ANALYSIS OF THE ROLE OF GLUCOCORTICOID RESPONSE ELEMENTS (GREs) OF HUMAN PAPILLOMAVIRUS TYPE 16 THROUGH CONSENSUS MUTATIONS OF GREs

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by

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ABSTRACT

The association of certain papillomaviruses with cancer of the uterine cervix has been established unequivocally by previous studies. In addition, steroid hormones, especially glucocorticoids and progesterone, have been implicated as an important cofactor in the oncogenesis. In this study, the role of glucocorticoid hormones in human papillomavirus type 16 (HPV16) expression and in the immortalization of cervical cells by HPV16 was analyzed.

To demonstrate the direct effect of the hormones on expression, the three glucocorticoid response elements (GREs) in the HPV16 regulatory region were mutated to the consensus GRE (cGRE) sequence, GGTACA(N)$_3$TGTTCT. The effect of these mutations on expression was assayed using the chloramphenicol acetyl transferase (CAT) reporter gene, which was placed under the control of the HPV16 enhancer with the cGREs, in pBLCAT2 vector. Activity of the CAT from these constructs was assayed in the HeLa cervical carcinoma cells. The CAT assays revealed that both single cGRE and triple cGRE mutations enhanced the expression of the reporter gene in the presence of glucocorticoid dexamethasone (dex). Similar results were obtained in the CAT assays when these constructs were transfected into primary baby rat kidney (BRK) epithelial cells.

To examine the molecular involvement of the GREs in the enhanced in vivo CAT activities from cGRE constructs, assays

for DNA-protein interaction were performed _in vitro_ using 22 base pair oligomers, representing the wild type GREs or cGREs at nucleotide (nt) 7385 and nt 7474, and HeLa whole cell protein extracts. Binding with the 22-mer representing the nt 7474 cGRE sequence in the mobility shift assays was more, in comparison with the wild type GRE 22-mer. A moderate difference between the binding ability of nt 7385 GRE and the cGRE 22-mers was evident. The results of UV crosslinking indicated that a HeLa cell protein of approximately 96 kDa, the size of the native glucocorticoid receptor (GR), interacted with the different GRE 22-mers. Southwestern blot assays using these 22-mers also suggested the interaction of the cGRE sequences with a protein, possibly the GR, which is known to be present in HeLa extracts.

The HeLa cervical cell-pBLCAT2 system was an efficient and quantitative model for dex-induced expression, possibly mediated by GR. To advance to a homologous promoter and the natural target of HPV infection, whole HPV16 constructs and primary cultures of ectocervical cells were chosen. The effect of the cGRE mutations on the expression of E6 and E7 viral oncogenes in these cells was analyzed by the sensitive _in situ_ hybridization assays. Strong hybridization signal for the E6-E7 oncogenes was displayed in the presence of dex, when the RNA from the cells transfected with the HPV16 genome construct containing cGREs, was analyzed. Next, these ectocervical cells, which are the appropriate cell type and the natural

target of steroid hormone action, were chosen for stable transfection of the whole HPV16 genome construct with triple cGREs. An immortalized population of cells, designated HEC-16cGRE, was established. These cells were morphologically distinct from the parental ectocervical cells. Analysis of the high molecular weight DNA in HEC-16cGRE cells by Southern blot indicated the presence of HPV16 DNA in an integrated form. The integrated HPV16 DNA was transcriptionally active, as shown by Northern blot assays which showed 4.5 and 2.3 kb transcripts, that contain mostly E6-E7. Interestingly, the viral RNA levels were about 2-fold higher in HEC-16cGRE than in HEC-16, which contains HPV16 with wild type GREs. These cells were not tumorigenic, as shown by soft agar assays and injection into nude mice. Further, the HPV16 genome construct with triple cGREs exhibited greater transformation of BRK cells with activated ras oncogene than the wild type HPV16 genome.

In conclusion, these results demonstrated an increase in expression and transformation from triple cGRE constructs. The enhanced expression was correlated with the specific protein-binding to the GREs. The HEC-16cGRE cells generated from HPV16 with cGRE mutations could be a valuable tool to test the models proposed earlier for HPV and hormone-mediated oncogenesis.

ACKNOWLEDGEMENTS

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A	=	Adenine
AP1	=	Activator protein 1
bp	=	Base pairs
BPV	=	Bovine papillomavirus
BRK	=	Baby rat kidney
BRL	=	Bethesda Research Laboratories
BSA	=	Bovine serum albumin
C	=	Cytosine
CAT	=	Chloramphenicol acetyltransferase
cGRE	=	Consensus glucocorticoid response element
CIN	=	Cervical intraepithelial neoplasia
CIP	=	Calf intestinal alkaline phosphatase
CK	=	Cytokeratin
CPM	=	Counts per minute
Dex	=	Dexamethasone
DEPC	=	Diethyl pyrocarbonate
D-MEM	=	Dulbecco's modified Eagle's medium
DNA	=	Deoxyribonucleic acid
DNase I	=	Deoxyribonuclease I
dNTP	=	Deoxy nucleotide triphosphate
DS	=	Double stranded
DTT	=	Dithiothreitol
E1A	=	Early region 1A
E1B	=	Early region 1B

EDTA	=	Ethylenediamine tetraacetic acid
EMSA	=	Electrophoretic mobility shift assays
ERE	=	Estrogen response element
FCS	=	Fetal calf serum
G	=	Guanine
GR	=	Glucocorticoid receptor
GRE	=	Glucocorticoid response element
GTF	=	General transcription factor
HEC	=	Human ectocervical cells
HEPES	=	N-(2-hydroxyethyl)-piperazine-N'-(2-ethane) sulphonic acid
HIV	=	Human immunodeficiency virus
HPV	=	Human papillomavirus
HRE	=	Hormone response element
kb	=	Kilobases
KBM	=	Keratinocyte basal medium
kDa	=	Kilodaltons
KGM	=	Keratinocyte growth medium
LCR	=	Long control region
LTR	=	Long terminal repeat
MMTV	=	Mouse mammary tumor virus
mRNA	=	Messenger ribonucleic acid
NF-1	=	Nuclear factor-1
nm	=	Nanometre
nt	=	Nucleotide position
ONPG	=	O-Nitrophenyl β-D-Galactopyranoside

ORF	=	Open reading frame
PBS	=	Phosphate buffer saline
PMSF	=	Phenylmethylsulphonyl fluoride
PR	=	Progesterone receptor
PRE	=	Progesterone response element
RNA	=	Ribonucleic acid
rpm	=	Revolutions per minute
SDS	=	Sodium dodecyl sulphate
SV40	=	Simian vacuolating virus 40
T	=	Thymine
T-antigen	=	Large tumor antigen
TE	=	Tris-EDTA
TEF	=	Transcription enhancer factor
TK	=	Thymidine kinase
TLC	=	Thin layer chromatography
UV	=	Ultraviolet

CHAPTER 1

INTRODUCTION

An infectious etiology for "verruca vulgaris" or common
warts was first identified by Ciuffo (1907) in filtered
homogenates from cutaneous benign tumors or warts. This
finding ruled out a bacterial or a protozoan etiology and
raised the possibility of a viral etiology. Subsequently, this
group of viruses was characterized and named papillomaviruses,
due to the nature of the lesions with which they are
associated (reviewed in Broker and Botchan, 1986). Much later,
the cottontail rabbit papillomavirus (CRPV) was isolated by
Shope and Hurst (1933). Moreover, CRPV-induced benign tumors
were observed to progress towards carcinomas (Rous and Beard,
1935). A large body of clinical, epidemiological and molecular
evidence has gathered over the years for the association of
papillomaviruses with human neoplasms, especially carcinomas
of the genital and respiratory mucosa. Through the pioneering
studies by zur Hausen's group and others, it was evident that
certain papillomaviruses are linked to the cancer of the
cervix (reviewed in zur Hausen, 1977, 1991).

1.1 Human papillomaviruses (HPVs)

1.1.1. Significance of HPVs and hormones in cervical cancer

Squamous cell carcinoma of the uterine cervix is the
second most common cancer of the female population in the

world, next only to cancer of the breast (Parkin et al, 1988; Brinton et al, 1993). The association of HPVs with cervical cancer is supported by numerous studies that have indicated the role of these viruses, especially the "high risk" HPV types 16 and 18, in the initiation of and progression to cancer (reviewed in zur Hausen and de Villiers, 1994 and McDougall, 1994a). While HPV16 is most frequently associated with squamous cell carcinoma, HPV18 has been implicated mostly in adenocarcinoma of the cervix (Lorincz et al, 1987; Wilczynski et al, 1988; Tase et al, 1988; Riou et al, 1990).

A prolonged latency between the HPV infection and malignant conversion implies the association of certain cofactors with the transformation of benign lesions to the cancerous condition. In addition to the viral etiology, steroid hormones have been proposed as an important cofactor for HPVs in malignant conversion (Stern et al, 1977; Brinton et al, 1993). Epidemiologically, the use of oral contraceptives, predominantly comprised of progestin and estrogen steroid hormones, was associated with an increased risk for the development of cervical cancer (Negrini et al, 1990; Brinton, 1991; Brinton et al, 1993). In addition, pregnancy which is invariably associated with increased progesterone levels, was also found to be an important risk factor for malignant conversion (Ferenczy, 1989; Bokhman and Urmancheyeva, 1989).

1.1.2 Classification and physical properties

The papillomaviruses have been taxonomically placed in the papovaviridae family along with mouse polyomavirus, simian vacuolating virus 40 (SV40), and the human BK and JC viruses (Melnick, 1962; Melnick et al, 1974). The genotyping of HPVs has identified and characterized more than seventy distinct types based on DNA hybridization analysis (reviewed in de Villiers, 1994). Further, the HPVs have been grouped according to the site of the lesions in which they were initially identified, namely the cutaneous and mucosal types. The mucosal types affecting the genital system were further classified as either "high risk" types (such as HPV 16,18,31,33,35,39,45,51,52,56 and 66) or "low risk" types (such as HPV 6 and 11) based on the likelihood of malignant progression of the lesions with which they are associated.

Electron microscopy has revealed that the papillomavirus particles are about 55 nm in diameter and have an icosahedral symmetry with 72 capsomers (the building blocks of the virus shell) (Howley, 1990). These viruses types have a closed circular double-stranded (ds) DNA genome that is complexed with histones and condensed into nucleosomes. This genome is enclosed in a capsid protein coat. Papillomaviruses are simple assemblies of a DNA genome and a few proteins and, unlike enveloped viruses, are devoid of a lipid membrane.

1.1.3 Genome organisation

Bovine papillomavirus type-1 (BPV-1) has served as a prototype for the study of genome organization of papillomaviruses and shares significant DNA sequence homology with those of HPVs (Howley, 1990). Since my study involves HPV16, I will discuss subsequent sections mainly in reference to this type. A circular map of HPV16 genome has been depicted in Fig.1.1. Broadly, the viral genome can be categorized into two regions: a) a coding region which is on only one strand and contains the open reading frames (ORFs) for viral early and late proteins and b) a non-coding region that spans between the 5' end of the early protein ORFs and the 3' end of the late protein coding region.

1.1.3.1 Early and late region open reading frames (ORFs) and proteins

The early proteins are involved in transactivation, transformation and viral DNA replication. These proteins are E1,E2,E4,E5,E6 and E7 (reviewed in Tomassino and Crawford, 1995). The E1 ORF encodes a protein that is primarily involved in DNA replication (Lambert, 1991). In vitro studies have shown that the E1 protein can complex with the E2 protein and the resulting protein-protein complex binds to the origin of viral DNA replication (Ustav et al, 1991; Yang et al, 1991; Hibma et al, 1995).

The papillomavirus E2 ORF encodes proteins that are

important and complex transregulators of viral transcription and DNA replication (reviewed in Ham et al, 1991). The E2 proteins can have both transactivation and repression functions depending on the context of the E2 protein binding sites in the enhancer/promoter regions of papillomaviruses (PVs) (Androphy et al, 1987; Knight et al, 1991). In addition, the nature of the E2 gene products determines their effect on expression (Choe et al, 1989; McBride et al, 1989, Vaillancourt et al, 1990). These studies showed that the full-length E2 protein mainly acts as transactivator whereas the two shorter E2 proteins expressed from alternative splicing act as transrepressors. Interestingly, the E2 protein of the high-risk HPVs can repress the expression of the E6 and E7 oncoproteins (Cripe et al, 1987; Thierry and Yaniv, 1987; Romanczuk et al, 1990). Further, it was observed that HPV16 DNA with the wild type E2 ORF immortalized primary human keratinocytes with less efficiency than the HPV16 genome harbouring mutations in the E2 ORF (Romanczuk and Howley, 1992).

The function of the E4 protein is likely to be necessary for viral maturation (Brown et al, 1988) in a way similar to the late proteins, although E4 is not present in the virus particle.

The E5 protein is the major transforming protein of BPV-1 (Schiller et al, 1986; Martin et al, 1989). The E5 protein of HPV16 has also been shown to have oncogenic potential,

Fig. 1.1. Circular map of the HPV16 genome.

The size in basepairs (bp) of the genome is indicated inside the map. The figure depicts ORFs for early (E1, E2, E4, E5, E6 and E7) and late (L1 and L2) proteins. The regulatory region or long control region (LCR) has been indicated between 5' end of early ORFs and 3' end of late ORFs. The numbers indicate the nucleotide positions in the genome.

HPV 16
7904 bp

4000 3000

5000

2000

6000

1000

7000 7900/1

LCR
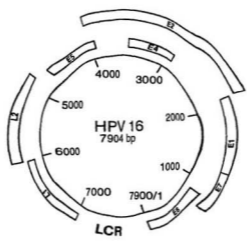
transforming cells partly through the modulation of the signal transduction pathway (s) of growth factors (Leechanachai et al, 1992; Pim et al, 1992; Banks and Matlashewski, 1993).

The E6 and E7 proteins are the potential oncoproteins of HPVs (reviewed in Vousden, 1990 and Howley, 1991). The E6 gene is transcriptionally active in human cervical carcinoma cells containing high risk genital types HPV16 and 18 (Baker et al, 1987). The E6 proteins of high risk HPVs, like the SV40 T antigen and adenovirus E1B, can complex with the p53 tumor suppressor (Werness et al, 1990; Scheffner et al, 1990). It has been observed that the E6 proteins of HPV types 16 and 18 stimulated the degradation of p53, resulting in the abrogation of the transactivation function of p53, which is necessary for the control of cell cycle progression (Crook et al, 1994). The transforming function of E6 was supported by studies that showed that both E6 and E7 proteins were necessary and sufficient for immortalizing primary human cervical cells, the natural target of the genital HPVs (Munger et al, 1989b; Hawley-Nelson et al, 1989).

The E7 oncoprotein is a multifunctional protein that is similar to adenovirus E1A protein in its ability to complex with the pRB tumor suppressor, induce DNA synthesis and cooperate with activated ras in the transformation of rodent cells (Dyson et al, 1989; Munger et al, 1989a; Barbosa et al, 1991; Heck et al, 1992). While both E6 and E7 were necessary to transform primary human fibroblasts and keratinocytes, E7

protein alone was sufficient to transform rodent cells in cooperation with ras (Storey et al, 1988; Barbosa et al, 1991). The oncogenic nature of E6 and E7 proteins of high risk HPVs in transformation assays correlates with the clinical association of these HPV types with anogenital cancers, whereas the E6 and E7 proteins of low risk HPVs had poor or no transforming capacity in the same assays.

The late proteins L1 and L2 are encoded by the late region in the HPV genome. While L1 is the major capsid protein, L2 is the minor capsid protein (Zhou et al, 1991; Kirnbauer et al, 1992; Rose et al, 1993).

1.1.3.2 Long control region (LCR)

The HPV regulatory region, which is also known as long control region (LCR), is localized to a region of about 700 basepairs (bp), extending between the 3' end of late genes and the 5' end of early genes. The LCR has many cis-acting elements that control viral replication and gene expression (Sibbet and Campo, 1990; Chong et al, 1990, 1991; Apt et al, 1993). The transcription of viral early and late proteins is initiated from a single promoter at nucleotide position (nt) 97 at the 3' end of the LCR (Smotkin and Wettstein, 1986) and therefore the promoter site was designated P97.

The LCR contains elements that bind tissue-specific and ubiquitous regulatory factors (Fig.1.2). The tissue-specific expression of the P97 promoter was assumed to be mediated by

a cell-type-specific or keratinocyte dependent (KD) enhancer. This could possibly explain the strict epitheliotropic nature of HPV infection (Cripe et al, 1987). Further, the KD enhancer region, that was mapped to an 88 nucleotide fragment between nt 7631 to 7718 of HPV16, was found to contain binding sites for several factors. An example of this is the cytokeratin (ck) motif, TTTGGCTT, that is also present in the promoter regions of cellular genes expressed in keratinocytes (Blessing et al, 1987). However, it was later reported that this ck motif does not bind a unique epithelial-specific factor. Instead, it was one of several binding sites for nuclear factor-1 (NF-1) with a common TTGGC motif (Cripe et al, 1990; Chong et al, 1991).

DNase I footprinting analyses have identified a number of specific recognition sequences for cellular factors within the HPV16 LCR (Fig.1.2). The most important of these are the binding motifs for the transcription factors, activator protein 1 (AP1), nuclear factor-1 (NF-1), octamer-binding factor (Oct-1), SP1 and transcriptional enhancer factors -1 and -2 (TEF-1 and TEF-2) (Gloss et al, 1989a; Chong et al, 1991). Also, one more important cis-acting element identified in the HPV16 enhancer is the binding sites for glucocorticoid and progesterone receptors, which mediated hormone-induced expression (Gloss et al, 1987; Chan et al, 1989; Chong et al, 1990; Mittal et al, 1993a and 1993b). Binding sites in the LCR for the transcription factor YY1 have been identified in high

Fig. 1.2. Schematic representation of regulatory long control region (LCR) of HPV16.

