ENHANCER ACTIVITY OF HUMAN PAPILLOMAVIRUS TYPE 11 IN MOUSE EMBRYONAL CARCINOMA CELLS

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ENHANCER ACTIVITY OF HUMAN PAPILLOMAVIRUS TYPE 11 IN MOUSE EMBRYONAL CARCINOMA CELLS

By

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

Faculty of Medicine
Memorial University of Newfoundland
January, 1994

St. John's Newfoundland

This thesis is dedicated

to the memory of my sister-in-law

late Lakshmi Kanthi Kasinadhuni.

ABSTRACT

The activity of minimal constitutive enhancer (MCE) (nucleotides 7657-7870) of human papillomavirus 11 was studied in undifferentiated and retinoic acid-differentiated P19 mouse embryonal carcinoma cells. The enhancer was active in RA-differentiated cells but not in undifferentiated cells. The *in vivo* activity was correlated with *in vitro* DNA-protein interactions by DNaseI protection assay. Four protected regions (I-IV) were detected with RA-differentiated nuclear extracts, while protection was observed with undifferentiated nuclear extracts for the regions III and IV. To further study the functional importance of the protected regions, deletion mutant enhancers were tested for their *in vivo* activity. Mutant enhancers were active like wild type in RA-differentiated but not in undifferentiated cells. Deletion of protected region I and a 9 base pair sequence of the imperfect NF1 palindrome of protected region II of HPV11 MCE displayed activity close to that of wild type MCE. This suggests that the major constitutive enhancer activity is exhibited by the minimal sequences of MCE (nucleotide 7785-7870) in P19 RA-differentiated cells. The mutant enhancer (nucleotide 7811-7870) which lost both protected regions I and II showed only 50% of the wild type activity. This indicates that sequences displaying major constitutive activity of HPV11 MCE are

present in the protected region II, although functional synergism appears to be important for full activity of the enhancer. The role of entire protected region II by itself including the 9 base pair sequence which was deleted in the minimal enhancer sequences, was examined for its *in vivo* activity. The protected region II alone showed substantial *in vivo* activity in RA-differentiated cells which was 90-fold higher than the residual activity observed for undifferentiated cells. The *in vivo* activity of protected region II in RA-differentiated-dependent manner was correlated with *in vitro* DNA-protein interactions. The protected region II showed an extra DNA-protein complex with RA-differentiated nuclear extracts in the gel retardation assay. The formation of this complex was competed by the imperfect NF1 palindromic sequences but not by the NF1 half-site sequence of protected region II. This suggests that certain factor(s) in RA-differentiated cells specifically binds the imperfect NF1 palindrome. The DNA-protein interaction was specific for HPV11 imperfect NF1 palindrome as the complex formation was not abolished either by polyoma JC virus perfect NF1 palindrome or its mutant sequence. This suggests the existence of family of NF1 proteins in P19 RA-differentiated cells that show different binding specificities for the NF1 binding sequences of imperfect and perfect palindromic nature. As the extra RA-differentiated DNA-protein complex was specific for the

imperfect NF1 palindrome, UV cross-linking was performed to determine the number of proteins interacting with it. At least six proteins were found to interact with the imperfect NF1 palindrome and three of them were specific for RA-differentiated nuclear extracts. The exact nature and binding sites of these three proteins for the functional importance of protected region II in RA-differentiated cells require further investigation.

ACKNOWLEDGEMENTS

I am extremely indebted to both Dr. Verna Skanes, Assistant Dean, Research and Graduate Studies, Faculty of Medicine and the supervisory committee member Dr. Banfield Younghusband whose timely help, support and invaluable suggestions encouraged me to give a shape to this thesis. I express my sincere gratitude to my supervisor Dr. Mary Pater for her guidance during research work and thesis preparation. I am very much grateful to Dr. Gary Paterno for serving as supervisory committee member and for his invaluable comments on the draft of this thesis. I would like to thank Dr. Alan Pater for his guidance during research work.

I am very much thankful to my colleague and friend, Kotlo Umesh Kumar not only for reading the draft of this thesis but also for his help in many ways. I thank my colleagues Dr. Rakesh Mittal, Dr. Belaguli, Raghuram and Miss. Christine Kisaka. I thank Rao P. Varada for his help in preparing the figures. The company of Umesh Kumar, Balaji Balasa, Devi Reddy, Srinivas Denduluri, Narayanan Rajgopal, Annaji, Dr. C.V.V Prasad and Dr. K.S.R. Prasad is highly appreciated.

I hereby acknowledge the encouragement given by the professors in India, Dr. T. Suri babu, Dr. Krishna Swamy and Dr. P. Rama Rao. My special thanks are to my friend Dr. Suryanarayana Vulimiri (Houston) for his moral support.

The lack of vocabulary fails to express the stupendous weight of my heartfelt gratitude for the moral support extended by my parents, my loving and caring wife Srilakshmi, my brothers Giri and Kanth, my sisters Vani and Anu, brothers-in-law and sisters-in-law. I remember the memory of my uncle, late B. Rama Rao without his help I would not have got the necessary education for the higher studies.

Last but not least, the financial support provided by the School of Graduate Studies and Faculty of Medicine throughout my study period is highly acknowledged.

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AP1	Activator protein 1
bp	base pairs
BPV1	Bovine papillomavirus 1
BRL	Bethesda Research Laboratories
BSA	Bovine serum albumin
BUDR	Bromodeoxyuridine
C	Celsius
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
C/EBP	CCAAT/Enhancer binding protein
CTF	CCAAT-binding transcription factor
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonuclease I
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EC	Embryonal carcinoma
EDTA	Ethylenediaminetetra-acetic acid
HPV	Human papillomavirus
kb	Kilo base pairs
kd	Kilo daltons
MCE	Minimum constitutive enhancer
MEM	Minimum essential medium

mRNA	Messenger RNA
NCR	Noncoding region
NF1	Nuclear factor 1
NFA	Nuclear factor 1-associated factor
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
RA	Retinoic acid
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SV40	Simian virus 40
TAF	TBP-associated factor
TBP	TATA box-binding protein
TEF	Transcription enhancer factor
TLC	Thin layer chromatography
UD	Undifferentiated
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 TRANSCRIPTIONAL CONTROL IN EUKARYOTES:

1.1.1 Regulatory elements:

In eukaryotes, regulation of mRNA synthesis is an important means to regulate gene expression. The frequency of initiation of mRNA synthesis depends upon different characteristic cis-regulatory elements that modulate the rate of transcription of active genes. The regulatory elements thus identified are called promoter elements and enhancer elements. The promoter elements are the regulatory elements that determine the site of initiation and are responsible for basal levels of transcription. The enhancer elements increase or reduce the rate of transcription from the promoter. These elements are located at distant positions from the promoter elements (Dynan, 1989; Mitchell and Tjian, 1989; Hernandez, 1993).

The promoter elements in eukaryotes were first identified for chicken ovalbumin and adenovirus-2 major late genes (Gannon *et al.* 1979; Corden *et al.* 1980). The promoter elements have an AT-rich region, TATAAA designated as the TATA box located at 25-30 basepairs upstream from the start site of initiation of transcription, and one or more sequence elements

of 8-12 basepairs designated as upstream promoter elements (UPEs). Some of the upstream promoter elements have CCAAT boxes and GC boxes located from 30-110 basepairs upstream of the start site of initiation of transcription. The UPEs act regardless of their orientation with respect to the TATA box. The strength of UPEs depends upon the number and type of UPEs. The CCAAT boxes and GC boxes are known to bind transcription factors which modulate the transcription carried out by the general transcriptional machinery (Maniatis *et al.* 1987; Mitchell and Tjian, 1989).

A simple functional promoter element known as an initiator (Inr) was identified within the transcription start site for the lymphocyte-specific terminal deoxynucleotidyltransferase gene which lacks a TATA box. The initiator element specifies the start site for initiation of transcription for those genes lacking a TATA box (Smale and Baltimore, 1989).

The enhancer elements contain modules of discrete DNA sequence motifs that specifically interact with activator or repressor proteins. They were first identified in SV40 virus by Banerji *et al.* (1981). The enhancer elements can act over long distances and are orientation independent. Some modules of enhancers, for example, those of the heat-shock regulatory element can not act from a distance but are active when they are tandemly duplicated. The principle of tandem duplication

can not be applied to all regulatory elements since multiple copies of UPE CCAAT box do not stimulate transcription at a distance. Structurally UPEs and enhancers are similar but there are operational differences based upon the number and arrangement of transcription factor recognition sequences. The enhancers that respond to environmental stimuli such as those for heat shock, heavy metals, viral infection, growth factors or hormones are called inducible enhancers. The inducible enhancers were identified, for example, for genes of metallothionein, β-interferon, heat-shock proteins, *c-fos,* steroid hormone responsive enhancers of mouse mammary tumour virus and moloney mouse sarcoma virus and the glucocorticoid responsive enhancer element (GRE) of human papillomavirus 16 (Maniatis *et al.* 1987; Mitchell and Tjian, 1989; Gloss *et al.* 1987).

