transcription factor, E2F, is the candidate target for viral oncoprotein action (Huang et al., 1993). It has been shown that the hypophosphorylated form of pRB binds to E2F (Chellappan et al., 1991, 1992). Thus, it is postulated that, following an HPV infection the E7

protein binds to pRB and releases E2F, which then activates the genes involved in cell proliferation and DNA replication (Morris et al., 1993b). Release of E2F from pRB complexes was also correlated with the differential pRB binding efficiencies of the high and low risk HPV E7 oncoprotiens (Wu et al., 1993). E2F binding sites have been found in a group of cell proliferating genes, such as c-myc and c-myb (Mudrij et al., 1990) and also in genes involved in DNA replication, such as DNA polymerase alpha (Pearson et al., 1991) and dihydrofolate reductase (Blake and Azizkhan, 1989). The hypothesis proposed is that viral oncoproteins release growth-promoting transcription factors from pRB complexes. Other mechanisms, involving cyclin-dependent kinases, cyclin A and p107 (a pRB related product) have also been suggested (reviewed in Nevins, 1992). All these studies supports a model in which the proliferating status of a given cell is controlled by cellular regulatory proteins. These proteins in turn modulate the activity of E2F and perhaps other transcription factors. Viral proteins would function by driving E2F into different multiprotein complexes, each activating and/or repressing a given set of growth promoting and/or inhibiting genes.

Another tumor suppressor gene of interest is the p53 gene product, a nuclear phosphoprotein and a negative regulator of cell proliferation. This protein interacts with a number of different DNA tumor virus-encoded proteins (reviewed in Levine, 1990). It has been shown that, like the SV40 LT Ag and adenovirus E1B, HPV 16 E6 protein interacts with the p53 gene product (Werness et al., 1990). The biological properties of HPVs were correlated with their abilities to associate with p53. Thus,

E6 of the high risk (16 and 18) but not the low risk HPV types (6 and 11) were capable to interact with p53 (Werness et al., 1990). However, unlike the SV40 and adenovirus counterparts, HPV E6 binds to p53 and targets its degradation via the ubiquitin-dependent pathway (Scheffner et al., 1990). In contrast, another study has shown that E6 proteins of both the high risk and low risk categories are capable to interact with p53, but degradation occurs only by the high risk HPV E6 protein (Crook et al., 1991c). Also, HPV transformed cells did not contain detectable levels of p53 protein despite the presence of p53 mRNA (Matlashewski et al., 1986). Like pRB, p53 is a negative regulator of cell growth (reviewed in Marshall, 1991) and is found to be mutated in many tumors (Levine et al., 1991, Nigro et al., 1989). p53 is probably involved in regulation of DNA replication (Gannon and Lane 1987) and acts as a transcriptional activator (Weintraub et al., 1991). In support of the suggestion for the role of pRB and p53 in the etiology of cervical cancer, it has been shown that all HPV positive cell lines had the wild type pRB and p53 gene, whereas the HPV negative lines contained mutated forms (Scheffner et al., 1991, Wrede et al., 1991, Srivastava et al., 1992, Crook et al., 1991a). A similar inverse correlation was observed between HPV positivity and somatic mutations in the p53 gene in naturally occuring primary anogenital tumors (Crook et al., 1991d, Crook et al., 1992). Inactivation of p53 gene as an essential step in HPV-mediated oncogenesis was further substantiated by the demonstration that only the mutant form of p53 could potentiate the transforming function of E7 (Crook et al., 1991b) and specific loss of p53 expression in HPV E6 immortalized cells (Band et al., 1991). From these

biochemical studies, Howley has postulated that inactivation or loss of these negative growth regulators would constitute a prime mode of HPV-mediated or non HPV-mediated oncogenesis (Howley et al, 1991). However, recent studies have also indicated that in primary cervical cancers, inactivation of p53 gene by mutations is a rare event which does not correlated with HPV status in these cancers (Crook and Vousden, 1992; Fujita et al., 1992; Choo and Chong, 1993). In addition, these studies did not address the expression levels of p53 protein, independent of the HPV status in primary tumors. Hence, further studies will be required to establish this correlation and disparity.

2.3.4 Viral induced chromosomal instability and abnormalities as cause of cervical cancer.

Chromosomal abnormalities are the oldest, and as yet, the only consistent observation made of cancer cells. It was rightly postulated by Boveri in 1914, even before the discovery of DNA and point mutations, that cancer would be caused by abnormal chromosomes (reviewed in Sandberg, 1990). It is very interesting to note that most benign and low grade cervical lesions contain viral DNA in the extrachromosomal form, whereas in most cervical carcinomas the viral DNA is found integrated and covalently linked to the host cell genome (Durst et al., 1985; Pater et al., 1986; Lehn et al., 1988; Cullen et al., 1991). The random integration events, can lead to activation of cellular oncogenes (Riou et al., 1988, 1990a, 1990b; Ocadiz et al., 1987; Couturier et al., 1991; Iwasaka et al., 1992), loss of chromosomal regions carrying

putative tumor suppressor genes (Rosl et al., 1988; Atkin and Baker, 1988) or chromosomal abberations causing illegitimate rearrangements or recombinations creating new cellular genes with novel functions (Duesberg, 1987). Chromosomal regions 8q24 and 12q13 are frequent integration targets for HPV 16 and 18 transformed cells (reviewed in Lazo et al., 1992). A few reports have also stressed some preferential integration sites for HPV 16 and 18 transformed cells at fragile sites (Cannizzaro et al., 1988; Popescu and Dipaolo, 1989, 1990). In addition, viral DNA integration in most cases disrupts the E1-E2 region (Schwarz et al., 1985, Baker et al., 1987) which is believed to lead to deregulation of viral gene expression from the inhibitory influences of the E2 ORF (Schneider-Maunoury et al., 1987; Choo et al., 1987).

Chromosomal instability in HPV-immortalized cells have been observed and this lack of genomic stability most likely plays an important role in the progression of lesions (Durst et al., 1987b, Kaur and McDougall 1988, Woodworth et al., 1990a). Interestingly, aneuploidy has been described for HPV 16 or 18 positive lesions (Crum et al., 1985) but not for the those of low risk HPV types (Fu et al., 1981). More recently, it has been shown that HPV 16 E7, but not E6, is capable of inducing chromosomal abnormalities in mouse and human keratinocytes (Hashida and Yasumoto, 1991). It is postulated that virus-induced genetic instability could be an important determinant of progression of HPV related lesions (zur Hausen, 1991) and may be dependent on viral oncoprotein expression at an early stage of HPV infection (Lorincz et al., 1990).

2.3.5 Role of HPV E5 ORF in transformation.

HPV research has to date concentrated largely upon the two major transforming genes, E6 and E7, for obvious reasons: first, integration events of the viral genome, often seen in cervical carcinomas, results in the deletion and disruption of the E5 region (Schwarz et al., 1985; Baker et al., 1987). Secondly, it was only recently discovered, due to previous sequencing errors, that HPV is capable to encode an E5 protein (Halbert and Galloway, 1988). However, the first observation can still be questioned since some in vivo studies have suggested that HPV DNA can remain episomal and intact until very late stages of malignancy (Matsukura et al., 1989; Durst et al., 1992). Furthermore, there is no evidence to exclude a role of HPV E5 in the early stages of cervical cancer. Some recent studies have addressed the role of E5 gene in transformation. However, I will first briefly discuss BPV E5 which, unlike HPV, is the major transforming protein for this virus. BPV E5 is a small hydrophobic protein localized in the membrane within the golgi apparatus (Schiller te al., 1986; Schlegel et al., 1986; Burkhardt et al., 1989). It is thought to act through the EGF receptor by extending the half life of the receptor (Martin et al., 1989) and also possibly by activation of the PDGF receptors (Petti et al., 1991). Recently it was shown that BPV E5 complexes with a protein component of the vacuolar ATPases which are present in cellular compartments involved in the internalization and processing of growth factor receptors (Goldstein et al., 1990, 1991). Thus BPV E5 appears to modulate signal transduction from at least two growth factor receptors.

HPV E5 also encodes a very hydrophobic protein of about 10 kDa (Halbert

and Galloway, 1988). Initial studies had indicated that HPV E5 may also contain a weak transforming activity (Bedell et al., 1989). In addition, HPV type 6 E5 gene was capable to transform NIH3T3 cells and murine epidermal keratinocytes which formed tumors in nude mice (Chen and Mounts., 1990; Leptak et al., 1991). To examine the role of E5 and EGF receptors, two seperate studies have demonstrated that HPV 16 E5 gene cooperates with the EGF receptor to promote anchorage-independent growth of NIH3T3 cells (Pim et al., 1992; Leechnachai et al., 1992) and it appears to act by induction of the c-fos gene. No role has yet been assigned to E5 in the immortalization of primary cells, except that it induces proliferation of primary keratinocytes (Storey et al., 1992). Since E5 may act by inducing c-fos, it is possible that c-fos upregulates the expression of viral genes through AP-1 binding elements present in the regulatory regions of several HPV types (Chan et al., 1990). Another interesting finding was the existence of a cellular homologue to E5. This protein was found to have homology with conserved regions of several viral and cellular growth factors (Kahn et al., 1992). In light of these findings it remains a possibility that the HPV E5 gene could play an important role in the early stages of oncogenesis by modulating signal transduction and HPV gene expression.

2.4 Role of co-factors in HPV-mediated transformation.

Role of co-factors have been increasingly emphasized in the multi-factorial etiology of papillomavirus related tumors. For example, CRPV require additional stimuli, such as exposure to chemical carcinogens, before tumors occur in animal models

(Rous and Friedewald, 1944). Ultraviolet radiation was considered to be a co-factor in the malignant progression of HPV 5 and 8 containing cutaneous carcinomas in patients with Epidermodysplasia Verrucciformis (Jablonska et al., 1972).

Similarly, in the development of cervical carcinomas, the long delay between onset of infection and development through the successive stages of increasing dysplasia, points to the involvement of additional factors in this multistep process. It was postulated that papillomaviruses may act as promoter-like agents acting in synergy with carcinogenic initiators, such as cigarette smoking or Herpes Simplex Virus infection (zur Hausen, 1982). Indeed epidemiological data has supported the involvement of co-factors in the developement or progression of cervical cancers (reviewed in Brinton, 1992). Smoking has been identified as an important risk factor (Vessey 1986, Trevathan et al., 1983; Winkelstein, 1990) and tobacco metabolites have been demonstrated to be selectively increased in cervical secretions (Sasson et al., 1985; Holly et al., 1985). Chemical carcinogens can synergise with HPV 16 and 18 to fully transform human keratinocytes (Li et al., 1992; Garrett et al., 1993). Similarly, in the presence of a chemical carcinogen, NIH3T3 cells could be fully transformed by HPV 16 but not by the low risk HPV type 6 DNA, indicating a differential cooperation between HPV and carcinogens (Mitrani-Rosenbaum and Tsvieli, 1990 and 1992). In vivo evidence of the requirement of additional mutagenic stimulation was provided by the report of Sasagawa et al. (1992) in which recombinant HPV 16 E6/E7 in a retroviral vector was injected into the vagina or cervix of mice. Invasive squamous cell carcinomas were only induced in the presence

of either a tumor promoter (TPA) or a mutagen (MNNG). Herpes Simplex Virus infection has long been proposed to be a co-factor in HPV-mediated oncogenesis (reviewed in Rapp and Reed, 1976). It was also considered as the cause of cervical cancers until HPV was discovered. Recently, it was shown that HSV 2-induced tumorigenicity in HPV-immortalized human keratinocytes (DiPaolo et al., 1990). The role of mutagenic co-factors also fits very well with the "CIF" theory of zur Hausen (zur Hausen, 1989a), in which it is assumed that such factors may be involved directly or indirectly in inactivating the putative CIF gene/s.

One of the more important co-factors involved in the progression of cervical cancer is steroid hormones (Stern et al, 1977). The role of hormones has recently been emphasized in several studies (Pater et al., 1988, 1990; Durst et al., 1989; Mittal et al., 1993a). Epidemiologically, oral contraceptive pills have been shown to be an important risk factor for the developement of invasive cervical neoplasia (reviewed in Brinton, 1992). In addition, duration of oral contraceptive used was associated with this increased risk (Peritz et al., 1977; Brinton et al., 1990; Negrini et al., 1990). In addition, pregnancy, a condition associted wih elevated levels of progesterone, is also shown to be a risk factor for the development of cevical cancer (Haker et al., 1982; Ferenczy, 1989; Bokhman and Urmancheyeva, 1989). As discussed earlier, steriod hormones could increase the transforming potential of HPV type 16 DNA in rodent epithelial cells (Pater et al., 1988, 1989; Crook et al., 1988) and also in human cells (Schlegal et al., 1988; Durst et al., 1989; Sexton et al., 1993). Steriod hormones, dexamethasone and progesterone have also been shown to be essential for the

expression of HPV 16 RNA in primary human cervical cells (Mittal et al., 1993a). The role of steroid hormones gained a lot of attention, both epidemiologically and experimentally, when it was found that the regulatory region of several oncogenic HPV types contained hormone response elements in their regulatory regions (Chan et al., 1989).

2.5 Objective of this study.

Our laboratory had previously reported that steroid hormones, glucocorticoid and progesterone, markedly enhances transformation of primary baby rat kidney (BRK) epithelial cells by HPV type 16 DNA in cooperation with activated ras oncogene (Pater et al., 1988, 1989). Since BRK cells are epithelial in origin and do not require hormones for growth, it is a good system to critically assess and examine the role of hormones in HPV-mediated transformation. Moreover, HPVs are strictly epitheliotropic viruses and therefore BRK cells are suitable to study transformation by HPVs. It was hypothesized that this hormone-dependent transformation could occur due to increased expression of viral oncoproteins by hormones, which was thought to act through the hormone response element (GRE/PRE) located in the HPV 16 regulatory region (Pater et al., 1988; Gloss et al., 1987). Alternatively, the effect of hormones could have been indirect by modulating other cellular transcription factors.

The objective of my study was to address the molecular mechanisms of hormone-dependent transformation of BRK cells by HPV 16 DNA. To achieve this

goal I pursued the initial hypothesis of a direct role of hormones and examined the involvement of a known GRE at nt position 7640 of the HPV 16 genome (Gloss et al., 1987) by using site-directed mutatgenesis. In the search for a possible direct mechanism, I found two other putative GRE-like sequences, upstream to the one that had previously been characterised. These two other novel GREs were also experimentally mutated and their role in hormone-induced transformation was investigated (chapter 3).

Steroid hormones, glucocorticoids and progesterone, have been shown to be involved in the regulation of HPV 16 gene expression (Chan et al., 1989). Therefore, it was essential to demonstrate the role of these GREs in the transcriptional regulation of HPV 16 (chapter 4) and the levels at which this regulation occurs (chapter 6). For this purpose, different combinations of GRE mutations were used in the context of the HPV 16 enhancer, cloned upstream to a bacterial chloramphenicol acetlytransferase (CAT) reporter gene, and tested for the role of GREs in glucocorticoid induction. Transient CAT assays are very suitable for this purpose. This assay directly assays the in vivo effects of hormones and the cloned enhancer on the expression of the CAT gene. Since HPV is a strictly epitheliotropic virus, it was suitable to use HeLa cells, a human cervical carcinoma keratinocyte cell line, for transfections and transient expression assays. In addition, I wished to characterise the newly identified GREs for their ability to respond to glucocorticoids and to examine if they are competent for in vitro DNA-protein interactions. Since the GRE at nt position 7640 is a composite GRE with an overlapping AP-1 binding

site, a P19 embryonal carcinoma cell line was used to study the role of cellular oncogenes c-jun and c-fos in hormonal regulation at this element (chapter 5). In addition, cultured primary human ectocervical cells, the natural host tissue of HPV infection, were used to demonstrate the functional involvement of all three GREs in glucocorticoid-mediated HPV transcription (chapter 6).

Chapter 3

Molecular mechanisms of glucocorticoid-dependent transformation of primary rodent cells by HPV type 16 DNA

3.1 Introduction

To date, the following experimental studies have indicated an important role and involvement of hormones in HPV-mediated oncogenesis: **a).** HPV oncoproteins expressed from strong heterologous promoters do not require hormones for transformation of rodent cells (discussed earlier in chapter 2). **b).** RU486, a competitive inhibitor of the glucocorticoid receptor mediated trans-activation, results in a dose-dependent inhibition of glucocorticoid induced transformation of BRK cells (Pater et al., 1991). **c).** HPV 11 DNA is inactive in transforming BRK cells (Pater et al., 1988). However, duplication of its LCR which contains a hormone responsive GRE (Chan et al., 1989), confers dexamethasone-dependent transformation of these cells by HPV 11 (Rosen and Auborn, 1991). This activity could have been due to increased expression of the E6 and E7 viral oncoproteins. **d).** Immortalization of primary epithelial keratinocytes by HPV 16 is markedly increased in the presence of glucocorticoids (Schlegel et al., 1988; Sexton et al., 1993).

In the present study, I have examined the molecular mechanism of steroid hormone-induced transformtion of rodent epithelial cells by HPV 16 and EJ-ras. It is very clear from experimental data that other co-factors are required for HPV-

mediated oncogenesis. Understanding the mechanisms of these effects in a rodent cell transformation system would not only provide insights into but also strategies to prevent or to inhibit progression of the disease. For this purpose, mutations in the hormone response element located at nt position 7640 of the HPV 16 genome were undertaken by using site-directed mutagenesis. Mutations were created that would destroy the hormone receptor binding site or convert it into a consensus sequence. In vitro co-transformation assays were done in primary rodent cells using these mutants in the context of the full length HPV genome.

3.2 Materials

Restriction endonucleases were obtained from New England Biolabs, Bethesda Research Laboratories (BRL) or Boehringer Mannheim. T4 DNA polymerase and ligase, calf intestinal phosphatase (CIP) and reverse transcriptase were purchased from BRL, Boehringer Mannheim, and Life Sciences, respectively. Radioactive [^{35}S]-dATP for dideoxy-sequencing was purchased from Amersham. The dideoxy-sequencing kit was obtained from United States Biochemical Coorporation (Sequenase version 2.1). Flow Laboratories supplied the tissue culture medium, Dulbecco's modified media (DME), penicillin-streptomycin, phosphate buffered saline (PBS) and the trypsin-EDTA. Bockneck Laboratories supplied the bovine fetal calf serum. Synthetic oligonucleotides were purchased from the Regional DNA Synthesis Lab, University of Calgary and mutagenesis was performed using the mutagene kit

from Biorad. BRK cells were prepared from inbred fischer rats in our laboratory. HPV 16 DNA was a kind gift from Dr. Herald zur Hausen.

3.3 Methods

3.3.1 Plasmids and site-directed mutagenesis.

Table 3.1 illustrates the positions of all the three GREs, the wild type and mutated sequences and their percentage homology with the consensus GRE. Site-directed mutagenesis was performed using appropriate oligonucleotides, the Biorad mutagenesis kit and the uracil incorporation method of Kunkel (Kunkel, 1985). Briefly, mutagenesis was performed on a 2554 bp BamHI-KpnI fragment (nt 6150-880) subcloned into the pTZ19 vector (termed pTHBK) provided in the kit and following the manufacturer's instruction. All mutations were screened and confirmed by dideoxy sequencing (Sanger et al., 1977). To avoid any fortuitous mutations in the HPV coding region, a SphI-PpuMI (nt 7467-112) fragment containing mutations of the GRE at position 7640 was subcloned into a similarly digested wild type pTHBK construct. To insert the mutagenised fragment into the context of the whole HPV genome, a BamHI-NcoI fragment (nt 6150-863) from the mutants were ligated into a partial BamHI-NcoI digested wild type HPV genome. Mutants HPV 5 and HPV 6 created a BamHI and NsiI restriction site and mutations were pre-screened by restriction analysis and confirmed by sequencing, and were similarly ligated into the whole HPV genome. Double and triple GRE mutants were constructed by using

Table 3.1. Wild type (WT) and mutated glucocorticoid response elements present in the regulatory region of human papillomavirus 16 plasmids. Nucleotide sequences and their positions in the HPV 16 genome for the first nucleotide of each WT glucocorticoid response element are indicated. Mutations introduced by site-directed mutagenesis are indicated by lower case letters and the WT nucleotides of these mutants are indicated by dashes.

	Homology to consensus glucocorticoid response elements		
Plasmids	83%	75%	75%
	7389	7474	7640
pHPV (WT)	GCTACATCCTGTTTT	GGCACAAAATGTGTT	TGTACATTGTGTCAT
pmHPV2	---------------	---------------	--gt-------a---
pmHPV4	---------------	---------------	g----------tc-*
pmHPV5	---gg------a---	---------------	---------------
pmHPV6	---------------	----------ca--	---------------

* Consensus glucocorticoid response element.

the appropriate single stranded substrate for site-directed mutagenesis and one or two rounds of hybridization with appropriate oligonucleotides.

3.3.2 Preparation of primary baby rat kidney cells.

Five day old inbred Fischer rats were used to prepare primary kidney epithelial cells. Briefly, rats were decapitated and their kidneys removed through incisions made in the posterior sub-costal (kidney area) region. The kidneys were then cleaned free of connective tissue and washed five times in sterile PBS. Kidneys were then minced using a scalpel and trypsinized for about 1 hour using trypsin-EDTA at a volume of 1 ml/ pair of kidneys. The trypsinized cells were then suspended in DME containing 10% FCS, passed through a piece of sterile guaze and then plated at a concentration of 10^5 cells/60 mm tissue culture dish.

3.3.3 In vitro Transformation Assays.

For in vitro transformation assays BRK cells were fed with fresh medium (DME plus 10% FCS) and an hour later 5 μg each of HPV 16 and activated form of human c-Ha ras oncogene were cotransfected according to a modified calcium phosphate precipitation method (Chen and Okayama, 1988). Briefly, 1 ml of a $CaCl_2$/DNA mix (50 μl of 2.5M $CaCl_2$ plus DNA in dH_2O) was slowly mixed with 1 ml of 2X N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES)-buffered saline (50 mM BES, 280 mM NaCl and 1.5 mM $Na_2HPO_4.2H_2O$) and incubated for 10-20 minutes at room temperature. The mixture (500 μl/dish) was then slowly added into the dishes

with gentle swirling and left for 12-16 hours at 37°C in a humified incubator at a CO_2 concentration of 5%. The cells were then washed two times in PBS and fed with medium containing 10 % FCS in the presence or absence of 100 nM dexamethasone as described previously (Pater et al., 1988). Forty eight hours later the media was changed to DME with 2 % FCS with 0 or 100 nM dexamethasone. Foci of transformed colonies appeared in 3-4 weeks.

3.4 RESULTS

3.4.1 Effect of mutations at the previously characterised GRE on dexamethasone induced transformation of BRK cells.

The GRE at nt position 7640 was mutated to generate a loss-of-function mutation, or changed into a consensus GRE sequence (Table 3.1). With the wild type HPV 16 genome an approximately three-fold increase was observed in transformation in the presence of dexamethasone (Dex) (table 3.2). The loss-of-function mutation (pmHPV2) retained its response to dexamethasone in transformation assays and the response did not significantly differ from that of the wild type sequence. The consensus GRE mutant (pmHPV4) resulted in an overall increased frequency of transformation in the absence and/or presence of dexamethasone. As for the wild type construct, a three-fold induction was also observed in the presence of dexamethasone for this mutant. However, the number of colonies that appeared

Table 3.2. Effect of mutations of the known GRE at nt position 7640 on steroid hormone-mediated transformation of primary BRK cells by HPV 16 and activated EJ-ras oncogene.