The LCR and the ORFs are indicated around the circular map of HPV16. The LCR is shown enlarged below the circular map. The LCR contains sites for binding of several cellular proteins and the viral E2 protein, as listed below. The binding sites for these transcription factors have been depicted by the corresponding symbols. The site and the direction (arrow) of the initiation of transcription at the promoter P97 have been indicated. The positions of the GREs have been indicated by the first nucleotide of the palindrome in the LCR. YY1 (rev) refers to those YY1 sites which are in reverse orientation.

HPV 16

E7
E6
E1
LCR
E2
L1
E5
L2

7385 7474 7640 P97

TEF-2
AP1
Sp1
E2
YY1
YY1 (Rev)

GR/PR
NF-1
TEF-1
Oct-1
MSPF
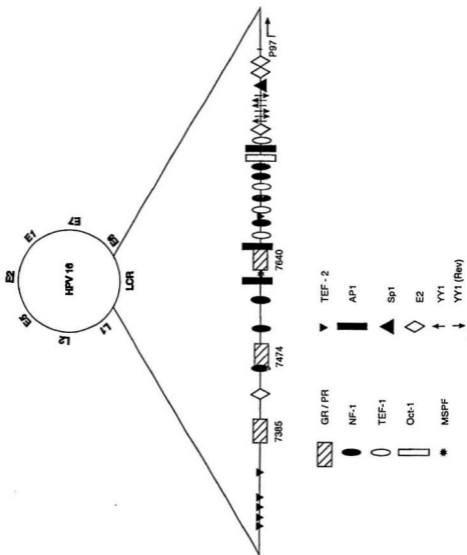
risk HPV16 and HPV18 (Bauknecht et al, 1992; May et al, 1994). In the enhancer region of HPV16, a regulatory element that is dependent on the methylation status to bind to a cellular factor, methylation sensitive papillomavirus transcription factor (MSPF), has been reported (List et al, 1994).

On the whole, the HPV LCR exhibits typical features, such as AT-rich regions, RNA polymerase II promoter, constitutive and inducible transcriptional enhancers containing binding sites for viral E2 transactivator/repressor and several cellular factors.

1.1.4 HPV life cycle

The viral replicative cycle was found to be closely related to the differentiation pattern of infected epithelial cells (Broker and Botchan, 1986; Howley, 1990). The virus is likely to reach target cells of squamous epithelium and columnar epithelium of ectocervix and endocervix, respectively, through mechanical injury (Meisels and Fortin, 1976; Reid et al, 1984). This allows access of the virus to the basal cells, wherein the viral DNA is maintained in an episomal state in a low copy number. The viral DNA amplifies as a consequence of differentiation of host cells. This differentiation triggers late gene expression and capsid assembly in the superficial layers of the stratified epithelium (Lambert, 1991). The HPV infections, especially high risk types, in the cervix is often associated with a set

of premalignant changes in the epithelium referred to as cervical intraepithelial neoplasia (CIN) (Rader et al, 1990). This CIN is further classified as CIN I through CIN III in the increasing order of histological abnormalities. In CIN type I or genital warts, a slightly altered differentiation pattern is observed which is indicated by the presence of the undifferentiated basal cells in 1/3 portion of the epithelium (Hurlin et al, 1991; Blanton et al, 1991). On the other hand, in CIN type III and in carcinomas, the viral DNA usually gets integrated with the host genome and the entire cervical epithelium manifests an undifferentiated state (McCance et al, 1988).

A number of laboratory systems have been developed for the propagation of HPVs. Keratinocytes immortalized by HPVs were initially considered for the study of viral infection. However, due to the subsequent loss of epithelial features such as differentiation, study of viral replication and maturation of viral particles remained elusive. Subsequently, using nude mice xenografts of HPV11-induced benign lesions, a productive replication of this papillomavirus type could be achieved (Kreider et al, 1987). Further, the W12 cell line that contains HPV16 DNA in an episomal state in about 100 copies per cell was found to be a useful system for virus propagation in athymic mouse grafts (Stanley et al, 1989). The production of virus-like particles was also achieved using a recombinant vaccinia expression system that allows generation

of pseudovirions by viral L1 and L2 proteins (Zhou et al, 1991). Also, the production of BPV-1 infectious virions in cells containing episomal DNA with overexpression of L1 and L2 proteins has been reported (Zhou et al, 1993).

Subsequently, an organotypic (raft) culture system has been employed to study the differentiation pattern of human foreskin keratinocytes containing HPVs (McCance et al, 1988). This system, which allows the study of the stratification and differentiation of epithelia, has been found to be a model system to analyze the altered differentiation pattern in HPV-containing cells (reviewed in Meyers and Laimins, 1994). Further, HPV16-induced differentiation abnormalities in these cultures were comparable with the CIN (Pecoraro et al, 1989; Blanton et al, 1991; Merrik et al, 1992). Raft cultures of HPV-immortalized cells, which resemble in vivo lesions, have been ideal to study progression of HPV-induced lesions in vitro (Hurlin et al, 1991). Further, the HPV type 31b exhibited differences in expression in monolayer and in raft cultures (Hummel et al, 1992). This particular study revealed evidence for the initiation of transcription from an alternative differentiation-dependent promoter in raft cultures, but not in monolayer cells.

1.2 Regulation of gene expression of HPV16

The major level of regulation of gene expression is at the stage of initiation of transcription. For the

epitheliotropic nature of HPVs, the regulation of initiation
of transcription is a crucial aspect of the cell-specific gene
expression. In general, the initiation of transcription occurs
at a defined start site or promoter region by RNA polymerase
II in association with a complex set of proteins termed
general transcription factors (GTFs), including TFIIA, TFIIB,
TFIID, TFIIE, TFIIF and TFIIH (reviewed in Tjian and Maniatis,
1994; Zawel and Reinberg, 1995). The basal level of
transcription is augmented by sequence-specific transcription
activators that bind to the distinct enhancer regions
(Mitchell and Tjian, 1989). In fact, these transcription
factors might establish an active link with the basal
transcription machinery, thereby leading to activated
initiation of transcription.

The HPV16 LCR is approximately 1000 bp, comprised of a
constitutive enhancer region of 400 bp. The LCR contains a
proximal region containing the P97 promoter, for the
transcription of both early and late genes (Smotkin and
Wettstein, 1986; Smotkin et al, 1989, Taniguchi et al, 1993).
A TATA box, 39 bp upstream of the start codon of E6 gene, acts
as the site for the formation of the initiation complex for
the early and late genes. Initiation of transcription is
facilitated by the binding of the SP1 transcription factor to
a GC-rich region slightly upstream of the TATA box (Gloss and
Bernard, 1990; Tan et al, 1992, 1994). About 400 bp further
upstream of the SP1 site is the main transcriptional

activation region of HPV16. This enhancer region harbours <u>cis</u> acting elements for both cell-specific and ubiquitous transcription factors (reviewed in Bernard and Apt, 1994).

1.2.1 Role of <u>trans</u>-acting cellular and viral factors

DNase I footprinting and mobility shift assays have identified binding sites for ubiquitous factors such as NF-1, Oct-1, AP-1, SP1 and TEF-1 and TEF-2 (reviewed in Hoppe-Seyler and Butz, 1994). Strikingly, although these factors are ubiquitous in nature, the epithelial specificity of expression is apparently imparted by synergism and differing concentrations.

Several NF-1 binding motifs seem to be important for the transcriptional activity of the viral enhancer (Gloss <u>et al</u>, 1989b; Chong <u>et al</u>, 1990, 1991; Apt <u>et al</u>, 1993, 1994). However, in the context of the HPV16 promoter, the presence of several half palindrome (TTGGC) NF-1 sites with low affinity for NF-1, suggests only a marginal role of NF-1 for epithelial-specific activation of transcription. Nevertheless, the expression pattern of HPV16 from alternatively spliced forms of NF-1 in epithelial and fibroblast cells provide some evidence for the role of NF-1 sites in cell-type specific regulation of expression (Apt <u>et al</u>, 1993; 1994). Further, Taniguchi <u>et al</u> (1993) reported the mapping of a cell-type specific regulatory region upstream of the previously proposed KD region. This region was found to consist of three consensus

binding sites for NF-1 that were important for epithelial-specific expression. Some evidence is available for the role of TEF-1 in cell-specific activation, possibly through a cell-specific coactivator for TEF-1 (Ishiji et al, 1992). However, it may not be the sole factor for epithelial specificity.

The NF-1 motifs bind factors that also cooperate with those binding to the AP-1 elements in the context of a heterologous promoter (Chong et al, 1990). The involvement of AP1 is supported by the presence of multiple AP1 sites in both HPV16 and HPV18 and may be important in signalling cascade, ultimately modulating transcription of HPV genes (Chan et al, 1990; Chong et al, 1991; Thierry et al, 1992; Bernard and Apt, 1994; Peto et al, 1995). The promoter proximal SP1 site is likely to be important for regulation through an interplay between enhancer binding factors and SP1 in activation (Gloss et al, 1990; Tan et al, 1992; 1994).

Negative regulation of HPV16 E6/E7 promoter by cellular factor YY1 has been reported (Bauknecht et al, 1992; May et al, 1994). The derepression of viral oncogenes as a result of deletions in the YY1 transrepressor could be an alternate mode of deregulated HPV expression (May et al, 1994). Interestingly, methylation of certain regions of the HPV16 enhancer could also be a negative regulatory mechanism of transcription, mediated by methylation-sensitive factor MSPF (List et al, 1994). This recent study revealed that the CaSki cervical carcinoma cell line, containing multiple copies of

integrated HPV16 DNA, had methylated and inactive regions in the enhancer of some copies of HPV16.

Papillomaviral E2 protein is an important regulator of HPV expression (reviewed in Ham et al, 1991). Although the BPV-1 E2 protein acts both as activator and repressor (Thierey and Yaniv, 1987), the E2 protein of HPVs regulates expression mainly as a repressor of E6 and E7 expression, as well as its own expression (Cripe et al, 1987; Gloss et al, 1987; Phelps and Howley, 1987, Chin et al, 1988; Thierry and Howley, 1991). The E2 protein of BPV-1 could also cooperate synergistically with cellular factors, such as AP1, glucocorticoid receptor (GR) and NF-1, to activate transcription (Monini et al, 1991; Gauthier et al, 1991). E2 might negatively regulate epithelial-specific activation due to displacement of activators from the promoter/enhancer region, since E2 binding sites overlap motifs for cellular factors (Tan et al, 1992; 1994). However, transactivation by E2 mediated through E2 binding motifs in the LCR from a heterologous, but not the homologous promoter, has been reported (Marshall et al, 1989). Further, Bouvard et al (1994) reported that the full length E2 of HPV16 acted as a transcriptional activator in cervical keratinocytes.

Overall, there has been no single factor identified so far that can be held responsible for cell-type specific expression of HPV16 enhancer. Instead, a multitude of factors may contribute additively or synergistically to the enhancer

activity and epithelial specific expression. Interestingly, the lack of E2 negative regulation due to the disruption of the E2 ORF upon integration of the HPV genome into the host chromosomal DNA in high risk HPVs, could lead to enhanced expression of HPV oncogenes (Schwarz et al, 1985; Smotkin and Wettstein, 1986; Sang and Barbosa, 1992; Romanczuk and Howley,1992).

1.2.2 Role of glucocorticoid steroid hormones

Glucocorticoid hormones, a subgroup of the steroid hormones, modulate several physiological functions in different cells. The action of these hormones usually involves their influence on gene expression. Predominantly, glucocorticoids have a positive regulatory effect on the expression. However, they regulate cellular activities by negative effects also (reviewed in Beato et al, 1991). In general, the effect of these hormones on gene expression is at the level of initiation of transcription. Glucocorticoid hormones gain entry into the cell through the cell membrane by passive diffusion and bind to a specific intracellular glucocorticoid receptor (GR) (Yamamoto and Alberts, 1976). The GR has an intracytoplasmic location in association with a set of heatshock proteins in the absence of hormones (reviewed in Pratt et al, 1992 and Smith and Toft, 1993). The GR acquires nuclear localization upon hormone stimulation and in the nucleus it regulates gene activity through binding to specific

DNA regulatory elements called glucocorticoid response elements (GREs) (Payvar et al, 1983; Scheidereit and Beato, 1984).

1.2.2.1 Glucocorticoid receptor (GR)

The GR belongs to the superfamily of ligand-inducible intracellular receptors for steroid hormones (reviewed in Evans, 1988 and O'Malley, 1990). In the cytoplasm, GR exists as an inactive molecule in association with the hsp90 (Sanchez, 1992), hsp70 (Sanchez, 1990; Srinivasan et al, 1994) and hsp56 heatshock proteins (Sanchez, 1990). Upon hormone stimulation, the GR dissociates from the heatshock proteins (Mendel et al, 1986; Denis et al, 1988).

Molecular cloning of GR cDNA and subsequent analysis of the protein structure revealed that the GR consists of four main domains that are involved in ligand binding, dimerization, DNA binding and transcription regulation, respectively (Dellweg et al, 1983; Carlstedt-Duke et al, 1987). Among these domains, the DNA binding domain is the most conserved region (Hollenberg et al, 1985; Giguere et al, 1986). This domain of approximately 70 amino acids has eight cysteine residues that are tetrahedrally coordinated with two zinc ions to form zinc finger motifs (Rusconi and Yamamoto, 1987; Hollenberg et al, 1985; (Freedman et al, 1988; Hard et al, 1990). The zinc finger motifs have been found in a number of DNA-binding regulatory proteins such as transcription

factor IIIA of Xenopus, chicken estrogen receptor and v-erb A oncogene product (Miller et al, 1985; Weinberger et al, 1985; Krust et al, 1986). The zinc finger organization was found to be important for the structural integrity and DNA-binding function of GR (Pan et al, 1990; Miyamoto et al, 1991; Zilliacus et al, 1992). The transactivation domain of GR has been mapped to the amino terminus and to a small region in the carboxy terminus of the receptor molecule that overlaps the ligand binding domain (Giguere et al, 1986; Guiochon-Mantel et al, 1989). Further, the transactivation domain has been mapped to a 41-amino acid core region of the GR (Dahlman-Wright et al, 1994). Supershift assays using antibodies against the activation core region demonstrated the importance of this region of the GR in the activation of transcription (McEwan et al, 1994).

1.2.2.2 Glucocorticoid response element (GRE)

The specific binding of glucocorticoid-activated receptor to its cognate DNA element was shown initially for the mouse mammary tumor virus (MMTV) provirus, which has served as a classic model for the study of hormonal regulation of gene expression (Huang et al, 1981; Payvar et al, 1983; Kalff et al, 1990). The MMTV long terminal repeat (LTR) region containing the hormonal regulatory region (reviewed in Truss and Beato, 1993) has been used to demonstrate hormonal regulation of expression of a transfected gene. Using chemical

protection assays and DNase I footprinting analysis, an imperfect palindrome of 15 bp has been shown to be the DNA binding site for the activated GR, termed GRE (Beato et al, 1989). Subsequently, it was demonstrated that the receptor interacts with the GREs present in the regulatory region of many cellular genes (Payvar et al, 1983; Bruzdzinsky et al, 1993; Lee and Tsai, 1994; Rozansky et al, 1994; Murasawa et al, 1995). Similar studies with the purified progesterone receptor (PR) also yielded a DNA binding pattern akin to that of the GR (Ahe et al, 1985). Induction by glucocorticoid or progesterone hormones decreased when certain conserved bases of the 15-mer palindrome were mutated (Cairns et al, 1991; Truss et al, 1992), confirming that the specific binding of the receptor to its respective DNA binding element was necessary for the hormone response.

A comparison of the many natural GREs occurring in the regulatory region of viral genomes and cellular genes identified the consensus sequence for these elements as GGTACA(N)₃TGTTCT (N is a nonconserved nucleotide) (Beato et al, 1989). The TGTTCT hexanucleotide in the 15-mer sequence is highly conserved and is likely to play an important role in the interaction with the receptor (Scheidereit and Beato, 1984). The GGTACA left hexanucleotide is less conserved except for the C. This consensus sequence displays an imperfect dyad symmetry, suggesting that a dimer of the receptor interacts specifically with the 15-mer GRE motif (Ahe

et al, 1985). This same element can also mediate induction by progesterone (Chalepakis et al, 1988).

1.2.2.3 Mechanism of regulation of transcription by glucocorticoids

A multitude of pathways may be involved in hormone-induced expression. For example, the ligand binding to the receptor transforms the inactive receptor into an activated DNA binding protein that induces activation of transcription (reviewed in Evans, 1988; Beato, 1989 and Cato et al, 1992). However, the hormone receptor may bind to a hormone response element (HRE) in the absence of hormone in vitro, whereas in vivo the receptor-DNA binding depends on the presence of the ligand (Groyer et al, 1987). This could be explained by the dissociation of the receptor from the hsp90 protein which otherwise renders the unliganded receptor inactive in vivo (Groyer et al, 1987).

Upon hormone stimulation, the receptor acquires DNA-binding ability that may be dependent on topological features of the HRE. In general, a supercoiled state of DNA is required for the interaction of steroid receptors with the respective binding sites. This is especially true in the case of the PR which is strictly dependent on the DNA topology of the transfected construct containing the hormone response sequences (Truss et al, 1993). However, glucocorticoid hormone response was found to have less stringent requirement for

supercoiled DNA topology (Carballo and Beato, 1990). Further, glucocorticoid and progesterone inducibility of the cloned MMTV-LTR region is reduced significantly along with a decrease in cooperative DNA binding, when any of the four conserved half palindrome HRE motifs TGTTCT is mutated (Chalepakis et al, 1988). Also, a cooperative functional interaction between receptor molecules bound at the HRE region was revealed, suggesting the role of multiple HREs in hormonal regulation of the MMTV LTR (Chalepakis et al, 1988).