The enhancers which are active only during specific developmental stages or only in specific tissues are called temporal and tissue-specific enhancers respectively. For example, immunoglobulin genes have B-cell specific enhancers whereas enhancers for some *Drosophila* genes are active only in different tissues and at different stages of fruitfly development (Maniatis *et al.* 1987).

The cis-acting regulatory elements in yeast are known as upstream activation sites (UASs) and have properties which are similar to mammalian enhancers (Guarente, 1983).

Some of the cis-acting elements known as silencers were identified in the repetitive sequences of the rat insulin gene. These sequences can block the activity of enhancers (Mitchell and Tjian, 1989; Laimins *et al.* 1986).

1.1.2 GENERAL TRANSCRIPTIONAL MACHINERY:

Three different RNA polymerase enzymes namely RNA polymerases I, II and III carry out transcription of different sets of genes in eukaryotes. Messenger RNA (mRNA) synthesis cannot be carried out by RNA polymerase II alone but rather requires a set of transcription factors called 'general transcription factors' (GTFs). RNA polymerase II along with GTFs are necessary for basal levels of transcription. Transcription is activated or repressed by sequence-specific factors binding to the recognition sequences of upstream promoter elements and enhancers. Weil *et al.* (1979) observed that purified RNA polymerase II cannot initiate transcription unless the *in vitro* system is supplemented with crude nuclear extracts rich in different transcription factors. Subsequently, seven general transcription factors known as TFII A, B, D, E, F, H and J were identified. With the exception of TFIIJ, all other GTFs were purified to homogeneity and the cDNAs encoding TFII B, D, E and F were cloned (Hoffmann *et al.* 1990; Ha *et al.* 1991; Peterson *et al.* 1991; Sopta *et al.* 1989; Finkelstein *et al.* 1992). TFIID is a

multiprotein complex composed of TATA-box binding protein (TBP) and TBP-associated factors (TAFs). The TBP encoding genes were cloned from different organisms and are highly similar (Hernandez, 1993). TBP along with RNA polymerase II and other GTFs can mediate basal transcription whereas TFIID is essential to respond to transcriptional activators. In order to form a preinitiation complex, all the GTFs along with RNA polymerase II assemble at the TATA box for initiation of transcription like the formation of closed complex at bacterial promoters (Hernandez, 1993; Ham *et al.* 1992; Zawel and Reinberg, 1992; Dynlacht *et al.* 1991; Tanese *et al.* 1991).

1.1.2.1 TFIID as the first general transcriptional factor to interact with the promoter:

TFIID is the first general transcription factor which recognises and binds the TATA box in a sequence-specific manner. TFIID is a 750 kd multiprotein complex composed of a 38 kd (in humans) TATA-box binding protein (TBP) and eight polypeptides known as TBP-associated factors (TAFs) of approximately 250, 150, 120, 100, 80, 75, 65 and 60 kd (Pugh and Tjian, 1992; Hisatake *et al.* 1993). The TBP and TAFs are physically associated forming the TFIID complex. TBP brings basal levels of transcription, whereas TAFs are necessary for transcriptional activation by the activators. The TBP has a conserved carboxy-terminal domain and has 180 basic amino

acids. This domain is 80% identical in different organisms. The C-terminal domain is essential for determining species specificity, DNA-binding and response to transcriptional activation signals. The C-terminal domain is rich in lysines and can form an α-helix which is important for protein-protein interactions and DNA-binding (Hoffman *et al.* 1990; Cormack *et al.* 1991; Reddy and Hahn, 1991; Hernandez, 1993).

Zhou *et al.* (1993) observed that the C-terminal domain interacts with two major TAFs. Such an interaction supports *in vitro* transcriptional stimulation by different activation domains and can even support transcription from TATA-less promoters. TFIID binds in the minor groove of DNA and induces DNA bending which may facilitate formation of the preinitiation complex and interactions with other transcription factors (Horikoshi *et al.* 1992; Lee *et al.* 1991; Starr and Hawley, 1991). TBP is required by all three RNA polymerases as well as for transcription from TATA-box lacking promoters (Hernandez, 1993; Cormack and Struhl, 1992; Zhou *et al.* 1992; Zenzie-Gregory *et al.* 1992).

1.1.2.2 Other general transcription factors necessary for transcription preinitiation complex:

TFIIA binds the promoter after TFIID binding and stabilizes the TFIID-DNA interaction generating the TFIIDA complex at the promoter. It was hypothesized that this causes

some conformational change in the promoter DNA after its binding to TFIID. TFIIA is an heteromer composed of 3 polypeptides of 34, 19 and 14 kd (Reinberg *et al.* 1987; Cortes *et al.* 1992).

TFIIB binds to the TFIIDA complex formed at the TATA box providing a scaffold for the binding of RNA polymerase II. The cDNA encoding TFIIB was cloned. The cDNA sequence indicates that it encodes a protein composed of a 76 amino acid repeat at its C-terminal domain and a hydrophilic tail at the N-terminus. The C-terminus contacts DNA whereas the tail participates in protein-protein interactions. It was observed that an acidic activator VP16 interacts with the N-terminus of TFIIB (Ha *et al.* 1991; Lin and Green, 1991; Lin *et al.* 1991).

TFIIF is a heteromeric factor also known as RNA polymerase II-associated factor 30/74 (RAP 30/74). It consists of two polypeptides of 30 and 74 kd whose cDNAs were cloned. The small subunit of TFIIF i.e. RAP 30 recruits RNA polymerase II to the TFIIDAB complex and prevents non-specific binding of RNA polymerase II to DNA. It was thought to play a role in the unwinding of DNA strands since it has ATP-dependent helicase activity. The role of RAP 30 is reminiscent of the characteristics of bacterial sigma factors (Sopta *et al.* 1989; Greenblatt, 1991; Flores *et al.* 1991; Killen and Greenblatt, 1992; Aso *et al.* 1992; Finkelstein *et al.* 1992).

TFIIE enters the TFIIDAB.POLII.F complex and is important

for the formation of a stable preinitiation complex. It is a heteromer of 57 kd TFIIEα and 34 kd TFIIEβ. The cDNAs encoding both of these subunits were cloned. The sequences indicated that TFIIEα has leucine repeats, zinc fingers and helix-turn-helix motifs whereas TFIIβ has characteristic sequence motifs found in ATPases and helicases. These features of TFIIE α and β suggest that they are important for its interactions with DNA as well as RNA polymerase II and other GTFs (Peterson *et al.* 1991; Sumimoto *et al.* 1991; Ohkuma *et al.* 1991).

The identity of transcription factor TFIIH was established as a multisubunit factor consisting of 5 polypeptides (92, 62, 43, 40 and 35 kd). It was observed that TFIIH can phosphorylate the carboxy-terminal domain of RNA polymerase II but it is not known which subunit has the kinase properties. TFIIJ is an activity known to enter the preinitiation complex after TFIIH (Lu *et al.* 1992; Flores *et al.* 1992).

1.1.3 RNA polymerase II:

This is a 8-10 subunit enzyme able to polymerize ribonucleotides into mRNA using a DNA template by interacting with GTFs and elongation factors. It functions perhaps in conjunction with other enzymes such as helicases and topoisomerases. The *in vivo* activity of RNA polymerase II was attributed to its largest subunit of 240 kd which exists as

phosphorylated, nonphosphorylated and degraded forms (Corden *et al*. 1985; Saltzman and Weinmann, 1989).

The largest subunit has a distinct structural domain called the carboxy-terminal domain (CTD) or 'tail' which consists of tandem repeats of a seven amino acid consensus sequence. The 7 amino acid repeat varies in number in different organisms. This seems to be related to the complexity of the organisms. The CTD which is unique to eukaryotes has 52 repeats in mammals. Most of the current research related to polymerase II is focussed on the CTD since CTD was found to be essential for cell viabilty (Corden *et al*. 1985; Saltzman and Weinmann, 1989; Corden, 1990).

The CTD of the nonphosphorylated form was found to be phosphorylated by TFIIH when RNA polymerase II is present in the preinitiation complex (Lu *et al*. 1992). Previously it was observed that phosphorylation brings a conformational change in the CTD which is essential for transcription initiation (Cadena and Dahmus, 1987; Zhang and Corden, 1991). But recent findings from Serizawa *et al*. (1993) indicate that phosphorylation and basal levels of transcription are not coupled. They point out that probably all the essential GTFs and some unidentified factors may be important for CTD-phosphorylation related transcription initiation.