Primary BRK cells were transfected with the indicated HPV 16 plasmids and activated EJ-ras and were cultured in the absence (-) or presence (+) of 100 nM dexamethasone. Colonies were counted after 4 weeks of selection in DME supplemented with 2% FCS. The number for each experiment represents the total number of colonies in sixteen 60 mm tissue culture plates.

[a]Wild type non induced (- Dex) transformation levels are normalized to 1.0 ± Standard deviation and values for mutated plasmids are given relative to wild type, to allow comparison of the experiments for the mutant HPV genomes.

[b]Induction levels are indicated as the +Dex:-Dex ratio.

Plasmid	Number of colonies														
	- DEXAMETHASONE						+ DEXAMETHASONE						Transformation[a]		
Expt.No:	1	2	3	4	5	6	1	2	3	4	5	6	- Dex.	+ Dex.	Induction[b]
pHPV16(WT)	12,	15,	10,	16,	18,	16	42,	48,	34,	39,	52,	50	1.00±0.20	3.0±0.3	3.0
pmHPV2	10,	10,	12,	10,	14,	12	38,	42,	38,	32,	40,	46	0.78±0.11	2.7±0.3	3.5
pmHPV4	18,	25,	14,	28,	35,	30	68,	70,	50,	78,	114,	110	1.70±0.5	5.6±1.7	3.3

was about 2 times more and the colonies appeared about a 7-10 days earlier compared to the wild type genome, indicating that this mutant is more effective than the wild type HPV in transforming BRK cells.. Retention of a glucocorticoid response with mutations of the known GRE lead me to speculate that either dexamethasone had an indirect effect in addition to a direct effect on transformation or that there were other unidentified GREs present in the regulatory region of the HPV 16 genome. An increased frequency of transformation by the consensus GRE mutant, confirmed that hormones have a direct effect. Overall, the results from this experiment indicated the known GRE at nt position 7640 was not sufficient to account for the dexamethasone induced transformation of BRK cells.

3.4.2 Identification of two novel GREs in the regulatory region of HPV 16 DNA.

The HPV 16 regulatory region was reexamined with a computer search for the existence of other putative GREs. Two more GRE like sequences 5' to the one already known were found. These two putative GREs were located from nt positions 7385-7499 and 7474-7488 and were strikingly similar to the one present at nt position 7640 (Table 3.1). Importantly, the GRE present at 7640 is not a typical GRE and diverges from the consensus GRE by three nucleotides (Pater et al., 1988). The two newly identified putative GREs also diverge from the consensus sequence, but at different positions.

3.4.3 Role of the two newly identified GREs in dexamethasone induced transformation of BRK cells.

To examine the possible involvement of these two putative GREs, site-directed mutagenesis was performed as shown in Table 3.1. The mutated GRE constructs at nt positions 7385 and 7474 are referred to as mHPV5 and mHPV6, respectively, in the context of the intact HPV 16 genome. For in vitro transformation assays only a double GRE mutant (pmHPV56) and a triple GRE mutant (pmHPV256) were used to examine the direct role of these GREs in glucocorticoid induced transformation. As HPV 16 DNA transforms BRK cells at a low frequency (average of 3-4 colonies per 60 mm dish), it was difficult to use all the possible combinations of GRE mutations in transformation assays and to arrive at a rational conclusion. Instead, a double GRE mutation (pmHPV56) was used to address whether the known GRE at nt position 7640 is sufficient by itself to respond to dexamethasone for transformation. This mutant retained a dexamethasone response and resulted in a two-fold increase in transformation (table 3.3) as compared to the three-fold observed for the wild type HPV genome. This retention of a dexamethasone response was significant, considering the low transforming activity of HPV 16 in BRK cells. It is not possible to comment on the relative contribution of the three GREs for transformation but it appears that the GREs at nt position 7385 and 7474 are functional because plasmid pmHPV2 did not significantly differ from the wild type HPV DNA in transformation assays (table 3.2). A triple mutant (pmHPV256) containing mutations at all the three GREs was then used. As shown in Table 3.3

Table 3.3. Effect of mutations of the all three GREs on dexamethasone induced transformation of BRK cells.

Primary BRK cells were transfected with the indicated HPV 16 plasmids as in table 3.2. Colonies were counted after 4 weeks of selection in DME supplemented with 2% FCS. The number in each experiment represents the total number of colonies in sixteen 60mm tissue culture plates. Colonies with the consensus GRE mutant (pmHPV4) gave rise to early appearing colonies compared to the other plasmids.

[a]Wild type non-induced (- Dex) transformation levels are normalized to 1.0 and values for mutated plasmids are given relative to wild type, to allow comparison of the experiments for the mutant HPV genomes.

[b]Induction levels are indicated as the +Dex:-Dex ratio.

Plasmid	Number of colonies								Transformation[a]		
	- DEXAMETHASONE				+ DEXAMETHASONE						
Expt.No:	1	2	3	4	1	2	3	4	- Dex.	+ Dex.	Induction[b]
pHPV16(WT)	22,	20,	16,	18	50,	55,	48,	45	1.0±0.14	2.6±0.4	2.6
pmHPV56	10,	10,	8,	10	18,	25,	16,	20	0.5±0.05	1.0±0.2	2.0
pmHPV256	18,	24,	16,	20	24,	27,	14,	22	1.0±0.18	1.1±0.3	1.1

this mutant almost completely abolished the wild type dexamethasone response. Results from the triple mutant demonstrated that dexamethasone induced transformation might occur through all three GREs and is most likely to be a direct effect since there was no significant residual dexamethasone response after destroying all three GREs.

In the following study, I have further confirmed the role of the three GREs for gene expression and also characterized the two newly identified GREs for specific in vitro DNA-protein interactions.

Chapter 4

Regulation of HPV type 16 gene expression in HeLa cells by steroid hormones and characterisation of two novel GREs in the HPV regulatory region

4.1 Introduction

Cervical carcinoma is one of the most common cancers in the world and second only to breast cancer as the leading cause of cancer related mortalities in women (Parkin et al., 1988). As discussed in previous sections, a large body of evidence suggests a causal role of human papillomaviruses in the pathogenesis of various human malignancies, especially cervical carcinoma. However, it is also clear that HPV infection alone is not sufficient to account for the transforming abilities of these viruses and that other cofactors and cellular genetic events are required for a fully malignant phenotype. More recently, attention has been focussed to study the various cofactors involved in HPV-induced cancers. In my previous study I had attempted to address the role of one of the physiologically important cofactor, steroid hormones, in HPV-mediated transformation of cultured rodent cells.

In this chapter, the direct role of steroid hormones in HPV gene expression was examined in detail to explain some of the results obtained in the transformation assays. In this regard two novel GREs were found, in addition to a previously characterised GRE at nt position 7640. Mutations created in the known and the two

newly identified GREs were used to ask if they are required for induction of HPV 16 gene expression by hormones. For this purpose, the full enhancer fragments of the HPV 16 DNA, containing all three GREs, were cloned upstream to a reporter CAT gene driven by the tk promoter in pBLCAT2 expression vector (Luckow and Schutz, 1987). Different combinations of the three GRE mutations were used to study their role in hormone induction. In addition the two newly identified GREs were further characterised for their ability to respond to glucocorticoids and to interact with the steroid hormone receptor.

4.2 Materials

Some of the materials used in this study have already been described in previous sections. Thin layer chromatography (TLC) plates and X-ray films were purchased from Kodak. Acetyl coenzyme A, 0-Nitrophenyl β-D-Galactopyranoside (ONPG), and protease inhibitors antipain, pepstatin A and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma. [^{32}P]-dCTP and [^{35}S]-methionine were obtained from Amersham. The glucocorticoid receptor-expressing (pHGO) and β-galactosidase-expressing (pCH110) plasmids were a generous gift from Dr. P. Chambon, whereas, the plasmid pBLCAT2 was obtained from Dr. B. Luckow.

4.3 Methods

4.3.1 Plasmids.

Site-directed mutagenesis and construction of the GRE mutations have been described earlier in chapter 3. For the pCPH series of constructs, a pStI-HpHI (nt 7007-7770) fragment of the wild type and mutated HPV genomes was blunt ended by T4 DNA polymerase and cloned in the correct orientation into a reverse transcribed BamH1 site of the pBLCAT2 vector (Luckow and Shutz, 1987). The pCD series of constructs were prepared by cloning a 232 bp blunt-ended DraI-DraI (nt 7522-7754) fragment of the wild type and mutated HPV genomes into the reverse transcribed BamH1 site of pBLCAT2 vector.

Double-stranded oligonucleotides with XbaI overhangs and corresponding to the GREs at position 7385 (GRE5WT) and 7474 (GRE6WT) were annealed in buffer containing 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, and 50 mM NaCl, kinased with T4 polynucleotide kinase and then ligated into the Xba1 site in pBLCAT2 vector as a monomer. The sequence of the double stranded (ds) oligonucleotides used in this study is as follows with the GRE shown in bold and XbaI overhangs in lower case letters :

GRE5WT 5' ctagATTT**GCTACATCCTGTTTTTGT** 3'
3' TAAA**CGATGTAGGACAAAAA**CAgatc 5'

GRE6WT 5' ctagATTT**GGCACAAAATGTGTTTTT** 3'
3' TAAA**CCGTGTTTTACACAAAAA**gatc 5'

The HPV 16 sequences for GRE5WT and GRE6WT were from nt 7381-7402 and 7471-7488, respectively.

4.3.2 Cell lines and culture.

All the cell lines used in this study (HeLa, CV1) were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS).

4.3.3 Transfections and transient CAT assays.

For transfections HeLa cells were cultured in DME containing 5% dextran-coated charcoal treated FCS. Dextran-coated charcoal treatment was done according to Horwitz and McGuire (1978). Ten μg of the indicated CAT construct were transfected, by the calcium phosphate precipitation method (Sambrook et al., 1989), into 70% confluent cells with 2 μg HGO (glucocorticoid receptor expressing plasmid) and 5 μg pCH110 (a β-galactosidase expressing plasmid as an internal control for efficiency of transfection) to a total of 20 μg DNA per 100 mm tissue culture plates. Transient assays were performed as previously described (Gorman, 1982) and normalized with equal amounts of β-galactosidase activity. β-galactosidase activity was determined according to the protocols by Pharmacia. Briefly, extracts were mixed with Z-buffer (60 mM Na_2PO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, 50 mM β-mercaptoethanol, pH adjusted to 7.0) in a total of 200 μl reaction volume. Reactions were initiated by adding 40 μl of ONPG (4 mg/ml in 100 mM phosphate buffer, pH 7.0) and incubating at 37^{o}C untill a faint yellow colour appears. Reactions were stoped at this time by adding 100 μl of 1M Na_2CO_3. Absorption was measured at A_{420} and β-galactosidase activity/ml extract was measured as per the calculations given below:

$$\frac{\beta\text{-galactosidase units}}{\text{ml extract used}} = \frac{A_{420}/0.0045}{\text{Reaction time (min) X Volume (ml)}}$$

4.3.4 Preparation of whole cell extracts.

Preparation of whole cellular extracts were according to Tasset et al. (1990). Briefly, cultured cells were scraped and collected in Falcon tubes, washed twice in PBS and lysed with equal pellet volumes of high-salt extraction buffer (0.4 M KCl, 20 mM Tris-HCl pH 8.0, 2 mM DTT, 20% glycerol and protease inhibitors: 5 mM PMSF and 1μg/ml each of pepstatin A and antipain) by three cycles of feeze-thaw in liquid nitrogen and ice, respectively. The homogenate was microcentrifuged at 4^{o}C for 15 min and supernatant aliquoted, flash frozen in liquid nitrogen and then stored at -70^{o} C. Protein concentration was measured by the method of Bradford (1976).

4.3.5 Preparation of probes.

For mobility shift and south-western blotting, GRE5WT and GRE6WT ds oligonucleotides were end labelled by filling in the Xba1 overhangs using [^{32}P]-dCTP and reverse transcriptase.

Probes for UV-crosslinking were prepared by nick-translating GRE5WT and GRE6WT ds oligonucleotides in the presence of 0.15mM bromodeoxyuridine and [^{32}P]-dCTP.

4.3.6 Mobility Shift Assays and UV-crosslinking.

Mobility shift assays were done according to the methods of Chadosh et al. (1988) with slight modifications. Binding reactions of 10 μl were carried out in buffer containing 12 mM Hepes (pH 7.9), 4mM Tris-HCl (pH 7.9), 10% (w/v) glycerol, 0.1 mM EDTA, 1 mM DTT, 5 μg of poly (dI-dC), 60 mM KCl, $1x10^4$ CPM of end-labelled probes and the indicated amounts of cellular extracts for 20 min at room temperature. Free and protein bound DNA were seperated on 4% nondenaturing polyacrylamide (acrylamide:bisacrylamide, 29:0.5, v/v) gels which were run at 4°C and a constant voltage of 100 V in 22 mM Tris-borate, 0.5 mM EDTA. Gels were then dried and autoradiographed. Where indicated, radioinert specific and non-specific competitors were included in binding reactions.

For UV-crosslinking, binding reactions of 25 μl were done as described for mobility shift assays using 15 μg of poly (dI-dC), $5x10^5$ CPM of radiolabelled probes and the indicated amounts of cellular extracts for 20 min at room temperature and then exposed to UV irradiation under the Fotodyne UV lamp (maximum emmission wavelength 310 nm and maximum intensity of 7,000 fw/cm^2) for 15 min. The reactions were stopped by adding equal volumes of 2X SDS-loading buffer (100mM Tris-HCl pH 6.8, 200mM DTT, 4% SDS, 20% glycerol and 0.2% bromophenol blue). The reactions were not treated with DNAaseI and micrococcal nuclease, since the size of the probe used was less than 50 base pairs. To determine the molecular weight sizes of the protein/s interacting with the probe, the reaction mixture was boiled for 3 min and then subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel at a constant voltage of 100 volts. The gel was then dried on a 3mm Whatman

paper and exposed for autoradiography.

4.3.7 South-Western Blot Analysis.

South western blotting was done according to the methods of Silva et al. (1987) with some minor modifications. The indicated amounts of cellular extracts were seperated on a 7.5% denaturing SDS-PAGE gel and transferred to a BA85 nitrocellulose membrane by wet electro-transfer in Tris-Glycine buffer (25mM Tris-HCl, 190mM glycine). The filters were blocked in binding buffer (containing 5% nonfat dry milk in 50 mM NaCl/10 mM Tris-HCl,pH 7.4/1 mM EDTA) in a heat sealable plastic bag overnight (12-15 hrs) and then probed, in the same buffer (containing 0.25% nonfat dry milk), using 10^6cpm/ml of the indicated end-labelled Oligonucleotides (GRE5WT and GRE6WT) for 2-3 hours at room temperature. After binding, the filter was washed in four 30 min washes in the binding buffer, air dried and then exposed for autoradiography.

4.4 RESULTS

4.4.1 Role of the three GREs in dexamethasone induced HPV 16 gene expression in cervical keratinocytes.

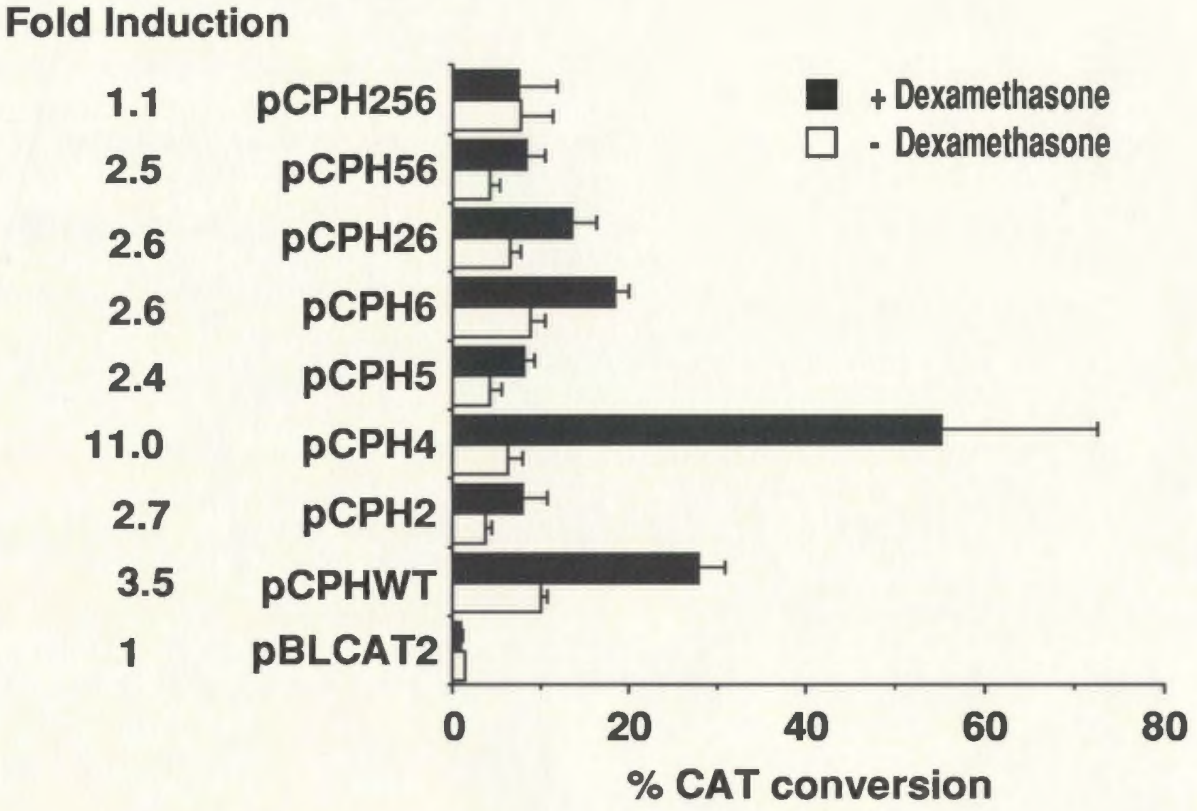
To examine the functional role of steroid hormones in inducing HPV 16 gene expression in a transient assay system, the full enhancer fragment of HPV 16 DNA

were used. These enhancer fragments were cloned in the CAT expression vector, pBLCAT2. Single, double and triple GRE mutants were transfected in the cervical keratinocyte cell line (HeLa) and harvested for the expression of CAT gene in the absence or presence of 100 nM dexamethasone. The wild type construct (pCPHWT) showed an approximately three-fold activity in the presence of dexamethasone (fig. 4.1), which is in agreement with the results for transformation assays (table 3.2). As shown in fig 4.1 single GRE mutant pCPH2, retains a 2.7-fold response to dexamethasone, whereas pCPH4, which contains the consensus GRE mutation results in a considerably higher induction (more than ten-fold), compared to the wild type construct. Mutants pCPH5 and pCPH6 were still responsive to dexamethasone even though the level of induction had decreased. Double GRE mutants pCPH26 and pCPH56 resulted in a lower level of inducibility but did not completely eliminate induction. The triple mutant (pCPH256) completely abolished dexamethasone induced HPV gene expression. These transient gene expression studies support the conclusion that all three GREs are responsive to dexamethasone and supports the suggestion that glucocorticoid induced transformation of BRK cells is due to increased HPV 16 gene expression by steroid hormones (Pater et al.,1988, 1990).

4.4.2 Effect of an "A to C" conversion at the +5 position of the nt 7640 GRE.

The GRE at nt position 7640 is a composite GRE (cGRE) which contains an overlapping AP-1 binding site (Table 4.1, Chan et al., 1990). In the consensus GRE mutant the overlapping AP-1 motif is destroyed and therefore it was possible that the

Figure 4.1. Glucocorticoid induction of gene expression from HPV enhancer region containing different combinations of the three GRE mutations. Ten μg of the indicated CAT constructs were transiently transfected in HeLa cells in addition to a β-galactosidase expression vector as described in Methods. pCPHWT refers to the wild type PstI-HphI fragment of HPV 16 enhancer region. Constructs containing mutations at the three GREs have been shown in table 3.1 and are numbered accordingly. pCPH2 and 4 are single GRE mutants, pCPH26 and 56 are double GRE mutants and pCPH256 is a triple GRE mutant. CAT values are expressed as a percentage of acetylated chloramphenicol conversion and has been normalized with equivalent amounts of β-galactosidase activity present in the extracts. Induction (fold induction) by dexamethasone for the various plasmids has been corrected for the observed downregulation of CAT expression by dexamethasone for the control plasmid pBLCAT2.

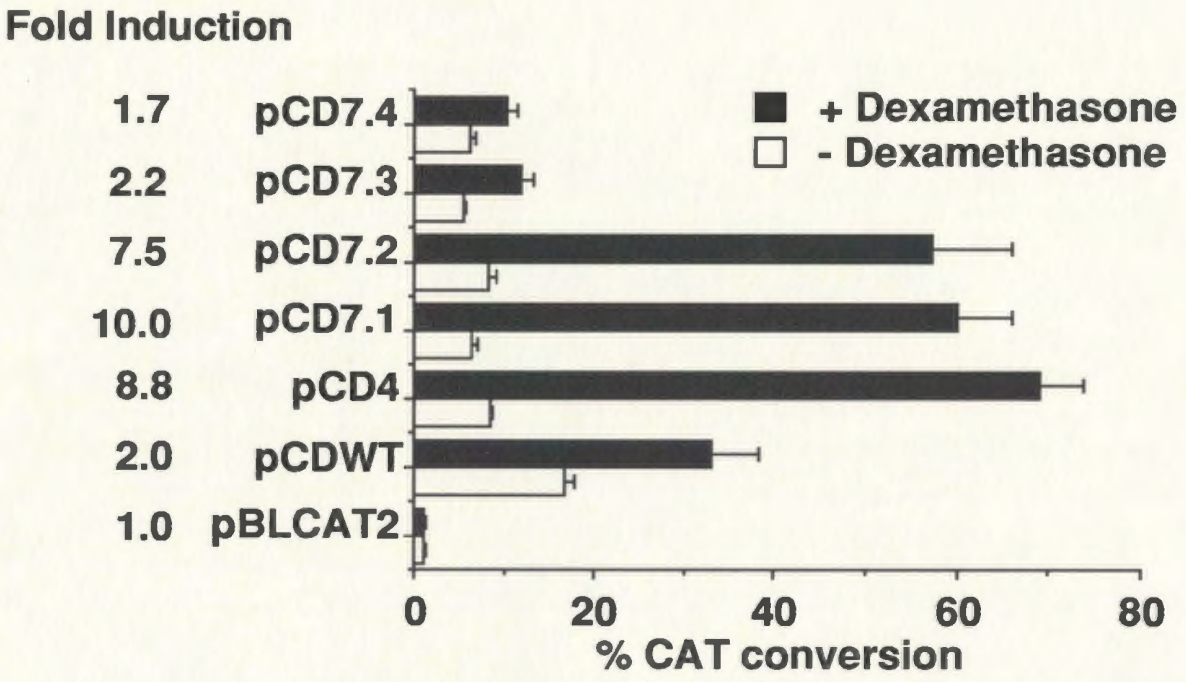

Fold Induction
1.1
2.5
2.6
2.6
2.4
11.0
2.7
3.5
1
pCPH256
pCPH56
pCPH26
pCPH6
pCPH5
pCPH4
pCPH2
pCPHWT
pBLCAT2
+ Dexamethasone
- Dexamethasone
0
20
40
60
80
% CAT conversion

observed induction levels with this mutant could be either due to higher binding affinity of the glucocorticoid receptor, or as suggested previously, due to release from possible steric hinderance or mutual competitive inhibition between the GR and AP-1 factors (Chong et al., 1990). In search for suitable explanations for the very high levels of hormone induction observed with the consensus GRE mutation (pCPH4) and to examine the possibility of a competitive inhibition between GR and AP-1, several different mutations were created in the overlapping AP-1 motif without disrupting the nucleotides important for receptor interaction (shown in table 4.1). A DraI-DraI (nt 7522-7754) HPV 16 enhancer fragments containing only the nt 7640 GRE, and either wild type or the indicated AP-1 mutations, were cloned in the pBLCAT2 vector. These plasmids were tested for dexamethasone induction of CAT expression in transiently transfected HeLa cells. As shown in fig. 4.2 only those plasmids with AP-1 mutations containing the "C" at position +5 of the GRE (pCD7.1 and pCD7.2) resulted in dexamethasone induction comparable to the consensus GRE mutant (pCD4), whereas, constructs (pCD7.3 and pCD7.4) containing an "T" (table 4.1) did not. In addition, these constructs gave lower levels of induction compared to the wild type sequence in pCDWT. Although these four constructs had the mutated form of AP-1 motif, only those with a "C" at +5 position gave high induction levels by dexamethasone, indicating that this effect could be due to increased affinity of the GR to bind to this sequence and not due to loss of AP-1 motif. If a competitive inhibition model existed, one would expect to see similar induction levels with all four mutants. Supporting this view is the finding that a "C" at position +5

Table 4.1. Mutations created in the AP-1 motif overlapping the GRE at nt position 7640. The pCD series of plasmids represents a DraI-DraI fragment (7522-7754) of the HPV 16 enhancer region. Shown are the sequences of the consensus GRE mutation and consensus AP-1 binding site. The GRE in the wild type HPV construct (pCDWT) and the consensus GRE mutation (pCD4) is shown in bold and the nucleotides important for AP-1 binding in the concensus AP-1 sequence is shown double-underlined. Mutations created in the overlapping AP-1 motifs in pCD7.1 to pCD7.4 are underlined.