Upon binding of the GR or the PR to DNA, activation of expression could be brought about by: i) The influence of the bound receptor in promoting/inhibiting the binding of other transcription factors to the promoter. These effects may vary from synergism or cooperativity to mutual interference. ii) Alteration in chromatin structure so as to favour activation through a DNA conformational change, making the regulatory region accessible to other transcription factors.

Synergism or cooperativity terms, which are used interchangeably, describe a response from two or more regulatory DNA elements or factors that is more than the sum of the response of the individual component (reviewed in Ptashne, 1988). This phenomenon has been demonstrated for the GR, whereby it interacted with the yeast GAL4 transactivator to activate transcription (Kakidani and Ptashne, 1988; Wright and Gustafsson, 1991). The interaction of GR with the GTFs, especially with TFIIB, has been reported (Klein-Hitpas et al,

1990; Ing et al, 1992). TFIID has been found to be another
target for steroid receptor—mediated interaction and
transactivation (Bron et al, 1993).

The activated GR bound to GRE as a dimer could synergize
with either another GRE (Ankenbauer et al, 1988; Strahle et
al, 1988; Tsai et al, 1990; Oshima and Simons, 1993) or other
cis acting elements of factors, such as octamer binding
factor-2A (Oct-2A), CACCC box factors and Ets transcription
factors (Weiland et al, 1991; Espinas et al, 1994). There is
evidence for synergism between GR/PR and NF-1 for induction
by glucocorticoid and progesterone hormones (Schule et al,
1988; Kalff et al, 1990). It was observed in these studies
that the induction of expression was abolished upon mutation
of the NF-1 binding site. However, induction was observed in
another instance, wherein there was a lack of any NF-1 binding
site between the HRE and promoter in similar assays
(Bruggemeier et al, 1990), suggesting alternative NF-1-
independent pathways of induction. Recently, the role of the
pRB tumor suppressor in potentiating glucocorticoid-activated
transcription has been reported (Singh et al, 1995).

Several studies have reported the interaction of GR with
other transcription factors (Mordacq et al, 1989; Diamond et
al, 1990). This interaction could modulate the transactivation
function of GR through interference. The classic example of
this is the interaction between GR and the AP-1 family of
transcription factors at their respective DNA elements that

overlap with one another called composite elements (Miner and Yamamoto, 1992). This type of interaction could either positively or negatively regulate the hormone-mediated expression (Yang-Yen et al, 1990; Schule et al, 1990; Touray et al, 1991; Berko-Flint et al, 1994; Mittal et al, 1994). Further, the effect of the AP-1 factors on hormone response has been ascribed to the members of the AP-1 family, generally c-jun and c-fos, that can form either hetero or homodimers (Chiu et al, 1988; Jonat et al, 1990).

The other mechanism for hormonal regulation is the role of chromatin structure in hormone-induction. DNase I protection assays revealed that the nucleosomal structure spanning the GRE altered upon hormone stimulation (Zaret and Yamamoto, 1984). Also, the binding of the receptor modified the nucleosomal structure to give NF-1 access to the promoter site (Cordingley et al, 1987). Recently, Truss et al, (1995) reported that hormones could induce rearrangement of the nucleosomal structure that could allow binding of other transcription factors to the MMTV regulatory region simultaneously. Thus, it is likely that hormone-induced receptor binding to cognate DNA somehow modifies DNA conformation and allows the subsequent interaction of other transcription factors in the promoter/enhancer region.

1.2.2.4 Role of glucocorticoid hormones in HPV expression

Progesterone and glucocorticoid hormones have been shown to have important roles in the regulation of high risk HPVs, especially HPV16 (Chan et al, 1989; Chong, 1990). This is supported by the protection of GREs in the HPV regulatory region in DNase I footprinting assays and by the demonstration of specific binding of GR/PR (Chan et al, 1989). The essential role of dexamethasone (dex), a synthetic glucocorticoid, in the transformation of rodent cells by HPV16 along with an activated ras oncogene, through an increase in expression of viral oncogenes E6 and E7 was reported (Pater et al, 1988; 1992b). Interestingly, the SV40 early region, under the control of the regulatory region of low risk HPV11 containing a near-consensus GRE sequence, could transform BRK cells in dex-dependent manner (Pater et al, 1988).

As mentioned earlier, the HPV16 cell-specific constitutive enhancer contains binding sites for the GR known as GREs (Chan et al, 1989). In these studies, the TGTACA(N)$_3$TGTCAT GRE sequence at nt 7640 of HPV16 LCR has been shown to bind to the GR/PR in transient expression assays. This GRE has been shown to activate transcription by a heterologous promoter in the context of the whole LCR as well as a 15-mer GRE oligonucleotide placed upstream of a basal promoter (Gloss et al, 1987). Further, the HPV16 GRE mediated an increase in transcripts encoding E6 and E7 oncogenes in S1 nuclease mapping of RNA from SiHa cervical carcinoma cells

(Chan et al, 1989).

Loss-of-function mutations of the GRE at nt 7640 of HPV16, led to the identification of two additional GREs in the regulatory region (Mittal et al, 1993a and 1993b). Transient expression assays in HeLa cervical cells suggested the regulatory role of hormones through the three GREs, independently or in combination. In addition, characterization of these three GREs by in vitro binding assays and by transformation assays in the above studies implicated a significant role for glucocorticoid hormones in HPV-mediated oncogenesis. The positive regulatory role of the c-jun protooncogene on dex-induced expression through the composite GRE at nt 7640 (that overlaps an AP1 motif) suggested the importance of activated GR in association with other regulatory factors (Mittal et al, 1994).

Further, GRE-like sequences were also identified in other genital HPV types like HPV6,11,18 and 33 (Chan et al, 1989). This is suggestive of a role for physiological factors such as the steroid hormones in the modulation of HPV expression. On the whole, the modulation of HPV expression from GRE/PREs in genital HPV types is likely to correlate with an increased risk for cervical cancer associated with the hormones of pregnancy and oral contraceptives (Ferenczy, 1989; Brinton, 1991). The role of these hormones could be at the hormone responsive oncogenic HPV target site in the transformation zone of the cervix, where the ectocervical squamous cells

merge with the simple endocervical cells (Ferenczy and Winkler, 1987).

1.3 Immortalisation and transformation by HPVs *in vitro*

A great amount of information has been obtained concerning multistep carcinogenesis through the studies involving the DNA tumor viruses, adenoviruses, polyomaviruses, herpesviruses and papillomaviruses, in animal and human cells. These studies have unravelled the functions of the viral and cellular oncogenes that are central part of the control of cell growth and viral-cellular interactions. Indeed, the human papillomaviruses have served as important tools in the understanding of epithelial cell carcinogenesis. The availability of recombinant viral subgenomic segments and appropriate cell systems has enabled the study of the development of cancer in viral-infected cells. While many types of the papillomaviruses cause benign proliferative lesions in host tissue, certain high risk types have been linked to preneoplastic and neoplastic conditions (Durst et al, 1985; Lorincz et al, 1987). Interestingly, the potential of these viruses to induce tumors can be studied in experimental systems by immortalization and transformation assays.

Chiefly, cultured human foreskin and cervical epithelial cells have been employed in immortalization assays using recombinant HPV genomes (McCance et al, 1988; Kaur and

McDougall, 1989; Pecoraro et al, 1989; Rader et al, 1990). In these studies, cells transfected with HPV16 or HPV18 DNA acquired an indefinite life span and distinct morphological manifestations suggestive of the potential of these cells for epithelial transformation. However, in the same studies, the low risk HPV6- and 11-transfected cells underwent senescence. Further, immortal cell lines harbouring HPV16 DNA exhibited abnormal differentiation patterns in histological studies in vitro using raft cultures that could mimic CIN in vivo (McCance et al, 1988). These studies possibly reflect more closely the different cellular alterations occurring in the initiation of the carcinogenic process in the natural host tissue. However, these immortal cells are not true representatives of cancerous cells, as these cells did not have tumorigenic potential in anchorage independent growth and nude mice tumorigenicity assays (Pecoraro et al, 1989; Tsutsumi et al, 1992; 1994).

The lack of tumorigenicity of cells immortalized by HPVs implies the necessity of additional events for the malignant conversion. Usually, HPV-immortalized human cell lines are not tumorigenic, except for the rare cases of progression to invasive tumors upon extensive in vitro passaging (Hurlin et al,1991; Pecoraro et al, 1991). In most cases, cysts or small tumor nodules form which have a tendency to regress eventually. Interestingly, the transfected HPV genome was transcriptionally active and viral oncogenes E6 and E7 were

continuously expressed in these cell lines (Schwarz et al, 1985; Smotkin and Wettstein, 1986). When cervical carcinoma tissues were analyzed, there was a significant increase in expression of the cellular oncogenes, c-myc and c-ras (Riou et al, 1985, 1987). This was also observed in cell lines derived from cervical carcinomas (Durst et al, 1987b). Subsequently, malignant transformation mediated by HPV16 in cooperation with the activated ras was successfully demonstrated in rodent cells (Matlashewski et al, 1987; Pater et al, 1988; Mittal et al, 1993b) and in human epithelial cells (Durst et al, 1989). These studies constituted landmark evidence for the tumorigenic potential of these HPVs in an experimental model that could correlate with the HPV-associated human cervical neoplasia.

1.3.1 Role of E6 and E7 oncogenes in immortalization and transformation

A consistent feature of the HPV-containing immortalized and transformed cells, as well as of cervical carcinomas, is the retention and expression of the E6 and E7 oncogenes (Kaur et al, 1989; Hawley-Nelson et al, 1989). In malignant lesions and cell lines derived from cervical carcinomas, the HPV DNA is integrated into the cellular genome in a single copy or in multiple copies (Matsukura et al, 1986; Durst et al, 1987b). Although this integration event is random for the cellular genome, a general pattern of HPV integration sites is usually

observed, in which the viral genome is disrupted between E1 and E2 ORFs (Durst et al, 1985; 1987a). Eventually, only E6 and E7 ORFs are maintained and expressed continuously (reviewed in McDougall, 1994a and Tomassino and Crawford, 1995). Further studies in primary human keratinocytes demonstrated that both the E6 and E7 are required for immortalization (Barbosa et al, 1989; Kaur et al, 1989). Although the E6 gene alone did not immortalize the cells, E7 alone under the control of a strong heterologous promoter was sufficient for immortalization (Halbert et al, 1991). Moreover, under these conditions, both the proteins could very efficiently extend the cellular life span.

The HPV16 E7 was sufficient to induce colony formation and growth in soft agar with ras in a number of established rodent fibroblast cell lines and to immortalize primary rodent cells (Kanda et al, 1987; Tanaka et al, 1989; Crook et al, 1989). However, for full malignant transformation, the cooperation with another oncogene such as an activated ras or fos was necessary (Matlashewsky et al, 1987; Storey et al, 1988; Phelps et al, 1988). It was also shown that HPV16 E6 can cooperate with ras in transformation assays in primary mouse cells (Storey and Banks, 1993). In conclusion, the E6 and E7 proteins of high risk HPV types can induce immortalization in primary epithelial cells. However, full malignant transformation required a second factor, such as an activated ras oncogene, in in vitro assays.

Analysis of cervical carcinomas harbouring HPV 16 or HPV 18 DNA revealed that the E7 transcript is more abundant in these tissues than that in low risk HPV-containing lesions (Sousa et al, 1990). In high risk HPV types, both E6 and E7 are transcribed from a single promoter upstream of E6 (Smotkin and Wettstein, 1986). Alternative splicing within E6 can give rise to three distinct mRNA species that encode full length E6 and E7 and two truncated E6 proteins (Smotkin and Wettstein, 1986; Seedorff et al, 1987). Further, the presence of an intron in E6 was found to facilitate translation of E7 proteins (Sedman et al, 1991). The lower levels of E7 led to a low frequency of transformation and higher levels of E7 resulted in a high frequency of transformation (Liu et al, 1995). It was observed that the 124 nucleotides at the amino terminus of E7 coding sequences of HPV16 enabled HPV11 to transform cells (Pater et al, 1992a). Based on these studies, the oncogenic potential of HPVs is apparently determined by the intrinsic properties of E6 and E7 (whether it is a high or low risk type) as well as by the levels of these proteins.

The E7 oncoprotein is a nuclear phosphoprotein of 98 amino acids (Greenfield et al, 1991). A remarkable study that identified and characterized the transformation function of E7 was the report that this protein had striking structural and functional similarities with other DNA tumor virus transforming proteins (Phelps et al, 1988; Dyson et al, 1992). The amino terminal region of E7 shares significant homology

with conserved regions 1 and 2 (CR1 and CR2) of adenovirus E1A oncoprotein and a homologous region of the SV40 large tumor (T) oncoprotein. Mutational analysis of these regions abrogated _in vitro_ transformation by E7 (Phelps et al, 1988). The region of E7 homologous to CR2 contains a domain involved in binding to the tumor suppressor protein, pRB, a cell cycle regulator (Buchkovich et al, 1989; Munger et al, 1989a). One of the best understood mechanisms of the function of pRB in cell cycle regulation is its interaction with the family of E2F transcription factors, which in turn participate in the control of expression of several genes essential for cell cycle progression (Chellappan et al, 1991,1992; Morris et al, 1993; Melillo et al, 1994). The E7 proteins of HPV16 and 18 bind hypophosphorylated pRB that leads to the release of E2F factors, which in turn upregulate certain genes, for instance b-myb, leading to uncontrolled cell growth (Munger et al, 1989b; Sang and Barbosa, 1992; Munger and Phelps, 1993). It is interesting to note that E7 proteins of high-risk HPVs bind to pRB with higher affinity than that of low risk HPVs, which further consolidates the functional importance of the pRB-E7 interaction for malignant transformation (Barbosa et al, 1990; Imai et al, 1991).

The HPV E7 also associates with another pRB-related cellular protein, p107, which functions in the regulation of cell growth (Davies et al, 1993; Lam et al, 1994). Mutations in conserved amino acids in the pRB-binding domain of E7 led

to decreased efficiency of transformation of rodent fibroblasts (Phelps et al, 1992). However, only a small effect on immortalization of primary human keratinocytes was observed in the above studies, suggesting that pRB is functionally involved in transformation rather than immortalization. It could be speculated, based on this and other studies (Zhu et al, 1993; Mayol et al, 1993), that E7 binding to p107 may be sufficient for immortalization while E7-pRB complex formation is essential for transformation.

The E6 protein is a small nuclear protein and it can associate with the tumor suppressor gene product p53, similar to the SV40 large T-antigen and adenovirus E1B proteins (Barbosa et al, 1989). It is now well established that p53 can control cell proliferation by regulation of transcription of cellular genes involved in cell cycle control. The wild type, but not the mutant p53, was shown to bind to the regulatory region of the WAF1 gene, whose product induces cell cycle arrest through its action as a cyclin-dependent kinase inhibitor (Xiong et al, 1993; EL-Deiry et al, 1993). Interestingly, the E6 protein of high risk HPVs associates with p53 and promotes its degradation in a ubiquitin-dependent pathway (Scheffner et al, 1990; Werness et al, 1990; Sedman et al, 1992). However, in these studies, low risk type HPV E6 did not stimulate degradation of p53. The inactivation of p53 by E6 observed in vitro in these studies may correlate with reduced p53 levels in vivo in HPV-containing cervical

carcinoma cell lines, such as SiHa, CaSki and HeLa (Crook et al, 1991, 1992). Further, the presence of low levels and mutations of p53 in cervical and other tumors was reported (Nigro et al, 1989; Crook et al, 1991,1992). The human keratinocytes expressing HPV16 E6 had markedly reduced p53 levels, which were not elevated upon exposure to DNA-damaging agents which under normal circumstances induce p53 (Demers et al, 1994; Bristow et al, 1994).

A recent report suggested that the degradation of p53 by E6 alone did not stimulate cell growth in keratinocytes, and indicated that E6 may have additional cellular target(s) which are necessary to deregulate growth, apart from p53 (Ishiwatari et al, 1994). This was further supported by the observation that E6 of HPV16 and 18 could interact with multiple proteins, one of them having a kinase activity (Keen et al, 1994). Thus, it appears that E6 and E7 proteins attack two or more targets in cell cycle control and these attacks initiate a dynamic alteration in cell cycle, leading to the manifestations of abnormal cell growth.

1.3.2 Role of cofactors in HPV-mediated transformation and oncogenesis

Apart from the inactivation of p53 and pRB tumor suppressors by E6 and E7 transforming proteins, respectively, other endogenous cellular mechanisms might be involved in progression to cancer (reviewed in Herrington, 1995). These

mechanisms may include chromosomal abnormalities, amplification of cellular oncogenes and rearrangement in chromosomes. Although high risk HPVs have malignant potential, in most cases the hyperplastic lesions produced by these HPVs do not attain the neoplastic condition. Rather, these lesions regress. The prolonged latency between HPV infection and the appearance of cervical malignancy implies a role for additional cofactors and/or events occurring after HPV infection. Possible exogenous cofactors have been reviewed (McDougall, 1994b; Herrington, 1995; Khare et al, 1995). Several studies have indicated that smoking is a risk factor in the development of cervical cancer (Daling et al, 1992; Brisson et al, 1994; Kenny, 1994). Studies have shown the presence of carcinogens from tobacco smoke, for instance nitroso compounds, in the cervical mucosa of females manifesting cervical dysplasia (Simons et al, 1995). Garret et al, (1993) employed primary and HPV18-immortalized human keratinocytes to study carcinogenic conversion by HPV and nitrosomethylurea (NMU). It was observed that only cells containing both HPV18 and NMU were transformed.