Studies using a reconstituted transcription system showed functional evidence that CTD interacts with high molecular

form TFIID for the formation of preinitiation complex (Conway *et al.* 1992). The amino terminal domain of RNA polymerase II having zinc finger motifs was thought to be involved in DNA binding. But the model proposed by Suzuki (1990) suggests that CTD forms β-turns and binds by intercalating with DNA.

1.1.4 Transcriptional activators and repressors modulate the levels of transcription:

Various DNA binding proteins were identified in eukaryotic cells and found to bind specific sequence motifs of the cis-regulatory elements thereby modulating the levels of transcription. DNA binding proteins can be grouped into four families based upon their DNA-binding domains namely zinc fingers, leucine zippers, homeo domains and basic amino acid-rich CCAAT box binding domains (Pabo and Sauer, 1992; Mitchell and Tjian,1989). For example, the steroid, thyroid and retinoic acid receptors, GAL4 and SP1 have zinc finger domains whereas the transcription factors AP1 and C/EBP have leucine zippers. CTF/NF1 binds to the CCAAT motifs. Factors like Oct-1 and Oct-2 have homeodomains. Some proteins such as muscle cell-specific MyoD, upstream stimulatory factor (USF) and C-Myc have helix-loop-helix(HLH) motifs. These proteins bind DNA by either dimerization or heteromerization. There are number of other DNA binding proteins such as SV40 large T-antigen, transcription enhancer factor (TEF), papillomavirus E2

protein, TFIID and human p53 tumour suppressor gene product which do not come under any particular family (Mitchell and Tjian, 1989; Herr, 1991; Harrison, 1991; Cole, 1991).

Transcription factors activate transcription by possessing different transactivation domains such as glutamine-rich (SP1), proline-rich (CTF/NF1) and acidic amino acid-rich (VP16) domains (Sigler, 1988; Mitchell and Tjian, 1989; Ptashne and Gann, 1990).

Some of the transcription factors such as AP1 can also act as repressors by directly competing for the same sequence element recognised by glucocorticoid and retinoic acid receptors. Repression can also be due to mutual inhibition of DNA binding of AP1 and glucocorticoid receptors through protein-protein interactions (Schule *et al.* 1990a; Schule *et al.* 1990b; Yen *et al.* 1990). Active transcriptional repressors like human kruppel-related factor YY1 directly downregulates transcription (Shi *et al.* 1991). A novel repressor of 19 kd known as Dr1 is phosphorylated and interacts with TBP thereby inhibiting the association of TFIIA or TFIIB with TBP, leading to the repression of both basal and activated levels of transcription (Inostroza *et al.* 1992).

Currently, research on transcription factors is focussed on how cell specificity is contributed by different tissue-specific transcription factors and their coactivators. For example, previously it was thought that B-cell specific Oct-2

was important for transcriptional activation from immunoglobulin promoters. Recent studies showed that a tissue-specific, promoter-specific and Oct-1 and Oct-2 specific coactivator, OCA-B, might be the major determinant for B-cell specific transcription of immunoglobulin promoters. But the questions that arise are whether Oct-2 is physiologically important for the activation of transcription by OCA-B and whether there are any additional sequence requirements that determine the B-cell specific function of Oct factors and their coactivators (Luo *et al.* 1992).

Three principal models have been proposed for the positive regulation of transcription factors. The DNA-looping model is most favoured. This model is gaining importance since it was demonstrated that the factor, SP1, activates transcription through protein-protein interactions when the intervening DNA loops out (Su *et al.* 1991).

1.1.4.1 CTF/NF1 family of transcription factors:

The sequences (G)CCAA(T) and TGG (A/C)N_5 GCCAA are important regulatory elements found in the regulatory region of a number of RNA polymerase II-regulated cellular and viral genes. The factors that bind these sequences and regulate transcription are known as CCAAT-binding transcription factors or the nuclear factor 1 (CTF/NF1) family of proteins

(Rosenfeld and Kelly, 1986; Jones *et al.* 1988; Mitchell and Tjian, 1989).

Several distinct proteins exist as CTF/NF1 in eukaryotic cells. These factors can be encoded by the same gene by differential RNA splicing giving rise to proteins of 52-66 kd as observed in human HeLa cells (Santoro *et al.* 1988) Different genes encode CTF/NF1 proteins of 29-33 kd in chicken liver tissue (Rupp *et al.* 1990). Sequence alignments of different CTF/NF1 proteins revealed certain conserved domains despite the differences observed in the amino-terminal and carboxy-terminal domains (Mermod *et al.* 1989; Rupp *et al.* 1990). At least 6×10^4 NF1 binding sites were estimated to exist in the human genome (Gronostajski *et al.* 1984).

Jones *et al.* (1987) purified NF1 to homogeneity and observed that NF1 activates adenovirus gene transcription and stimulates adenovirus DNA replication *in vitro*. Later on it was observed that the domains responsible for transcriptional activation and replication are different. The positively charged N-terminal domain was found to be responsible for DNA binding and replication whereas the C-terminal proline-rich activation domain was necessary for transcriptional activation. The DNA binding domain has the characteristic features necessary for dimerization (Mermod *et al.* 1989).

The HeLa CCAAT-binding protein CP1 has two heterologous

subunits which can functionally complement the yeast transcription factors HAP2/HAP3 complex and they recognise the same DNA binding sequences. The proteins CP1, CP2 and NF1 are different factors. Each protein binds to its recognition sequence with affinity that is three orders of magnitude higher than the binding of the others. This indicates that different CTF/NF1 proteins have different DNA binding specificities probably because of heteromerization (Chodosh *et al.* 1988a; Chodosh *et al.* 1988b; Mitchell and Tjian, 1989).

CTF/NF1 recognition sequences are the potential binding sites for other proteins like CCAAT/enhancer binding protein (C/EBP). The binding of C/EBP to the CTF/NF1 motifs was found to be involved in transcriptional activation (Jones *et al.* 1988). McQuillan *et al.* (1991) showed that cells which do not express NF1 proteins can still stimulate transcription from NF1 binding sites because of the existence of functionally distinct NF1 related proteins in different cells.

Nuclear factor 1 was shown to be important for cell specific enhancer function of adipocytes (Graves *et al.* 1991). The glial cell-specific expression of the neurotropic JC virus regulatory region was shown to be dependent on the NF1 binding sites (Kumar *et al.* 1993).

The proteins that bind the CCAAT motif need not be transactivators since some of them may act as negative regulators. An example of this is for the sperm histone H2B-1

gene promoter of sea urchin embryos (Jones et al. 1988).

1.1.5 Role of coactivators and different TFIID complexes for transcriptional activation:

Studies using the TFIID fraction confirmed that additional factors known as coactivators are necessary to bridge the transactivators SP1 or NF1 with the general transcriptional machinery transcriptional activation (Pugh and Tjian, 1990; Dynlacht *et al.* 1991; Tanese *et al.* 1991). Similarly, coactivators in yeast were identified and termed as mediators or adaptors (Kelleher *et al.* 1990; Flanagnan *et al.* 1991). The coactivators for NF1 were purified from TFIID complex and found to mediate activation by the chimeric glutamine-rich activator, SP1 (Pugh and Tjian, 1990; Dynlacht *et al.* 1991). The copurification of TBP and coactivators suggested that part of the coactivator function is contributed by different TAFs associated with TBP (Pugh and Tjian, 1990; Dynlacht *et al.*1991). Coactivator activity, for example, upstream stimulatory activity (USA), is necessary for transactivation by SP1 and USF and this USA is not tightly associated with TBP (Meisterernst *et al.* 1991).

The human TAF_{II} 250 of TFIID was cloned and found to be identical to the cell cycle regulatory *ccg1* gene. This suggests that TAF_{II} 250 may be involved in the activation of

genes that control cell cycle progression (Ruppert *et al.* 1993; Hisatake *et al.* 1993).

Different TAFs act as mediaters between TBP and transactivators forming a single TFIID complex called holo-TFIID complex (Zhou *et al.* 1992; Zhou *et al.* 1993). But Brou *et al.* (1993) reported that there are at least two distinct TFIID populations composed of both common and unique TAFs. It was shown that chimeric GAL4-TEF1 activates transcription from two different TFIID populations. One is unique to GAL4-TEF1 activation and the other one is common for transcriptional stimulation by three different activators including GAL4-TEF1. The existence of different TFIID complexes for different transactivators needs to be further investigated.

1.2 PAPILLOMAVIRUSES:

Papillomaviruses are small, nonenveloped, circular, double stranded DNA viruses of about 8kb in size. They belong to the subfamily papillomavirinae of Papovaviridae. Initial studies on cottontail rabbit papillomavirus (CRPV) and a rare human disease, epidermodysplasia verruciformis, indicated that papillomaviruses might be important for the development of epithelial neoplasias. Subsequently a large body of information accumulated about the role of papillomaviruses in causing benign and malignant cancers. Bovine papillomavirus 1 (BPV1) is the most extensively studied virus and it served as

prototype virus for understanding the molecular studies of papillomaviruses (Giri and Danos, 1986).