Plasmids	Sequence
consensus (pCD4)	**GGTACA**TTG**TGTTCT**
consensus AP-1 motif	TGAGTCA
pCDWT (7640)	**TGTACA**TTG**TGTCAT**
pCD7.1	GCTGTAC
pCD7.2	GATGTAC
pCD7.3	GCTGTAT
pCD7.4	GATGTAT

Figure 4.2. Glucocorticoid induced CAT gene expression from plasmids containing mutations of the AP-1 motif overlaping the composite GRE. CAT assays and transfections were done as described in figure 4.1. CAT values were normalized with equal amounts of β-galactosidase activity and dexamethasone fold induction was normalized with the control plasmid, pBLCAT2.


Fold Induction
1.7
pCD7.4
2.2
pCD7.3
7.5
pCD7.2
10.0
pCD7.1
8.8
pCD4
2.0
pCDWT
1.0
pBLCAT2
+ Dexamethasone
- Dexamethasone
0
20
40
60
80
% CAT conversion

of a GRE palindrome is important for high affinty receptor binding (Noordeen et al., 1990). However, the above mentioned experiments do not address the function of this AP-1 binding motif in glucocorticoid mediated induction of the wild type HPV 16 cGRE. This question has been dealt with in greater detail in chapter 5 of this thesis and the possible role of jun/fos oncogenes in glucocorticoid induction has been addressed.

4.4.3 Functional analysis of the newly identified GREs for gene expression and specific DNA-Protein interactions.

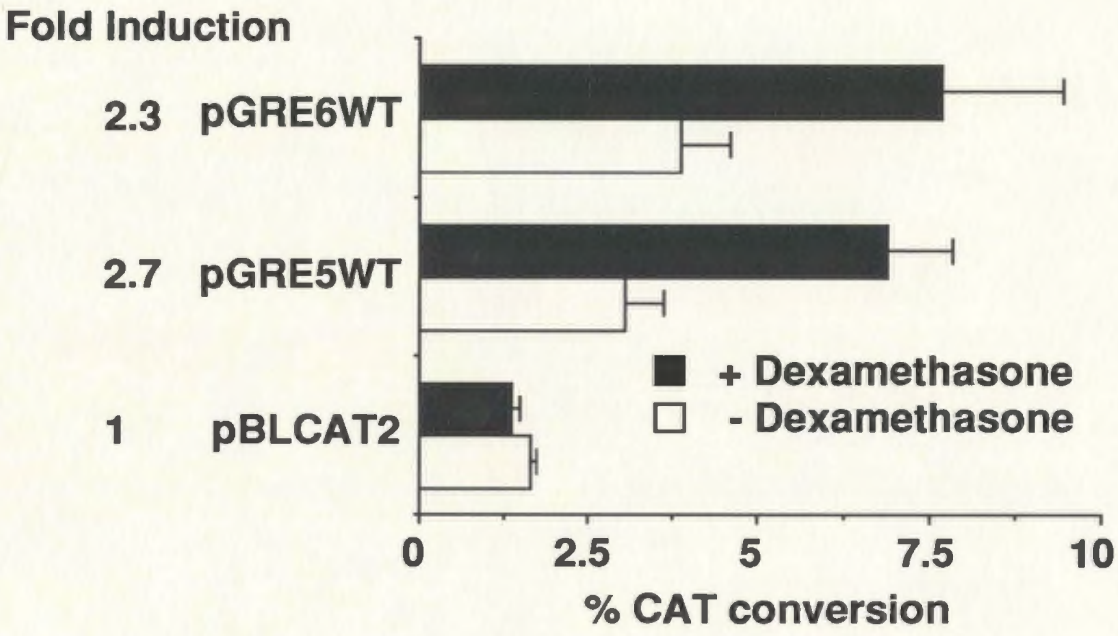
(a). Transient assays.

To ascertain glucocorticoid induction by the two GREs at nt positions 7385 and 7474, double stranded oligonucleotides corresponding to both elements (GRE5WT and GRE6WT) were cloned upstream to the CAT expression vector, pBLCAT2. These constructs were then tested for their ability to confer a hormone-induced CAT gene expression in HeLa cells. As shown in fig. 4.3, both sequences resulted in more than 2.5-fold induction, indicating that both putative GREs are in fact glucocorticoid responsive sequences.

(b). Mobility shift assays.

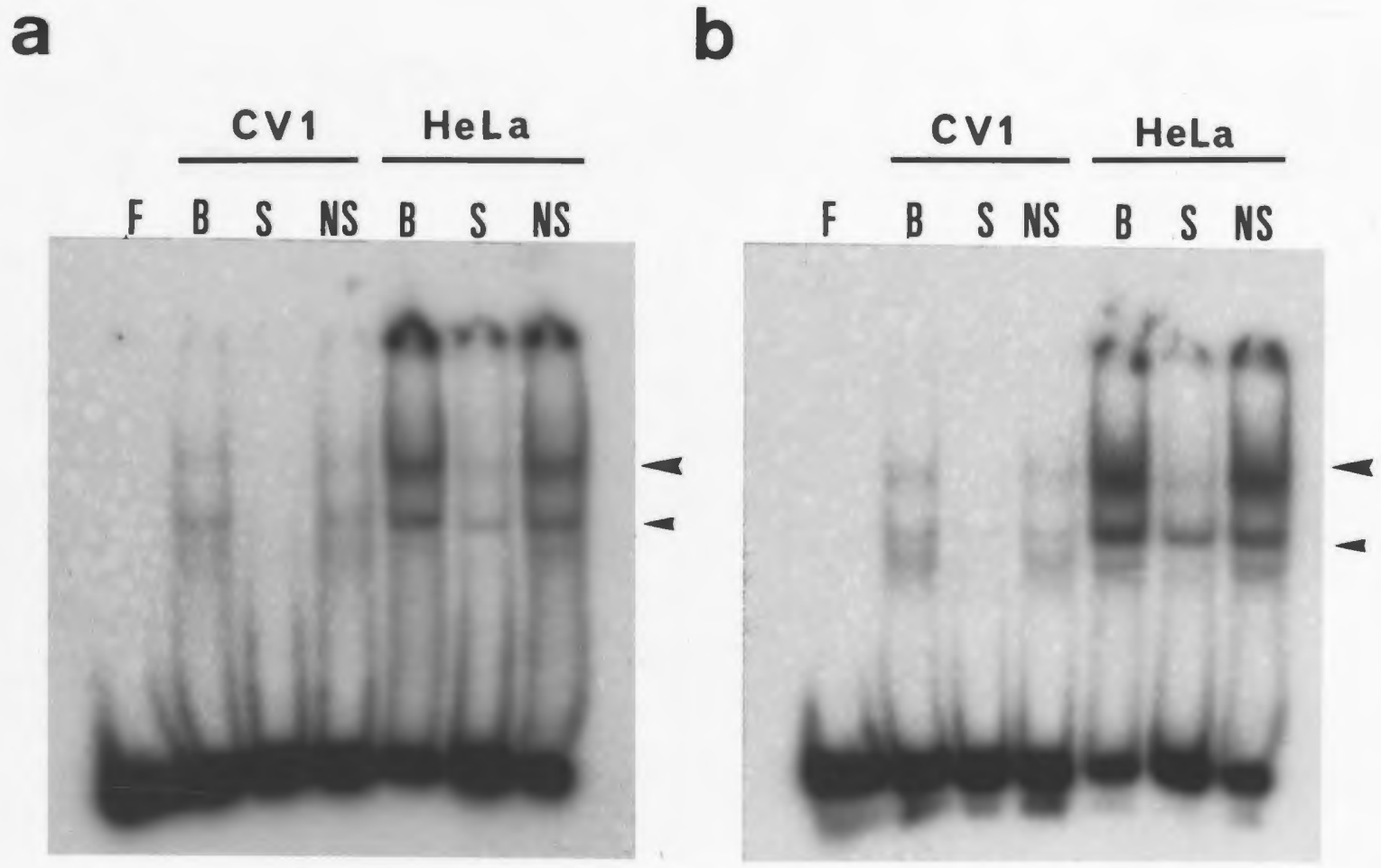
Mobility shift assays were done using GRE5WT and GRE6WT double-stranded oligonucleotides as probes and crude cellular extracts from HeLa cells and from a

Figure 4.3. Glucocorticoid induced gene expression from plasmids containing GRE5WT and GRE6WT ds oligonucleotides in a cervical carcinoma cell line. Ten μg each of pBLGRE5WT and pBLGRE6WT were transfected along with β-galactosidase internal control plasmid into HeLa cells. CAT values were normalized to equal amounts of β-galactosidase activity and fold induction by dexamethasone was normalized to the control plasmid, pBLCAT2.


Fold Induction
2.3 pGRE6WT
2.7 pGRE5WT
1 pBLCAT2
+ Dexamethasone
- Dexamethasone
0
2.5
5
7.5
10
% CAT conversion

monkey kidney cell line CV1. CV1 cells were used as a control as a source of low glucocorticoid receptor levels compared to HeLa cells (Pearce and Yamamoto, 1993). As shown in fig. 4.4 both GRE5WT and GRE6WT bound to a protein present in the HeLa cell extracts resulting in a retarded band. Similarly, retarded bands of identical mobility were also observed from CV1 extracts, but as is clear from fig. 4.4, the intensity of these bands were much lower than that obtained from HeLa cell extracts. Specificity of binding was confirmed in both cases using a 1000-fold molar excess of the homologous unlabelled oligonucleotide as a specific competitor. A region II NF1 binding motif of the JC virus was utilized as a non-specific competitor (Kumar et al., 1993). Gel shift assays demonstrated formation of a specific complex (shown by arrows in fig. 4.4). A second faster migrating complex was also observed even in the presence of specific competitor. Analogous banding patterns have been observed in previous studies using enriched partially purified glucocorticoid receptor preparations (Nemoto et al., 1990). Although similar banding patterns obtained from two distinct probes points out that both might be interacting with a common protein, it cannot be ruled out that some other host cellular factor(s) also interacts with an unknown overlapping sequence(s). Due to the unavailability of the glucocorticoid receptor monoclonal antibody it was not posssible to conclusively say that the protein interacting with the two sequences is the glucocorticoid receptor. However, these results indicated that host protein(s) in cellular extracts from HeLa cells forms a specific complex with the two GREs.

Figure 4.4. Mobility shift assays with GRE5WT and GRE6WT oligonucleotide probes. 10 μg protein of either CV1 or HeLa cellular extracts were incubated with $1x10^4$ cpm (0.1 ng) of end-labelled GRE5WT (a) or GRE6WT (b) and analysed on a 4% polyacrylamide gel as described in methods. A 1000-fold molar excess (100 ng) of non-radioactive homologous specific (S) or heterologous non-specific competitors (NS) containing human JC virus NF1 motif were used in the binding reactions. F = Free (no protein); B = Bound (no competitor). Arrows indicate the formation of specific complexes.


a
CV1
HeLa
F
B
S
NS
B
S
NS
b
CV1
HeLa
F
B
S
NS
B
S
NS

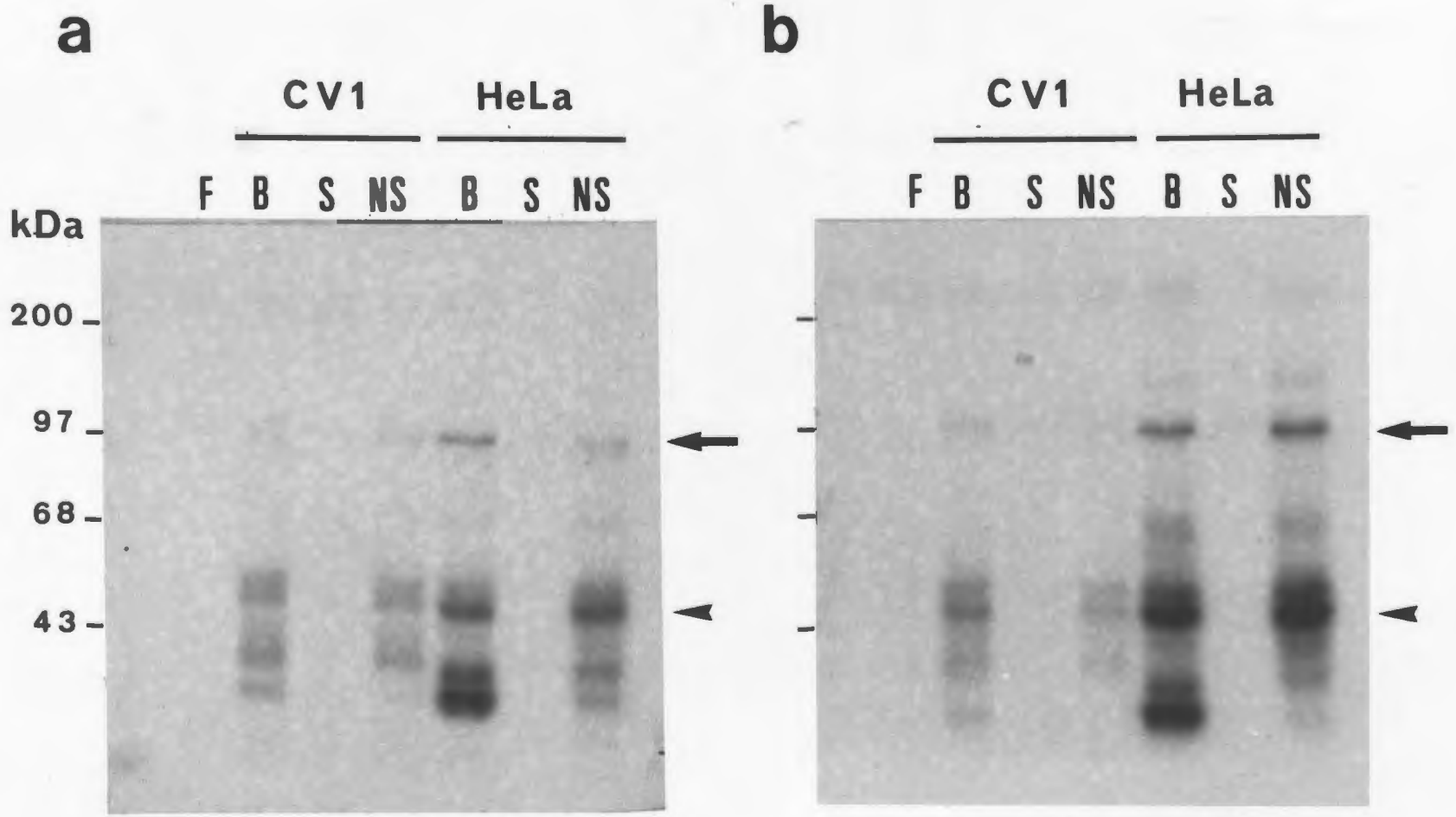
(c). UV-crosslinking.

To further continue with the assumption that glucocorticoid receptor is the most likely protein interacting with these sequences, a more sensitive method of UV-crosslinking was used. The same cellular extracts were used to examine the molecular sizes of the proteins binding to the GREs in the gel shift assays. As shown in fig. 4.5, two major bands were detected, one about 96-kDa and the other 45-kDa in size (indicated by arrows in fig. 4.5). Both oligonucleotides gave an exactly similar pattern from CV1 or HeLa cellular extracts, though in CV1 extracts the bands were considerably weaker. Specificity of binding was further confirmed using non-radioactive specific and nonspecific competitors as indicated. Since the 96-kDa band corresponds to the native size of the glucocorticoid receptor phosphoprotein (Hollenberg et al., 1985), the results strongly indicate that the receptor protein is capable to interact with both GREs. The 45-kDa and a few slower migrating bands could be degradation products of the receptor molecule. Similar and several other proteolytic degradation products have been commonly described in the literature and frequently observed in receptor purification studies (Govindan and Sekeris, 1978; Singh and Moudgil, 1985; Govindan and Gronemeyer, 1984). The marked similarity of banding patterns combined with competition analysis suggest that both GREs are competent for receptor interaction.

(d). South-western blot analysis.

Selective and specific protein interactions to the GRE oligonucleotides was further

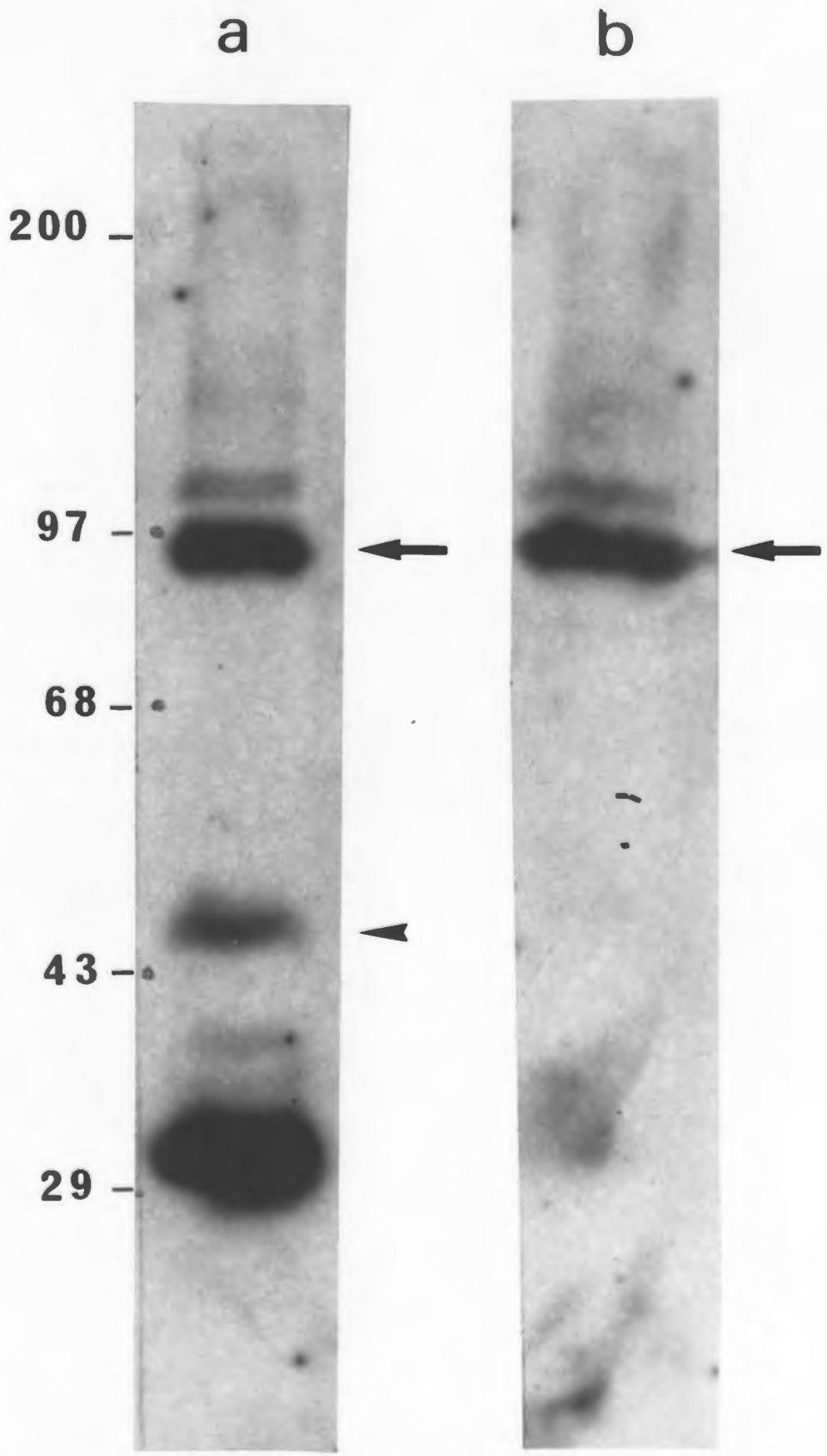
Figure 4.5. UV-Crosslinking with GRE5WT and GRE6WT oligonucleotide probes. $5x10^4$ cpm of nick translated and 5'-bromodeoxyuridine incorporated GRE5WT (a) and GRE6WT (b) probes were incubated with 30 μgs of either CV1 or HeLa cellular extracts, UV irradiated and then subjected to SDS-PAGE. A 1000-fold molar excess (800 ng) of non-radioactive homologous oligonucleotide (S) or heterologous DNA containing the JC virus NF1 motif were used in the binding reactions (NS) as competitors. F = Free (no protein); B = Bound (no competitor). Arrows indicate the 96-kDa and 45-kDa proteins respectively. High molecular weight standard protein markers are indicated.


a
CV1
HeLa
F B S NS B S NS
kDa
200
97
68
43
b
CV1
HeLa
F B S NS B S NS

studied using a southwestern blot analysis in which HeLa cellular extracts were separated by SDS-PAGE, immobilized on a nitrocellulose membrane and then probed with end-labelled oligonucleotides corresponding to the two GREs. As expected and shown in fig. 4.6, both oligonucleotides bound to a 96-kDa protein, the glucocorticoid receptor, present in the extracts (Hollenberg et al., 1985). With GRE5WT, a 45-kDa band also appeared (fig. 4.6), although this was not a consistent finding with either probe. Another very prominent band of about 29-kDa size was also observed in cellular extracts with the GRE5WT probe. This band most likely represents histones present in the whole cellular extracts. Depending on the washing conditions this particular band was variably present sometimes using either probe. It is likely that both GREs are binding the glucocorticoid receptor because although they do not share an identical sequence, they do give similar patterns of interactions with three different assays.