There are several reports of the involvement of other biological agents in association with HPVs in HPV infection and oncogenesis. Herpesviruses, which also cause persistent genital infections similar to HPVs, have been implicated in HPV-associated cervical cancers. Human herpes virus-6 (HHV-6) enhanced the expression of HPV early proteins and induced

rapid tumor formation by HPV-transformed cervical cells in nude mice (Chen et al, 1994a and 1994b). A few other studies have reported the association of Epstein-barr virus, human immunodeficiency virus (HIV) and cytomegalovirus (CMV) with HPV-associated neoplasia (Scurry and Wells, 1992).

1.3.2.1 Role of steroid hormones in HPV-associated oncogenesis

Among several risk factors associated with HPV-induced oncogenesis, the steroid progesterone and glucocorticoid hormones have been implicated as important cofactors for cervical carcinogenesis, based on epidemiological, clinical and experimental studies. This has been supported by studies relating to the increased risk of cervical cancer in oral contraceptive users and multiparous individuals (reviewed in zur Hausen, 1991; Schneider, 1993; Pater et al, 1994; Herrington, 1995). Specific hormones, especially dex and progesterone hormones, have been implicated in HPV-induced transformation. In fact, steroid hormones from oral contraceptives facilitated HPV16 cooperation with ras oncogene in transformation assays, and the transformation could be specifically abolished by the RU486 steroid antagonist (Pater et al, 1990, 1991). Dex glucocorticoid hormone increased the potential of HPV16 DNA to transform rodent epithelial cells (Crook et al, 1989; Pater et al, 1988, 1990, 1992b) and also human cells (Schlegal et al, 1988; Durst et al, 1989; Sexton et al, 1993).

As mentioned earlier, several genital HPV types harbour GREs in their regulatory region and these GREs may mediate hormone induction of expression (Gloss et al, 1989a; Chan et al, 1989). In the SiHa cervical carcinoma cell line, which contains a single copy of HPV16 in an integrated form, the dex enhanced the expression of E6 and E7 transforming genes (Chan et al, 1989). In addition, two more GREs were identified in the HPV16 LCR and these GREs were functional in hormone-induced transformation in rodent epithelial cells (Mittal et al, 1993b). Further, all these GREs are likely to mediate dex-induced expression of E6 and E7 in cervical cells in the context of the HPV16 promoter as well as in the context of a heterologous promoter.

1.4 Hypothesis and objectives

Based on the above reports, it is clear that the glucocorticoid dex and progesterone steroid hormones have a role in the HPV-mediated oncogenic process. This is supported by the observation that the presence of hydrocortisone glucocorticoid hormone in the medium enhanced the transformation of keratinocytes by HPV16 and ras oncogene (Durst et al, 1989). Previously, it has been proposed that HPV16 might mediate hormone-induced expression in cervical cells through multiple GREs (Mittal et al, 1993a and 1993b).

In the studies of this thesis, to further investigate the direct role of hormones in expression and HPV16-mediated

immortalization, the three GREs were converted to functional consensus sequences. This would clearly evaluate whether dex can induce expression directly or indirectly due to the consensus GRE sequences. The use of consensus sequence elements to characterize transcriptional regulation of homeoproteins in the development of Drosophila (Yamamoto et al, 1992; Cavarec and Heidmann, 1993) and to characterize specific DNA elements in nematode Cenorabditis elegans (Vanwye et al, 1991) has been reported. Also, the consensus sequence of GREs in the regulatory region of several cellular genes have been useful to study glucocorticoid-mediated expression (Rozansky et al, 1994; Jenab and Inturrisi, 1995).

Earlier it was hypothesized that dex increases the E6-E7 message of HPV16 through the binding of GR to GREs (Chen et al, 1989; Mittal et al, 1993b). Further, it was also speculated in a model that there is a "second hit," such as chromosomal aberrations or ras mutations, in addition to an increase in E6-E7 message, that may lead to an aggressive state of CIN (Mittal, 1993). It was shown in another study that a GRE at nt 7640 position is a composite element that overlaps an AP1 binding site and is regulated by c-jun and c-fos in a dex-dependent manner (Mittal et al, 1994). Furthermore, it was hypothesized in this laboratory that the HPV16 genome of dex-resistant clones of transformed cells, uses different transcription start site(s) compared to dex-dependent cells (Pater et al, 1993). With this background

information, I initiated my work with the following objectives:

1. The consensus mutants would be used to study the dex-induced and noninduced HPV16 expression and also to correlate the _in vivo_ expression with _in vitro_ binding studies.

2. HPV16 whole genome bearing cGREs would be further employed to immortalize the ectocervical cells, the natural target of HPVs and steroid hormones. This construct would also be used to analyze the effect of dex on transformation of baby rat kidney (BRK) epithelial cells.

The purpose of the immortalization and transformation assays is two-fold: i) The cells harbouring HPV16 DNA would be used to examine changes in morphology and differentiation ii) The immortalized and transformed cells containing HPV16 with triple cGREs would be useful to analyze the role of HPVs and hormones in different stages of oncogenesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The keratinocyte serum-free growth medium and Dulbecco's modified Eagle's medium (D-MEM) were purchased from GIBCO-BRL. GIBCO-BRL supplied fetal calf serum (FCS) and trypsin-EDTA. Penicillin-streptomycin was purchased from ICN Biomedicals. Cervical biopsy specimens for the preparation of primary ectocervical cells were kindly provided by Dr. M. Parai of Grace Hospital, St. John's, Newfoundland.

HPV16 DNA plasmid was a kind gift from Dr. Harald zur Hausen. The pBLCAT2 expression vector was a gift from Dr. B. Luckow. The glucocorticoid receptor-expressing (pHGO) and δ-galactosidase-expressing (pCH110) plasmids were a generous gift from Dr. P. Chambon. Oligonucleotides used in this study were obtained from General Synthesis and Diagnostics, Toronto.

Restriction endonucleases were obtained from New England Biolabs and GIBCO-BRL. The T4 DNA polymerase and ligase, calf intestinal alkaline phosphatase (CIP) and reverse transcriptase were supplied by GIBCO-BRL, Boehringer Mannheim and Life Sciences, respectively. The DNase I and E.coli DNA polymerase were supplied by GIBCO-BRL. $[\alpha^{32}P]dCTP$, $[\alpha^{35}S]dATP$ and $[^{14}C]$chloramphenicol were purchased from Amersham. Random primer DNA labelling system and Bionick labelling system were supplied by GIBCO-BRL. The dideoxy sequencing kit (Sequenase

version 2.1) was obtained from United States Biochemical Corporation. Thin layer chromatography (TLC) plates and X-ray films were purchased from Kodak.

Biotrace HP membranes for Northern and Southern blot were obtained from Gelman Sciences. The BA85 membranes for Southwestern blot were purchased from Schleicher & Schuell. Tissue culture plates and other items for culturing cells were obtained from Fisher and Nunc.

Dexamethasone (dex), acetyl coenzyme A, diethyl pyrocarbonate (DEPC), o-nitrophenyl δ-D-galactoside (ONPG) and protease inhibitors, aprotinin, antipain, pepstatin A, leupeptin and phenylmethylsulfonyl fluoride (PMSF), were obtained from Sigma. The dNTP, poly dI-dC, nick columns and mobility shift assay kit were purchased from Pharmacia. Lipofectin, hydrocortisone, Biotin-7-dATP, nick translation kit and the DNA detection kit were obtained from GIBCO-BRL.

2.2 METHODS

2.2.1 Plasmids, site-directed mutagenesis and DNA sequencing

The plasmid construct, pTHBK4, containing HPV16 nt 6150-880 with the consensus GRE (cGRE) sequence at nt 7640, was constructed by R. Mittal in this laboratory. Using this construct, cGRE mutations at nt 7385 and nt 7474 were generated by site-directed mutagenesis, according to the procedure recommended by Biorad, the supplier of the mutagenesis kit. Single-stranded oligonucleotides representing

cGRE sequences were used for this purpose, to obtain the pTHBK489, the triple cGRE construct. These mutations were screened by restriction analysis and confirmed by dideoxy sequencing (Sanger et al, 1977) or by chemical sequencing in ambiguous cases (Maxam and Gilbert, 1977).

To insert the mutated segments into pBLCAT2 expression vector, a Sal I-digested and DdeI blunt-ended (Sal I at polylinker of pTZ19 vector and Dde I at nt 7761 of HPV16) fragment of pTHBK489 was subcloned into pBLCAT2 (linearized at Bam HI and blunt ended and then digested with Sal I), in the correct orientation. The p1XGRE(C)CAT, which contains 232 bp of HPV16 Dra I-Dra I (nt 7522-7754) fragment containing the nt 7640 cGRE in the pBLCAT2 vector was prepared by R. Mittal. To insert the cGRE mutations into whole HPV16 plasmid, a BamH I-Nco I fragment (nt 6150-863) from the mutants was ligated into HPV16 plasmid, partially digested with Bam HI and digested with Nco I. The nomenclature of all these constructs in pBLCAT2 and HPV16 plasmids and the GRE sequences in each construct are indicated in Table 3.1.

2.2.2 Cell culture

All the cell lines used in this study were maintained and cultured in this laboratory. The cell lines, HeLa, CaSki, J2, SiHa and C33A (Pater et al, 1985; Tsutsumi et al, 1994 and Yokoyama et al, 1995) were propagated in Dulbecco's modified Eagle's medium (D-MEM). The cervical cells derived from

primary cultures and immortalized cells were grown in keratinocyte growth medium.

2.2.2.1 Transfection of HeLa cells by different pBLCAT2 constructs

The HeLa cells were plated in 100 mm tissue culture dishes and after 8 hours of plating the cells were transfected with the indicated constructs, according to the calcium-phosphate precipitation method (Gorman et al, 1982). Briefly, 10 μg indicated CAT constructs were cotransfected into 70% confluent cells with 2 μg pHGO and 5 μg pCH110, adjusted to a total of 20 μg DNA with pBR322 vector in 100 mm tissue culture plates (as described in Mittal et al, 1993b) and incubated at 37°C at 3% CO_2 for 4 hours. The plates were subjected to glycerol shock using 1.5 ml 15% glycerol for one minute. The cells were washed with phosphate buffer saline (PBS) three times to remove glycerol. The cells were fed with D-MEM containing 10% FCS and with or without 10^{-6} M dex from a 100X stock in 100% ethanol and incubated at 37° C at 5% CO_2 for 48 hours.

2.2.2.2 Transfection of HPV16 whole genome constructs into ectocervical cells

Primary human ectocervical cells (HEC) were cultured from the cervical specimens obtained by punch biopsies that were found to be free of cervical intraepithelial neoplasia (CIN)

by histological studies. Primary cultures were initiated by the method described by Boyce and Ham (1985). Initially, the cervical tissue in punch biopsies was washed with solution A containing keratinocyte basal media (KBM, Clonetics) supplemented with antibacterial and antifungal agents. Later this tissue was incubated with collagenase (GIBCO-BRL) (650 units/ml) for two hours to separate epithelium and stroma. The epithelium was separated, finely minced with a scalpel and trypsinized (0.025% trypsin solution) for three minutes at room temperature. Subsequently, trypsin was inactivated by adding PBS containing 10% FCS. The cells were collected by centrifugation, resuspended in KGM and plated in 100 mm tissue culture plates.

For transient transfections, the primary ectocervical cells were seeded onto coverslips that were placed in 30 mm tissue culture plates and incubated until the cells became 70% confluent. The cells were transfected with HPV16 plasmids containing either wild type GREs or cGREs by the method of lipofection as described by Felgner et al (1987). Briefly, 3 μg HPV16 plasmid and 1 μg pHGO were each diluted in serum-free KBM and incubated with diluted lipofectin reagent for 10 minutes. The DNA-liposome complexes thus obtained were introduced into the cells and incubated at 37^0C in a CO_2 incubator. After five hours, the DNA-lipofectin solution was removed and cells were supplemented with KGM either with or without 10^{-6} M dex and incubated for 48 hours.

The HEC cultures initiated from cervical biopsy tissue, as described above, were used for immortalization of these cells with the HPV16 plasmid carrying three cGRE mutations. First, the cells were grown on 60 mm tissue culture plates and transfected with 10 μg pHPV3XGRE(C) and 6 μg pHGO plasmids by lipofection. The KGM used for culturing these cells was supplemented with 1 μg/ml hydrocortisone after transfection. The transfected cells were passed when they reached confluence, usually once in 3-4 days. The untransfected cells underwent senescence upon culturing for 7-8 passages. The HPV16-transfected cells continued to grow upto passage 18, at which stage they underwent a crisis period for 3-4 weeks. Those cells whose life span was extended after this period were cultured regularly and the cells obtained were designated HEC-16cGRE.

2.2.3 Chloramphenicol acetyl transferase (CAT) assays

CAT assays were performed according to Gorman et al (1982). The transfected cells were harvested after 48 hours of incubation. The cells were washed with PBS twice and collected into Eppendorf tubes in 1 ml PBS using a rubber policeman. The cells were pelleted by spinning for one minute at 10,000 rpm at 4^0C and the pellet was dissolved in 0.25 M Tris HCl (pH 7.8). These cells were lysed by three cycles of freezing and thawing. The lysed cells were then spun in a microfuge for 5 minutes at 4^0C. The supernatant was collected for assaying CAT

activity.

The reaction mix of 20 μl for the CAT assays included 14 μl 1 M Tris-HCl (pH 7.8), 1 μl (0.1 μCi) ^{14}[C]chloramphenicol, 1 μl distilled water and 4 μl 8 mM acetyl coenzyme A. This mix was added to 10 μl cell lysate and incubated at 37^{0}C for one hour. The reaction was stopped adding 0.5 ml ethyl acetate and vortexing the contents of the tubes. The resulting organic top layer was transferred to a fresh Eppendorf tube and dried in a vacuum aspirator (Savant). The [^{14}C]chloramphenicol and acetylation products thus obtained were dissolved in 15 μl ethyl acetate and vortexed for one minute and spotted on silica gel TLC plates. The acetylated and nonacetylated forms of [^{14}C]chloramphenicol were separated by ascending chromatography in chloroform-methanol (95:5). The TLC plates were exposed to Kodak X-ray film at room temperature for 3 days. The quantitation of acetylated and nonacetylated [^{14}C]chloramphenicol was done by counting the radioactivity of the respective spots in a liquid scintillation counter. The results were calculated as the percentage of total [^{14}C]chloramphenicol that was converted to acetylated form in comparison with the nonacetylated form. These data were normalized with β-galactosidase activity determined by the protocol recommended by Pharmacia.

2.2.4 DNA-protein interaction assays

2.2.4.1 Preparation of HeLa whole cell extracts and probes

Whole cell extracts were prepared following the procedure described by Tasset et al, (1990). Briefly, confluent HeLa cells in 100 mm tissue culture dishes were scraped with rubber policeman and collected in Falcon tubes. Cells were washed with PBS and lysed with two pellet volumes of whole cell extract buffer [0.4 M KCL, 22 mM Tris-HCl (pH 8.0), 2 mM DTT, 20% glycerol and protease inhibitors: 5 mM PMSF and 1 mg/ml of each of aprotinin, leupeptin, pepstatin A and antipain] by three cycles of freezing and thawing in liquid nitrogen and ice, respectively. The cell lysates were microfuged at 10,000 rpm at 4°C for 15 minutes. The resulting supernatant was aliquoted and flash-frozen in liquid nitrogen. The protein concentration was estimated by the method of Bradford (1976) using a kit from Biorad. The extracts were stored at -70°C until used.

The sequence of the double stranded (ds) 22-mers used for DNA-protein interaction assays is given below. The GRE palindrome is underlined on one strand and XbaI overhangs are at the 5' ends in lowercase letters. The mutated bases in the palindrome are in lowercase letters. GRE8WT and GRE9WT indicate wild type HPV16 sequences corresponding to nt 7381-7402 and nt 7470-7491, respectively. GRE8C and GRE9C are similar to GRE8WT and GRE9WT, except for the consensus sequence of the GRE palindrome.

```
GRE8WT    5'  ctagATTTGCTACATCCTGTTTTTGT  3'

          3'  TAAACGATGTAGGACAAAAACAgatc  5'

GRE8C     5'  ctagATTTGgTACATCCTGTTcTTGT  3'

          3'  TAAACcATGTAGGACAAgAACAgatc  5'


GRE9WT    5'  ctagATTTGGCACAAAATGTGTTTTT  3'

          3'  TAAACCGTGTTTTACACAAAAAgatc  5'

GRE9C     5'  ctagATTTGGtACAAAATGTtcTTTT  3'

          3'  TAAACCaTGTTTTAGAagAAAAgatc  5'
```

For electrophoretic gel mobility shift assays (EMSA) and
Southwestern blot assays, the ds oligonucleotides were
endlabelled by filling in the 5' XbaI overhangs with [^{32}P]dCTP
and reverse transcriptase.

Probes for UV crosslinking were prepared by
nicktranslating the ds oligonucleotides in the presence of
0.15 mM bromodeoxyuridine, [^{32}P]dCTP and E. coli DNA polymerase
I.

2.2.4.2 Electrophoretic mobility shift assays (EMSA)

Binding reactions for the mobility shift assays were done
using mobility shift assay kit from Pharmacia, following
manufacturer's recommendations. Free DNA and DNA-protein
complexes were separated on 3% nondenaturing polyacrylamide
(acrylamide: bisacrylamide, 29:1 w/w) gels at 4^0 C with 35

milliamperes current in 22 mM Tris-borate, 0.5 mM EDTA.

2.2.4.3 Southwestern blot assays

Southwestern blot assays for interaction of HeLa whole cell extracts with GRE oligonucleotides were performed as per the procedure of Silva et al, (1987) with a few modifications. One hundred μg whole cell extracts were separated on 7.5% SDS-PAGE and transferred to BA85 nitrocellulose membranes by electrotransfer in Tris-glycine buffer (25 mM Tris-HCl, 190 mM glysine). The membranes were blocked in binding buffer (5% nonfat skimmed milk powder in 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA) in a heat-sealable plastic bag overnight and then probed in the same buffer containing 0.25% nonfat dry milk, using 10^6 cpm/ml endlabelled oligonucleotides for 3 hours at room temperature. Then, the filters were washed four times in the binding buffer. Finally, the filters were air-dried and subjected to autoradiography.