Papillomaviruses are highly species specific and induce squamous epithelial tumours and fibroepithelial tumours in their natural hosts. It has not been possible to propagate these viruses in tissue culture and the mode of viral replication is not understood precisely (Howley, 1990).

Papillomaviruses are strictly epitheliotropic in nature and infect the basal layers of the epithelium. The viral life cycle is complete only in the upper layers of differentiating keratinocytes. All viral replication functions are restricted only to terminally differentiated cells (Howley, 1990). The genomic organization of BPV1 was first studied because the viral DNA can be relatively easily obtained from animal lesions. Subsequently, over 68 types of human papillomaviruses were isolated (Payne *et al.* 1993) from different human lesions and were characterised as infectious agents of cutaneous, oral and anogenital lesions (de Villiers, 1989).

Human papillomaviruses (HPVs) show strict epitheliotropism like other papillomaviruses. They are involved in the development of benign adult and juvenile laryngeal papillomas and genital condylomata acuminata or genital warts. The benign lesions caused by the low risk HPVs (example: HPV11 and HPV6) rarely progress to malignancy. The high risk viruses for example HPV16 and HPV18 are known to be

associated with the malignant genital cancers especially cervical carcinoma. Different human papillomaviruses sporadically or regularly infect the genital tract, hence sometimes one or more HPVs can be observed in the lesions (zur Hausen, 1989).

1.2.1 Genomic organization of papillomaviruses:

DNA sequence comparison of different human papillomaviruses led to the establishment of at least eight early genes (E1-E8) and two late genes (L1 and L2), although minor variations do occur among different family members. All the different early and late genes are located only on one strand giving rise to early and late translational open reading frames (ORFs). Overall, the genomic organization is conserved with respect to the size and location of open reading frames among different papillomaviruses. The open reading frames E3 and E8 are not established in HPVs as seen in BPV1. Transcription of papillomaviruses is complex because of the presence of multiple promoters, different splice sites and differential synthesis of mRNA species in different cells. Additional open reading frames are also present in HPVs such as E5a and E5b in HPV11 (Howley, 1990; Giri and Danos, 1986; Chiang *et al.* 1991).

Papillomaviruses have an untranslated regulatory region consisting of enhancer and promoter elements between the stop

codon of L1 ORF and first codon of E6 ORF. The regulatory region is known as noncoding region (NCR) or upstream regulatory region (URR) or long control region (LCR). The size of the regulatory region varies among different HPVs. The enhancer and promoter elements of the NCR respond to different cellular and virally encoded proteins. The 3′ extremity of NCR is GT-rich in genital papillomaviruses whereas it is AT-rich in other viruses. This 3′ end has a polyadenylation signal for the late transcripts. Episomal replication and maintenance sequences are also located at the same position. The 5′ end of the NCR is conserved and contains repeats of the palindromic sequence, $ACCGN_4CGGT$, a characteristic of papillomaviruses and typical promoter elements (Giri and Danos, 1986). Viral E2 proteins interact with this sequence (Howley, 1990).

1.2.2 Role of viral and cellular factors in the oncogenicity of human papillomaviruses:

The malignant cancers associated with HPVs have integrated viral DNA and the majority of such tumours possess viral transcripts of E6 and E7 ORFs. The E6 and E7 proteins are considered as viral oncoproteins particularly in the case of high risk HPVs and they are found to interact with cellular factors such as retinoblastoma protein (pRb) and p53. The E6 protein binds and degrades the tumour suppressor gene product,

p53, whereas the E7 protein interacts with pRb. The precise regulation of cellular DNA replication and mitotic cycle by E6-p53 and E7-pRb interactions is not clearly defined. They appear to play major roles because such interactions are not essential for benign proliferative lesions caused by low risk HPVs like HPV6 and HPV11 (zur Hausen, 1991). These types of viral and cellular interactions might play a role in the instability and chromosomal changes leading to the progression from the latency state to malignancy induced by high risk HPVs. In cases of malignancy by low risk HPVs, mutational events set by exogenous factors such as smoking might play a pivotal role (zur Hausen, 1991).

The importance of chromosome 11 which harbours neither p53 nor pRb genes was notably observed to play an important role in the regulation of HPV transcription (zur Hausen, 1991). Deletion of chromosome 11 was found to suppress HPV transcription in nontumorigenic cervical carcinoma-fibroblast hybrid segregants whereas loss of chromosome 11 did not affect HPV transcription in tumorigenic segregants (zur Hausen, 1991). The SV40 small t-like factor i.e., PR55β, the regulatory subunit of protein phosphatase 2A (PP2A), was found to transactivate HPV gene transcription in the chromosome 11 deleted fibroblasts (Smits *et al.* 1992).

The observations made by Swift *et al.* (1987) suggested that a neuroblastoma cell line supported enhancer function of

HPV18. The HPV16 DNA has been observed in the plasma cell tumour of cervix. This raised an interesting question whether HPVs can also infect cells of lymphoid origin. It is not known whether the plasma cell malignancy observed in the above case was a sequel to the primary infection of epithelial cells by HPV16 (Payne *et al.* 1993). Recently, Arbeit *et al.* (1993) observed the expression of E6 mRNA and E7 oncoprotein in neuroepithelial tumours of transgenic mice. These different observations suggest that ubiquitous and cell-specific host factors might be responsible for the transcription of HPV genes. The identification of tissue-specific enhancers and their cognate transcription factors has shed some light on the importance of epithelial cell-specific and ubiquitous transcription factors in the transcriptional regulation of HPV viral gene expression in epithelial tissues.

1.2.3 Noncoding regions of human papillomaviruses 16 and 18:

1.2.3.1 Transcriptional regulation by HPV16 enhancer elements:

The noncoding region (NCR) of HPV16 is located between the 3′ end of L1 ORF and 5′ end of E6 ORF (nucleotide 7150-82). The NCR controls the viral gene expression from p97 promoter (Seedorf *et al.* 1985; Gloss *et al.* 1987). The NCR of HPV16 contains inducible and cell-specific enhancer elements (Fig.1). Three viral E2-binding motifs acting as inducible

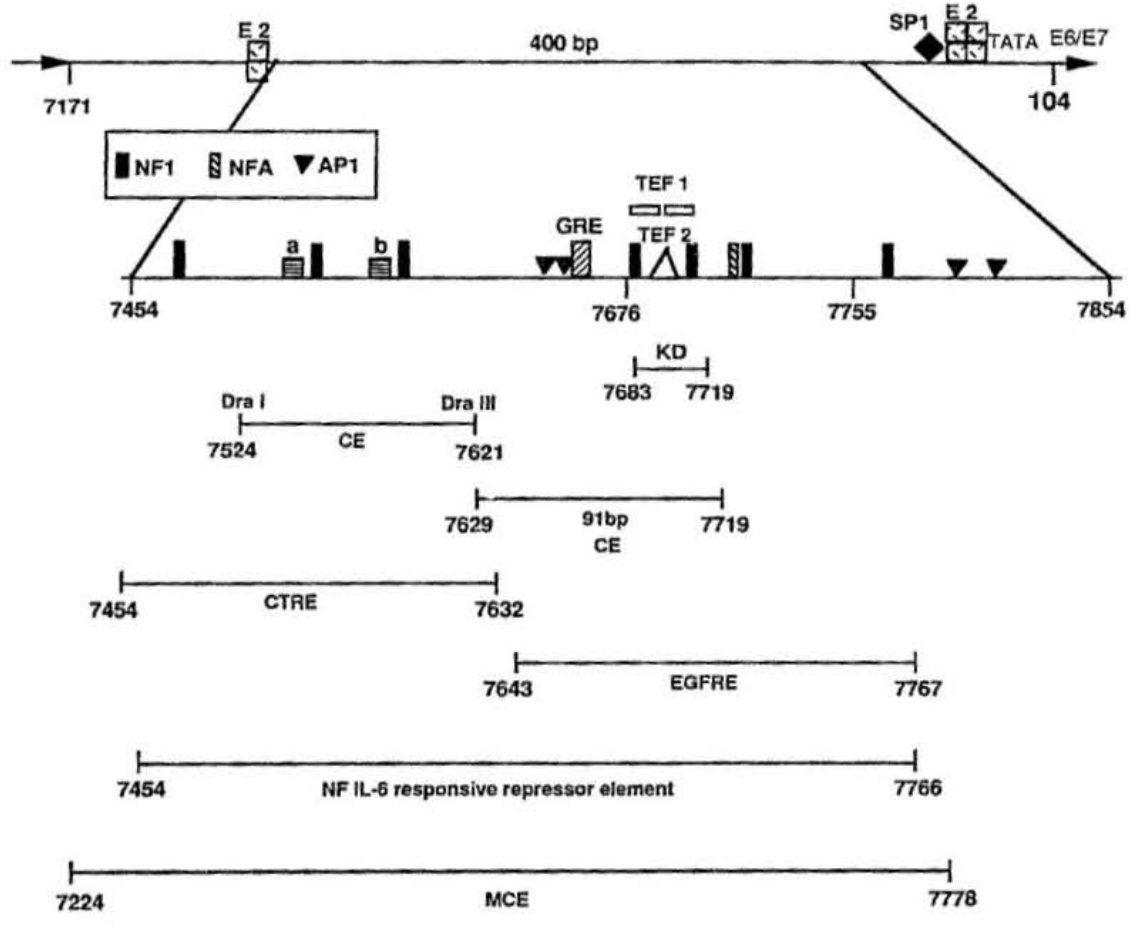
enhancers were identified upstream of the p97 promoter which responds to the transactivation and repression by the viral E2 proteins (Sousa *et al.* 1990; Phelps and Howley, 1987; Cripe *et al.* 1987). An epithelial cell-specific enhancer of 400 base pairs (nucleotide 7454-7854) was identified in HeLa (HPV18 integrated cell line) cells. This enhancer is located upstream of the two promoter-proximal E2-binding motifs. Hence this enhancer is also termed to be an E2-independent enhancer (Chong *et al.* 1991). The cell-specific enhancer also consists of a glucocorticoid or progesterone responsive element (GRE or PRE) (nucleotide 7640-7654). The GRE was found to be essential for glucocorticoid-dependent oncogenic transformation of baby rat kidney epithelial cells (Pater *et al.* 1988; Chan *et al.* 1989).