In the following study, I have examined the function of the overlapping AP-1 motif at the nt 7640 GRE and the role of the c-jun and c-fos protooncogenes in glucocorticoid-mediated induction of HPV expression.

Figure 4.6. South-Western blotting with GRE5WT and GRE6WT probes using HeLa cellular extracts. 300 μg of whole cell extracts from HeLa cells were seperated by SDS-PAGE, transferred on a BA85 nitrocellulose membrane and probed with $1x10^6$ cpm/ml of end-labelled GRE5WT (a) or GRE6WT (b). A prominent 96-kDa band (arrow) and a 45-kDa band (arrow head) were observed for GRE5WT. A smaller 29-kDa protein also bound to the GRE5WT probe, which most likely represents binding to histones. High molecular weight standard protein markers are indicated.


a
b
200
97
68
43
29

Chapter 5

Role of c-jun and c-fos protooncogenes in glucocorticoid-mediated induction of HPV 16 gene expression.

5.1 Introduction

Eukaryotic gene regulation is a complex process which is usually regulated at the level of transcription and involves a multitude of transcription factors interacting with specific DNA sequence elements located upstream to or within the promoter region (McKnight and Tjian, 1986; Dynan, 1989; Maniatis et al., 1987). Recently, novel patterns of gene expression have been demonstrated by regulatory factors belonging to different families that seem to interact with each other at DNA sequence elements possessing weak or low-affinity binding sites. One such element that has attracted some recent attention is a "composite GRE" (cGRE), which consists of binding sites for the glucocorticoid receptor and AP-1 protein and was first described in the rat proliferin gene promoter (Diamond et al., 1990). As discussed earlier in chapter 1, section 1.6, glucocorticoid receptors are members of the steroid hormone receptor superfamily. AP-1 protein, on the other hand, comprises of a distinct set of products encoded by a gene family characterized by the presence of a DNA binding dimerization motif called the leucine zipper (reviewed in Landschulz et al., 1988). The transcription factor AP-1 is generally regarded as a homodimer of c-jun or a heterodimer of c-jun/c-fos oncogenes (Chiu et al., 1988) and binds with high affinity

to an AP-1 motif, also called TPA response element (Lee et al., 1987). More recently, several studies have demonstrated the physical and functional interaction of glucocorticoid receptor and the AP-1 class of proteins, which modulates transcription by a variety of different mechanisms (reviewed in Schule and Evans, 1991 and references therein). The rat proliferin gene cGRE is regulated either positively or negatively, depending on whether the glucocorticoid receptor interacts with c-jun homodimers or c-jun/c-fos heterodimers (Diamond et al., 1990; Miner and Yamamoto, 1992). In addition, cell type-specific factors belonging to other members of the steroid hormone receptor and the AP-1 family, regulates transcription from the proliferin cGRE in diverse ways, quite often with opposite effects (reviewed in Miner and Yamamoto, 1991; Pearce and Yamamoto, 1993).

The nt 7640 GRE in the HPV 16 LCR is actually a cGRE with an overlapping AP-1 binding motif (Chan et al., 1990). The objective of this study was to examine the role of this cGRE and the AP-1 class of proteins, c-jun and c-fos, in the glucocorticoid-mediated activation of HPV 16 gene expression. For this purpose, I have used the embryonal carcinoma cell line, P19, which is thought to contain none or very low levels of AP-1 activity (de Groot et al., 1990). The full-length HPV 16 enhancer containing the wild type nt 7640 GRE or mutated forms of this element were cloned upstream to a CAT reporter gene to address the role of AP-1 in glucocorticoid-mediated activation. In addition, a definitive role of the cGRE was established using enhancer fragments containing only this element.

5.2 Materials.

P19 cells were obtained from Dr. H. Hamada. The c-jun and c-fos expressing plasmids were a kind gift from Dr. P. Chambon. Flow Laboratory supplied the α-minimal essential media (α-MEM). The rest of the materials used in this study have been previously described.

5.3 Methods.

5.3.1 Plasmids

All the plasmids used in this study have been described earlier in Chapter 4, section 4.3 (table 5.1).

5.3.2 Cell culture, transfections and CAT assays.

P19 cells were maintained in α-MEM supplemented with 10% heat-inactivated fetal calf serum. For transfections, cells were passed in 60 mm tissue culture plates in α-MEM containing 5% heat inactivated fetal calf serum. 12-16 hours later cells were fed with the same media and transfected 1 hour later, by the calcium phosphate precipitation method, with the indicated CAT construct, a glucocorticoid-receptor expressing plasmid, a β-galactosidase expressing plasmid as an internal control and the indicated amounts of c-jun and c-fos expressing plasmids. Cells were glycerol shocked after 4 hours for 3 minutes and fresh media was added with 0 or 100 nM dexamethasone. Cells were harvested 48 hours after transfection for CAT assays (Gorman et al ., 1982). The values are given as a percentage of CAT conversion

Table 5.1. Plasmids and mutations created at the nt 7640 cGRE. Shown are the wild type and mutant composite GRE sequence. Nucleotides important for AP-1 binding are double-underlined in the wild type sequence, whereas, the mutations created are shown underlined. The GRE palindrome represents sequences from nt 7640-7654 of the HPV genome.

* Partially destroyed AP-1 motif.

** Completely destroyed AP-1 motif.

Plasmids	cGRE Sequence	Motif destroyed
pCPHWT/ pCDWT	TGTACAT**TGT**G**TCA**T	none
pCPH2/ pCD2	TGG**T**C**A**TTGTG**A**CAT	GRE and AP-1*
pCPH4/ pCD4	**G**GTACATTGTGT**T**C**A**T	AP-1*
pCD7.2	TGTACAT**GA**TGT**AC**T	AP-1**

after normalizing with equal amounts of β-galactosidase activity present in the extracts.

5.4 Results

5.4.1 Effect of c-jun and c-fos in glucocorticoid-mediated induction of wild type HPV 16.

To examine the role of c-jun and c-fos in glucocorticoid-mediated activation of HPV 16, I have used the pCPHWT construct which contains the full-length wild type HPV 16 enhancer. As shown in fig. 5.1a, increasing amounts of c-jun resulted in a progressive increase of the basal level non-induced activity. Addition of dexamethasone resulted in a further increase in expression, indicating that c-jun confers a positive hormone-response to the wild type HPV 16 enhancer. However, the presence of c-fos, either transfected alone or with c-jun, inhibited this positive response. Thus, like the rat proliferin gene, c-jun and c-fos behaves as selectors of hormone response. No significant effect of c-jun or c-fos was observed on the hormone response of the control plasmid, pBLCAT2 (fig. 5.1b).

5.4.2 Effect of c-jun and c-fos in glucocorticoid-mediated activation of mutated HPV 16.

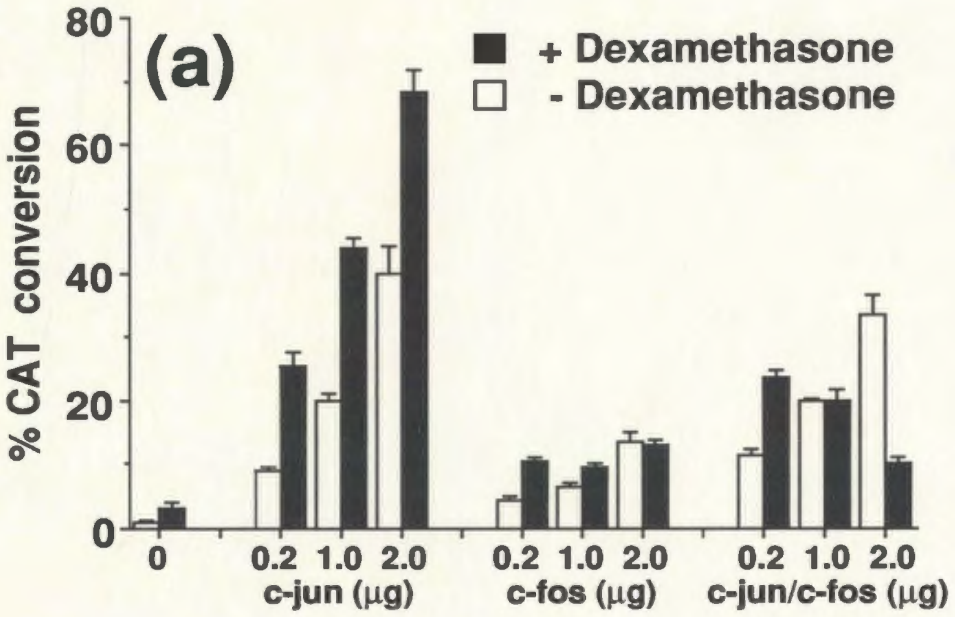
Next, I used the pCPH2 and pCPH4 plasmids to examine if the above observed

effect was mediated through the nt 7640 cGRE. In pCPH2, both the GR-binding and AP-1 motifs are destroyed, whereas in pCPH4, the cGRE is converted into a simple consensus GRE with the AP-1 motif partially destroyed (table 5.1). Thus, the loss-of-function mutation of both the GRE and overlapping AP-1 motif in pCPH2 was used to establish whether this sequence is required for AP-1-regulated hormone response of the HPV 16 enhancer. The pCPH4 construct was used to ask if this particular AP-1 motif is involved. As shown in fig. 5.2, cotransfection of increasing amounts of c-jun with either construct fails to confer a positive dexamethasone-mediated activation, as observed for the wild type construct. In addition, presence of c-fos inhibited hormone response. These results indicate that the positive glucocorticoid response by c-jun is mediated by the nt position 7640 cGRE (pCPH2, fig. 5.2a), and also, the overlapping AP-1 motif appears to be required for this effect (pCPH4, fig. 5.2b). It also appears that in the presence of low concentrations of c-jun, both pCPH2 and pCPH4 responded to dexamethasone. This result might indicate that the adjacent AP-1 motif at nt 7629 which is only 4 base-pairs away from the cGRE might have some influence on hormone response.

5.4.3 Effect of c-jun and c-fos on glucocorticoid-mediated regulation of cGRE containing plasmids.

Since there are three GREs and three AP-1 motifs in the HPV 16 LCR (Chan et al., 1990; Mittal et al., 1993), it was important to examine the effects of c-jun and c-fos in constructs that do not contain the two other GREs at nt position 7385 and 7474.

Figure 5.1. Effect of c-jun and c-fos on glucocorticoid-mediated induction of wild type full-length enhancer. P19 cells were transfected with 5 μg of the reporter plasmid pCPHWT (a), or the control plasmid pBLCAT2 (b), 2 μg of GR expressing plasmid, 1 μg of β-galactosidase-expressing plasmid and the indicated amounts of AP-1-expressing plasmids. Cells were harvested after 48 hours and CAT activity determined as the percentage conversion of [^{14}C]-chloramphenicol to acetylated forms. CAT values have been normalized relative to the β-galactosidase activity.


(a)
+ Dexamethasone
- Dexamethasone
% CAT conversion
80
60
40
20
0
0
0.2 1.0 2.0
c-jun (μg)
0.2 1.0 2.0
c-fos (μg)
0.2 1.0 2.0
c-jun/c-fos (μg)

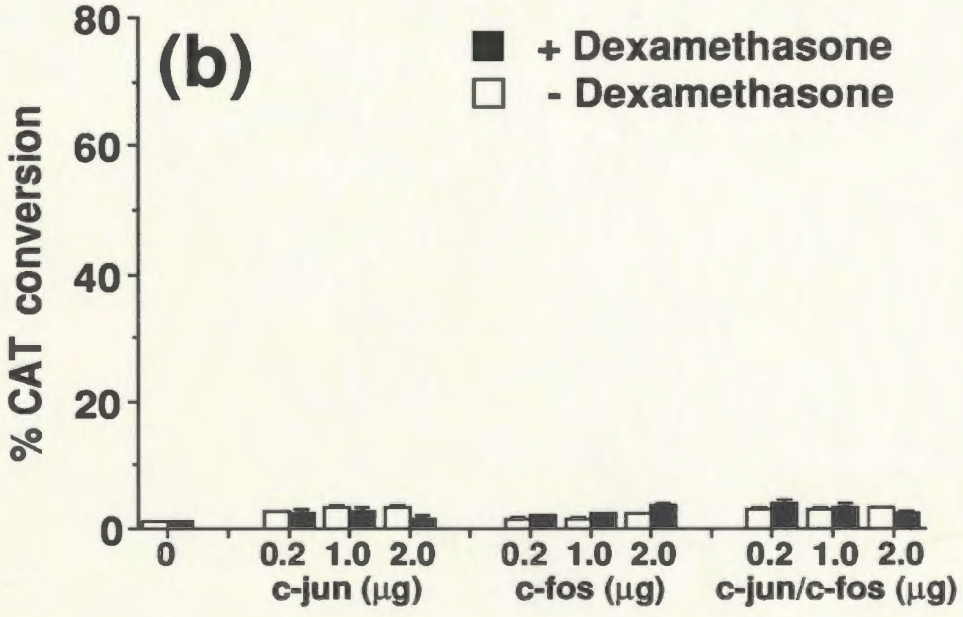
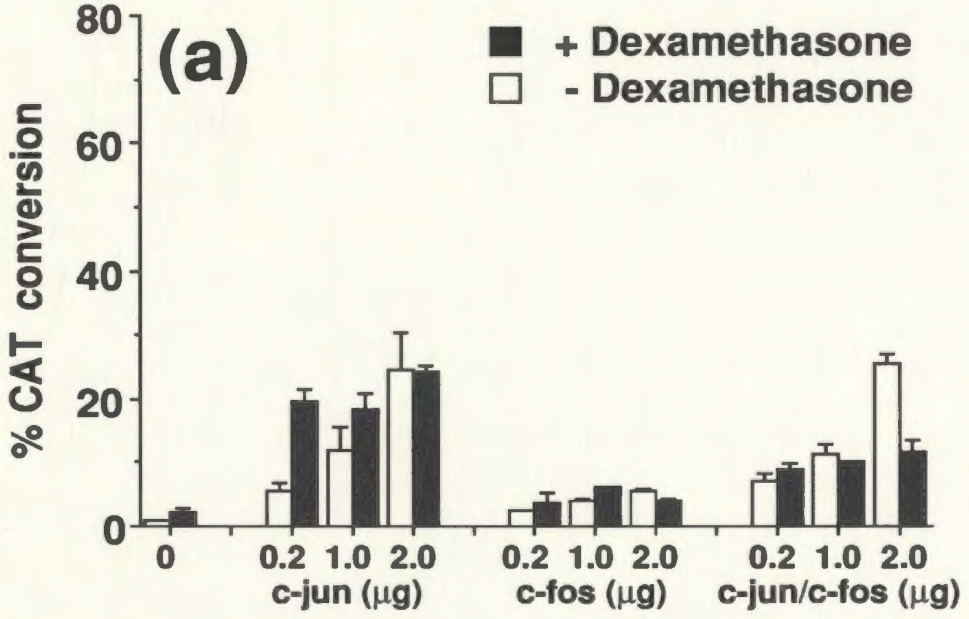
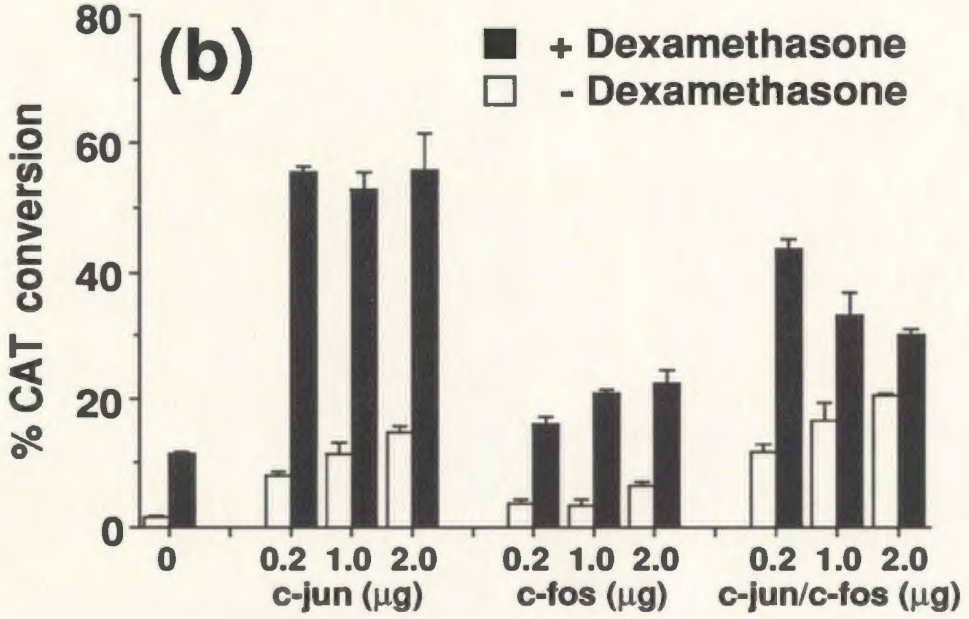

(b)
+ Dexamethasone
- Dexamethasone
% CAT conversion
80
60
40
20
0
0
0.2 1.0 2.0
c-jun (μg)
0.2 1.0 2.0
c-fos (μg)
0.2 1.0 2.0
c-jun/c-fos (μg)

Figure 5.2. Effect of c-jun and c-fos on glucocorticoid-mediated induction of mutant full-length enhancer. P19 cells were transfected with the reporter plasmid pCPH2 (a), and pCPH4 (b). Labels are as for fig. 5.1.


(a)
+ Dexamethasone
- Dexamethasone
% CAT conversion
80
60
40
20
0
0
0.2 1.0 2.0
c-jun (μg)
0.2 1.0 2.0
c-fos (μg)
0.2 1.0 2.0
c-jun/c-fos (μg)

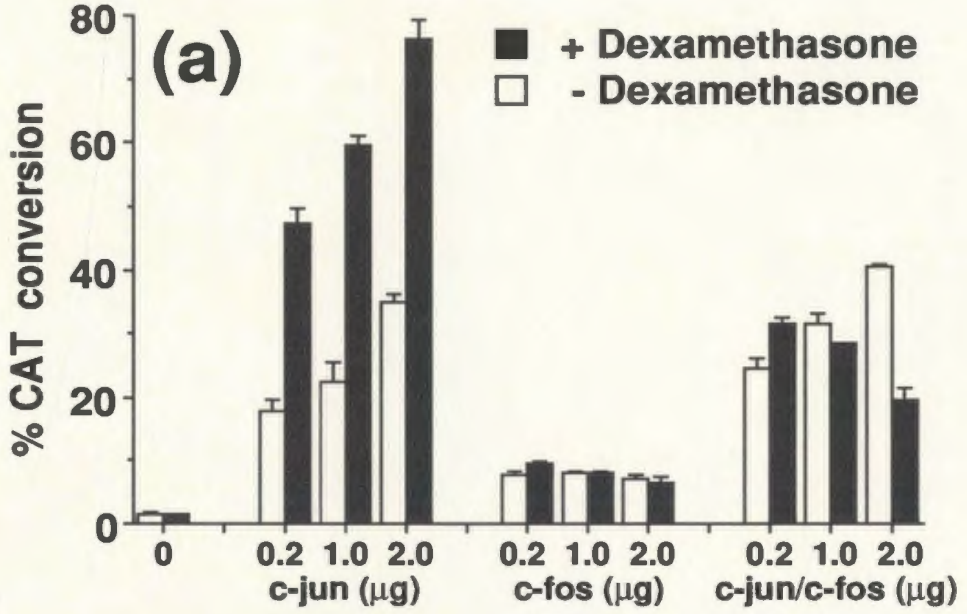

(b)
+ Dexamethasone
- Dexamethasone
% CAT conversion
80
60
40
20
0
0
0.2 1.0 2.0
c-jun (μg)
0.2 1.0 2.0
c-fos (μg)
0.2 1.0 2.0
c-jun/c-fos (μg)

For this purpose the pCD-series of constructs, that contains only the nt 7640 GRE were used. These constructs also contained an additional consensus AP-1 motif from nt position 7629-7636, that is 4 base-pairs from the cGRE. However, mutations of the AP-1 motif overlapping the nt 7640 GRE were useful to establish a definitive role of the cGRE in hormonal regulation of HPV expression.

As shown in fig. 5.3a, the wild type cGRE in pCDWT gave results that were similar to the full-length HPV enhancer containing plasmid (pCPHWT, fig. 5.1a). Increasing amounts of c-jun increased the basal level activity which was further increased in the presence of dexamethasone. c-fos, on the other hand inhibited this positive regulation by hormones. Interestingly, the wild type sequence was not responsive to glucocorticoids in the absence of transfected c-jun (fig. 5.3a). Activation by dexamethasone was observed in the presence of c-jun, indicating that this particular GRE is functional only in the presence of c-jun. No significant dexamethasone-activation was observed in the presence of c-fos or c-jun/c-fos. Instead, c-fos inhibited the positive dexamethasone response mediated by c-jun. In contrast to these results, the full-length enhancer was responsive to dexamethasone even in the absence of transfected AP-1 (pCPHWT, fig. 5.1a), indicating that the other two GREs at nt position 7385 and 7474 were probably responsible for this induction.

Mutations of both the GRE and AP-1 in pCD2 confirmed that the positive effect of dexamethasone is mediated through the cGRE (fig. 5.3b). Partial destruction of the AP-1 motif in pCD2 and pCD4 resulted in a similar loss of

Figure 5.3. Effect of c-jun and c-fos on glucocorticoid-mediated activation of wild type and mutant cGRE containing plasmids. P19 cells were transfected with the reporter plasmids pCDWT (a) and pCD2 (b), pHGO and a β-galactosidase-expressing plasmid. CAT activity was determined as for fig. 5.1.