2.2.4.4 Ultraviolet (UV) crosslinking assays

Binding reactions for UV crosslinking were done similar to the EMSA, except that 5 μg of poly (dI-dC) and 5×10^5 cpm of radiolabelled probes were used. The binding reaction was at room temperature for 30 minutes and then exposed to UV irradiation at the maximum emission wavelength of 310 nm under a Fotodyne UV lamp for 30 minutes (Ausbel et al, 1994). The binding reactions were stopped by adding equal volumes of 2X

SDS-loading buffer [100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 20% glycerol and 0.2% bromophenol blue]. The reaction mixture and high range molecular weight markers (GIBCO-BRL) were boiled for 5 minutes and then separated by electrophoresis on a 7.5% SDS-PAGE at 100 volts. The gel was dried and subjected to autoradiography.

2.2.5 In situ hybridization assays for RNA from transiently transfected ectocervical cells

In situ hybridization assays for viral gene expression in transfected ectocervical cells from primary cultures were performed following the method described by Lawrence and Singer (1986). Briefly, the transfected cells were fixed in 4% paraformaldehyde in PBS with 5 mM MgCl$_2$ after 48 hours of incubation. These cells were treated with 50% formamide, 2X SSC (1X SSC is 0.15 M sodium chloride, 15 mM trisodium citrate, pH 7.0) at 65°C for 10 minutes. The probe for these assays was prepared by nick-translating the nt 501–863 (Hpa II and Nco I) fragment of HPV16 genome coding E6 and E7, in the presence of biotin 7-dATP. The probe was dissolved in 100% deionized formamide and denatured at 95°C for five minutes and brought to a final concentration of 50% formamide, 2X SSC, 0.2% BSA, 10% dextran sulfate and vanadyl-sulfate ribonucleotide complex RNase inhibitor. The coverslips were incubated with 5-10 μg/μl probe overnight and the signal was detected using the BRL DNA detection kit. The results were

evaluated with a Leitz Diaplan microscope.

2.2.6 Characterization of immortalized ectocervical cells
2.2.6.1 Analysis of high molecular weight DNA by Southern blot assays

Analysis of high molecular weight DNA from HEC-16 (established by K. Tsutsumi) and HEC-16cGRE cells was as described by Sambrook et al (1989). Briefly, the cells were collected from confluent monolayer cultures by scraping with a rubber policeman using PBS and pelleting by centrifugation. The cells were then suspended and incubated in extraction buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA, 20 μg/ml pancreatic RNase, 0.5% SDS) at 37^0 C for one hour. Proteinase K was next added to a final concentration of 100 μg/μl and the mixture was incubated for 3 hours at 50^0C. The DNA was then extracted by phenol-chloroform extraction, followed by ethanol precipitation.

For Southern blot analysis, 10 μg DNA was digested with the indicated enzymes, electrophoresed through 1% agarose gel and transferred to Biotrace HP membranes by capillary transfer. Next, the membrane was baked at 80^0C for two hours and then incubated in prehybridization buffer (1% non-fat dry milk, 0.5 M NaH$_2$PO$_4$, 7% SDS, pH 7.2) in a sealable bag at 65^0C for three hours. The membrane was hybridized in the same buffer with 1 X 10^6 cpm/ml Bam H1-linearized, ^{32}P-labelled HPV16 DNA probe by random priming, at 65^0C overnight. After

hybridization, the membrane was washed twice with 2X SSC, 0.1% SDS at room temperature for 15 minutes and twice with 0.1X SSC, 0.1% SDS at 42°C for 10 minutes. Then, the membrane was air-dried and exposed to X-ray film.

2.2.6.2 Analysis of RNA from immortalized cells by Northern blot assays

The total RNA from the cells was extracted according to the procedure described by Sambrook et al, (1989). Briefly, the cells were washed with PBS and lysed with lysis solution (4 M guanidinium isothiocyanate, 0.5% sodium-N-lauryl sarcosinate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol). The lysed cells were collected and high molecular weight DNA was disrupted with a syringe fitted with a 21 gauge needle. The RNA was recovered after cesium chloride centrifugation at 30,000 rpm for 20 hours, followed by ethanol precipitation.

For Northern blot assays, 20 µg total RNA was mixed with sample buffer (20 mM MOPS, 0.5 mM EDTA, 1.5 mM formaldehyde, 50% formamide) and separated on 1% denaturing gel (1% agarose in 20 mM MOPS, 8 mM sodium acetate and 0.66 mM formaldehyde) at 40 volts overnight in running buffer (0.4 M MOPS, 100 mM sodium acetate, pH 8.0). The gel was then washed two times with DEPC-treated water to remove the formaldehyde. The RNA was transferred to Biotrace HP membrane and hybridized with ^{32}P-labelled HPV16 DNA probe, as described for Southern blot.

2.2.6.3 Differentiation in organotypic (raft) cultures

The raft system was used to evaluate the pattern of differentiation of the immortalized cells, following the method described in Meyers et al, (1992). Initially, the 3T3 J2 fibroblast cells were cultured using E-medium (which contains 0.4 μg hydrocortisone glucocorticoid as described in McCance et al, 1988). Then the gel raft was made by mixing $3X10^5$ J2 fibroblasts with collagen mix containing type I collagen, 10X reconstitution buffer (100 mM sodium bicarbonate, 200 mM HEPES) and 10X E-medium (8:1:1 v/v/v) and plating the 3 ml gel mix in each of 6-well tissue culture plates. After solidification of the gel, the cells were supplemented with 2 ml E-medium and incubated at 37^0 C until the fibroblasts grew to confluence in the collagen gel. Next, $3X10^5$ cervical cells were seeded on the fibroblast-collagen gel, immersed in 2 ml medium and grown to a confluent monolayer. These rafts were raised on to stainless steel grids to allow the cervical cells to differentiate into epithelia from the surface of the air-liquid interface at the surface of the gel. The gels were freshly surrounded with E-medium on every second day for 12 days. To analyze the histology, these specimens were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned and stained with hematoxylin and eosine at the Department of Pathology, Faculty of Medicine, Memorial University of Newfoundland, St. John's.

2.2.6.4 Anchorage independent (soft agar) growth and tumorigenicity assays

The anchorage independent growth and tumorigenicity assays were initiated for HEC-16cGRE cells as described in Tsutsumi et al (1994). The SiHa cervical carcinoma cells were used as a positive control. For soft agar assays, a 0.7% agarose basal layer was initially prepared in 2X D-MEM and dispensed in 5 ml volume into 60 mm plates. The cells (1×10^5) were suspended in 0.35% overlay agar and overlaid on the basal layer. After each week, fresh D-MEM was added on the agar gel. Colony formation was examined for 16 weeks. For tumorigenicity assays, the HEC-16cGRE cells were trypsinized and suspended in PBS. These cells (1×10^7) were injected into each of 3 one-month-old-female immunocompromised nude mice subcutaneously at three different sites on the dorsolateral lumbar region . These mice were observed for tumor formation for 16 weeks.

2.2.7 In vitro transformation assays

Inbred Fisher rats were used to prepare primary baby rat kidney (BRK) cells for transformation assays. The primary cells were cultured from five-day-old rats as described previously (Mittal, 1993). Briefly, rats were decapitated and an incision is made on the ventral lumbar region. Kidneys were separated from the connective tissue and washed three times in sterile PBS. Kidneys were then finely minced using a scalpel and trypsinized for one hour in trypsin-EDTA. The trypsinized

kidney cells were suspended in D-MEM with 10% FCS and passed through a piece of sterile gauze. The cells thus obtained were plated at a concentration of 10^5 cells per 60 mm tissue culture dishes.

For transformation assays, the BRK cells were fed with fresh D-MEM. After an hour 5 μg each of HPV16 construct and EJ-ras plasmid (EJ c-Ha-ras-1, Pater et al, 1988) containing an activated form of human ras oncogene were cotransfected as described in the section 2.2.2.1. Then the cells were fed with media containing 10% FCS and either with or without 10^{-6} M dex. After 48 hours the media was changed to D-MEM with 2% FCS with 0 or 10^{-6} dex. These cells were maintained in D-MEM with 2% FCS with or without dex until the foci appeared. Foci of transformed colonies were counted after 4 weeks.

The CAT constructs were transfected into primary BRK cells according to the method described in section 2.2.2.1.

3.1 Effect on expression by cGRE mutations in HPV16 regulatory region

In the regulatory region of HPV16, glucocorticoid response elements (GREs) were identified earlier from studies performed in this and other laboratories (Chan et al, 1989 ; Mittal et al, 1993b). These studies suggested a role for the GREs in steroid hormone-mediated expression. The previous studies dissected the role of a GRE at nt 7640 by mutagenesis and transient expression assays. In this study, the role of this GRE and the other two GREs identified recently are studied further. For this purpose, the three GREs in the enhancer region of the HPV16 at nt 7385, nt 7474 and nt 7640 were mutated to the consensus sequence, GGTACA(N)$_3$TGTTCT by site-directed mutagenesis. To study the glucocorticoid-mediated expression, a fragment of HPV16 DNA, nt 6150-7761, containing the HPV16 enhancer region with the consensus GRE (cGRE) mutations, was cloned upstream of the tk basal promoter of the enhancerless pBLCAT2 vector, as shown in Table 3.1. The HeLa cervical carcinoma cells and BRK epithelial cells were used for the transfection of the CAT expression vector constructs, since the expression of HPVs is epitheliotropic in nature. Previous studies have shown that these cells supported expression from the HPV16 enhancer (Chan et al, 1989). The efficiency of transfections was normalized by the transfected

Table 3.1 Wild type (WT) and consensus (C) glucocorticoid response element (GRE) sequences in various constructs

	Glucocorticoid response element sequences[a]		
Plasmid[c]	7385[b]	7474	7640
pHPV(WT)	GCTACATCCTGTTTT	GGCACAAAATGTGTT	TGTACATTGTGTCAT
pHPV3XGRE(C)	- g - - - - - - - - - -c-	- - t - - - - - - - - - -tc-	g - - - - - - - - - - - -tc-
p1XGRE(C)CAT			g - - - - - - - - - - -tc-
p1/3XGRE(C)CAT	- - - - - - - - - - - - - - -	- - - - - - - - - - - - - - -	g - - - - - - - - - - - -tc-
p3XGRE(WT)CAT	GCTACATCCTGTTTT	GGCACAAAATGTGTT	TGTACATTGTGTCAT
p3XGRE(C)CAT	g - - - - - - - - - - -c-	- - t - - - - - - - - - -tc-	g - - - - - - - - - - - -tc-

[a] The palindrome sequence of GREs of wild type is shown in capital letters. The consensus mutations created by site-directed mutagenesis are in lower case letters and the other wild type nucleotides are indicated by dashes.

[b] The position of the first nucleotide of each GRE in the HPV16 genome is indicated.

[c] Wild type [pHPV (WT)] and triple consensus [pHPV3XGRE(C)] GREs are in whole HPV16 plasmid. CAT reporter plasmids contain single consensus GRE [p1XGRE (C)CAT], one consensus GRE and the other two wild type GREs [p1/3XGRE(C)CAT], triple wild type GREs [p3XGRE(WT)CAT] and triple consensus GREs [p3XGRE(C)CAT].

ß-galactosidase plasmid internal control.

3.1.1 CAT assays in HeLa cells

As shown in Fig.3.1 and 3.2, there was an increase in expression of the CAT reporter from the construct, p3XGRE(WT)CAT, containing wild type GREs in the presence of dex. In this case, there was a 2.9-fold induction (+dex/-dex) of expression. The dex had little effect on the basal levels of expression in the control vector pBLCAT2. The construct p1XGRE(C)CAT, that contains a single cGRE at nt 7640, showed a remarkable 9.4-fold induction of CAT expression in presence of dex. A slightly higher level of induction of 11.0-fold was observed with the p1/3XGRE(C)CAT plasmid that contains a single cGRE at nt 7640 and the two other wild type GREs at nt 7385 and nt 7474. Interestingly, the uninduced (-dex) levels of CAT from the two constructs containing a single cGRE were significantly lower than that of the wild type construct.

The GRE in the HPV16 regulatory region at position nt 7640, TGTACAT<u>TGTGTCAT</u>, is a composite element that overlaps an AP1 binding motif (underlined TGTGTCA) (Chong <u>et al</u>, 1990,1991; Mittal <u>et al</u>, 1994). The mutation of this GRE into the consensus sequence, gGTACAT<u>TGTGT</u>tcT (as shown in Table 3.1), created a simple GRE with a disruption of the overlapping AP1 binding site. Next, the two additional GRE sequences at nt 7385 and nt 7474 also were mutated to consensus sequence, resulting in triple cGRE sequences in the

Fig. 3.1. Effect of HPV16 consensus GRE (cGRE) mutations on hormone response in CAT assays.

Ten μg indicated CAT reporter constructs were cotransfected with 2 μg pHGO GR expression plasmid in HeLa cells. The ß-galactosidase expression vector pCH110 was used as an internal control for the efficiency of transfection. The CAT expression vector constructs containing different cGRE mutations are shown in Table 3.1. The + and - indicate presence and absence, respectively, of 10^{-6} M dex, a synthetic glucocorticoid.

pBLCAT2⁻ ⁺   p3XGRE(WT)CAT⁻ ⁺   p1XGRE(C)CAT⁻ ⁺   p1/3XGRE(C)CAT⁻ ⁺   p3XGRE(C)CAT⁻ ⁺
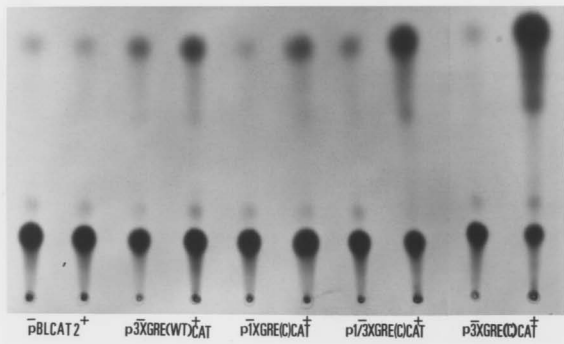
Fig. 3.2. Bar graphs for expression from cGRE mutants.

The CAT activities of different CAT expression
plasmids shown in Fig.3.1 are represented graphically after
normalizing with δ-gal activity of the pCH110 cotransfected
plasmid internal control. Fold induction by dex for
different plasmids was calculated as the ratio of normalized
% CAT activity in the presence of dex (+) to the % CAT
activity in the absence of dex (-) for the respective
plasmids after adjusting with the CAT activity of the
control pBLCAT2 vector in the absence of dex to 1.0. The
error bars represent the standard deviation of the results
of three independent experiments.

Fold induction

22.5	p3XGRE(C)CAT
11.0	p1/3XGRE(C)CAT
9.4	p1XGRE(C)CAT
2.9	p3XGRE(WT)CAT
1.0	pBLCAT2

+ DEX
- DEX

% CAT activity
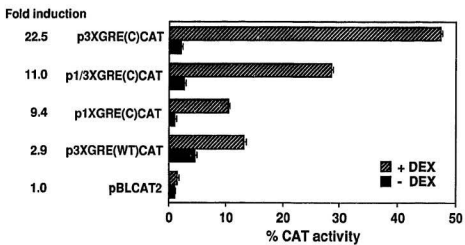
whole HPV16 enhancer. This construct with the three cGREs, which apparently do not have any influence from overlapping sequences for the two additional GREs, was subsequently used to study dex-mediated expression. When these sequences in the pBLCAT2 vector were analyzed for the effect on CAT expression, there was a 22.5-fold induction of expression by dex with p3XGRE(C)CAT (Fig. 3.1 and 3.2). In contrast, there was only a 2.9-fold induction of expression by the wild type GRE-containing plasmid, p3XGRE(WT)CAT (Fig.3.1 and 3.2).

3.1.2 CAT assays in BRK cells

A similar pattern of expression from the different CAT constructs was also observed in primary baby rat kidney (BRK) epithelial cells as shown in Table 3.2. Although the fold-induction levels were not as high as in HeLa cells, the cGRE constructs exhibited higher CAT activities than those of the wild type constructs in the presence of dex. Similar results were reported earlier for the expression of the CAT reporter under the control of dex-inducible GRE construct of MMTV-LTR in BRK cells (Pater et al, 1993)

Overall, the results of the CAT assays for single and triple cGRE constructs suggested that there was a combined effect of all the three GREs in glucocorticoid-induced transient expression through a heterologous tk promoter in pBLCAT2 vector. Consistently, the uninduced levels of expression were significantly lower in cGRE constructs than

Table 3.2 Expression from different CAT constructs in primary baby rat kidney (BRK) cells

Plasmids	GRE (s)	Fold Induction[a]
pBLCAT2	Nil	1.0+/- 0.1[b]
p3XGRE(WT)CAT	Three wild type GREs	2.5+/- 0.4
p1XGRE(C)CAT	cGRE at nt 7640	4.6+/- 0.3
p1/3XGRE(C)CAT	cGRE at nt 7640 and the two other wild type GREs	4.9+/- 0.2
p3XGRE(C)CAT	Three cGREs	7.1+/- 0.6

[a] The fold induction of expression of CAT was calculated as described for Fig. 3.2.

[b] The results shown were from three independent experiments.

that of the uninduced wild type construct.

3.2 Effect of dex on expression of E6-E7 genes of HPV16 with triple cGREs in ectocervical cells

To examine the effect on expression in the context of the whole HPV16 genome, the HPV16 enhancer region containing triple cGRE mutations was inserted into a whole HPV16 genome plasmid. HPV16 plasmid constructs with wild type GREs or triple cGREs were cotransfected with the human glucocorticoid receptor (GR) expression plasmid pHGO into cells derived from primary cultures of the ectocervix. In situ hybridization for viral RNA was used to evaluate the expression of the E6-E7 oncogenes from the homologous HPV16 promoter/enhancer region. The CaSki cervical carcinoma cells and untransfected ectocervical cells served as positive and negative controls for in situ hybridization of viral mRNA, respectively.