The epithelial cell-specific enhancer of 400 base pairs has recognition sequences for transcription factors such as NF1, TEF1, TEF2, Oct-1, AP1 and unidentified factor, for example, nuclear factor 1-associated factor (NFA) (Chong *et al.* 1990; Chong *et al.* 1991; Ishiji *et al.* 1992; Taniguchi *et al.* 1993). The complex array of overlapping recognition sequences for transcription factors include 7 NF1 sites, 3 AP1 sites, a TEF2 binding site overlapping with two TEF1 sites. One of the NF1 sites partially overlaps with one of the TEF1 binding sites. A 37 base pair fragment (nucleotide 7683-7719) of the 400 base pair enhancer region was observed to be active

Fig.1. Schematic representation of different enhancer elements of HPV16 noncoding region and the interacting transcription factors.

CE: This term is used for the purpose of this illustration. This represents the epithelial cell-specific enhancer elements (nucleotide 7524-7621 and 7629-7719) (Chong *et al.* 1991). KD: This term is used for the purpose of this illustration. This represents TEF1 and its coactivator-mediated keratinocyte dependent enhancer (Ishiji *et al.* 1992). CTRE: Cell-type dependent regulatory element identified with HPV-associated epithelial cell lines (Taniguchi *et al.* 1993). EGFRE: Epidermal growth factor responsive element (Yasumoto *et al.* 1991; Taniguchi *et al.* 1993) MCE: Minimal constitutive enhancer (Marshall *et al.* 1989) used in this study.

E2: Viral E2 protein; NF IL-6: Nuclear factor for Interleukin-6; GRE: Glucocorticoid responsive element; NFA: unidentified factor called nuclear factor 1-associated factor; a and b : unknown factor interactions.


E 2
400 bp
SP1
E 2
TATA
E6/E7
7171
104
NF1
NFA
AP1
TEF 1
GRE
TEF 2
a
b
7454
7676
7755
7854
KD
7683
7719
Dra I
Dra III
7524
CE
7621
7629
91bp
CE
7719
7454
CTRE
7632
7643
EGFRE
7767
7454
NF IL-6 responsive repressor element
7766
7224
MCE
7778

and found to interact with two TEF1 proteins that require a limiting coactivator in keratinocytes (Ishiji *et al.* 1992). The two fragments with nucleotides from 7524-7621 and 7629-7719 (91bp) having potential binding sites for NF1 were also found to be active in epithelial cells but not in fibroblasts. The NF1 species that are responsible for the expression from these fragments act in an epithelial cell-specific fashion (Apt *et al.* 1993).

Another inducible enhancer known as epidermal growth factor responsive element (EGFRE) was tentatively mapped between 7643 and 7767 base pairs. This element was observed to downregulate the expression of E6/E7 mRNA (Yasumoto *et al.* 1991; Taniguchi *et al.* 1993). The enhancer element from 7454-7632 base pairs was identified as cell-type dependent regulatory element (CTRE) in HPV-associated epithelial cell lines but not in HPV-free epithelial cell lines. The CTRE has three NF1 binding sites. Mutational analysis indicates that all three NF1 sites synergistically activate transcription for complete activity of the CTRE (Taniguchi *et al.* 1993). The nuclear factor responsible for interleukin expression (NF-IL6) represses HPV16 NCR by binding to the sequences from 7454-7766 base pairs. This suggests the role of NF-IL6 as the host's defence mechanism to counteract the viral infection (Kyo *et al.* 1993).

Previously Marshall *et al.* (1989) identified the

sequences from nucleotide 7224-7778 in the NCR of HPV16 as the minimal constitutive enhancer required for cell-specific expression. This was used in the preliminary experiments of this study. It was proposed that the epithelial cell specificity is brought about by the cooperative interaction of different ubiquitous and cell-specific transcription factors binding to their cognate binding sites (Chong *et al.* 1991). The NF1 binding sites and a subset of NF1 proteins were shown to play a major role in the epithelial cell-specific expression of HPV16 and probably other genital papillomaviral genes (Apt *et al.* 1993).

1.2.3.2 Transactivation and repression by enhancer elements of HPV18:

The noncoding region of HPV18 is 828 base pairs in length (nucleotide 7134-105) extending between the stop codon of L1 ORF and the first codon of E6 ORF. The NCR of HPV18 has a typical TATA box and transcription starts from p105 promoter. The NCR has a CCAAT box-like sequence (CAAT) upstream of the TATA box as seen in HPV 11 and HPV16 (Cole and Danos, 1987; Thierry *et al.* 1987; Bernard *et al.* 1989). Based upon the response to virally encoded proteins, other stimuli and cellular factors, the enhancer elements can be classified into inducible, constitutive and cell-specific enhancer elements (Fig.2). Four copies of E2-responsive sequences are present in

the NCR of HPV18. Two of these sites are situated between TATA and CCAAT boxes and repress the promoter activity in keratinocytes in the presence of BPV1 E2 or HPV18 E2 proteins. These sites activate transcription of heterologous promoters by the E2 proteins when present distant from the promoter (Bernard *et al.* 1989).

The second inducible enhancer is viral E6-dependent enhancer (IE6) located 500 base pairs upstream of the E6 cap site (Gius *et al.* 1988). A 30 base pair region designated as IETPA is responsible for phorbol ester activation of HPV18 gene expression from a heterologous promoter (Gius and Laimins, 1989). Another inducible enhancer i.e., glucocorticoid or progesterone responsive element (GRE/PRE) (nucleotide 7839-7853) responds to glucocorticoids or progesterone and mediates transcriptional activation (Chan *et al.* 1989). The minimal constitutive enhancer (MCE) is a 230 base pair fragment (nucleotide 7508-7738) and is active only in certain cervical carcinoma cell lines, a neuroblastoma cell line and some fibroblast cell lines (Swift *et al.* 1987; Gius *et al.* 1988; Marshall *et al.* 1989).