(a)
+ Dexamethasone
- Dexamethasone
% CAT conversion
80
60
40
20
0
0
0.2 1.0 2.0
c-jun (μg)
0.2 1.0 2.0
c-fos (μg)
0.2 1.0 2.0
c-jun/c-fos (μg)

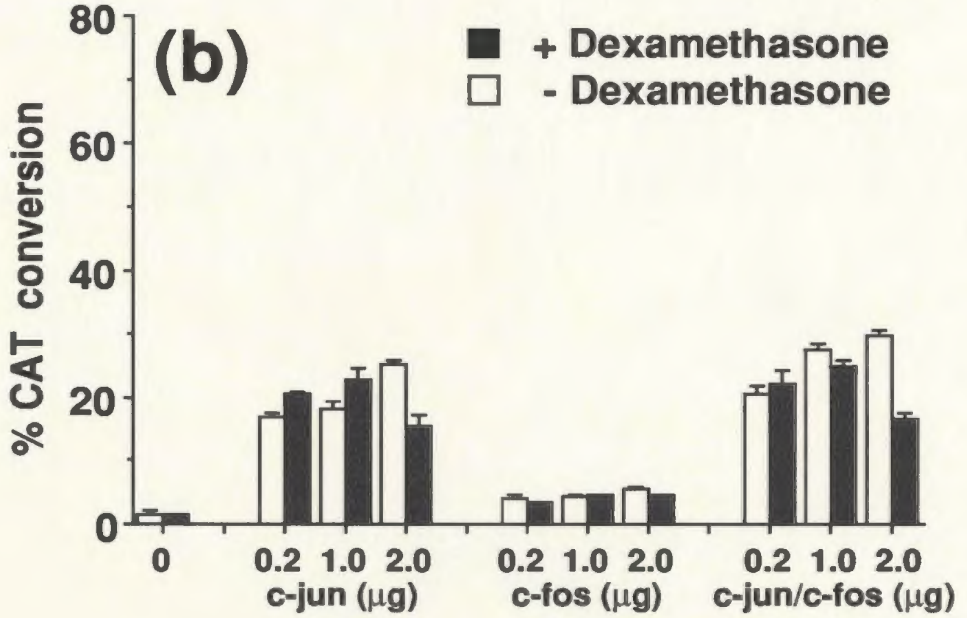
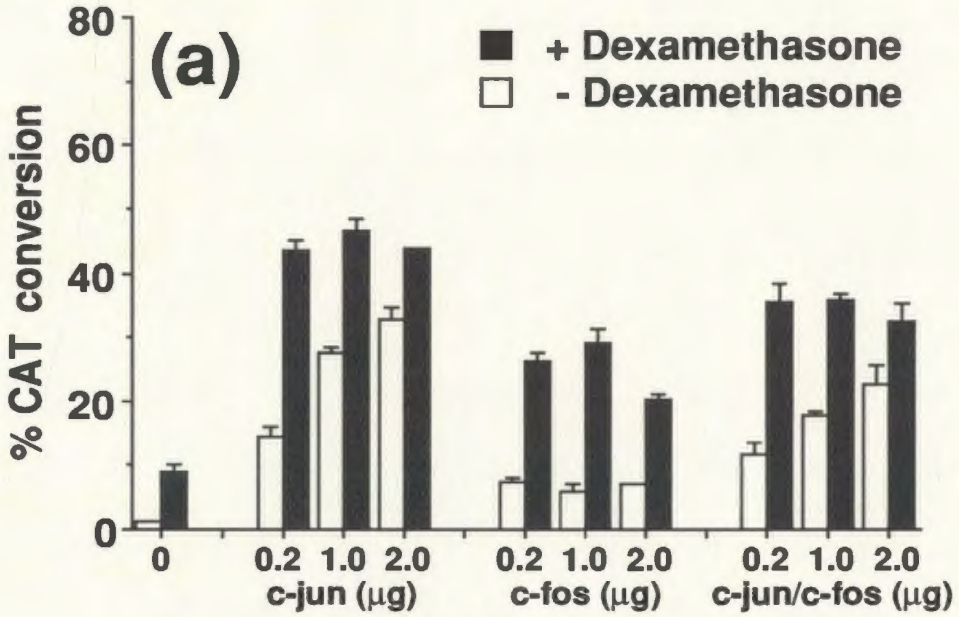
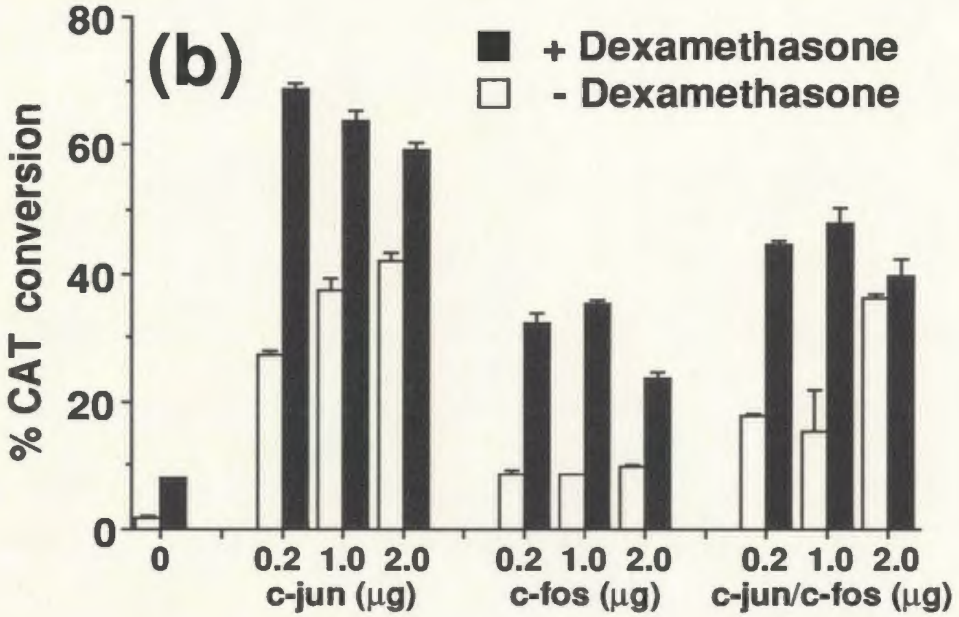

(b)
+ Dexamethasone
- Dexamethasone
% CAT conversion
80
60
40
20
0
0
0.2 1.0 2.0
c-jun (μg)
0.2 1.0 2.0
c-fos (μg)
0.2 1.0 2.0
c-jun/c-fos (μg)

Figure 5.4. Effect of c-jun and c-fos on glucocorticoid-mediated activation of mutant cGRE containing plasmids. P19 cells were transfected with the reporter plasmids pCD4 (a) and pCD7.2 (b).


(a)
+ Dexamethasone
- Dexamethasone
% CAT conversion
80
60
40
20
0
0
0.2 1.0 2.0
c-jun (μg)
0.2 1.0 2.0
c-fos (μg)
0.2 1.0 2.0
c-jun/c-fos (μg)


(b)
+ Dexamethasone
- Dexamethasone
% CAT conversion
80
60
40
20
0
0
0.2 1.0 2.0
c-jun (μg)
0.2 1.0 2.0
c-fos (μg)
0.2 1.0 2.0
c-jun/c-fos (μg)

hormone-regulation by c-jun (fig. 5.3b and 5.4a). Enhancement of transcription by GR from simple GREs in pCD4 and pCD7.2 was not significantly affected by transfection of c-jun (fig 5.4). However, unlike the wild type construct (pCDWT), the pCD4 mutant responded to dexamethasone without any transfected c-jun. This result has clearly indicated that cGREs require c-jun for positive regulation, whereas, simple GREs do not. To establish a definitive role of the AP-1 motif, I have used another mutation, pCD7.2 (table 5.1, Chan et al., 1990), which completely destroys the AP-1 motif overlapping the cGRE. Similar results were also obtained by this mutant indicating that the AP-1 motif is required for positive dexamethasone regulation by the c-jun protooncogene (fig 5.4b). Interestingly, all three cGRE mutations, pCD2, pCD4 and pCD7.2, responded to c-jun and c-jun/c-fos in enhancing the basal level activity with increasing amounts of these proteins (fig 5.3 and 5.4). This enhancement occurred even with the partial or complete loss of AP-1 motif in the above mentioned constructs and indicated that the induced basal levels could be due to the adjacent AP-1 motif at nt 7629. Another interesting observation is that transfected c-fos alone stimulated CAT expression above the basal expression from all plasmids, although c-fos alone does not dimerize and has no AP-1 activity. This suggests that P19 cells express a protein from the jun family with low intrinsic activity (den Hertog, et al., 1992) and heterodimerizes with c-fos to form functional AP-1.

Chapter 6

Human papillomavirus type 16 gene expression in primary cervical keratinocytes: Role of progesterone and glucocorticoids.

6.1 Introduction

As previously discussed, HPV 16 is the most prevalent high-risk HPV strongly associated with high grade cervical intraepithelial neoplasia (CIN) and invasive cervical carcinomas. In addition, HPV 16 DNA-containing lesions also have a strong tendency to progress through successively higher grades of CIN (Weaver et al., 1990; Kadish et al., 1992). Significantly, the DNA of HPV 16 is episomal in benign and low grade CIN lesions, while in most cervical tumors and all cervical tumor cell lines it is integrated into the host genome (Durst et al., 1985; Cullen et al., 1991; Durst et al., 1983; Pater et al., 1985). These integration events have been shown to cause oncogenic changes in viral (zur Hausen, 1991; Smits et al., 1991; Durst et al., 1987a) and cellular (Durst et al., 1987b) gene expression. Progression from low grade dysplasia to invasive carcinoma is likely to depend on the cumulative effect of a number of other cellular genetic changes (Sreekantaiah et al., 1988; Riou et al., 1988). Hence, it is essential to understand not only the events occurring at the early stages of HPV infection, but also the viral and/or host regulatory factors and the physiological signals (such as steroid hormones) that modulate responses of these regulators. Normal increases of these hormones, especially progesterone, during

pregnancy and female ovulatory cycles or nonphysiological increases as seen for oral contraceptive users, can result in disturbances of the normal controls of HPV expression by cellular regulators.

The objective of this study was to address the role of the three GREs in modulation of HPV type 16 gene transcription in primary human ectocervical keratinocytes, the natural host cell for HPV infection. In chapter 4 and 5, I studied the functional requirement of HPV 16 GREs in the context of heterologous promoters. The levels at which hormones directly regulate HPV 16 expression from its own promoter has not been addressed. To achieve this goal, HPV 16 whole genomes with various combinations of mutated GREs were prepared and transfected into cultured primary human cervical cells. Due to the limited supply of human cells, in situ hybridization was used to detect the presence of virus-specific mRNA as a function of HPV 16 gene expression.

6.2 Materials

Cervical tissues for preparation of ectocervical cells were kindly provided by Dr. J. Williams, L. Simms and the staff of St. Clare's Mercy hospital, St. John's, Newfoundland. All tissue culture was performed with keratinocyte growth media (KGM) obtained from Clonetics, San Diego, CA. Lipofectin, Biotin-7-dATP, nick-translation kit and the DNA detection kit were obtained from BRL (Gaithersberg, VA). Biotin-11-dUTP was obtained from Enzo Diagnostic Inc., New York, NY.

RU486 was a kind gift from D. Martini, Roussel Uclaf, Romainville, France. Ki67 monoclonal antibody was obtained from Dakopatts, High Wycombe, Bucks, U.K. Biotrace HP membranes for Southern blotting were obtained from Gelman Sciences.

6.3 Methods:

6.3.1 Plasmids

Constructions of plasmids (HPV genomes with wild type or mutated GREs) used in this study have been described in chapter 3. The constructed mutations are shown in Table 6.1.

6.3.2 Preparation and transfection of primary cervical cells

Ectocervical epithelial cell cultures were derived from cervical specimens obtained from hysterectomies performed for benign conditions and shown to be free of cervical intraepithelial neoplasia by histological examination. Primary cultures were initiated by G. Jin in our laboratory and done according to the methods of Turyk et al. (1989). Briefly, cervical tissues were washed twice in solution A containing keratinocyte basal media (KBM) supplemented with penicillin, streptomycin and amphotericin B. Using a punch biopsy, small tissue explants containing both stroma and epithelium were removed from the ectocervical region and digested in collagenase for 2 hours. The epidermis was subsequently seperated, minced with a scalpel and trypsinized for 3 minutes at room temperature in a 1:1 ratio of trypsin-EDTA in PBS. Trypsin

Table 6.1. Wild type (WT) and mutated GREs present in the regulatory region of human papillomavirus 16 plasmids. Nucleotide sequences and their positions in the HPV 16 genome for the first nucleotide of each WT GRE are indicated. Mutations introduced by site directed mutagenesis are indicated by lower case letters while the WT nucleotides of these mutants are indicated by dashes.

Homology to consensus the glucocorticoid response element

Plasmids	83%	75%	75%
	7389	7474	7640
pHPV (WT)	GCTACATCCTGTTTT	GGCACAAAATGTGTT	TGTACATTGTGTCAT
pmHPV2	---------------	---------------	--gt-------a---
pmHPV4	---------------	---------------	g----------tc- *
pmHPV26	---------------	----------ca--	---gt------a---
pmHPV25	---gg------a---	---------------	--gt-------a---
pmHPV56	---gg------a---	----------ca--	---------------
pmHPV256	---gg------a---	----------ca--	--gt-------a---

* Consensus GRE.

digestion was stopped by adding PBS containing 10% FCS and cells were briefly centrifuged and plated in KGM in 100 mm tissue culture plates. For transfection, cells were seeded onto coverslips (22 X 22 mms) placed in 30 mm tissue culture plates, incubated for 48 hours in hydrocortisone-free KGM and transfected by lipofection using 3 μg of the indicated HPV genomes and 10 μl of lipofection solution. After transfection, cells were incubated in hydrocortisone-free KGM or media containing the indicated hormones (dex., prog. or dex. plus RU486) for another 48 hours before preparing them for in situ hybridization detection of HPV RNA.

6.3.3 In situ hybridization

In situ hybridization detection of viral message was done as described by Lawrence and Singer (1986). Briefly, the cells grown on coverslips were fixed in 4% paraformaldehyde in PBS containing 5 mM $MgCl_2$ and either were used immediately for hybridization or stored in 70% ethanol for later use. The cells on coverslips were rehydrated for 10 minutes in both PBS plus 5 mM $MgCl_2$ and 0.2 M Tris-Hcl pH 7.4, 0.1 M Glycine. Prior to hybridization the cover slips were transferred to 50% formamide, 2X SSC and incubated at 65^{o}C for 10 min. The probe was prepared from HPV 16 genomic DNA (BamHI fragment) nick-translated in the presence of biotin-7-dATP or biotin-11-dUTP, precipitated in the presence of 20 μg tRNA and 20 μg sheared salmon sperm DNA and dissolved in 100% deionized formamide. After heat denaturation the probe was brought to a final concentration of 50%

formamide, 2X SSC, 0.2% BSA, 10% dextran sulfate and vanadyl-sufate ribonucleotide complex RNAase inhibitor. The probe was used at a concentration of 5-10 ng/μl and detection was with alkaline phosphatase using the BRL DNA detection kit. The results shown are typical for cells observed in a minimum of three independent experiments.

6.3.4 Indirect immunofluoresence

For indirect immunofluoresence assays, HPV 16 transfected ectocervical cells were incubated in either hydrocortisone-free KGM or media containing a 1:5 molar ratio of 100 nM dexamethasone to RU486 for 48 hours. Cells were fixed in 50% acetone-methanol and processed for immunofluoresence according to Bartek et al (1990), using a 1:500 dilution of Ki67 monoclonal antibody.

6.3.5 Hirt extraction and Southern blot analysis

Ectocervical cells were plated in 60 mm tissue culture dishes, transfected by lipofection with 10 μg of wild type HPV 16 plasmid and incubated in KGM in the presence of 0 or 100 nM dexamethasone. After 48 hours, cells were washed twice in PBS and then lysed in 500 μl Hirt lysis buffer (0.01M Tris-HCl pH 8.0, 0.01M EDTA and 0.6% SDS) for 15 min at room temperature (Hirt, 1967). NaCl was added to a final concentration of 1M and samples were placed at 4 ^{o}C for 12-16 hrs. The mixture was microcentrifuged at 8000 rpm for 90 min to pellet cellular DNA and debris. The supernatants were extracted once with phenol and phenol-chloroform

and twice with chloroform, and precipitated overnight at -20 °C with three volumes of 95% ethanol. Low molecular weight (LMW) DNA of the supernatant was resuspended in 25 μl of 1X TE. High molecular weight (HMW) genomic DNA was also extracted from the cellular pellet by resuspending and incubating the pellet in digestion buffer (100 mM NaCl, 10 mM Tris-HCl pH 8, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K) for 12-16 hrs at 50°C, extracted once with phenol and phenol-chloroform and ethanol precipitated. The recovered DNA was resuspended in 25 μl of 1X TE.

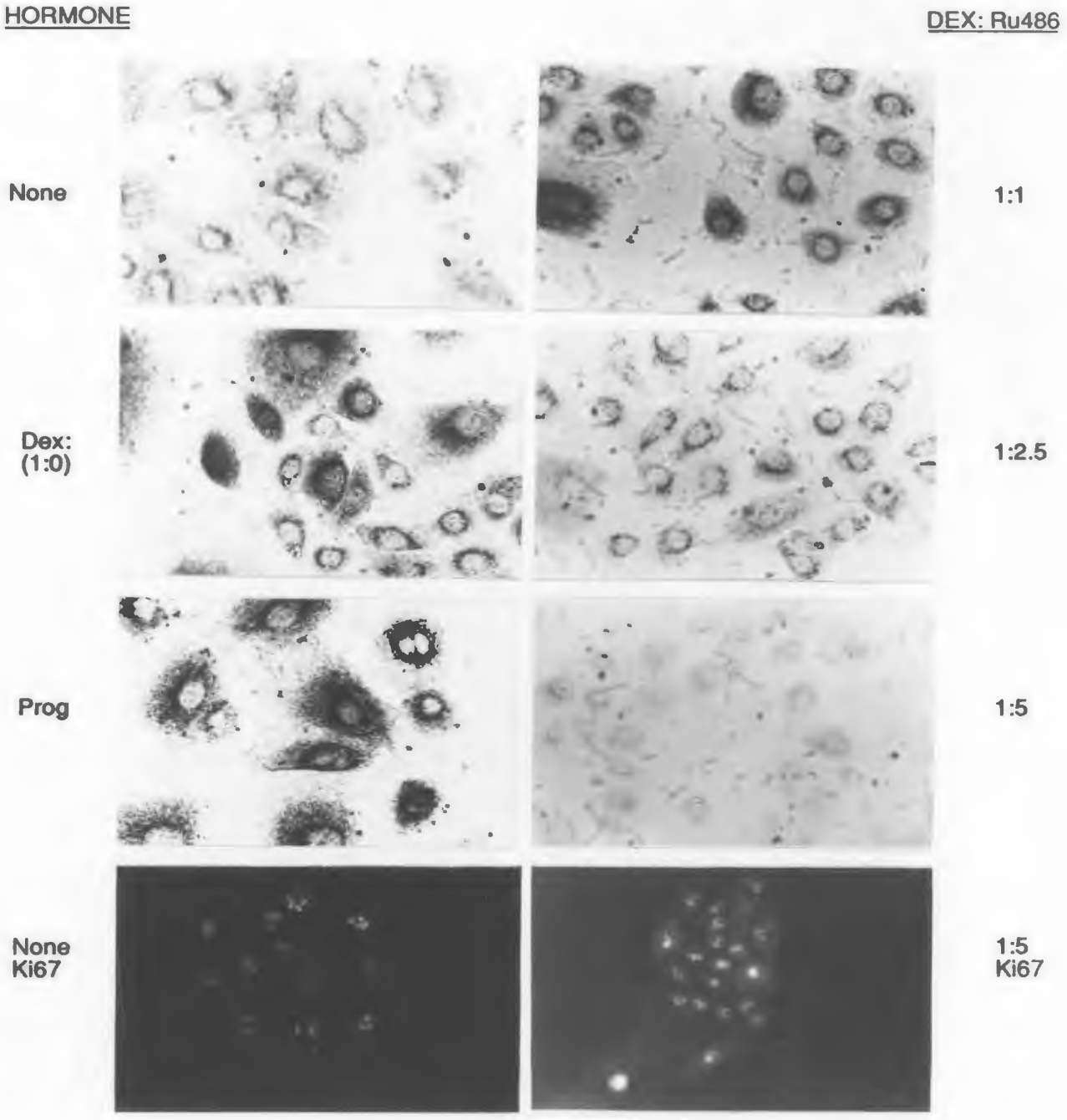
Southern blotting was performed according to the protocols provided by Gelman Sciences. Equal amounts of LMW and HMW DNA were digested with indicated restriction enzymes, electrophoresed on a 1% agarose gel, transferred to Biotrace HP membranes, baked at 80°C for 1 hr, prehybridized for 30 min at 65°C in hybridization buffer (containing carnation 1% non-fat dry milk, 1 mM EDTA, 7% SDS and 0.5 M NaH_2PO_4) and probed in the same buffer with a nick-translated ([^{32}P]-dCTP labelled) BamH1 fragment of HPV 16 DNA. After washing the filter in wash solution I (40 mM NaH_2PO_4, 1 mM EDTA, 5% SDS and 0.5% skimmed milk) and wash solution II (20 mM NaH_2PO_4, 1 mM EDTA and 1% SDS), it was exposed to a Kodac X-ray film at -70°C with intensifying screens.

6.4 RESULTS

6.4.1 In situ detection of viral message transcribed from HPV 16 genomes containing wild and/or mutant GRE sequences.

Intact HPV 16 genomes were introduced by lipofection into cultured human ectocervical cells and viral gene expression was monitored by in situ hybridization. In the absence of hormones there was no detectable RNA signal (Fig. 6.1, left panel). However, a marked increase in the levels of HPV RNA was observed after treatment with either dexamethasone (Dex) or progesterone (Prog). To examine whether the absence of detectable HPV gene expression in hormone-free medium involved a block in cell proliferation, expression of nuclear antigen for Ki67 monoclonal antibody was used (Gerdes et al., 1983). Positive Ki67 staining verified the presence of dividing cells in cultures grown in the absence of steroid hormones (Fig. 6.1, bottom left panel). These results indicated that neither the presence of HPV 16 episomes nor the transfection process prevented cell-cycling, and the induction of viral RNA by hormones was not due to release of cells from growth arrest, but was specific for HPV expression. The ability of the in vitro model system to distinguish different expression levels was examined. First, the consensus GRE-mutated plasmid was used to examine the mutations effect on HPV transcription. Compared to the wild type HPV 16, this mutant, pmHPV4, displayed a much greater level of gene transcription after induction by either hormone (Fig. 6.2). The ability of these assays to detect

Figure 6.1. Effects of hormones and antiprogestin RU486 on HPV 16 gene expression in primary human keratinocytes as detected by in situ hybridization of viral mRNA. Ectocervical cells were transfected with wild type HPV 16 DNA and cultured in the absence of hormones (none) or in the presence of 100 nM dexamethasone (dex) or 100 nM progesterone (Prog, left panel). On the right, cells were grown in media with molar ratios of 100nM dexamethasone to RU486 (DEX:RU486) as indicated. Ki67 antibody staining, shown at the bottom, was for ectocervical cells cultured for 48 hrs in the absence of hormones or in the presence of 1:5 molar ratio of dexamethasone to RU486. Positively stained dark spots found predominantly in the cytoplasm, represents specific hybridization signals.


HORMONE
DEX: Ru486
None
1:1
Dex:
(1:0)
1:2.5
Prog
1:5
None
Ki67
1:5
Ki67

lower levels of hormone induction was also analyzed by using RU486, a progesterone and glucocorticoid hormone antagonist. This antagonist is known to inhibit hormone-induced expression by competetively interacting with the hormone receptors (Baulieu, 1991). Clearly, a progressive decrease in the level of HPV 16 wild type gene expression was observed with increasing concentrations of RU486 relative to dexamethasone (Fig. 6.1, right panels). Immunofluoresence assays for expression of Ki67 antibody reactive nuclear antigen showed that this decrease was not due to the loss of proliferative potential of the cells due to RU486 (Fig. 6.1, bottom right panel). These results illustrate that this in vitro model system could be used to detect expression levels which range between the undetectable uninduced level (none) and the higher than wild type induced level for the consensus mutant (pmHPV4). Therefore, this in vitro system was suitable to test the functional role of the three GREs for HPV 16 gene transcription.

Induction of HPV 16 gene expression in the presence of hormones, could have involved the direct interactions of hormone-receptor complexes with the previously identified GRE at nt 7640 on HPV 16 DNA. Therefore, a loss-of-function mutation introduced into this GRE, pmHPV2, (table 6.1), was tested for its ability to respond to hormones. This mutated HPV 16 genome remained responsive to both dexamethasone and progesterone (fig. 6.2), suggesting that hormone induced transcription also involved the two newly identified GREs. Alternatively, hormones might have been acting directly or indirectly through other viral or cellular sequences.