When the ectocervical cells were transiently transfected with the HPV16 DNA constructs containing wild type GREs (WT) or triple cGREs (3XcGRE) (Fig. 3.3), the signals for the E6-E7 transcripts were significantly higher in the dex-induced (+dex) cells compared with the dex-untreated (-dex) cells. An analogous observation was made previously, (Mittal et al, 1993a) when ectocervical cells were transfected with pHPV16 and probed with whole HPV16 for the viral RNA. Further, in this study, the cells transfected with HPV16 carrying cGRE mutations displayed stronger signals to dex-induced E6-E7

Fig. 3.3. Effect of dex on E6-E7 expression in cultured human ectocervical cells.

The HPV16 plasmid constructs with wild type GREs (WT) or triple cGREs (3XcGRE) were transfected into ectocervical cells by lipofection, fixed after 48 hours and probed with biotinylated E6-E7 HPV16 region DNA probe. CaSki cells that contain and express HPV16 DNA were used as a positive control and untransfected (control) ectocervical cells were used as a negative control. The transfected cells were incubated with hydrocortisone-free medium either with 10^{-6} M dex (+dex) or without dex (-dex). Magnification of light microscopy: X 100

CaSki

Control

3x cGRE
+dex

3x cGRE
−dex

WT
+dex

WT
−dex
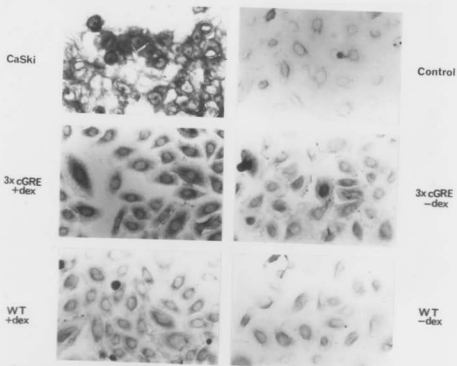
message (Fig. 3.3), in comparison with the cells transfected with the wild type HPV16 plasmid in the presence of dex. Although qualitative, the levels of expression of E6-E7 were significantly higher in dex-induced and 3XcGRE-transfected cells than those of WT.

3.3   In vitro DNA-protein interaction assays with wild type GRE and cGRE oligonucleotides

Glucocorticoid hormones regulate gene expression by interacting with intracellular GR. The binding activates GR, which in turn binds to specific recognition motifs, the GREs, to modulate the initiation of transcription of target genes (Beato et al, 1989; Dahlman et al, 1994; McEvan et al, 1994). In the results described above, the dex-mediated activation of expression through the GREs was demonstrated. To determine whether or not factor (s) bind to the GRE oligonucleotides and to characterize the amount and nature of factor(s) possibly binding to the wild type and cGREs of HPV16, DNA-protein interaction assays were performed. The in vitro interaction assays are important to identify specific binding of protein(s) to consensus mutant oligonucleotides and to correlate with the expression pattern observed in vivo in CAT expression and in situ hybridization assays. Here, DNA-protein interaction assays were performed for the two GREs at nt 7385 and nt 7474, which are simple GREs, unlike nt 7640 GRE (Mittal et al, 1993b).

3.3.1 Electrophoretic mobility shift assays (EMSA)

The EMSA were performed using GRE8WT, GRE8C, GRE9WT and GRE9C ds oligonucleotide probes and whole cell extracts from HeLa cells as described in Materials and Methods. The GRE-protein interactions were demonstrated by the presence of two to three retarded complexes of GRE oligonucleotide probes with HeLa whole cell extracts (B lanes, Fig.3.4 and 3.5). A 1000-fold molar excess homologous radioinert oligonucleotide as specific (S) competitor completely abolished the DNA-protein complexes for all four oligonucleotide probes (Fig. 3.4 and 3.5). An Oct-1 binding motif, used as a nonspecific (NS) competitor, had little effect on the retarded complexes. Earlier studies showed the possible binding of protein molecules as monomers and dimers to their cognate GRE sequences in the EMSA (Chalepakis et al, 1990, Mittal et al, 1993b). As shown in Fig.3.4, there was a quantitative difference in the protein-DNA complexes formed between GRE8WT and GRE8C oligonucleotides. Considerably, more binding was observed with the GRE8C oligonucleotide in comparison with the GRE8WT oligonucleotide.

The GRE9WT and GRE9C probes showed distinct features in their binding patterns (Fig.3.5). The total protein binding of the GRE9C probe to HeLa protein extracts was significantly higher than that of GRE9WT. In addition, a predominant low mobility complex appeared with GRE9C, whereas two to three faster migrating complexes were observed with GRE8WT, GRE9WT

Fig. 3.4. EMSA for nt 7385 wild type GRE (GRE8WT) and consensus mutant (GRE8C) oligonucleotides.

Ten μg proteins from HeLa whole cell extracts were incubated with 20,000 cpm of endlabelled GRE8C (nt 7385 cGRE) or GRE8WT (nt 7385 wild type GRE) probes. The figure shows the mobility pattern of GRE-protein complexes in polyacrylamide gel electrophoresis using 3% nondenaturing gels. Lanes: F, free DNA (without protein); B, bound (with protein); S, as in lane B plus 1000-fold molar excess of unlabelled homologous specific competitor; NS, as in lane B plus 1000-fold radioinert nonspecific competitor containing an Oct-1 binding motif. The arrowhead indicates the free probe and the arrows indicate bound complexes.
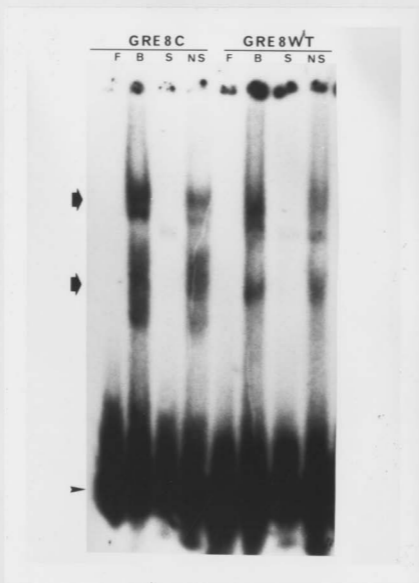
3.5. EMSA for nt 7474 wild type GRE (GRE9WT) and consensus mutant (GRE9C) oligonucleotides.

The conditions for the assays were as described for Fig. 3.4. The lane designations are similar to Fig.3.4. The arrowhead and the arrows indicate the free probe and specific complexes, respectively. A thick arrow on the top indicates a low mobility complex with GRE9WT probe.
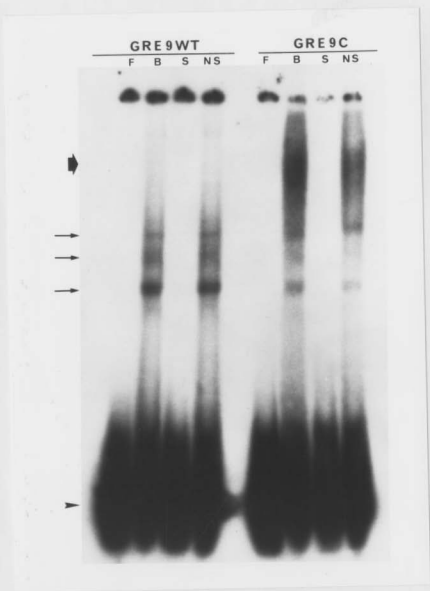
and GRE8C probes. This difference in binding ability could reflect the respective affinity of protein binding to the specific wild type and consensus sequences. Perhaps, this difference could also be due to the GRE flanking sequences.

Although specific binding of protein-GRE sequences was demonstrated, the above studies did not determine whether the bound protein is specifically the steroid receptor and/or how many polypeptides were present. To characterize specific protein(s) binding to cGREs, UV crosslinking and Southwestern blot assays were performed.

3.3.2 Southwestern blot assays

To characterize the protein(s) in the DNA-protein complexes observed in EMSA, HeLa whole cell extracts were resolved on SDS-PAGE gels, transferred to nitrocellulose membranes and then probed with GRE8C and GRE9C radiolabelled oligonucleotides. A protein of approximately 96 kDa, the size of the native GR (Hollenberg et al, 1985), interacted with both probes (Fig.3.6). In these assays, predominantly a 96 kDa protein was detected. These results were similar to the observations for wild type nt 7385 and nt 7474 GREs (Mittal et al, 1993b).

3.3.3 UV crosslinking assays

To further characterize the GRE-protein interactions, UV crosslinking assays were performed. These assays allow the

Fig. 3.6. Southwestern blot assays for nt 7385 GRE8C and nt 7474 GRE9C oligonucleotides.

One hundred μg whole cell extracts from HeLa cells were separated by SDS-PAGE, transferred to nitrocellulose membrane and then probed with 1×10^6 cpm/ml endlabelled GRE8C (A) and GRE9C oligomers (B). The arrow indicates a 96 kDa band that interacted with the GRE probes. High molecular weight standard protein markers are indicated by numbers for their sizes in kDa.
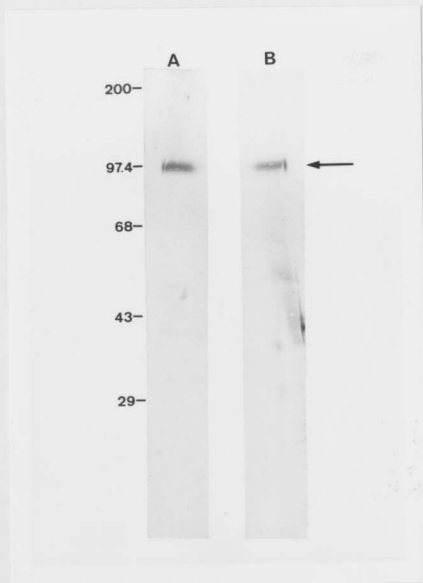
characterization of the binding of DNA to proteins with multiple subunits or to protein-protein complexes (Ausbel et al, 1994). Such DNA-protein interactions would be undetected in Southwestern blot assays due to the dissociation of proteins during denaturing gel electrophoresis. Also, the sizes of the higher order complexes and possibly their interactions would not be identified by EMSA.

As shown in Fig.3.7, GRE8WT and GRE8C probes, corresponding to the wild type and cGRE at nt 7385 respectively, crosslinked to a protein of about 96 kDa (indicated by arrow), the size of the native GR. Another UV crosslinked product variably observed was of about 43 kDa size (arrowhead). A few additional intermediate size bands were observed in these assays. These bands ranging in sizes below 96 kDa may represent proteolytic products of GRE-binding protein as reported previously in cellular extracts (Singh and Moudgil, 1985). There was a moderate difference in binding ability of proteins in HeLa cell extracts to the GRE8C and GRE8WT probes. The band corresponding to the 96 kDa protein was more prominent with the GRE8C probe than that with the GRE8WT oligo (Fig. 3.7).

For the nt 7474 GRE, shown in Fig. 3.8, a band predominantly of about 96 kDa was found to bind to wild type GRE9WT and consensus GRE9C probes. A striking, quantitatively higher binding was observed to the GRE9C probe than to the GRE9WT probe. This differential affinity of binding of GRE9WT

**Fig. 3.7. UV-crosslinking assays with GRE8WT and GRE8C
oligonucleotides.**

Thirty μg protein extracts from HeLa cells were
incubated with 1×10^5 cpm uniformly labelled GRE8WT or GRE8C
probes in presence of 0.15 mM bromodeoxyuridine and
$[^{32}P]dCTP$ and UV-irradiated. The crosslinked products were
resolved on SDS-PAGE gels. The sizes (in kDa) of different
markers used are indicated by numbers. The arrow and
arrowhead indicate the major crosslinked protein (96 kDa)
and possibly its degradation product (43 kDa), respectively.
F, Free probe; B, bound; S, specific competitor; NS,
nonspecific competitor.

	GRE8WT				GRE8C			
	F	B	S	NS	F	B	S	NS

Fig. 3.8. UV crosslinking assays with GRE9WT and GRE9C oligonucleotides.

The conditions for the assays and description of the results are as shown for nt 7385 GRE probes (Fig.3.7). Here, predominantly a 96 kDa crosslinked product, indicated by the arrow, was observed.

| GRE 9C | | | | GRE 9WT | | | |
| F | B | S | NS | F | B | S | NS |

-200

-97.4

-68

-43

-29
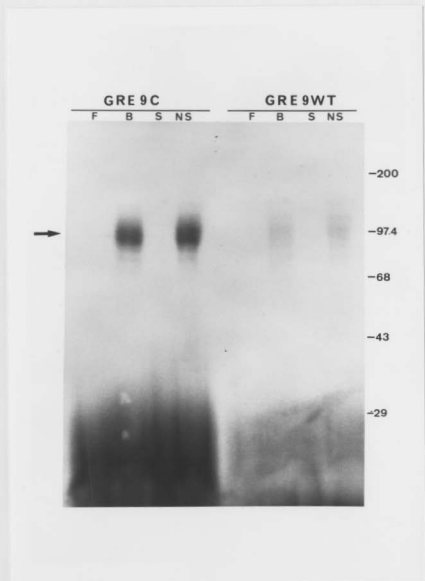
and GRE9C to HeLa protein extracts in UV-crosslinking was quantitatively comparable to that in EMSA (Fig.3.5). These results suggest that a protein of 96 kDa in HeLa cell extracts, probably the GR, could bind to the GRE probes and also the affinity of GRE-protein interaction appears to be enhanced by cGRE mutations, especially for the nt 7474 cGRE in the GRE9C probe. These results were substantiated with the specific and nonspecific competitors used in EMSA, although not with the Southwestern blots.

On the whole, the results of the three assays for DNA-protein interaction (Table 3.3) indicated that a cellular protein of approximately 96 kDa size predominantly interacted with GRE and cGRE oligonucleotides representing the two GREs in the HPV16 genome. Consistently, more protein binding was observed with cGRE oligonucleotides than with wild type GRE oligonucleotides.

3.4 Characterization of ectocervical cells immortalized by triple cGRE-containing HPV16 DNA

Dex-dependent enhanced expression of HPV16 E6 and E7 oncogenes from the HPV16 enhancer/promoter harbouring triple cGRE mutations was seen in ectocervical cells (Fig. 3.3). This result provided the impetus to derive a population of immortalized cervical cells with such a construct. Therefore, the human ectocervical cells (HEC) from primary culture of cervical biopsy specimens that were histologically and

Table 3.3. The results of the DNA-protein interaction assays for the GRE oligomers

Oligomers	[a]Sequence	DNA-protein interaction assays		
		[b]EMSA	UV	SWB
GRE8WT	*tt*GCTACATCCTGTTTT	Two fast migrating complexes	96, 43kDa and intermeiate size bands	[c]ND
GRE8C	*tt*<u>GGT</u>ACATCCTGTT<u>CT</u>	Three complexes	96kDa band is more intense than with GRE8WT	96kDa
GRE9WT	*tt*GGCACAAAATGTGTT	Three complexes	predominantly 96 kDa band	ND
GRE9C	*tt*<u>GGT</u>ACAAAATGT<u>TCT</u>	Two low mobility complexes	More intense 96kDa band than with GRE9WT	96kDa

[a]The partial sequences of the 22-mers used in the assays are shown. The GRE palindrome in the oligomers is in capital letters and mutated bases are double underlined

[b]EMSA, UV and SWB indicate electrophoretic mobility shift assays, UV crosslinking assays and Southwestern blot assays, respectively.

[c]ND indicates the assays not performed on the respective oligomers.

The underlined five-nucleotide motif is NF-1 half binding site. The bases in italics indicate the left flanking nucleotides of the GRE palindrome in the oligomers.

pathologically normal, were transfected with a triple cGRE-containing whole HPV16 construct. The pBR322 vector was used as a negative control for immortalization. The hydrocortisone glucocorticoid was incorporated in the media throughout the cell culture.

The untransfected and the control vector-transfected cervical cells underwent senescence after passage 6. However, the cells transfected with pHPV3XGRE(C) plasmid continued to grow until passage 18, at which time they underwent a transient period of crisis for four weeks. During this period, these cells could not be subcultured. However, a small population of cells acquired an extended life span. Thereafter, these cells have been in culture for more than 60 passages. These cells were designated HEC-16cGRE. Compared with HEC-16, immortalized by HPV16 with wild type GREs in this laboratory previously (Tsutsumi et al, 1992), HEC-16cGRE cells were found to have reduced growth requirement. For example, these cells could be readily adapted to low serum-containing medium and these cells were not stringently dependent on keratinocyte growth medium for growth. These cells were analyzed for morphology, differentiation, viral high molecular weight DNA and viral RNA.

3.4.1 Morphology in monolayer cultures

The three types of human ectocervical cells, normal HEC, HEC-16 and HEC-16cGRE were compared for their morphology in

Fig. 3.9. Morphology _in vitro_ of immortalized cells in monolayer cultures.