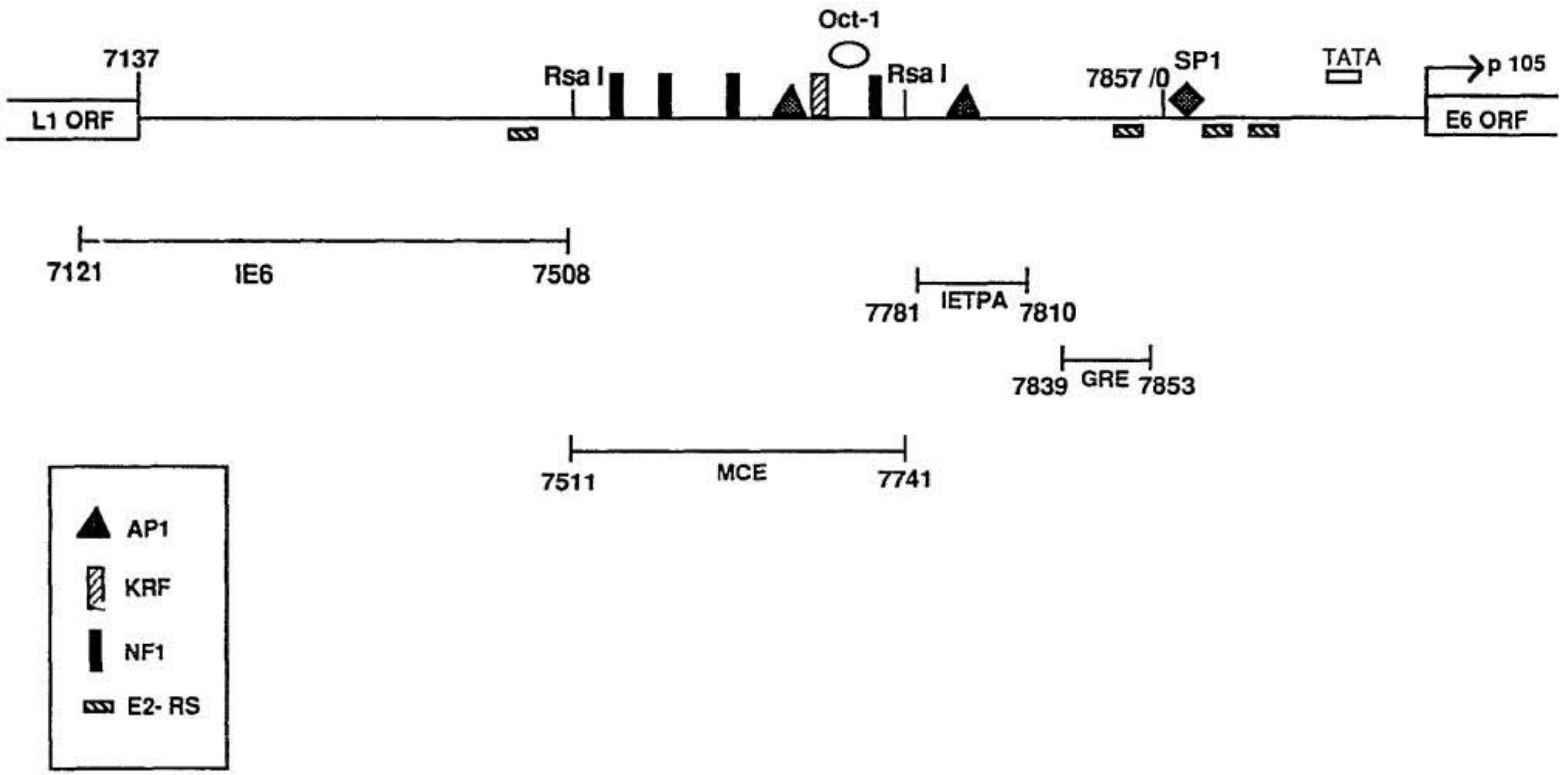
The transcription factor Oct-1 is known to repress transcription from a 138 base pair fragment of NCR. The Oct-1 does not repress heterologous promoters, but represses HPV18 enhancer. Its action is through protein-protein contacts (Hoppe-Seyler *et al.* 1991). A novel octamer binding protein of

Fig.2. Diagramatic representation of constitutive and inducible elements of HPV18 and DNA-protein interactions. IE6: Inducible enhancer for viral E6 protein (Gius *et al.* 1988). IETPA: Inducible enhancer for tetra decanoylphorbol acetate (Gius and Laimins, 1989).

MCE: Minimal constitutive enhancer (Marshall *et al.* 1989). GRE: Glucocorticoid responsive element; E2-RS: Viral E2 responsive sequences; KRF: Keratinocyte-specific transcription factor; Oct-1 is shown as lack of DNA binding (Hoppe-Seyler *et al.* 1991).

RsaI: restriction site for RsaI.

Arrow indicates transcription start site.


Oct-1
7137
Rsa I
Rsa I
7857 /0
SP1
TATA
p 105
L1 ORF
E6 ORF
7121
IE6
7508
7781
IETPA
7810
7839
GRE
7853
7511
MCE
7741
AP1
KRF
NF1
E2- RS

92 kd was identified and shown to activate HPV18 transcription in cell-cycle dependent manner (Royer *et al.* 1991).

A negative regulatory domain of 23 base pairs in length was identified upstream to CCAAT-box like sequence. It is known to bind YY1 repressor and found to repress the HPV18 p105 promoter activity in HeLa cells. This suggests that the distal AP1 binding site cannot activate p105 promoter due to the binding of the repressor to the negative regulatory element. This repression mechanism was suggested to be important in order to downregulate the activity of HPV18 NCR in premalignant latent stage (Bauknecht *et al.* 1992).

Mack and Laimins (1991) identified a keratinocyte specific transcriptional activator-1 (KRF-1). It was found to be necessary for the synergistic activation with AP1 from the constitutive enhancer. The two AP1 binding sites were shown to be important for JunB dependent activation of the p105 promoter in epithelial cell lines. However, it was suggested that other transcription factors may cooperate with AP1 in the tissue-specific expresssion of HPV18 (Thierry *et al.* 1992). The role of NF1 binding sites in the expression of HPV18 enhancer is not well established, although putative motifs for NF1 are present.

1.2.4 Human papillomavirus 11 (HPV11):

The DNA of human papillomavirus 11 (HPV11) was identified in the benign laryngeal papilloma and was subsequently characterised (Gissmann *et al.* 1982; Dartmann *et al.* 1986). HPV11 is mostly found in episomal form and is rarely associated with malignant lesions. It causes benign lesions of the respiratory tract including especially, the larynx. The benign lesions caused by HPV11 in external genitalia are called condylomata acuminata. HPV11 is also associated with preneoplastic cervical lesions and cervical carcinomas (Gissmann *et al.* 1983; Stoler *et al.* 1989; Sherman *et al.* 1992). HPV11 was found in papillomas of both juveniles and adults and in rare conditions it was found to be associated with squamous cell carcinomas (SCC) of lung and skin (Gissmann *et al.* 1983; Byrne *et al.* 1987; Cohen *et al.* 1992; Shah and Howley, 1990; Tsutsumi *et al.* 1989).

HPV11, unlike the other benign virus HPV6, is frequently present in respiratory papillomas especially in infants. The transmission of virus from mothers to infants is postulated to occur through birth canal. Juvenile-onset patients with laryngeal papillomas might acquire the virus *in utero* probably because of the infected birth canal but this mode of transmission is not frequent (Shah and Howley, 1990).

HPV11 like other papillomaviruses is strictly epitheliotropic, but the sites of infection and the degree of

oncogenicity by HPV11 vary among the oncogenic types such as HPV16 and HPV18 (Howley, 1990). Stoler *et al.* (1990) were able to induce experimental condylomata by HPV11 in nude mice. The lesions were indistinguishable from naturally occuring condylomata acuminata. Recently, *in vivo* and *in vitro* assembly of recombinant virus-like particles (VLPs) expressing L1 coat protein but not L2 was demonstrated using baculovirus system (Rose *et al.* 1993). Still, there is no feasible system to propagate this virus in large quantities.

1.2.4.1 Role of genomic alterations in the oncogenic potential of HPV11:

In a rare case of chronic laryngotracheobronchial papillomatosis and metastatic squamous cell carcinoma of lung, the noncoding region (NCR) of HPV11 was found duplicated and believed to act as mutagen to bring about chromosomal instability (Byrne *et al.* 1987).

There are significant variations in the E7 proteins of HPV11 and HPV16. In-frame insertion of 124 base pairs from the 5′ amino end of the coding sequence of HPV16 E7 ORF into the 5′ end of the E7 coding sequence of HPV11 rendered HPV11 genome transformation competent in the presence of ras oncogene (Pater *et al.* 1992). A unique variant of HPV11 (HPV11vc) isolated from verrucous carcinoma of male genital organs was found to transform neonatal rat kidney epithelial

cells in the presence of Ha-ras oncogene with comparable efficiency to that of HPV16 DNA. This HPV11vc isolate was shown to transform fibroblasts even in the absence of ras oncogene. This variant of HPV11 was found to contain 16 nucleotide changes in the noncoding region and E1, E2, E4 and E5 ORFs. This indicates that *in vivo* mutations of the viral genome might alter the oncogenic potential of HPV11 (McGlennen *et al.* 1992).