Mutated forms of the two newly identified GREs at position 7385 and 7474

Figure 6.2. Viral gene expression from plasmids with mutated hormone responsive elements. In situ hybridization was done as for figure 6.1, with the plasmids described in Table 6.1. The results were part of the experiment shown in figure 6.1. Consensus GRE mutation in pmHPV4 produced stronger staining signal, whereas a loss-of-fuction mutation at this site in pmHPV2 had no effect.

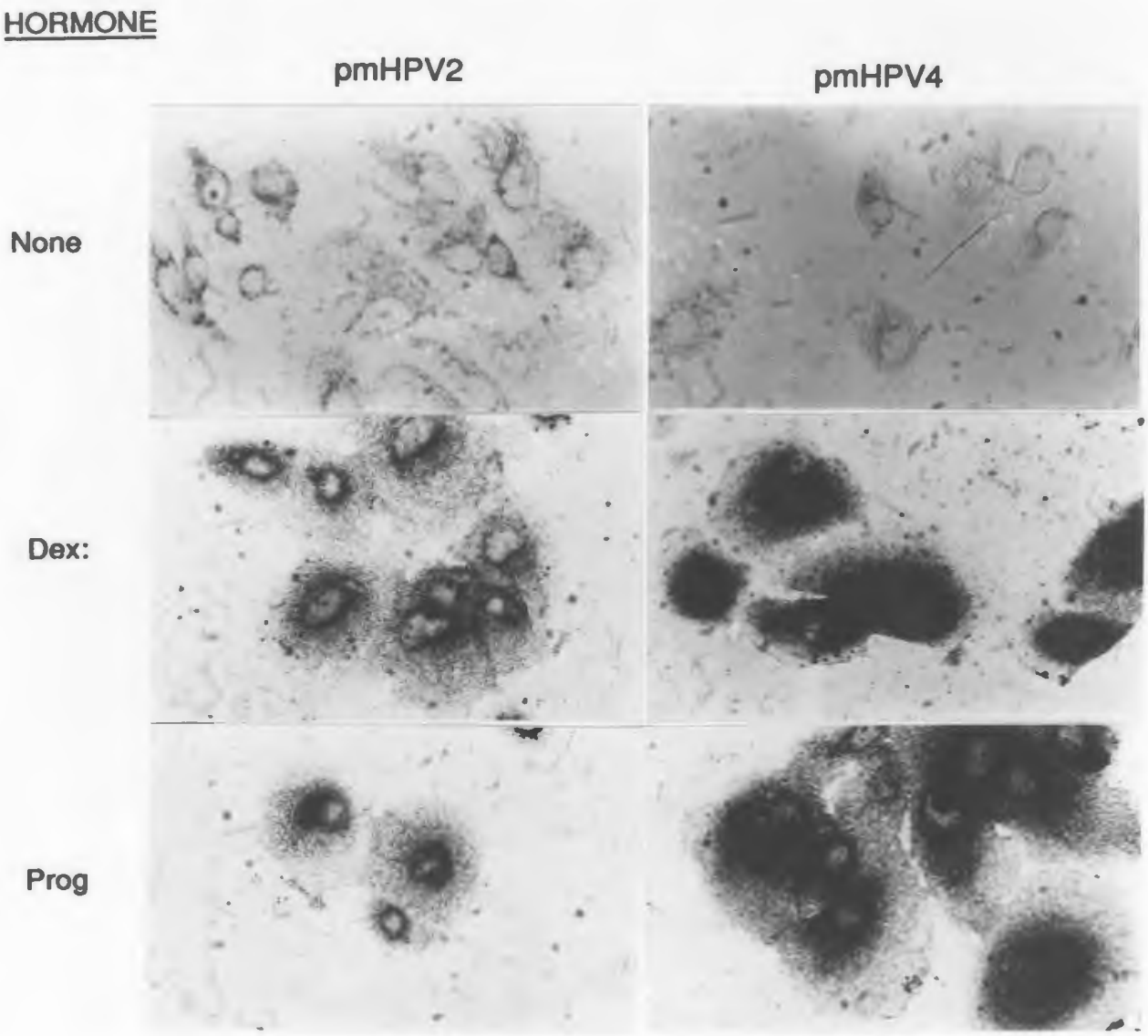
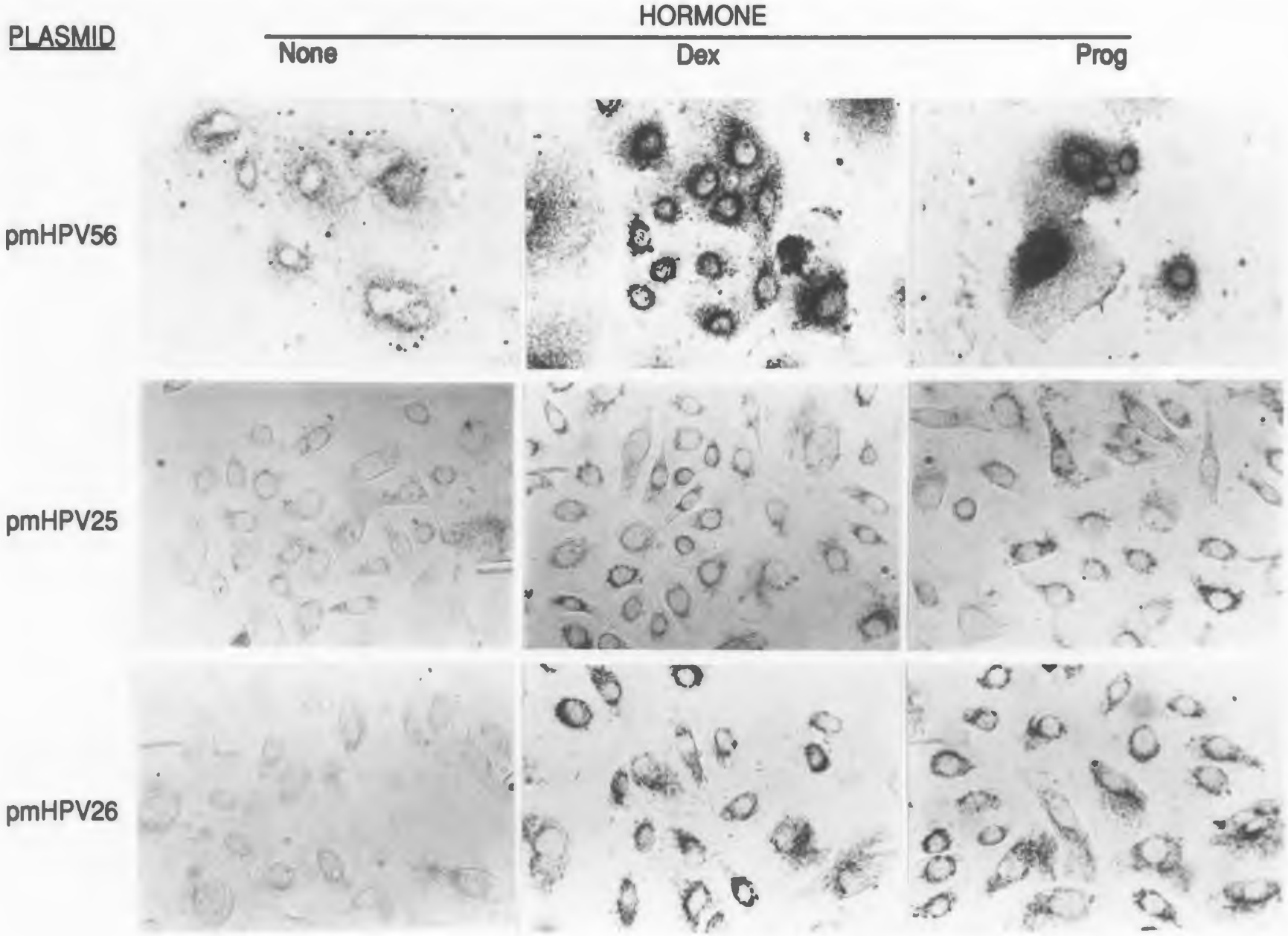

HORMONE
pmHPV2
pmHPV4
None
Dex:
Prog

Figure 6.3. Viral gene expression from plasmids with only one functional hormone response element. The results were part of the experiments shown in figure 6.1, with the plasmids described in Table 6.1. Positive staining was maintained in the presence of single elements in pmHPV26, pmHPV25 and pmHPV56, but the intact site at position 7474 in pmHPV25 gave a weaker signal. Dex = dexamethasone; Prog = progesterone.


PLASMID
HORMONE
None
Dex
Prog
pmHPV56
pmHPV25
pmHPV26

were next used to test whether or not these individual GRE-like sequences are functionally essential for hormone-induced HPV transcription. For this purpose, three double mutation plasmids containing only one intact GRE sequence (pmHPV26, pmHPV25 and pmHPV56, Table 6.1) were prepared. All three double mutants remained responsive to both dexamethasone and progesterone (Fig. 6.3), implying that all three elements are functional. The HPV 16 genome containing the wild type sequence at position 7474 only (pmHPV25) was induced to a qualitatively lower level than the other two plasmids. This suggested that the three sequences are differentially effective and it remained possible that other factors are involved in hormone induction. It is difficult to comment on the quantitative differences using this technique. However, qualitative differences were observed with each of the double mutants. To emphasize even more on the qualitative differences, the consensus GRE mutant at position 7640 (pmHPV4, fig 6.2) resulted in message levels which were far higher than the wild type HPV 16 sequence.

6.4.2 Effect of triple GRE mutations on hormone induced transcription of HPV 16 DNA.

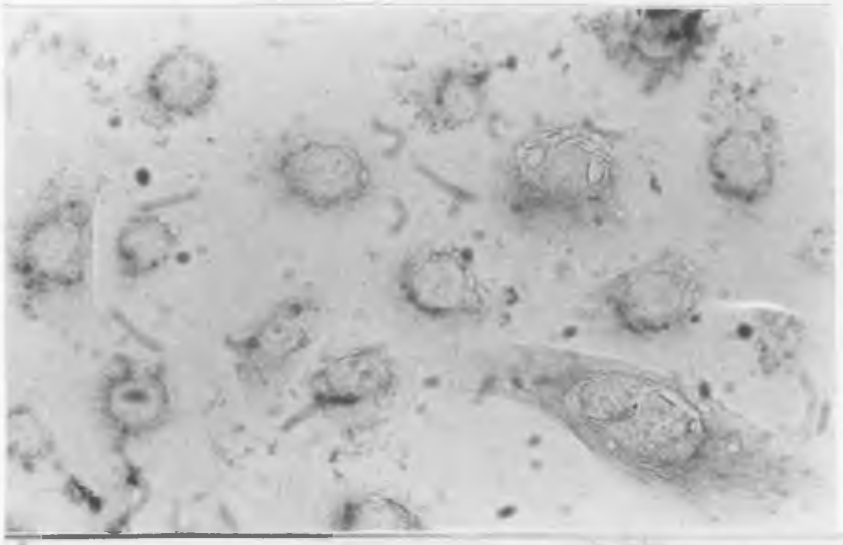
Since all three double mutation plasmids responded to either hormone, HPV 16 genomes containing loss-of-function mutations in all three potential GREs (pmHPV256, Table 6.1) were used. This construct, pmHPV256, failed to support any detectable induction of HPV 16 transcription by either hormone (Fig. 6.4). The results with the double and triple mutants have convincingly demonstrated a direct

Figure 6.4. Viral gene expression from a plasmid containing loss of function mutations in all three GREs. The results were part of the experiments shown in figure 6.1, with the plasmid described in Table 6.1. Mutations of all three GREs in pmHPV256 abolished positive staining for viral RNA in the presence of either dexamethasone (Dex) of progesterone (Prog).

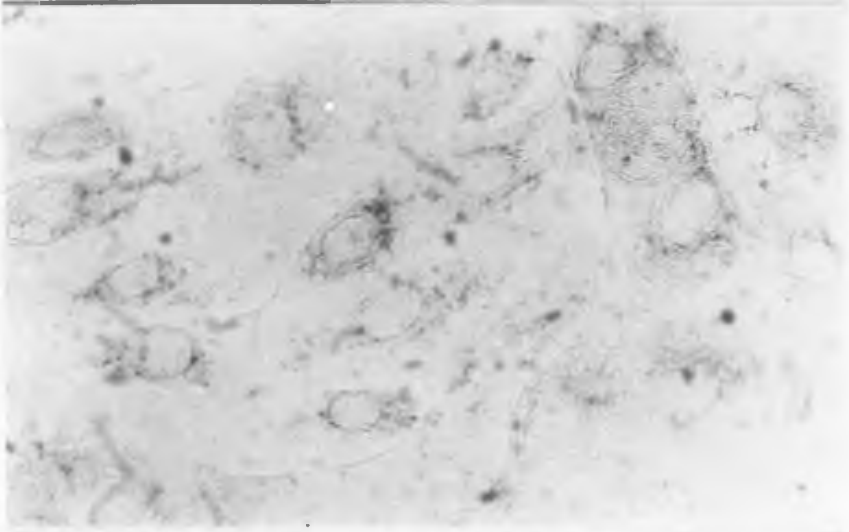
HORMONE

pmHPV256

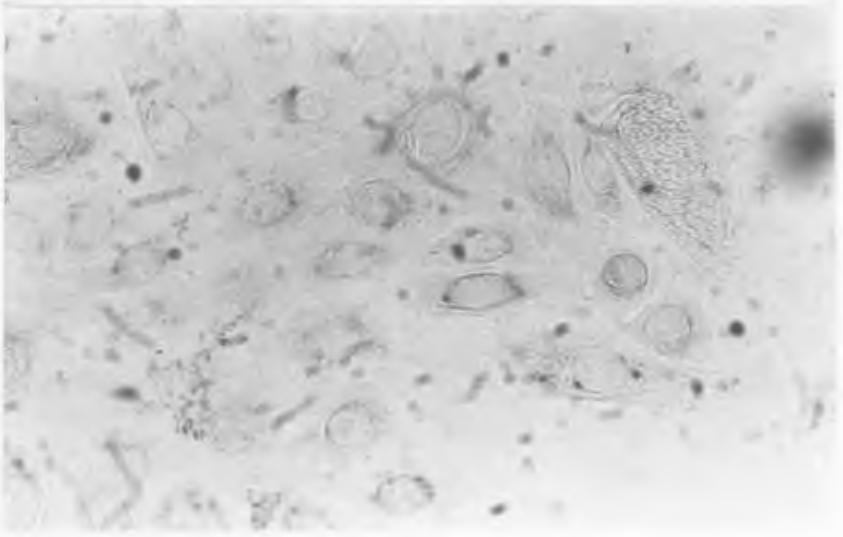
None



Dex:



Prog



role of all three GREs for hormone-mediated transcription of HPV 16 genes. Any one of these three elements were not only sufficient, but also appeared to be directly responsible for hormone-induced expression of HPV 16 transcription in human ectocervical cells.

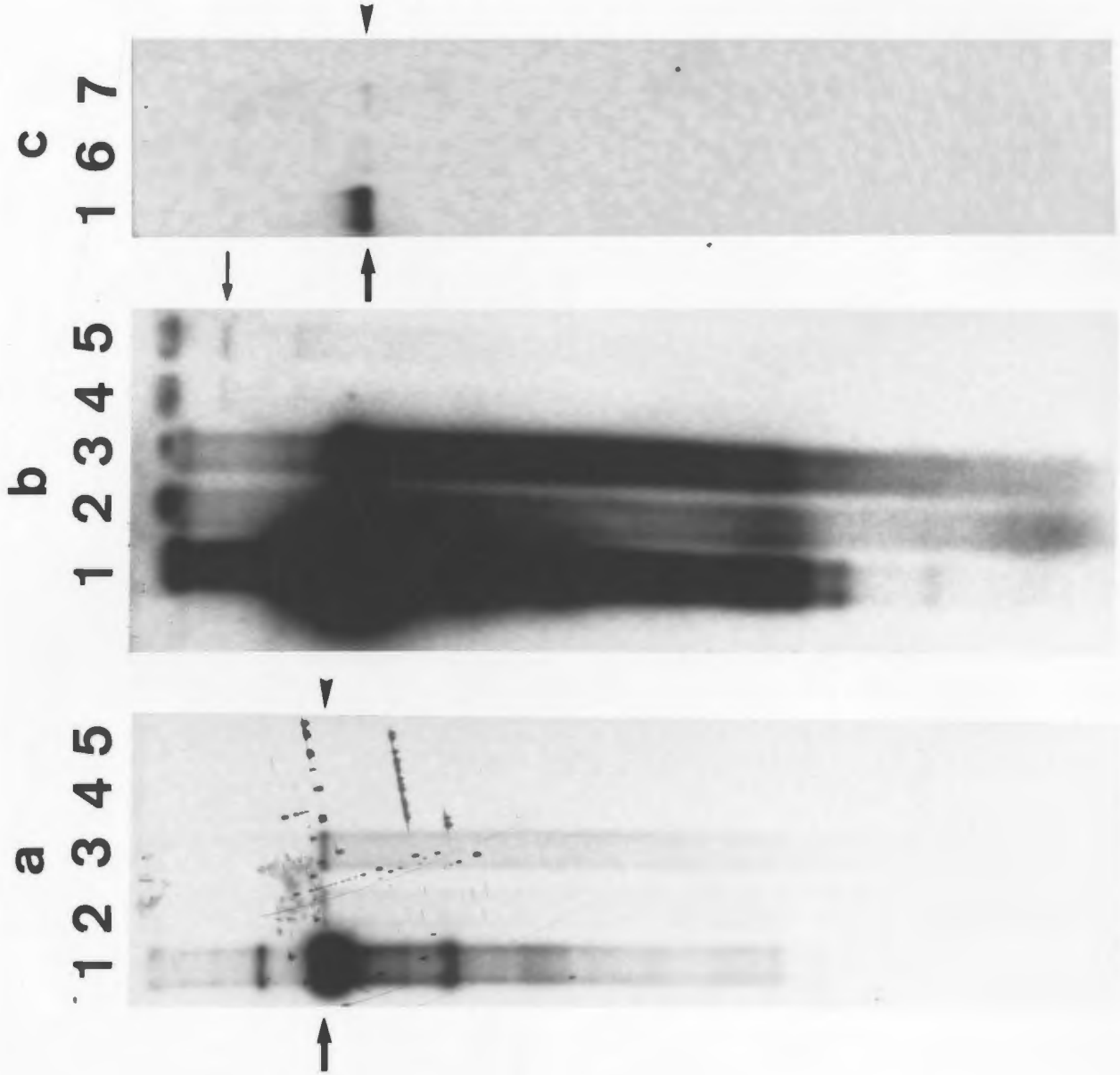
6.4.3 Physical state of HPV DNA in transfected ectocervical cells.

It is generally considered that transiently transfected DNA, initially remains in the episomal form in cells and is gradually lost over a period of time (Coffin, 1990). However, after longer cultures and appropiate selection, occasional colonies of cells would arise that have integrated the transfected DNA. To confirm that the transfected ectocervical cells contain only episomal form of HPV DNA, low (LMW) and high molecular weight (HMW) DNA was extracted from cells transfected with HPV 16 genomes. This was done after incubating the cells for 48 hrs in media with or without 100 nM dexamethasone. As shown in figure 6.5, cells harboured only episomal form of the DNA after digesting LMW DNA with a single cut (BamHI) restriction enzyme. HMW DNA digested with a non-HPV cutting enzyme (XbaI) revealed the presence of a high molecular weight band in a longer exposure. This band could be either integrated form of HPV or concatamerised form contaminated from the supernatant. However, digestion of the same DNA with the single cut, BamHI, demonstrated that HPV was most likely concatamerised and was present in the episomal form only (fig. 6.5c). It is interesting to note the slightly increased intensity of bands obtained for cells grown in the presence of dexamethasone. A

possible explanation has been discussed in chapter 7.

Figure 6.5. Hirt extraction and southern blotting for episomal presence of transfected viral DNA.

Primary ectocervical cells were transfected with the wild type HPV 16 DNA and cultured in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5 and 7) of 100 nM dexamethasone for 48 hrs. LMW (lanes 2 and 3) and HMW (lanes 4-7) DNA was extracted and digested with either BamHI (lanes 2, 3, 6, 7) or XbaI (lanes 4 and 5). Lane 1 is a marker containing HPV 16 DNA linearized with BamHI. Southern blots were probed by a nick-translated BamH1 fragment of HPV 16 plasmid. **b).** The gel in (a) was overexposed for 4 days to reveal a high molecular weight band (in thin arrows). **c).** HMW DNA was digested with single-cut BamHI restriction enzyme.


a
1 2 3 4 5
b
1 2 3 4 5
c
1 6 7

Chapter 7

DISCUSSION

7.1 Role of steroid hormones in HPV-mediated transformation.

It has been believed for a long time that the glucocorticoid-mediated transformation of primary rodent cells in culture occurs through a GRE known to be located in the enhancer of HPV 16 regulatory region (Pater et al., 1988, 1990; Crook et al., 1988; Gloss et al., 1987). This GRE has been characterised in detail and has been shown to be functional (Gloss et al., 1987; Chan et al., 1989). If this GRE was the only determinant of the observed increase in transformation of rodent cells in the presence of dexamethasone, then destroying this GRE should abrogate the hormone response. It is important to address the molecular mechanism by which this enhanced transformation occurs since steroid hormones have been implicated as an important risk factor in the outcome of cervical intraepithelial neoplasia and its progression to a fully malignant carcinoma (Stern et al., 1977; Beral et al., 1988; Hildesheim et al., 1990; Honore et al., 1991; Brinton, 1991). There have been numerous epidemiological studies that support this association and makes it important to understand and study the mechanism by which hormones act. In addition, a substantial body of experimental, clinical and epidemiological evidence indicates that hormones play an important role in the pathogenesis of many human cancers (Henderson et al., 1982). Hormone-related tumors account for more than

20% and 40% of all newly diagnosed cancers in males and females, respectively, in the United States (Henderson et al., 1991).

To study the molecular mechanisms of hormone-induced transformation of BRK cells, I generated two different mutations at the known HPV 16 nt 7640 GRE: one that completely destroys the glucocorticoid receptor binding site (pmHPV2) and another which converts this GRE into a consensus sequence (pmHPV4) (table 3.1). The latter mutant was used to examine whether or not a further increase in transformation can occur as a result of this change. My results clearly show that loss-of-function mutations of this GRE in pmHPV2 does not significantly affect the wild type positive dexamethasone response for transformation (table 3.2). A slightly reduced frequency of transformation was consistently observed with pmHPV2. It is difficult to determine small differences in transformation assays by HPV 16 because of low efficiency, experimental variability and appearance of secondary colonies. For pmHPV4, a marked increase in transformation was observed. This could be due to an increased affinity of the hormone receptor at this site. It is interesting to note that the wild type GRE at nt position 7640 is a composite GRE (cGRE) consisting of an AP-1 binding motif overlapping the GRE (Chan et al., 1990). Composite GREs are sequences that contains a GRE overlapping with a non-receptor transcription factor binding site (Diamond et al., 1990). These GREs have been described for several eukaryotic genes (reveiwed in Ponta et al., 1992). In pmHPV4 the overlapping AP-1 site is destroyed. The markedly increased transforming potential shown by pmHPV4 could be due to two possibile factors. First, it could be due to an increased affinity

of the glucocorticoid receptor to interact with this sequence resulting in a stronger response element. A second possibility is that binding of the AP-1 protein to the wild type sequence might inhibit or sterically hinder the binding of the glucocorticoid receptor. A mutation of AP-1 would then allow unrestricted binding of the receptor molecules and result in increased hormone response. Chong et al (1990) also found a marked increase in glucocorticoid response in transient assays when they mutated this AP-1 motif. They have suggested that mutations of this AP-1 site either allows the GR to bind to the GRE without competitive interference or, alternatively, the GR binds with a higher affinity to this sequence. Another significant observation is that this particular AP-1 motif does not respond to phorbol esters, such as TPA (Chan et al., 1990), unlike other AP-1 motifs (Lee et al., 1987). In addition, mutations introduced at this sequence resulted in an increased TPA induction of HPV 16 gene expression (Chan et al., 1990), indicating that this motif could be negatively acting. Thus, a third possibilty could be that AP-1 mutations in pmHPV4 resulted in the rescue of gene expression from this negative regulation. However, recent analysis of the complex nature of interactions of the GR and AP-1 at composite GREs have shown that both GR and AP-1 proteins can physically interact and bind to the cGRE as a protein complex with no competition involved (reviewed in Miner and Yamamoto, 1991; Konig et al., 1992). I have attempted to address the reasons for this observed increase in hormone induction by the consensus GRE mutant in chapter 4. In addition I have also probed into the role of AP-1 at this composite element in chapter 5.