The lanes are: **a**, Primary culture of HEC (human ectocervical cells); **b**, Secondary culture of HEC; **c**, HEC-16 and **d**, HEC-16cGRE. Magnification of light microscopy: X 100
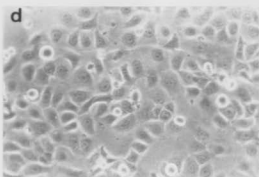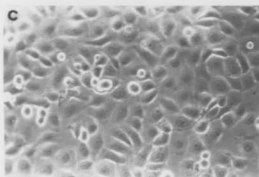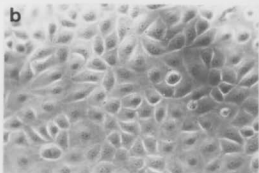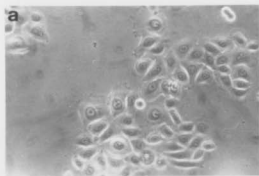
monolayer cultures at confluence (Fig. 3.9). Primary and secondary ectocervical cells were composed of keratinocyte-like polygonal cells forming a typical cobblestone monolayer (Fig.3.9 a and b), as described previously (Tsutsumi et al,1992, 1994). HEC-16cGRE cells (Fig. 3.9 d) were morphologically larger and flatter than primary and secondary cultures of HEC (Fig.3.9 a and b). Also, HEC-16cGRE cells (Fig. 3.9 d) were more pleomorphic than HEC-16 cells (3.9 c).

3.4.2 Morphology in organotypic (raft) cultures

Organotypic (raft) culture allows _in vivo_-like stratification and differentiation of keratinocytes, such as ectocervical cells, into an epithelium _in vitro_ (McCance et al, 1988). Therefore, epithelia from HEC-16 and HEC-16cGRE were analyzed with rafts. The untransfected HEC were used as a negative control (Figure 10 a), which showed normal epithelium with a single layer of dividing basal cells and differentiating superficial layers (McCance et al, 1988). On the other hand, the SiHa cervical carcinoma cells used as a positive control exhibited an extensive undifferentiated state marked by the presence of basal-like dividing cells throughout the epithelium with irregular stratification (Fig. 10 b)(Rader et al, 1990; Durst et al, 1991). Both HEC-16 and HEC-16cGRE raft cultures resembled low-grade dysplasia or CIN type I in organotypic cultures, as suggested by modest parabasal cell crowding, decreased cytoplasmic/nuclear ratio and mitotic

Fig. 3.10. Morphological differentiation of epithelia from immortalized cells in organotypic (raft) cultures.

The organotypic or raft cultures were initiated as described in the Materials and Methods. Cell differentiation is towards the top of each panel and the basal layer is towards the bottom. The panels are: **a**, untransfected HEC; **b**, SiHa; **c**, HEC-16 and **d**, HEC-16cGRE. The arrows indicate koilocytes. Original magnification of light microscopy is X 100.
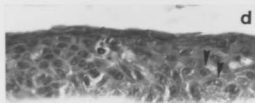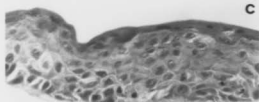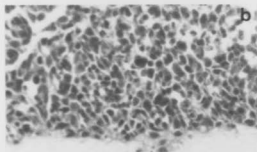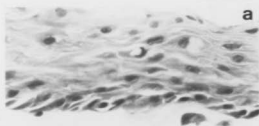
cells with more nuclear content (Fig. 10 c and d). In addition, HEC-16cGRE cells contained koilocytes i.e. the cells with abnormal nuclei and vacuolated cytoplasm (arrows, Fig. 10d), suggesting the altered differentiation characteristics of the epithelium.

3.4.3 Analysis of DNA by Southern blot assays

To determine the physical state of the HPV16 DNA, high molecular weight DNA was extracted from HEC-16, HEC-16cGRE and CaSki cells. These DNA samples were analyzed by Southern blot using full-length HPV16 DNA probe. HEC-16cGRE contained HPV16 DNA as shown by the high molecular weight DNA, undigested and BamHI-cleaved (Fig. 3.11: lanes, UD and 1, respectively). The restriction enzyme digestion pattern of DNA from HEC-16cGRE with noncleaving XbaI and multiple site-cleaving BamHI and PstI, revealed that the HPV16 DNA was integrated with host DNA in these cells. Further, the hybridization pattern showing the presence of a 1.78 kb BamHI-PstI fragment (Lane 1, BamHI+PstI) suggested that the E6-E7 region and the long control region (LCR) were retained in the immortalized cells (Durst et al, 1987a). The BamHI single site digestion revealed a more complex pattern, indicating multiple sites of integration for HEC-16cGRE (BamHI, lane 1) than HEC-16 (Panel b, lane B). This is consistent with the clonal selection of HEC-16 but not HEC-16cGRE.

Fig. 3.11. Analysis of high molecular weight DNA from immortalized cells.

The high molecular weight DNA from the cells was analyzed by Southern blot assays. Full-length HPV16 genomic DNA was used as probe. Molecular weights are indicated on the left in kb. The HPV16 genomic DNA (16) digested with indicated enzymes, was used as marker. The lanes are: Panel a: UD, undigested HEC-16cGRE; C, C33A as a negative control; 1, HEC-16cGRE; 2, CaSki as a positive control. Panel b: DNA from HEC-16 cells cleaved with BamHI and PstI (B/P), BamHI (B), and XbaI (X).
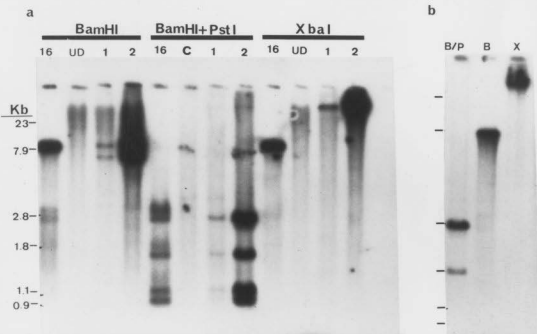
3.4.4. Analysis of RNA by Northern blot assays

To evaluate the expression of integrated HPV16 DNA in HEC-16cGRE cells, total RNA isolated from these cells was analyzed by Northern blot. The RNA samples were probed with whole HPV16 DNA probe. As shown in Fig. 3.12, the viral DNA was expressed predominantly as two transcripts of 4.5 kb and 2.3 kb that were comparable in sizes to the viral RNA from HEC-16 and CaSki cell lines (Tsutsumi et al, 1994). The additional 1.5 kb band observed in RNA from the CaSki cell line represented an E2 transcript (Smits et al, 1991) which was not evident in HEC-16 and HEC-16cGRE. The most abundant transcripts observed in HEC-16 and HEC-16cGRE were of 4.5 kb and 2.3 kb, which contain most of the E6-E7 transcripts (Choo et al, 1987; Durst et al, 1987a; Seedorf et al, 1987). Interestingly, the viral RNA levels upon densitometry analysis, were found to be 2-fold more in HEC-16cGRE than that of HEC-16, relative to the actin internal control (Fig. 3.12). These levels of RNA were detected at 1 μg/ml concentration of glucocorticoid hydrocortisone in the medium that was used for culturing ectocervical cells in monolayers.

3.4.5 Tumorigenicity assays

The oncogenic potential of HEC-16cGRE cells was of special interest, since the E6-E7 oncogenes were expressed at elevated levels (Fig. 3.3 and 3.12). Therefore, the cells were subjected to soft agar assays for testing anchorage

Fig. 3.12. Northern blot assays for expression of HPV16 DNA in immortalized cells.

Full-length HPV16 DNA was used as probe for hybridization with total RNA isolated from the indicated cells. Molecular weight markers are indicated on the right in kb. C33A cells were a negative control and CaSki cells were a positive control. The same blots were probed with γ-actin DNA as an internal control. Bottom panel: Densitometric analysis of the blots is shown by optical density (O.D.) values in three independent experiments. N.D. indicates not performed. The O.D. values for C33A control RNA indicates background radioactivity.
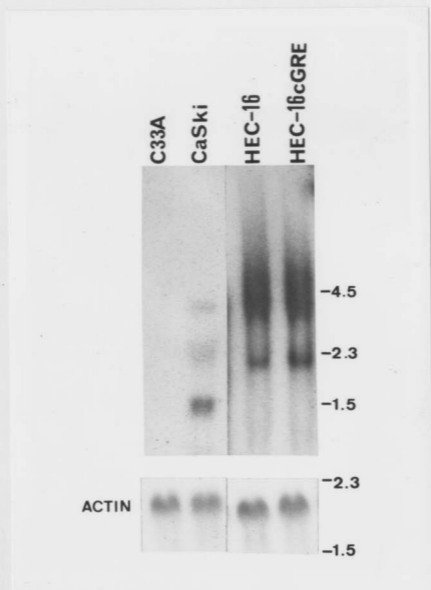
Cells	C33A	CaSki	HEC-16	HEC-16cGRE
O.D.	0.05+/-0.03	ND	3.0+/-0.5	6.4+/-1.2

independent growth. HEC-16cGRE cells failed to form colonies in agar over a period of four months of observation in triplicate assays. On the other hand, SiHa cervical carcinoma cell line exhibited soft agar colonies in two weeks. To test the _in vivo_ tumorigenic potential of the cells from HEC-16cGRE, immunocompromised mice were injected at three different sites. In correlation with the soft agar assays, HEC-16cGRE failed to produce tumors during the observation period of four months in duplicate experiments, while injection of SiHa cells caused tumors in mice in four weeks.

The results of the characterization of HEC-16cGRE by the above described assays are summarized in Table 3.4.

3.5 In vitro transformation assays

Previous studies established that HPV16 E6 and E7 oncogenes cooperate with activated ras oncogene in the transformation of baby rat kidney (BRK) cells (Storey et al, 1988; Pater et al, 1988, 1990, Mittal, 1993). Further, dex glucocorticoid increased transformation of BRK cells by HPV16 in the presence of ras (Crook et al, 1988; Pater et al, 1988, 1992b; Mittal et al, 1993b). To analyze the effect of cGRE mutations in the HPV16 genome on transformation, foci formation assays were performed. Foci were counted after 4 weeks of selection of BRK cells transfected with HPV16 constructs. As shown in Table 3.5, the number of foci formed were greater in BRK cells transfected with HPV16 with cGRE

mutations, in comparison with the wild type construct in the presence of dex. In addition, the foci appeared earlier with cGRE construct than with the wild type construct.

Table 3.4 Summary of the results of the characterization of HEC-16cGRE in comparison with HEC-16

Characteristics	HEC-16	HEC-16cGRE
Monolayer morphology	Large, flat cells	More pleomorphic than HEC-16
Epithelial differentiation	Parabasal crowding, mitotic cells	Parabasal crowding, high number of mitotic cells and koilocytes
Physical state of HPV DNA	integrated	mostly integrated
Expression of HPV DNA	HPV16 DNA was expressed	HPV16 RNA levels were two-fold higher than in HEC-16
Anchorage independent (soft agar) growth	Negative	Negative
In vivo tumorigenicity	Negative	Negative

Table 3.5 The results of foci formation in BRK cells by HPV16 constructs

Plasmid[a]	GREs	Transformation[b]		Induction[c]
		-dex	+dex	
pBR322	Nil	0	0	-
pHPV(WT)	Three wild type	1.0+/-0.3	2.8+/-0.2	2.8
pHPV3XGRE(C)	Three consensus	1.3+/-0.2	6.9+/-0.5	5.3

[a] pBR322 vector was used as a negative control for foci formation assays. pHPV(WT) and pHPV3XGRE (C) contain wild type and consensus GREs, respectively, in whole HPV16 genome.

[b] The foci were counted in five 60 mm dishes four weeks after transfections. The results are from three independent transfections. The average number of foci formed in wild type-transfected and noninduced (-dex) cells were normalized to 1.0. The values for pHPV1XGRE (C) plasmid were calculated relative to the normalized wild type values for comparison.

[c] Induction was calculated by the ratio of the normalized average number of foci in the presence of dex (induced) to the normalized average number of foci in the absence of dex (noninduced).

CHAPTER 4

DISCUSSION

Several lines of evidence have implicated the association of HPVs with human cervical cancer (reviewed in zur Hausen, 1977, 1991). In addition to HPVs, steroid hormones have been implicated as a cofactor in oncogenesis. Previous studies using loss of function mutations and DNase I footprinting analysis have indicated that glucocorticoid/progesterone hormones might mediate their effect through the three GRE-like sequences in the enhancer region of HPV16 (Gloss et al, 1987; Chan et al, 1989; Mittal et al, 1993a and 1993b).

In this study, the role of glucocorticoid steroid hormones in HPV expression and immortalization has been studied with constructs containing consensus mutations of GREs (cGREs) of HPV16. Studies have shown that constructs with cGRE sequences could be useful tools to analyze glucocorticoid-mediated expression from the regulatory region of many genes (Rozansky et al, 1994; Jenab and Inturrisi, 1995). Previously, transformation studies using mouse or rat kidney epithelial cells demonstrated that dex glucocorticoid enhanced efficiency of transformation in co-operation with an activated oncogene (Crook et al, 1988, 1989; Pater et al, 1988, 1990; Mittal et al, 1993b). In this study, cervical and BRK epithelial cells were used to examine the expression pattern from cGRE mutations. The cells harbouring HPV16 cGREs may constitute an ideal tool to analyze hormone-mediated transformation.

4.1. Glucocorticoid-mediated expression by triple cGRE mutations in cervical and BRK cells

The three GRE-like sequences, sharing varied degrees of homology to cGRE sequence, were used to study glucocorticoid-mediated expression. Initially, the GRE sequence identified at nt 7640 position (Gloss et al, 1987; Chan et al, 1989) was converted to a functional consensus sequence, GGTACA(N)$_3$TGTTCT. This resulted in mutation of an overlapping AP1 binding site (Chong et al, 1990; 1991). This particular plasmid construct p1XGRE(C)CAT, induced expression in the presence of dex to a significant fold in HeLa cells. Since the overlapping AP1 site is mutated in this construct, it is likely that the dex-mediated response is solely through the GRE. Moreover, in the absence of dex, this particular construct had lower expression than that of the wild type construct. Such types of mutually exclusive interactions between GR and AP1 factors have been reported earlier (Jonat et al, 1990; Diamond et al, 1990). However, the role of a neighbouring, but not overlapping, AP1 motif in this construct (Chong et al, 1990; Mittal et al, 1994; Peto et al, 1995) for activation cannot be ruled out in this case. Next, the p1/3XGRE(C)CAT construct, that contains a cGRE at nt 7640 and two nonconsensus GREs, was assayed for dex-induced CAT activity. The induction was only slightly higher than that of the p1XGRE(C) CAT construct.

When the CAT expression from the plasmid construct

containing all three consensus GREs was compared with that of wild type sequences in transient expression assays, the enhanced function mutants were more responsive to dex in comparison with the wild type construct. This suggests a cumulative effect of the disruption in the AP1 binding site at nt 7640 GRE and the consensus mutations of the other two GREs in the triple cGRE construct. The GRE sequence at nt 7474 has an overlapping NF-1 half consensus recognition site, TTGGC (Gloss et al, 1990; Sibbet and Campo, 1990), which is mutated to TTGGT in the cGRE mutation. However, Jones et al, (1987) reported that the mutated C base is not a conserved nucleotide in the consensus NF-1 sequence TTGGA/C(N)$_5$AGCCAA. It could be possible that the high level of expression observed in the case of the triple cGRE construct may be due to a lack of interference of AP1 factors for GR activation at nt 7640 and possible cooperative interaction with the factors binding adjacent to nt 7385 and nt 7474 GREs.

The CAT assays were performed to measure expression from a heterologous tk promoter in HeLa cells. The triple cGRE mutations in the context of a heterologous tk promoter in HeLa, a HPV18-containing cervical carcinoma cell line, enhanced expression of the reporter gene in CAT assays. Similar results were observed in BRK epithelial cells for CAT expression through the GRE constructs. Although the levels of induction were not as high as in HeLa cells, the dex induction was consistently more with cGRE constructs than with the wild

type constructs. The difference in levels of induction between HeLa and BRK cells is more likely due to the different species of cells.

Next, to examine the effect of cGRE mutations on expression of HPV E6 and E7 oncogenes from the intact HPV16 genome, cultured ectocervical cells were transfected with whole HPV16 plasmids carrying wild type GREs or triple cGREs. Dex glucocorticoid enhanced the levels of E6-E7 message, as evident by in situ hybridization of viral mRNA. This is in agreement with the earlier observation that progesterone and glucocorticoid hormones activated the expression of HPV16 in these cells (Mittal et al, 1993a). In my study, I have probed for the expression of the E6-E7 oncogenes as a direct measure of the oncogenic potential. Similarly, S1 nuclease mapping of RNA from SiHa cervical carcinoma cells, which harbour a single copy of HPV16, elicited an increase in transcripts encoding E6-E7 in the presence of dex glucocorticoid (Chan et al, 1989).

4.2 Protein(s)-binding to wild type GRE and cGRE oligonucleotides by DNA-protein interaction assays

The HeLa cells have been shown to support the expression of the cell-type specific enhancer/promoter of HPV16 (Gloss et al, 1987). This enhancer region contains several overlapping motifs, such as the binding sites for NF-1, GR and AP1 transcription factors, which may additively or synergistically

activate expression (Chong et al, 1990; Sibbet and Campo, 1990). Moreover, previous studies have shown that oligonucleotides representing the HPV16 GREs can induce expression in a dex-inducible manner (Gloss et al, 1989a; Mittal et al, 1993b). In the context of whole HPV16 enhancer/promoter a cooperative interaction between these factors can be envisioned.

After observing an enhanced expression from the cGRE mutations in the HPV16 enhancer, in vitro GRE-protein interaction assays were done. To correlate dex-induced increase of HPV expression in vivo with in vitro binding with cGRE sequence, DNA-protein interaction assays were performed. In EMSA, the oligonucleotide representing nt 7474 GRE displayed a stronger binding with HeLa protein extracts. There was a marked difference in terms of the amount of protein bound to the wild type and consensus oligonucleotide probes. Methylation pattern and interference assays (Scheidereit and Beato, 1984; Chalepakis et al, 1988, 1990) have demonstrated that a cytosine at the +5 position in the consensus palindrome, $G^{-6}G^{-5}T^{-4}A^{-3}C^{-2}A^{-1}(N)_3T^{+1}G^{+2}T^{+3}T^{+4}C^{+5}T^{+6}$, is very important for binding of GR and is highly conserved among GREs of regulatory regions of several cellular genes (Beato et al, 1989). However, this conserved C residue is lacking in both wild type nt 7385 and nt 7474 GREs (Sibbet and Campo, 1990; Mittal et al, 1993b).