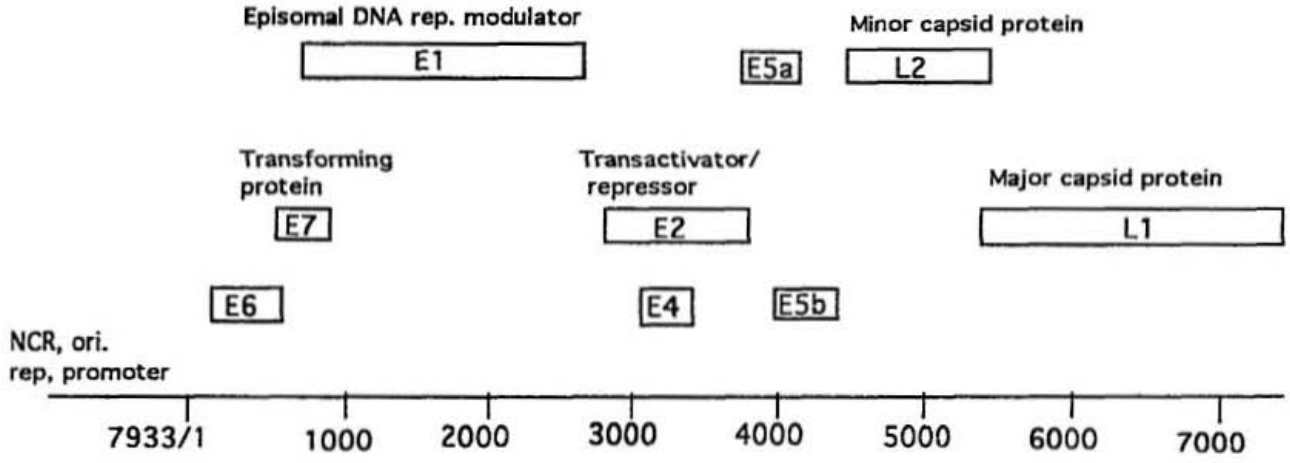
1.2.4.2 Transcriptional regulation by enhancer elements of HPV11:

The genome of HPV11 isolated from laryngeal papilloma consists of 7931 base pairs encoding E1, E2, E4, E5a, E5b, E6 and E7 early proteins and L1 and L2 late proteins. The region between L1 ORF and E6 ORF is the noncoding region consisting of different enhancer elements that regulate the viral gene expression (Dartmann *et al.* 1986). The different open reading frames and their possible functions are shown in figure 3.

The noncoding region of HPV11 starts within the L1 ORF and extends up to the first codon of E6 ORF. The NCR has a typical TATA box located at the nucleotide position 66. The CCAAT box has the sequence CAATCT similar to the CCAAT box-like sequence of HPV16 and HPV18 (Dartmann *et al.* 1986). The NCR has three constitutive enhancer elements namely CEI, CEII and CEIII. The inducible elements are E2-responsive sequences

Fig.3. Linearized map of HPV11.

Coding regions of different open reading frames (ORFs) are indicated as open boxes. The possible function of the different ORFs is also indicated.


Episomal DNA rep. modulator
E1
E5a
Minor capsid protein
L2
Transforming protein
E7
Transactivator/ repressor
E2
Major capsid protein
L1
E6
E4
E5b
NCR, ori. rep, promoter
7933/1
1000
2000
3000
4000
5000
6000
7000

(E2-RS) and the glucocorticoid responsive element (GRE) (Fig.4). The term constitutive enhancer was used simply based upon the differential expression of the elements in different cell types. The enhancer element, CEIII (nucleotide 7224-7493) is active in different epithelial and fibroblast cells (Steinberg *et al.* 1989; Auborn *et al.* 1989; Auborn and Steinberg, 1991).

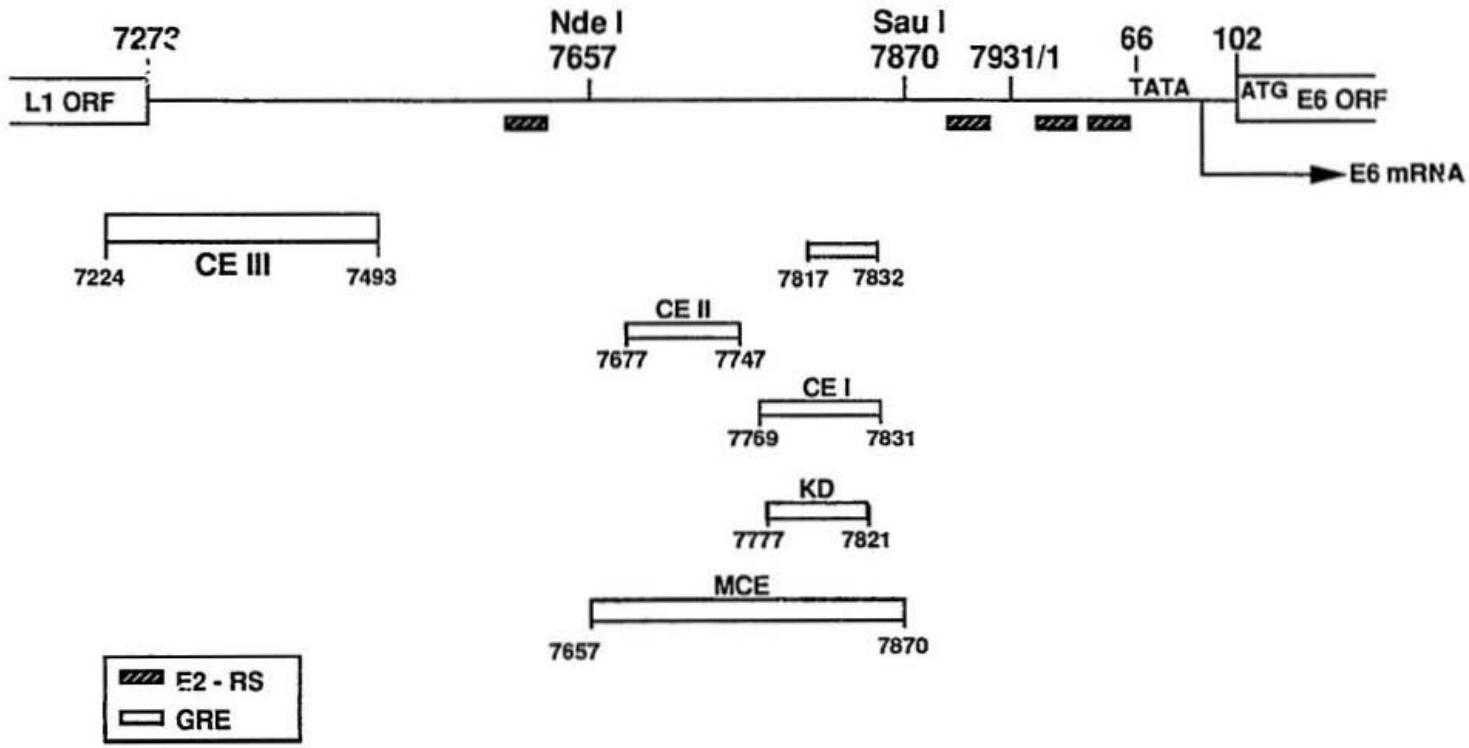
The constitutive enhancer element CEII (nucleotide 7677-7747) was found to be active in the cervical carcinoma cell lines C33A (HPV free) and HeLa (HPV18 integrated) and is relatively inactive in the monkey kidney epithelial cell line, CV-1 (Chin *et al.* 1989). The constitutive enhancer CEI (nucleotide 7769-7831) was shown to be active in CV-1 and primary keratinocytes but not in HeLa or C33A (Hirochika *et al.* 1988; Chin *et al.* 1988; Dollard *et al.* 1993).

Recently, Dollard *et al.* (1993) identified a 45 base pair region (nucleotide 7777-7831) which is active in a keratinocyte-dependent manner. The minimal constitutive enhancer (MCE), consisting of 213 base pairs (nucleotide 7657-7870), includes CEI and CEII. The MCE used in this study is required for epithelial cell-specific function (Marshall *et al.* 1989). The sequences of MCE are devoid of E2-responsive sequences. The minimal constitutive enhancer has recognition sequences for transcription factors CTF/NF1, AP1, C/EBP, NFA and homologous GT, Sph and P motifs (Hirochika *et al.* 1988;

Fig.4. Constitutive, inducible and cell-specific enhancer elements of HPV11.

CEI, CEII and CEIII: Constitutive enhancer elements as described previously (Chin *et al.* 1989; Hirochika *et al.* 1988; Dollard *et al.* 1993; Auborn *et al.* 1989; Steinberg *et al.* 1989). KD: The term is used for the purpose of this illustration and represents the keratinocyte-specific enhancer (Dollard *et al.*1993). MCE: Minimal constitutive enhancer (Marshall *et al.*1989) for epithelial cell-specific expression used in this study. GRE: Glucocorticoid responsive element; E2-RS: E2-responsive sequences.

The different nucleotide positions are not to scale. Arrow indicates the transcription start site E6 promoter.


7272
Nde I
7657
Sau I
7870
7931/1
66
TATA
102
ATG
L1 ORF
E6 ORF
E6 mRNA
CE III
7224
7493
7817
7832
CE II
7677
7747
CE I
7769
7831
KD
7777
7821
MCE
7657
7870
E2 - RS
GRE

Dollard *et al.* 1993).

The enhancer CEII was shown to bind CEII-binding proteins which are transcriptionally inactive in primary keratinocytes and active in C33A (Dollard *et al.* 1993; Chin *et al.* 1989). The CEII has recognition sequences for NF1 and putative binding motifs for AP1. The enhancer, CEI has several overlapping binding motifs for transcription factors such as C/EBP, NF1 and NF1-associated factor (NFA) (Dollard *et al.* 1993). The nature of proteins that bind to these sequences of CEI and CEII is still unclear.