Since mutations of the known GRE did not abrogate dexamethasone-induced transformation, other GRE-like sequences were searched for in the HPV 16 regulatory region. Two other GRE-like sequences were found by my computer search. One sequence was as divergent as and the second was less divergent than the originally identified nt 7640 GRE from the consensus GRE (table 3.1). To examine the likely involvement of these two other GREs in glucocorticoid-induced transformation, site-directed mutagenesis was performed to create loss-of-function mutations (Table 3.1). In order to directly address the involvement of these two other GREs, it was thought worthwhile to use only a double GRE mutation (pmHPV56) and a triple mutant (pmHPV256) for transformation assays. The double GRE mutant, pmHPV56, was responsive to dexamethasone and resulted in a two-fold increase in transformation in the presence of dexamethasone (table 3.3). The triple mutation in pmHPV256 eliminated the wild type dexamethasone response. Due to the insensitivity of the assay, only a semi-quantitative result was obtained.

My transformation results, probably addresses the very controversial issue of how steroid hormones in the form of oral contraceptive pills might act. One of the reasons for this controversy is due to the biases arising from epidemiological studies, including behavioural differences between women who use oral contraceptives and women using other forms of contraception (reviewed in Swan and Pettiti, 1982). So far it has been argued that oral pills have an indirect effect in either increasing the susceptibility of cervical tissue to HPV infection or increasing the persistence of HPV, and thereby explain the epidemiological finding that oral pills are associated with an

increased risk of developing cervical cancer (Vandenvelde and Beers, 1992). The alternative explanation was that steroid hormones act directly by increasing the expression of HPV 16 oncogene expression after infection (Lorincz et al., 1990; Gitsch et al., 1992; Mittal et al., 1993a).

In this regard, mammary gland tumor developement in mice infected with the hormone-responsive mouse mammary tumor virus (MMTV) is a good example of the relationship between hormones and viruses in the incidence and progression of tumors (Nandi and McGrath, 1973). A variety of hormonal stimuli, including steroid hormones and other peptide hormones, have marked effects on the incidence and progression of mammary tumors in mice (Bern and Nandi, 1961). Treatment of mice or primary tumor explants with glucocorticoids results in an accumulation of MMTV particles within the tumor tissue (Smoller et al., 1961). Using a raft culture system, steroid hormones have been shown to enhance MMTV particle formation (Yang et al., 1977). In addition to this glucocorticoid effect on particle production, a rapid increase in the steady state levels of intracellular viral RNA was also observed (Parks et al., 1974; Ringold et al., 1975a, 1975b; Scolnick et al., 1976). Subsequently, several studies have strongly supported the contention that the glucocorticoid induction of MMTV RNA, is indeed the primary hormonal response. Furthermore, it was also demonstrated that this induction is direct (Stallcup et al., 1978; Ucker et al., 1981) and is mediated through several GREs located in the MMTV regulatory region (reviewed in Truss et al., 1992). Drawing a parallel from the MMTV model, it is reasonable to hypothesize that increased HPV oncoprotein expression induced by

hormones would enhance transformation by the virus and confer a growth advantage for HPV-containing cells. It is also significant that the E7 gene product induces cellular DNA synthesis and progression of the cell cycle (Sato et al., 1989b; Banks et al., 1990a; Rawls et al., 1990). The present study has indicated that glucocorticoids act directly through three putative hormone responsive elements present in HPV 16 LCR. Another important consideration is that, in the context of naturally occuring cervical cancers, this hormone-induced expression should be relevant at an early stage of dysplasia where HPV is mainly found in the extrachromosomal form (Lehn et al., 1988; Cullen et al., 1991). It is postulated that steroid hormones may have an important role in the progression of early dysplastic lesions to a more malignant stage (Stern et al., 1977; Mittal et al., 1993a). Recent epidemiological studies have also strengthened this hypothesis and provide evidence for an important role of hormones in the outcome of cervical intraepithelial neoplasia (Herrero et al., 1990; Hildesheim et al., 1990; Honore et al., 1991; Debritton et al., 1993).

In addition to hormone-induced expression of viral oncoproteins, increased expression of other viral genes could also be important. Significantly, E1, E2 and E5 proteins can all be expressed, predominantly when HPV is in the early stage episomal form (Smotkin and Wettstein, 1986; Chow et al., 1987; Crum et al., 1988; Higgins et al., 1992), because integration events in transformed cells usually results in the disruption of these ORFs (Schneider-Maunoury et al., 1987; Choo et al., 1987). For example, increased expression of the HPV 16 E5 gene, especially at an early stage, could be a positive feedback mechanism for early gene expression, by modulating AP-

1 activity through growth factor-mediated signal transduction pathways (Leechanachai et al., 1992; Pim et al., 1992). Similarly, increased expression of E1 and E2 genes, could result in increased replication of the virus (Chiang et al., 1992a, 1992b; del Vicchio et al., 1992; Remm et al., 1992). As for the MMTV model, hormone-induced replication could play an important role in the virus life cycle. Supporting the role of increased E2 expression as an early stage event, is the demonstration that overexpression of E2 in rodent cells resulted in an extremely high frequency of transformation by HPV 16 (Lees et al., 1990). Another study has shown that a naturally-occuring isolate of HPV 16 with mutations in the E2 ORF, was unable to immortalize primary human epithelial cells (Storey et al., 1992). Cotransfection with an E2-expressing plasmid restored its immortalizing potential. However, contradicting results have also been observed as discussed in previous sections.

Early stage events almost certainly determines the final outcome of an HPV-infected cell (Durst et al., 1992) and I would presume that a complex sequence of events occur during these different stages. These events should be dependent not only on the expression levels, but, also on the transcription patterns of HPV genes (Sherman and Alloul, 1992, Sherman et al., 1992). One significant finding that emerged from studies done in our laboratory regarding dexamethasone resistant growth of HPV 16 transformed BRK cells, is the observation of differences in transcriptional initiation sites and patterns between dexamethasone sensitive and resistant transformed cells (Pater et al., unpublished observations). One can envision that glucocorticoid-induced gene expression, occuring in collaboration with other cell-

type or stage-specific host transcription factors, could result in preferential transcription and translation of certain viral ORFs. HPV mRNAs are generally polycistronic and differences in initiation sites and/or transcription patterns, could result in preferential translation of viral products, formed at a particular stage of HPV infection and important for disease progression. Clearly there is much more to be learned about the role of hormones in HPV 16-mediated transformation. However, it is useful to have the knowlege that hormones act directly through GREs in the HPV 16 regulatory region. Considering the recognized role of hormones in the progression of cervical cancer, the possibility of preventing this progression by inhibiting hormone-induced expression of viral oncoproteins by suitable anti-hormones becomes evident. Support for this comes from the demonstration of inhibition of glucocorticoid-enhanced transformation by the antiprogestin, RU486 (Pater et al., 1991). Similar strategies have been used for the treatment of breast cancers (Bakker et al., 1990) and inhibition of experimental tumor formation in mice (Bakker et al., 1987, 1990; Klijn et al., 1989).

However, before one can draw conclusions about the role of these newly identified GREs, it was important to characterize them and show that they are actually functional for expression and specific interaction with the glucocorticoid receptor. In the previous chapters, the functional requirement of these two GREs for steroid hormone-induced HPV gene expression has been addressed and both the elements have been further characterised for expression and DNA-protein interactions (chapter 4). In addition, I have also addressed the levels at which

hormone-induced expression occurs in primary cervical cells (chapter 6).

7.2 Role of steroid hormones in HPV type 16 gene expression.

The objective of this study (chapter 4) was to address the role of the three GREs in the glucocorticoid-induced expression of HPV in HeLa cells, a cervical carcinoma derived cell line. Also, the two GREs were characterised for expression and specific DNA-protein interactions. It has been previously shown that HPV 16 enhancer is responsive to dexamethasone and progesterone in transient gene expression assays. Bernard's group has characterised the GRE at position 7640 and has clearly shown that partially purified glucocorticoid receptor binds to this GRE (Gloss et al., 1987; Chan et al., 1989). However, mutations destroying this GRE did not completly eliminate dexamethasone response when they used a maximum enhancer fragment of the HPV 16 regulatory region. Instead, a two-fold induction was still observed. This induction could be due to the putative GRE at nt position 7474 since their constructs contained this GRE. In my study, the full length HPV 16 wild type enhancer or constructs containing single, double or triple GRE mutations were tested in transient gene expression assays in HeLa cells to establish their role in glucocorticoid-induced HPV gene expression. As shown in fig. 4.1 all single (pCPH2, 4, 5, and 6) and double (pCPH26 and 56) GRE mutations retained dexamethasone response, whereas, the triple mutant (pCPH256) abrogated induction. The consensus GRE mutant in pCPH4, resulted in more than a ten-fold induction compared to less than three-fold for the wild type sequence in pCPHWT. It is likely that these higher

levels of expression by the consensus mutant (pmHPV4) was responsible for the more efficiently transforming virus as shown in the transformation assays (Table 3.2). Results from transient assays support a direct role of the GREs in glucocorticoid-induced HPV 16 gene expression.

One very important question that remained unanswered was the observation of very high levels of expression with the consensus GRE mutant. It is important to consider here that this GRE is a composite GRE containing an overlapping AP-1 binding site. Two possibilities, that I have raised earlier in this chapter are conceivable. One possibility for increased expression, could be an increased affinity of the receptor for the consensus sequence. Another possibility could be the release of steric hinderance by AP-1 protein, thus allowing the receptor molecules to bind more efficiently. Evidence of such regulation by competitive inhibition of factors binding to composite hormone responsive elements have been described for several eukaryotic genes, such as the gonadotropin α-subunit gene (Akerblom et al., 1988), rat α-fetoprotein gene (Guertin et al., 1988; Zhang et al., 1991), human osteocalcin gene (Stromstedt et al., 1991), bovine prolactin gene (Sakai et al., 1988) and rat pro-opiomelanocortin gene (Drouin et al., 1989). In all of the above-mentioned examples steroid hormones negatively regulate expression of these genes by competitively inhibiting binding of positively acting cellular factors to binding sites overlapping the GREs (discussed in chapter 1, section 1.6.4). To examine either of the two possibilities, four different mutations were created in the AP-1 motif (fig. 4.2). It is presumed that all four mutants fail to bind AP-1, based on nucleotide sequences

important for AP-1 binding in the literature (Risse et al., 1989; Gaub et al., 1990; Okuda et al., 1990). As shown in figure 4.3, mutants pCD7.1 and pCD7.2, which have a C at nt position +5 of the GRE, result in CAT expression comparable to the consensus mutant pCD4. The latter construct also has a C at +5 position. The other two mutants, pCD7.3 and pCD7.4, however, fail to respond in a similar fashion even though the AP-1 binding site is mutated. This indicates that the C at position +5 is important for high levels of hormone induction and confirms results from previously done studies demonstrating the importance of this nucleotide in hormone induction and GR binding (Noordeen et al., 1990; Scheidereit et al., 1984, 1986). It is unlikely that the AP-1 motif creates steric hindrance for the binding of GR to this composite GRE. Instead, it is possible that some form of regulatory cross-talk occurs between GR and AP-1 at this composite GRE. This possibility has been further studied and is described in chapter 5 of this thesis.

Results from the transformation and transient assays strengthen the contention that all three GREs are involved in transformation and gene expression, but it still does not prove that the newly identified GREs are in fact authentic. To further characterize the two GREs, double-stranded oligonucleotides corresponding to both elements were inserted into plasmids used and tested for their ability to respond to dexamethasone in transient assays and also for specific DNA-Protein interactions in vitro. As shown in figure 4.4, both GRE5WT and GRE6WT, cloned in pBLCAT2 expression vector, were able to confer a more than two-fold induction with dexamethasone. This is probably the single most important evidence that both

putative GREs are in fact glucocorticoid response elements. Mobility shift assays done with these oligonucleotides demonstrated that these sequences bound to a highly specific protein or protein complex in HeLa cell extracts (fig 4.5), which constitutively expresses sufficient levels of glucocorticoid receptor. Similar complexes were also obtained in extracts from a monkey kidney cell line, CV1. However, the intensity of the retarded bands in CV1 extracts was far less than that obtained from equal amounts of HeLa cellular extracts and this confirms the fact that glucocorticoid receptor levels in CV1 cells are low (Pearce and Yamamoto, 1993). Further, UV-crosslinking experiments performed on the same cellular extracts demonstrated that at least two major proteins of 96-KDa and 45-kDa bind to both sequences. In addition, a few minor species in the 50 to 65-kDa range were also observed (Fig. 4.6). The 96-kDa protein corresponds to the size of the native glucocorticoid receptor (Hollenberg et al., 1985). The 45-kDa protein could be the major degradation product of the receptor (Singh and Moudgil, 1985) and the other slower migrating faint bands might represent other proteolytic degradation products. The 45-kDa proteolytic product has been shown to possess both the DNA binding and steroid hormone binding domains (Govindan and Gronemeyer, 1984; Singh and Moudgil, 1985; Smith et al., 1989a). This could explain its interaction with DNA in fig 4.6. However, it cannot be assumed that glucocorticoid receptor is the only protein interacting with these GREs and the possibility of other cellular proteins binding to these sequences cannot be excluded. The remarkable similarities of bands obtained from both mobility shift and UV-crosslinking experiments to two different DNA

sequences strongly suggests interaction with a common protein(s). Assuming an interaction of multiple proteins at both GRE5WT and GRE6WT, it is possible that they do so only in the form of a complex (as seen in mobility shifts or UV crosslinking) where protein-protein interactions are required for efficient binding. To exclude this possibility, proteins from HeLa cellular extracts were first seperated on a denaturing gel, transferred on a nitrocellulose membrane and then probed with radiolabelled GRE oligonucleotides (Fig. 4.7). This type of protein blotting analysis indicated that both GREs bound to a major 96-kDa protein which corresponds to the molecular size of the native glucocorticoid receptor (fig. 4.7). GRE5WT probe also occasionally bound to a faster migrating 45-kDa protein. In addition, a faint band of more than 97-kDa also appeared with both probes. However with the conditions employed and depending upon the amount of renaturation of the bound proteins, this band was not a consistently detectable finding. Denaturation of proteins by this method did not give reproducibly good results as far as interaction with the 45-kDa protein is concerned. However, the 96-kDa band always appeared with either oligonucleotides and resulted in an exactly similar pattern. Similar binding patterns were also obtained from HeLa cellular extracts, in which this technique was first described to study interactions of GR with the GRE (Silva et al., 1987). In their study too, a minor 45-kDa and a faint band above 97-kDa was also seen with the MMTV GRE. It is highly unlikely that the two sequences, in spite of being very different, produce an exactly similar pattern in South-western blots. From these experiments it can be confidently concluded that the glucocorticoid receptors bind to

these putative GREs in vitro.

The results in this study further clarify the results obtained for the transformation assays. It also addresses the issue of how steroid hormones, in the form of oral contraceptive pills, might act. According to my working hypothesis, steroid hormones most likely act directly by increasing the expression of HPV 16 oncogenes after infection. It has been demonstrated in several studies that the levels of expression of the transforming genes of HPV 16 are important for in vitro transformation of primary or established cells (discussed in chapter 2). HPV 11 DNA is inactive in transforming BRK cells in the presence of dexamethasone (Pater et al., 1989), although it contains a hormone responsive GRE in its regulatory region (Chan et al., 1989). Interestingly, duplication of the HPV 11 enhancer region rendered the viral DNA oncogenic in the presence of dexamethasone in BRK transformation assays (Rosen and Auborn, 1991). This suggests that one GRE was not sufficient to induce expression of HPV 11 oncogenes to support BRK transformation. This could have some biological significance and might explain the presence and requirement of several GREs in the regulatory region of the oncogenic HPV type 16 DNA. It is also suggestive of an important role of steroid hormones in the multistep model of cervical cancer.

7.3 Role of c-jun and c-fos in glucocorticoid-mediated expression of HPV 16.

As mentioned earlier in this chapter, the GRE at nt 7640 is a composite GRE with an overlapping AP-1 motif. The only other cGRE known to be able to interact with

both the GR and AP-1 proteins is the rat proliferin gene promoter cGRE (Diamond et al., 1990). Studies performed to establish the role of AP-1 in glucocorticoid-mediated induction have been done on sequence elements represented by oligonucleotides. To date, no data is available regarding the regulation of and role of the rat proliferin gene cGRE in the context of its whole enhancer. All studies done on this cGRE have used oligonucleotides (Diamond et al., 1990, 1990b; Miner and Yamamoto, 1992; Pearce and Yamamoto, 1993). Thus results obtained with cloned oligonucleotides do not necessarily address the biological significance of this element in expression of rat proliferin gene. It is very important to demonstrate that the effects one sees in isolated fragments also hold true in the context of an enhancer. Another important consideration is that not even a single study has shown by site-directed mutagenesis that both the GRE and overlapping AP-1 motif are indeed involved in this complex regulation. In this study, mutations of the nt 7640 GRE or its overlapping AP-1 motif were used to demonstrate their role in glucocorticoid-mediated regulation. It is clear from fig. 5.2 and 5.3 that, in the context of the full-length HPV enhancer, presence of c-jun stimulated the basal level activity which was further enhanced in the presence of dexamethasone. On the other hand, c-fos negatively regulated hormone induction. Mutations of the GRE and AP-1 site in pCPH2 and that of AP-1 in pCPH4 have undoubtedly demonstrated that both elements are required to observe this kind of regulation (fig. 5.3). In addition, smaller enhancer fragments of HPV 16 DNA, containing only the cGRE, directly addresses the functional requirement of c-jun for hormonal regulation (fig. 5.4a).

Interestingly, the pCDWT construct was inactive for dexamethasone-induction in P19 cells (fig. 5.4a), whereas, simple GREs in pCD4 and pCD7.2 were responsive to hormone-induction even in the absence of c-jun (fig. 5.4). This indicates that c-jun is essential for hormone regulation at this HPV cGRE. This is further supported by the evidence that pCDWT responds to dexamethasone in HeLa cells (fig. 4.3), but not in P19 cells. This difference is probably because AP-1 activity in HeLa cells is derived mainly from the c-jun protein (Vogt and Bos, 1990), whereas, P19 cells consists of very low levels or no AP-1 activity (de Groot et al., 1990). Similar results have been shown for the proliferin cGRE (Diamond et al., 1990). Furthermore, mutations at the GRE and/or AP-1 site abrogated this response (fig 5.3b and 5.4), indicating that both GR and AP-1 binding is essential. However, although I do not have direct evidence that this HPV cGRE is occupied by both the GR and AP-1, indirect evidence is available in the literature. Thus, it has been shown that the nt 7640 cGRE binds to the GR (Chan et al., 1989) and AP-1 (Cripe et al., 1990). Chan et al (1990) have demonstrated formation of at least 5 complexes at the cGRE in mobility-shift assays with HeLa cellular extracts. Competition analysis provided evidence that one of these complexes consists of AP-1 and it is likely that the GR also takes part to form any one of these retarded complexes.

I might also add that the HPV 16 cGRE is the second example in the literature where c-jun and c-fos function as selectors of glucocorticoid-mediated induction, as previously hypothesized by Yamamoto (Diamond et al., 1990). According to this hypothesis, the ratio of functional c-jun and c-fos present in cells

and not their absolute amounts determines whether a cGRE responds positively or negatively to hormones. Thus, an interesting cross-talk has unfolded in the regulation of HPV 16 expression by two different signalling pathways. It might also be possible that different members of the AP-1 and steroid-hormone receptor family behave differently in hormonal regulation of HPV 16 expression, as demonstrated for the regulation of proliferin cGRE (Pearce and Yamamoto, 1993). It would also be interesting to examine if AP-1 also regulates progesterone-induction of HPV 16 cGRE in a similar manner.

7.4 Effect of steroid hormones on HPV type 16 transcription.

In this study I have used cultured human ectocervical cells as a model system to examine the early events and the role of steroid hormones during HPV 16 infection. Transient transfection of whole HPV 16 genomes in cervical keratinocytes were assumed to resemble an HPV infection. Expression of HPV 16 RNA in this model system required either progesterone or glucocorticoid hormones (fig. 6.1). The progressive decrease in transcription of HPV genes by the hormone antagonist, RU486, demonstrated that this response was mediated by the hormone receptor.

It has been suggested that hormones promote cervical cancer (Stern et al., 1977; Beral et al., 1988; Bokhman and Urmancheyeva, 1989; Hildesheim et al., 1990; zur Hausen, 1991; Brinton, 1991; Honore et al., 1991; Bosch et al., 1992). This might occur through enhanced HPV oncogene expression in the target cells due to

their release of growth arrest by hormones. The Ki67 result (Fig. 6.1) suggests that this does not apply in this system, but does not exclude the possibility that a block in cell cycling in vivo is overcome by hormonally induced HPV 16 gene expression. Alternatively, hormones might first modulate the expression of other cellular genes, which then regulate HPV gene expression. However, the markedly enhanced induction observed with the consensus GRE mutant (pmHPV4, Fig. 6.2), suggested a direct hormone action. Direct induction of viral gene expression was confirmed, as there was no detectable viral message when all the three HPV hormone responsive elements were mutated (pmHPV256, Fig. 6.4). In addition, all three double GRE mutations were also responsive to dexamethasone and progesterone, indicating that all three hormone response elements are independently functional and each one of them is sufficient for steroid hormone-dependent expression of HPV 16 mRNA in human ectocervical keratinocytes. In addition to the direct transcriptional role of hormones, it is possible that hormones could also be playing a role in stabilization of viral mRNA. In several systems, steroid hormones have been shown to be involved in the post-transcriptional stabilization of mRNA (Paek and Axel, 1987; Peterson et al., 1989; Nielsen and Shapiro, 1990; Mendelson and Boggaram, 1991; Pilkis and Ganner, 1992). However, in the case of HPV, our laboratory has already looked into the possible role of steroid hormones in post-transcriptional stabilization of HPV mRNA (Belaguli et al., 1992 unpublished data). Using actinomycin-D chase experiments, no significant differences were observed in the half life of HPV 16 E7 mRNA, either in the presence of dexamethasone or progesterone. Thus the

increased RNA signals observed in the presence of hormones is likely to be at the level of transcription.