The oligonucleotides representing nt 7385 wild type GRE

and cGRE exhibited moderate to no difference in binding. Further, the nt 7385 GRE shares more homology (83%) to consensus sequence than that of the other two wild type GREs (75%). It is possible that the complexes formed with nt 7385 oligonucleotides might have dissociated due to the gel conditions. Chalepakis et al (1990) reported that high affinity binding of the receptor to its recognition sequence requires flanking sequences. It is likely that the flanking sequences may have a role in proper DNA-protein interactions. The GRE oligonucleotides used in this study, had different flanking sequences although the GRE-palindrome sequence was the same.

A prominent low mobility complex was observed with nt 7474 cGRE oligomer in comparison with the wild type GRE oligomer. In the nt 7474 GRE sequence, the TTGGC sequence is one of the half NF-1 binding sites (Sibbet and Campo, 1990). This was mutated to TTGGT in the cGRE. Meisterernst et al (1988) observed that the NF-1 members of transcription factors have more affinity of binding to the complete consensus sequence rather than either to the right or to the left half consensus sequence. Moreover, DNA-protein interactions have shown that NF-1 binds poorly to the half consensus site TTGGC in the regulatory region of HPV16 (Chong et al, 1990, 1991; Apt et al, 1993). Therefore, the specific protein bound with high affinity to the nt 7474 cGRE sequence is more likely to be the GR rather than NF-1 protein. Alternatively, there could

be a cooperative effect for binding and activation between the receptor and NF-1 and/or other factors binding to the adjoining cis-acting elements. This is true in case of the MMTV regulatory region that is regulated by glucocorticoid/progesterone hormones (Bruggemeier et al, 1991; Truss et al, 1992; Cavin and Buetti, 1995; Lee et al, 1995). However, in UV crosslinking assays only a 96 kDa band was detected with oligonucleotide representing nt 7474 GRE and no other protein suggestive of NF-1 was detected. This further supports the observation that the specific steroid receptor might bind with a high affinity to the nt 7474 cGRE.

The results for UV crosslinking were in excellent agreement with EMSA. The amount of cellular protein crosslinked to the nt 7474 cGRE was found to be more than that for wild type sequence and there was a moderate difference for protein binding to the oligomers of nt 7385 wild type and consensus GRE sequences. The 96 kDa protein found to bind the GREs in UV crosslinking is likely to be GR (Hollenberg et al, 1985). Interestingly, the HeLa cells do not have significant levels of progesterone receptor that also binds to GRE/PRE (Cato et al, 1986). Therefore, it is possible that the protein binding to the cGRE probes may be GR. However, supershift assays with monoclonal antibody for GR were not performed. These assays would be ideal for the identification of the GRE-binding protein. Nevertheless, Southwestern blot assays also supported the results of UV crosslinking and EMSA.

The high-affinity binding of the receptor to GREs may activate expression (McEvan *et al*, 1994; Dahlman *et al*, 1994, 1995). In the MMTV-LTR regulatory region, a paradigm for glucocorticoid-mediated regulation of target genes, a cluster of high affinity TGTTCT half GRE binding sites have been shown to be important for glucocorticoid-mediated regulation (Chalepakis *et al*, 1988; Truss *et al*, 1992). In the case of the HPV16 GREs, it is quite possible that a high level of activation of the reporter gene through the GREs in the upstream regulatory region may correlate with the high affinity binding of the receptor to these elements. In this manner, the nt 7640 GRE, which is closer to the P97 promoter than the other two GREs, might be the effective site for activation of HPV16 expression. The effect of the auxiliary GREs would be to increase the binding of GR cooperatively to them and to the nt 7640 site, which in turn interact with the general transcription machinery to enhance the efficiency of initiation of transcription (McEvan *et al*, 1994; Dahlman *et al*, 1994, 1995).

4.3 Immortalization of cultured ectocervical cells and transformation of BRK cells with HPV16 triple cGRE construct

The immortalized HEC-16cGRE displayed an altered morphological pattern in monolayer and raft cultures in comparison with the control cells. Also, the morphological features were distinct compared with HEC-16, a clonal line of

cells containing HPV16 with wild type GREs. Although the heterogenous nature of HEC-16cGRE could be attributed to the polyclonal population of cells, HEC-16cGRE appears to be distinct. Morphologically, it is likely that cells harbouring HPV16 with cGRE sequences would exhibit an altered phenotype in comparison with HEC-16. This could be due to a differential effect of hydrocortisone glucocorticoid hormone in the medium for growth and/or expression from the wild type GREs and cGREs (von Knebel Doeberitz, 1988). It is conceivable that the effect of glucocorticoid hormones on growth properties and/or differentiation of cervical epithelial cells could be readily evaluated in HEC-16cGRE due to the consensus mutations. This is supported by the additional changes in the differentiation pattern of HEC-16cGRE, for example appearance of koilocytes, in comparison with the HEC-16. Moreover, the organotypic (raft) cultures mimic the physiological milieu of cervical epithelium which could be monitored for differential hormonal levels in different layers of the epithelium (Sanborn et al, 1976; Gorodeski et al, 1989; Meyers et al, 1992).

Analysis of the physical state of DNA in HEC-16cGRE revealed integration of viral DNA into the host genome. It is apparent that integration is likely to have taken place within the E1-E2 region, as suggested by Southern and Northern blot patterns. The E6-E7 region and the LCR are retained. The integration of the HPV DNA into the host genome observed in immortalized cells resembles the state of the HPV DNA in the

cervical lesions progressing towards neoplasia (Yokoyama et al, 1995; Daniel et al, 1995). Jeon et al (1995) reported that the integration of HPV DNA was associated with enhanced growth of cells and viral expression and the integration of viral DNA into host genome was favourable to the proliferating cells progressing to carcinoma. Retention of E6-E7 is necessary to maintain an altered growth phenotype of cells (Baker et al, 1987; Munger, et al, 1989b). Presence of the LCR implies that this region may be functional. It may drive expression through the viral promoter and several cellular factors may interact with it. It is possible that there could be alteration of expression through the viral LCR regulatory region by cellular factors since the viral DNA is in the integrated state (Cripe et al, 1987; Gloss and Bernard, 1990; Smits et al, 1991). Conversely, the viral LCR may influence the expression of neighbouring cellular genes (Drews et al, 1992), thereby leading to altered phenotypic changes of HPV16 DNA-harbouring cells. There may also be a loss of hormone response (von Knebel Doeberitz et al, 1991; Pater et al, 1993). HEC-16 and HEC-16cGRE cells could be valuable to examine these possibilities.

The expression levels of the E6-E7 oncogenes in HEC-16cGRE were two-fold more than that of the HEC-16 cells. On the other hand, in CAT expression assays in HeLa cells, a nearly 8-fold greater induction of the expression of the triple cGRE construct than that of the wild type construct was

observed in the presence of dex. This difference may be due to the use of a strong heterologous tk promoter (Nakamura et al, 1989; Ray et al, 1989; Zhang et al, 1994) to drive the expression of the CAT reporter gene from the HPV16 enhancer with cGREs. In situ hybridization assays using whole HPV16 constructs demonstrated a significant induction, although qualitative, by dex. It has been reported that HPV16 in transiently transfected cells exists mainly in an episomal state in which the viral genome might be positively regulated by glucocorticoid hormones (Mittal, 1993 and Mittal et al, 1993a). However, the RNA levels of HPV16 in HEC-16 and HEC-16cGRE in Northern blot assays were from the integrated HPV16 genome. This suggests that the mode of regulation of HPV expression in early and late stages of immortalization might be different. This is consistent with the previous report that the viral DNA integration disrupted the glucocorticoid-mediated expression of HPV16 and that the hormone-induced expression was restored after excising the HPV16 DNA from the flanking cellular sequences (von Knebel Doeberitz et al, 1991). Further, since E2 ORF appears to be disrupted, these cells may lack the E2 protein which has been shown to cooperate with cellular factors for activation of expression from the HPV16 regulatory region (Ushikai et al, 1994). Other possibilities are: first, the high levels of E7 and/or E6 oncoproteins may activate macrophages of the immune system that may lead to cytotoxicity of HPV-harbouring cells (Banks

et al, 1991); secondly, there may be a feedback or autoregulation by the papillomaviral early proteins, which has become clear for the paradigm small DNA tumor virus, SV40 (Rio et al, 1980; Wildeman, 1989).

4.4 Role of glucocorticoid/progesterone steroid hormones and GREs in oncogenesis

In this study, HPV16 genomic DNA has been mutated to a consensus sequence in its three GREs and an enhanced expression has been demonstrated. Glucocorticoid hormones increased the efficiency of transformation by HPV16 in association with an activated oncogene in rodent epithelial cells (Pater et al, 1988; Crook et al, 1988; Durst et al, 1989). In these studies, the hormone-mediated upregulation of transformation of HPV16-harbouring cells required an activated oncogene for malignant conversion of the HPV16-containing epithelial cells. Further, integration of HPV16 DNA into the host genome may bring about deregulation of viral expression. In addition, the subsequent cellular DNA alterations that are accumulated in the form of DNA amplification, genomic rearrangements and mutations could result in a breakdown in the intracellular surveillance mechanism culminating in neoplastic conversion (zur Hausen, 1991).

There are reports of the use of cervical cells in the study of multistep carcinogenesis induced mostly by the high risk genital HPV types 16 and 18. Studies involving the use of

other HPV-cofactors such as cigarette smoke and certain viruses other than HPVs have been reported (Garett et al, 1993; Chen et al, 1994a and 1994b). The role of steroid hormones as an HPV-cofactor assumes importance, since these hormones are intricately involved with the physiology of the female genital system (Sanborn et al, 1976). Additionally, the use of oral contraceptives and pregnancy can be important risk factors. It is interesting to note that genital type HPVs, namely HPV6, 11, 16, 18 and 33 contain GRE/PRE sequences that induce hormone-mediated expression in transient expression assays (Chan et al, 1989). The presence of hormone response elements in RNA tumor viruses, especially in retroviruses, and hormonal induction of expression of the target genes has been well documented, particularly in the case of MMTV regulation (Chalepakis et al, 1988; Kalff et al, 1990; Truss et al, 1993, 1995). The cGRE construct could be ideal to examine the hormone response in human papillomaviruses that are the only DNA tumor viruses that involve hormone-mediated regulation of expression.

It is reported frequently that HPV-immortalized cell lines are nontumorigenic (Pecoraro et al, 1989, 1991). It was apparent that HEC-16cGRE cells were not transformed as suggested by the results of anchorage independent (soft agar) growth and nude mice injection studies. This is consistent with the theory of multistep carcinogenesis, involving more than one or two factors in neoplastic progression (zur Hausen,

1991; Howley, 1991; Yokota and Sugimura, 1993). Consistently, the HPV16 DNA with cGRE mutations was more efficient in dex-mediated transformation of BRK cells than the wild type HPV16 genome. This reinforces the earlier hypothesis that high levels of E6-E7 oncoproteins along with an activated oncogene leads to malignant transformation. Alternatively, the neoplastic progression in cultured cells could be a slow process with progressive changes in HPV-harbouring cells (Pecoraro _et al_, 1991), which may be true with HEC-16cGRE cells. Therefore, these cells could not be conclusively said to be nontumorigenic at this stage.

Based on dex-mediated expression, HCE-16cGRE cells are unique in that the hormone response was more stringent in these cells due to the cGRE mutations and the cells may be under strict hormonal regulation. HPVs could take advantage of hydrocortisone or progesterone hormones for the modulation of expression by hormones under different physiological conditions to which the cervical cells are subjected. Although GRE-mediated regulation is likely to be a general feature for genital HPVs, the regulation of expression in the case of HPV16 by glucocorticoids or progesterone may be unique, in that only such high risk HPVs contain oncogenic E6 and E7 genes (Pater _et al_, 1988;,1992a; Barbosa _et al_, 1990; 1991). Therefore, the exogenous glucocorticoid/steroid hormones or endogenous progesterone levels could be crucial in the transforming activity of HPV16. Although HPV16 does not

contain cGRE sequences in the natural context, these mutations may constitute a tool to analyze dex-mediated expression. The cGRE-containing enhancer constructs clearly demonstrated that the glucocorticoid regulation of expression has important effects on oncogenesis.

Steroid hormones probably mediate their effect by increasing the expression of viral oncogenes through the direct interaction of the GR/PR with the GRE/PREs of HPV16. It is apparent that glucocorticoid/progesterone hormones play a major role in the initiation process in multistep carcinogenesis in association with HPV16. In other words, HPVs may be considered as initiating agents in carcinogenesis, while glucocorticoid/progesterone hormones can be described as cofactors or tumor promoters (Pater et al, 1988, 1994; Khare et al, 1995). Further, neoplastic progression and subsequent metastasis could involve additional targets and/or events. This is supported by my study using cGRE mutations that could upregulate viral gene expression. This might lead to increased susceptibility for genetic alterations (zur Hausen, 1991; Drews et al, 1992). Further, the hormone dependence of cells for growth and expression in immortalized and transformed cells and the emergence of hormonal resistance of these cells need to be analyzed in depth. Some potential avenues for pursuing this goal are outlined in the following section.

This study dealing with the role of glucocorticoid hormones in HPV-associated cervical oncogenesis offered an opportunity to examine several aspects of hormone-induced oncogenesis. Several questions, however, remain to be studied: first, stage(s) of carcinogenesis at which glucocorticoid and/or progesterone hormones play a pivotal role; secondly, whether the role of hormones is direct or indirect and third, the role of hormones in HPV expression upon integration of the HPV16 genome and the emergence of hormone independence.

Regarding the stage of action of steroid hormones in cervical carcinogenesis, it is clear from several studies that the continued expression of viral E6 and E7 oncogenes is necessary for the initiation and maintenance of the transformed phenotype of HPV-harbouring cells. The dex glucocorticoid hormone enhanced the transforming potential of the E6 and E7 genes (Crook et al, 1988; Durst, et al, 1989). Further, glucocorticoid hormones are likely to play a pivotal role in the regulation of expression of the E6 and E7 oncogenes through the interaction of the GREs of several HPV types (Gloss, et al, 1987; Chan et al, 1989). To address the potential roles that glucocorticoids may play after integration, viral oncogene expression in HEC-16cGRE can be assessed by quantitating the levels of E6-E7 transcripts in dex-treated and untreated cells from these cell lines by RNase protection assays (Pater et al, 1993). It is interesting to

analyze the expression from reporter constructs containing the enhancer/promoter region of HPV16 in these cells. von Knebel Doeberitz _et al_ (1991) analyzed the effect of dex on expression from the upstream regulatory region of HPV18 DNA in various HPV16 and HPV18-containing cell lines. It was observed that the dex-mediated effect could be altered upon integration of the HPV DNA into the host cells. Similarly, it is quite reasonable to examine the effect of the HPV16 enhancer harbouring multiple cGRE sequences, on expression of reporter constructs in HEC-16cGRE. Due to the presence of cGRE sequences the effect of hormones on expression in these cells would be more readily evaluated.

Another important aspect is the stage of oncogenic progression at which hormone-independence emerges. The growth and expression pattern of hormone-dependent and independent cell clones could be analyzed by differential display (Liang _et al_, 1993). This recently developed method allows one to study the alteration of gene expression patterns by comparing cDNA fragments of mRNA from two different cell populations. Therefore, this method could be ideal to analyze differential expression patterns. Another avenue to pursue is to analyze GR levels and function in hormone-dependent and independent cells. The functional integrity of the hormone receptor could be assayed using enhancer elements that require glucocorticoid receptor for dex-induction (Pater _et al_, 1993; Adcock _et al_, 1995). Further, GR-GRE binding assays could be performed

(Ausbel et al, 1994) in hormone resistant clones to investigate the mechanism of the emergence of glucocorticoid independence. In fact, the alteration in the affinity and in the threshold levels of the receptor for binding to the cognate DNA elements have been reported in steroid-resistant cell population (Okret et al, 1991; Adcock et al (1995). Alternatively, GR levels could be directly assessed using systems such as organotypic cultures and in situ hybridization assays. This could be associated with HPV expression in differentiating epithelium of the rafts (Radar et al, 1990). The immortalized cells can also be used to test the hypothesis proposed earlier whether there is any change in the transcription start site in dex-dependent versus dex-independent cells (Pater et al, 1993).

It is worthwhile to note that pregnancy and the use of oral contraceptives are important risk factors for cervical cancer (Stern et al, 1977, Ferenczy, 1989). This points to a role for synthetic progesterone or estrogen hormones, major components of contraceptive pills. Although the role of progesterone and glucocorticoid hormones in cervical oncogenesis has been studied, the role of estrogen hormones is still elusive. Nevertheless, Mitrani-Rosenbaum et al (1989) reported that estrogen induced the expression of the HPV16 DNA that is present in the SiHa cervical carcinoma cell line. Since estrogen response element (ERE) sequences have not been reported in the regulatory region of HPV16, the effect of

estrogen is likely to be indirect. The expression of progesterone receptor was found to be increased as a result of estrogen action (Kastner et al, 1990). Further, estrogen could induce expression of the c-jun oncogene (Weisz et al, 1990). Since the c-jun product has been shown to enhance the hormone response through the nt 7640 GRE (Mittal et al, 1994), a synergistic role of estrogen and progesterone/glucocorticoid hormones in HPV16 expression could be expected. The HPV16 plasmid construct containing cGREs and consensus EREs could be ideal to probe the synergistic action of these hormones for expression and transformation. Indeed, the role of the AP1 and the NF-1 transcription factors in association with the hormone receptors needs to be investigated further due to the overlapping binding sites for these factors.

CHAPTER 6

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