This in vitro model system has a number of advantages, including many similarities to the cervical tissue and cervical lesion that it models. Primary human ectocervical cells are natural targets for hormone action (Gorodeski et al., 1989) and are permissive for expression of HPV 16 (fig. 6.1). As discussed earlier, in benign and low grade dysplastic cervical lesions HPV DNA is principally found in an episomal form, whereas, in most cervical carcinomas it is found integrated in the host genome (Durst et al., 1985; Cullen et al., 1991;). Since, only full length HPV 16 genomes were transfected into the cervical cells, it would be reasonable to assume that this type of model mimics the natural early-stage HPV infection. As in benign lesions, HPV DNA in these transfected cells is also episomal (fig. 6.5), with all the viral ORFs under the control of their own regulatory region. This indicates that steroid hormones should have a pivotal role in early stages of HPV infection in which the bulk of HPV DNA is found in an episomal form.Another significant consideration is that progression to invasive cervical carcinoma almost always involves integration of viral DNA into the host chromosome (Durst et al., 1985; Schneider-Maunoury et al., 1987). It has been demonstrated by von Knebel Doebertiz et al. (1991) that these integration events can eliminate hormone-inducible expression of viral genes due to regulatory influences of flanking cellular sequences. In an episomal form, expression of the viral DNA will not be under the influence of cis acting cellular regulatory sequences and should always be responsive to hormones (von Knebel Doebertiz

et al., 1991). Based on these assumptions, it can be postulated that steroid hormones play an important role in the early stages of cervical carcinogenesis.

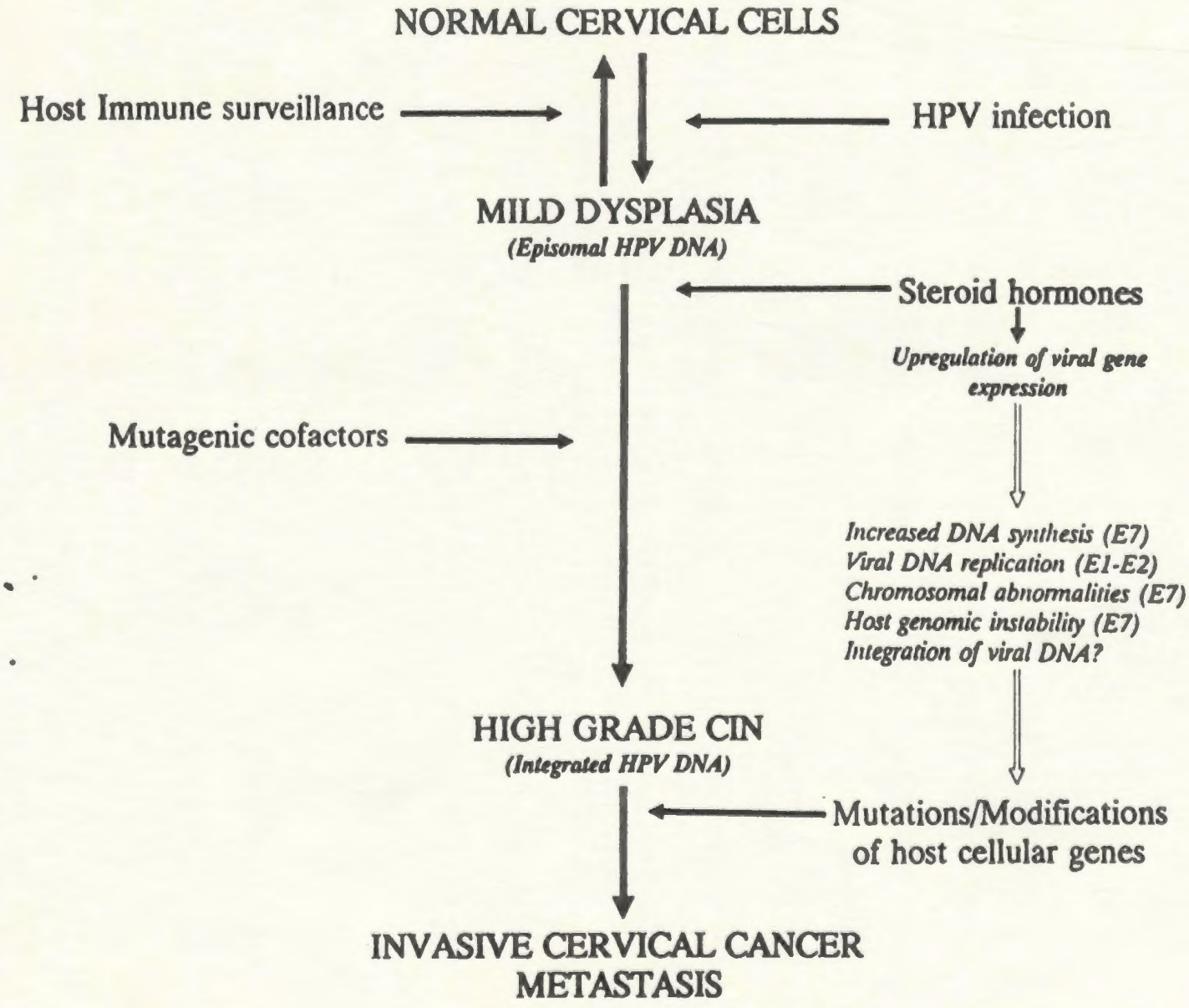
For HPV 16-associated lesions, progression occurs in approximately half of the cases and also the frequency of progression increases with disease severity (Fuchs et al., 1989; Lorincz et al., 1992; Weaver et al., 1990), suggesting that early stage events are pivotal for progression. One important early stage event is the ability of the virus to replicate in infected cells. Replication of HPV requires the host cellular replication machinery and the virally encoded E1 and E2 proteins (discussed in chapter 1, section 1.2.3 and 1.2.4). Also, the cellular transcription factor, E2F, is proposed to be the prime target of E7 protein function (discussed in chapter 2, section 2.2.3). It is thought that E7 functions by releasing the transcriptional regulatory E2F factor from pRb complexes. The evolution of this function in HPV, as well as in SV40, is not etiologically clear since there is no evidence that either one of these viruses uses the E2F factor for the transcription of viral genes. However, each of these viruses use host cell components for DNA replication. Interestingly, E2F is a critical factor for the expression of several proliferation associated genes, such as c-fos, c-myc, c-myb, and also for genes essential in DNA replication, such as DNA polymerase α and dihydrofolate reductase (discussed in chapter 2, section 2.2.3). Thus, as initially hypothesized by zur Hausen, it is possible that E7-mediated release of E2F from pRb complexes promotes cells entering the S phase, resulting in a favourable environment for viral DNA replication and, thus, viral growth. Expression of these viral regulatory genes in the absence of a lytic infection could

lead to continuous cellular proliferation and oncogenic transformation in concert with other cellular genetic events. Hormone-induced expression of E7, E1 and E2 proteins at early stages could, therefore, be an important factor for the virus to replicate. As discussed earlier in this chapter, increased replication of viral DNA would also indirectly result in increased transcription and expression of HPV genes. Considering the above speculation, it was interesting to observe that in transfected ectocervical cells and in the presence of dexamethasone, a stronger signal was detectable in Southern blot analysis of low molecular weight DNA (fig. 6.5). It is intriguing to consider that hormones could have caused transient replication of viral DNA in this system. Alternatively, this increase could be due to differences in transfection efficiency. However, recently, transient replication of whole HPV genomes was demonstrated in transfected cells (del Vicchio et al., 1992). In this study, interestingly, replication of HPV 16 genomes could be achieved only by overexpressing the viral E1-E2 proteins. However, overexpression of these proteins was not required for replication of HPV 6, 11 and 18 DNA. This study has clearly indicated that the HPV 16 promoter is not strong enough to express sufficient levels of the viral E1/E2 proteins and that other inducing factors (such as hormones) may be required. In addition, increased detection of HPV DNA in oral contraceptive pill users (Vandenvelde and Beers, 1992) and during pregnancy (Schneider et al., 1987; reviewed in Ferenczy, 1989) has been demonstrated in HPV-positive lesions. Schneider et al (1987) demonstrated a 10-fold higher number of viral copies in lesions from pregnant women, compared to non-pregnant. Similar results were also obtained

from another study by Hording et al (1990). It is likely that increased detection of HPV in these cases was due to replication of the viral DNA. Similarly, in oral contraceptive users and during pregnancy, increased recurrence of HPV 11 infection has been observed in clinical cases of condylomata acuminata (Franceschi et al., 1983; Daling et al., 1986). Although indirect, these studies indicate that hormone-induced viral DNA replication could account for the increased detection or HPV positivity in clinical lesions. In most epidemiological studies, increased detection of HPV has also been correlated with higher grades of clinical lesion, indicating that hormone-induced replication of virus could also be an important factor for progression of clinical disease (Negrini et al., 1990). This in vitro system, in my opinion, is a good model system to address the role of hormones in replication of transiently transfected HPV genomes.

In conclusion, based on results obtained from many different studies and my study on the role of steroid hormones as an important cofactor for HPV-mediated oncogenesis, I would like to propose the following model (fig. 7.1). It is well known that the E6/E7 oncoproteins are involved in cell biologic effects, such as growth promotion (Von Knebel Doebertiz et al., 1988, 1991), immortalization (Hawley-Nelson et al., 1989) and altered differentiation (McCance et al., 1988; Woodworth et al., 1990a, 1992a). More recently, the HPV 16 E7 oncogene has been shown to induce chromosomal abnormalities in transfected mouse and human keratinocytes (Hashida and Yasumoto, 1991; Swisshelm et al., 1992). Thus, E7 has the potential to induce the various chromosomal deletions, translocations, duplications and

Figure 7.1. Hypothetical model of the possible role of steroid hormones in the pathogenesis of cervical carcinoma.


NORMAL CERVICAL CELLS
Host Immune surveillance
HPV infection
MILD DYSPLASIA
(Episomal HPV DNA)
Steroid hormones
Upregulation of viral gene expression
Mutagenic cofactors
Increased DNA synthesis (E7)
Viral DNA replication (E1-E2)
Chromosomal abnormalities (E7)
Host genomic instability (E7)
Integration of viral DNA?
HIGH GRADE CIN
(Integrated HPV DNA)
Mutations/Modifications of host cellular genes
INVASIVE CERVICAL CANCER
METASTASIS

anueploidy typically observed in cervical carcinomas (Reid et al., 1984; Durst et al., 1987; Sreekantaiah et al., 1988; Smith et al., 1989b). In addition, hormone-induced expression of the viral E1 and E2 proteins will be important for viral replication. Considering these properties of viral proteins, I would like to propose that hormone-induced expression of the viral oncogenes in pre-neoplastic cervical cells would initiate altered cell growth, differentiation and chromosomal instability and abnormalities of the host genome (figure 6.6). These events could possibly be involved in facilitating or initiating integration of viral DNA into the host chromosome. Some of these random integration events would then deregulate expression of viral and cellular genes and probably initiate unkown pathways of progression into malignancy (zur Hausen, 1991). Similarly, increased replication of the virus at early stages might play an important role in progression of CIN lesions.

Another important consideration is that RU486 abolished steroid hormone-dependent expression of HPV 16 genes in ectocervical cells (Fig. 6.1). Our laboratory had previously reported that the antihormone, RU486, inhibits transformation of primary rodent cells by HPV 16 (Pater et al., 1990). This raises the issue of whether RU486 or other antiprogestins could be used for prophylaxis and/or treatment of HPV-induced lesions. The development of new and safe antiprogestins could provide the prospect of a treatment modality for HPV-induced early cervical lesions. Significantly, clinical trials have indicated a potential use of RU486 as an alternative form of contraception (Nieman et al., 1987). My in vitro model system could be utilized to assess some of these potential therapeutic agents.

In summary, my work has established a definitive and direct role of steroid hormones in HPV-mediated oncogenesis of rodent cells and in HPV gene expression in cervical cells. Further studies are required to establish the role of hormones in progression of early-stage lesions and are discussed in the next chapter for future studies.

Chapter 8

FUTURE DIRECTIONS

The studies initiated in this thesis are just a beginning towards the understanding of the role of hormones in HPV-mediated oncogenesis. The results shown for BRK transformation using HPV genomes with the GRE mutations are encouraging. However, a definitive role in the transformation process of cervical cells, the primary host cell of HPV infection, remains to be established. Although, many epidemiological, clinical and experimental studies support the hypothesis that hormones have a role at an early stage of the disease, further experimentation is required. The GRE mutations that I have generated in the HPV 16 LCR can provide useful information about the role of steroid hormones in HPV-mediated immortalization of primary human keratinocytes. A quantitative immortalization assay has been described by Schlegal et al (1988). In this study, the glucocorticoid hormone, hydrocortisone, was required for efficient immortalization of cultured primary human keratinocytes by HPV 16. In the absence of this hormone the immortalizing efficiency of HPV 16 DNA was about 5-fold less compared to those treated by hormone. More recently, similar results were also obtained for immortalization of oral keratinocytes by HPV 16 DNA (Sexton et al., 1993). However, it is not clear whether enhanced immortalization is due to the proliferative effects of hormones on cells (Gey et al., 1952; Rheinwald and Green, 1975) or is

related to hormone-induced expression of HPV oncogenes (discussed in chapter 7). These two different effects of hormones can be seperated and examined by using the different combinations of the GRE-mutated HPV 16 genomes in quantitative immortalization assays. For this purpose, I have designed and initiated the preparation of a triple consensus GRE mutation (termed pmHPV489), with all three GREs converted into consensus glucocorticoid receptor binding sites. The triple consensus GRE mutant (pmHPV489), loss-of-function triple GRE mutant (pmHPV256), and nt 7640 consensus mutant (pmHPV4) can be used to directly examine the role of glucocorticoids in HPV-mediated immortalization of primary human cells. It would be most appropriate to use primary ectocervical cells. However, primary human foreskin keratinocytes can also be used, because both cell types give identical results in immortalization assays (Sun et al., 1992). In this type of experiment, one would expect the pmHPV4 construct and the triple consensus GRE mutation to immortalize at a quantitatively higher frequency compared to the wild type HPV 16 genome. Similarly, the triple GRE mutant (pmHPV256) might be unable to immortalize or may do so at a very low frequency. Such experiments would indicate that hormones, which act directly through the GREs, are important for immortalization of these cultured cells. The results would also correlate very well with the BRK transformation assays and provide an appropriate model system to study the role of hormones in HPV-mediated oncogenesis.

Recently, many investigators have focused their attention on the histological features imparted by HPV-immortalized cells in vivo, after transplantation in nude

mice. Similar experiments done in our laboratory have recently demonstrated that HPV 16-immortalized human foreskin or ectocervical cells displayed mild dysplasia after transplantation in nude mice (Sun et al., 1992). It would be interesting to examine the effect of hormone treatment in mice, especially of progesterone, on the in vivo histology of these immortalized cells. Also, cells immortalized by the single consensus GRE mutant (pmHPV4) and the triple consensus GRE mutant could be similarly implanted in nude mice. It is possible that in vivo, higher grades of dysplasia would be observed with these mutated HPV genomes. Such experiments would directly address the role of hormones in transformation and also in the progression of HPV-infected lesions. A more suitable experiment to examine the role of hormones in progression would be to use the human cervical cancer-derived cell line, W12, which contains only episomal forms of HPV 16 DNA (Stanley et al., 1989). These cells displayed mild dysplasia after in vivo transplantation. It would be interesting to examine whether hormones injected into nude mice make any difference in the in vivo histological pattern of HPV-containing cells after transplantation. It is possible that treatment of nude mice with progesterone and/or glucocorticoids will result in higher grades of dysplasia. If so, this would be experimental evidence of progression of HPV-containing lesions by hormones. Similarly, experiments can be done using anti-progestins, such as RU486, to examine if these hormonal effects can be reversed.

Apart from a direct role in enhancing the expression of viral oncoproteins, hormones could also have an important role to play in HPV replication. The major

proteins involved in HPV replication are the E1 and E2 proteins (del Vicchio et al., 1992; Remm et al., 1992; Chiang et al., 1992a). Thus, increased hormone-induced expression of these proteins during the early stages of HPV-infected cervical lesions, which contain viral DNA mainly in the episomal form, could result in an increase of viral copy number. This increase in viral copy number could then indirectly cause an increase in viral transcription due to template effect. This prediction is supported from several clinical studies demonstrating an increased detection of virus and the presence of higher viral DNA copy number during pregnancy and among oral contraceptive users (Vandenvelde and Beers, 1992; Schneider et al., 1987; reviewed in Ferenczy, 1989). The in vitro cervical cell system described in chapter 6 is a suitable system to study transient replication of HPV genomes. Recent studies have also demonstrated transient replication of whole HPV genomes in transfected cells. Interestingly, one study by del Vicchio et al (1992) has shown that HPV 16 genomes were unable to replicate in the absence of co-transfected E1 and E2, whereas, those of HPV 6, 11 and 18 were able to. Their study has emphasized the role of other viral and/or cellular factors which could be involved in the induction of HPV gene expression after infection. It is possible that steroid hormone-induced expression of HPV 16 E1 and E2 proteins result in increased replication of viral DNA. In addition, zur Hausen (1989a, 1991) has proposed that one of the roles of hormones could be amplification of viral DNA at an early stage of HPV infection. Studies could be carried out using primary ectocervical cells to demonstrate the role of hormones in viral DNA replication. Cervical cells could be transiently transfected by HPV 16 wild

type or mutant GRE-genomes and low molecular weight DNA extracted at different times. Presence of DpnI-resistant plasmid would then indicate the presence of replicating DNA. The GRE mutants can be very useful to delineate the putative role of hormones in viral DNA replication, especially the consensus GRE mutations and the loss-of-function mutations. Significance of this effect on replication, if any, in HPV-mediated oncogenesis is speculative at this point. However, it is clear that any positive result would indicate an important role of hormones in the virus life-cycle.

At the molecular level, experiments could be initiated to examine the role of other cellular/viral factors, in addition to c-jun, NF-1 and OCT-1, in hormone-dependent transcription of viral genes. It is interesting to note that one of the HPV 16 E2 protein-binding sites (nt 7450-7461) is located near the nt 7474 GRE. To date no definitive function has been assigned to the role of the two E2 motifs located in the HPV 16-enhancer region. It is presumed that these two sites might be solely responsible for the transactivating properties of the E2 protein. The two E2-binding sites near the promoter region have been shown to be involved in repression of HPV transcription (Tan et al., 1993). In addition, Gauthier et al (1991) have demonstrated, using cloned cellular factor binding sites, that E2 can cooperate with other cellular transcriptional factors, such as NF-1, AP-1, and the GR. Mutational analysis of the E2-binding site at nt position 7450 and the nt 7474 GRE mutation might shed some light on the possible interaction of the E2 protein and GR in modulating HPV expression.

Another interesting area to study is hormone-independent transformation by

HPV 16. The HPV 16 transformed BRK cells obtained in the presence of hormones are dependent on these hormones for continous growth. However, upon withdrawal of hormones from the media, these transformed cells increasingly or over some time die and occasionally give rise to the appearance of colonies that are resistant to the growth stimulatory effects of glucocorticoids. Similarly, colonies that have appeared in the absence of dexamethasone could also be considered as glucocorticoid-resistant (results in chapter 2). Our laboratory has attempted to characterize the mechanisms involved in glucocorticoid resistance and have used hormone-resistant BRK transformed cells arising from a previously dependent cell line. Results have shown changes in the transcriptional initiation sites between the glucocorticoid-dependent and independent clones. It is clear from my studies that hormone-dependent transcription is directly through three GREs present in the HPV 16 regulatory region. It would be interesting to examine if the transformed colonies arising in the absence of dexamethasone also have changes in the transcription initiation sites compared to the ones obtained in the presence of hormone. In addition, the consensus GRE mutation in pmHPV4 and the triple consensus GRE mutations in pmHPV489 could be used to obtain transformed BRK cells that are more tightly regulated by hormones. Hormone-independent clones arising from these transformed cells could then be used to study the transcription and splicing patterns and compare them with their parental lines. Alternatively, clones arising in the absence of dexamethasone with these HPV constructs can also be used to examine such differences. These differences could be responsible for efficient translation of the E7 oncogene, which

is the major transforming gene of genital HPVs. It is reasonable to assume that differences in transcription and/or splicing patterns are responsible for the increased transforming frequencing of HPV in the presence of hormones (discussed in chapter 7). Although this speculation has so far not been experimentally tested, it remains a posibility. A quick method to examine such transcriptional differences is the use of RNA-polymerase chain reaction technique (Falcinelli et al., 1992).

In addition to the role of glucocorticoids and progesterone, it will also be interesting to examine the role of another steroid hormone, estrogen, in HPV-mediated oncogenesis. HPV gene expression in the HPV 16 containing cell line, SiHa, is induced by estrogens (Mitrani-Rosenbaum et al., 1989), although, no estrogen responsive element has been found or described in the HPV 16 LCR. This response could have been due to indirect effects, unlike the direct role of glucocorticoids and progesterone. So far, not many studies have addressed the role of estrogen in HPV-mediated oncogenesis. Importantly, the more commonly used forms of oral contraceptive pills contain both estrogen and progesterone as their major ingredients. This fact raises an interesting possibility of whether or not the combined effects of both hormones are largely responsible for the observed risk of developing cervical cancer in oral contraceptive users. A recent epidemiological study has shown that both the progesterone and estrogen components of oral contraceptives seem to be responsible for the observed risk associated with these pills (Vandenvelde and Beers, 1992). Estrogens could affect HPV gene expression by two different indirect mechanisms. First, it is known that estrogen treatment of target

cells, such as breast and oviduct, results in the induction of expression of progesterone receptors (Nardulli et al., 1988; Kastner et al., 1990). Estrogens could thus modulate HPV expression by induction of progesterone receptor expression in the target tissue. This hypothetical indirect mechanism can also result in synergistic effects of both hormones and can easily be tested in a transient gene expression assay using HPV enhancer regions cloned in CAT expression vectors. The breast carcinoma cell line, MCF-7, would be suitable to test this synergistic effect, since estrogen is known to stimulate progesterone receptor expression in these cells (Kastner et al., 1990). If results are positive then primary cervical cells should be used since they are also targets for estrogen action (Gorodeski et al., 1989). The second mechanism could be via induction of the cellular c-jun oncogene by estrogen (Wiesz et al., 1990; Chiappetta et al., 1992). As demonstrated in my present study, c-jun confers a positive response to dexamethasone in transient gene expression assays (chapter 5). However, it has also been demonstrated that estrogen induces expression of the c-fos protooncogene (Hyder et al., 1992), which negatively regulates hormone induction at composite GREs. Interestingly, the steroid hormones, progesterone and dexamethasone, but not, minerlocorticoids or androgens, inhibits estrogen-induced expression of c-fos (Kirkland et al., 1992). This indicates that the overall effect of estrogen and progesterone would be to enhance the expression of only c-jun. In this manner target cells will be more permissive for HPV expression in the presence of both hormones. Estrogen-mediated expresssion of c-jun can also directly stimulate HPV expression through the multiple AP-1 motifs in many HPV

LCRs (Chan et al., 1990; Thierry et al., 1992). As described earlier, nude mice implantation studies might provide in vivo evidence of any synergistic effect of both, estrogen and progesterone.

In conclusion, it would be challenging to demonstrate, in the context of the natural target cell, a definitive role of steroid hormones in HPV-mediated oncogenesis and progression. The role of estrogen in HPV-mediated oncogenesis and that of progesterone and dexamethasone in replication of the viral DNA should be initiated. Using several of the above mentioned in vitro and in vivo studies, it is possible to understand more about the role of hormones as an important cofactor in the HPV life-cycle.

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