The inducible element GRE is located (nucleotide 7817-7832) within the minimal constitutive enhancer and responds to both dexamethasone and progesterone (Pater *et al.* 1988; Chan *et al.* 1989).

The E2 proteins of HPV11 act as transcriptional regulatory factors and modulate viral gene expression from the E6 promoter by binding to the inducible elements i.e., E2-responsive sequences (E2-RS). The full length E2 protein has an amino terminal transactivation domain and carboxy terminal DNA-binding domain. Two other E2 viral proteins, E2-C and E1M^E2C are known to act as transcriptional repressors (Chin *et al.* 1988; Chiang *et al.* 1991). The full length E2 protein activates HPV11 E6 promoter in C33A (Chin *et al.* 1988).

The full length E2 protein can also repress the E6 promoter function when either CEI or CEII is deleted from the

NCR. But multimerization of either CEI or CEII in tandem overcomes the repression caused by the E2 protein in C33A cells(Chin *et al.* 1989). The E2 protein is a strong repressor of CEII and CEI in primary keratinocytes. But the cellular factors binding to CEII are functionally inactive in primary keratinocytes. Hence it has been proposed that E2 repression is abrogated when CEII proteins or their coactivators may be activated or available during the process of differentiation or neoplastic conversion (Dollard *et al.* 1993).

The full length E2 protein was found to be responsible for positive regulation of viral replication. The repressing E2 proteins did not support replication *in vitro* (Chiang *et al.* 1992).

1.3 Role of retinoids:

Retinoids are physiological regulators of growth and differentiation of a number of epithelial tissues. The retinoids such as retinol (vitamin A) and its metabolic derivative retinoic acid are important for normal embryogenesis (Glass and Rosenfeld, 1991). Retinoic acid not only acts as a morphogen but is essential to maintain the normal integrity of the epithelial tissues (de Luca, 1991). The *in vivo* experiments using vitamin A deficient mice indicated that the extensive keratinization may be a consequence of lack of physiological concentrations of

retinoids in the epithelial tissues. This confirms the role of retinoids in keratin gene expression (de Luca, 1991). Some of the studies indicated that at pharmacological doses, the retinoids inhibit tumorigenesis (de Luca, 1991). The retinoids exert their actions through different types of nuclear receptors namely retinoic acid receptors (RAR)- RARα, RARβ, RARγ and retinoid x receptors (RXR) (de Luca, 1991; Aneskievich and Fuchs, 1992).

Various nuclear receptors for retinoic acid control the expression of different cellular genes by binding to the retinoic acid responsive element (RARE) or RXR element (RXRE) located in the cellular genes. Recent studies have shown that nuclear receptor translocations gave rise to the concept that retinoids and their receptors might be involved in neoplasia (de Luca, 1991).

1.3.1 P19 mouse embryonal carcinoma cells:

The cells of P19 mouse embryonal carcinoma cell line are pluripotent in nature and can differentiate in cell culture into neuronal and muscle cell types when they are treated with retinoic acid (RA) and dimethylsulfoxide (DMSO), respectively (Rudnicki and McBurney, 1987). Different isoforms for the nuclear receptors-RARα, RARβ and RARγ were identified in P19 EC cells. The different isoforms can be seen in different levels either in RA-differentiated cells or uninduced P19

cells (Leroy *et al.* 1991; Zelent *et al.* 1991; Kastner *et al.* 1990).

The members of the gene family, AP1, are expressed differentially in P19 EC cells. The *c-jun* product is expressed at high levels in RA-differentiated cells whereas *junB* is expressed neither in undifferentiated nor in RA-differentiated cells. *JunD* expression remains the same irrespective of differentiation status (de Groot *et al.* 1990a). Ectopic expression of *c-jun* in the undifferentiated cells leads to differentiation and resembles the RA-differentiated derivatives both morphologically and biochemically. It was suggested that the effects of the *c-jun* product on RARβ gene transcription might be responsible for *c-jun* dependent-differentiation (de Groot *et al.* 1990b).

Two other transcription factors namely Oct-3 and Oct-4 are found at different levels in undifferentiated (UD) and RA-differentiated states of P19 cells. Oct-3 is expressed only in UD cells and its expression disappears in RA-differentiated cells. In contrast, Oct-4 was found to be induced by RA differentiation. Two cis-elements were identified in the transcriptional control region of *oct-3* gene namely RARE 1A and RARE 1B which showed no binding of nuclear receptors. Each of the two cis-elements were recognised by distinct cellular factors and their role in RA-differentiation is yet to be elucidated. However, it was documented that RARE 1A and RARE

1B are responsible for the RA-mediated repression of Oct-3 in P19 cells (Okamoto *et al.* 1990; Okazawa *et al.* 1991).

1.4 Statement of the research problem:

The initial objective of the study was to determine whether or not the minimal constitutive enhancer elements of HPV11, HPV16 and HPV18 described in 1.2, which are required for epithelial-specific function, are active in undifferentiated and retinoic acid-differentiated P19 mouse embryonal carcinoma cells.

Hypothesis:

P19 EC cells were used in this study in order to investigate whether HPV enhancers are active, like the members of the other subfamily, polyomavirinae of Papovaviridae (Nakshatri, 1990) and whether similar type of cellular factors were involved. Retinoic acid was chosen to differentiate P19 EC cells to study whether the differentiated cells can support the activity of HPV enhancers or not since retinoic acid is known to be a modulator of cellular transcriptional machinery (see 1.3.1).

The minimal constitutive enhancer of HPV11 was chosen as the model system for further studies with undifferentiated and RA-differentiated states of P19 mouse EC cells.

The subsequent objectives of the study were:

1. To find out the interaction of cellular factors with

the minimal constitutive enhancer (MCE) of HPV11 and to further correlate the *in vitro* DNA-protein interactions with *in vivo* activity.

2. To further delineate the minimal sequences of HPV11 MCE necessary for the *in vivo* acitivity in P19 EC cells.

3. To characterize the cellular factor interaction with some of the identified minimal sequences of MCE of HPV11 that show substantial *in vivo* activity.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS:

Restriction endonucleases and their 10X React buffers were purchased from Gibco-BRL. T4 DNA ligase and its 5X premix, mungbean nuclease, exonuclease III and calf intestinal phosphatase were also obtained from Gibco-BRL. Buffers (1X) for mungbean nuclease and exonuclease III were prepared according to the manual of Current Protocols in Molecular Biology (Wiley Interscience). Reverse transcriptase was obtained from Life Sciences. DNaseI and Acetyl coenzyme A (sodium salt) were purchased from Sigma.

$\alpha[^{32}P]$dCTP and $[^{35}S]$dATP were from Amersham. Nick columns and poly (dI-dC) double stranded sodium salt were purchased from Pharmacia. Thin Layer Chromatography (TLC) plates and X-ray films were supplied by Kodak. Retinoic acid (RA) was purchased from Sigma.

Alpha-MEM medium (α-MEM) and fetal calf serum (FCS) were supplied by Flow Laboratories Inc. Gibco-BRL supplied trypsin-EDTA. The P19 EC cell line maintained in the laboratory was kindly provided by H.Hamada. Omnifluor was purchased from New England Nuclear (NEN).

The dideoxy sequencing kit (Sequenase kit) was purchased

from United States Biochemical (USB) Corporation, Cleveland, Ohio. 5-Bromodeoxyuridine was obtained from Sigma. Synthetic oligonucleotides were purchased from the Regional DNA Synthesis Laboratory, University of Calgary.

The recombinant plasmids, pSV2CAT and pA10CAT maintained in the laboratory were provided by B. Howard. The HPV enhancer-CAT plasmid constructs pT1, pT3 and pT81 were as described by Marshall *et al.* (1989). The plasmid, HPV11 enhancer in pUC19 was provided by H. Nakshatri.

2.2 Recombinant plasmid constructs:

The negative control plasmid, pA10CAT, was as described by Laimins et al. (1982). It was constructed by joining the SphI/BamHI fragment of pA10 derived from pBR322 with the SphI/BamHI fragment of pSV2CAT so that it becomes a control plasmid having 21 base pair repeats and TATA box of SV40 upstream of the Chloramphenicol Acetyltransferase (CAT) gene coding sequences, but lacks SV40 enhancer sequences. The plasmid, pA10CAT has a BglII site immediately upstream of the 21 base pair repeats.

The positive control plasmid, pSV2CAT, was as described by Gorman *et al.* (1982). It serves as a positive control in the transfection assays since it has SV40 enhancer sequences which are absent in the negative control plasmid pA10CAT.

The human papillomavirus enhancer-CAT constructs pT1, pT3

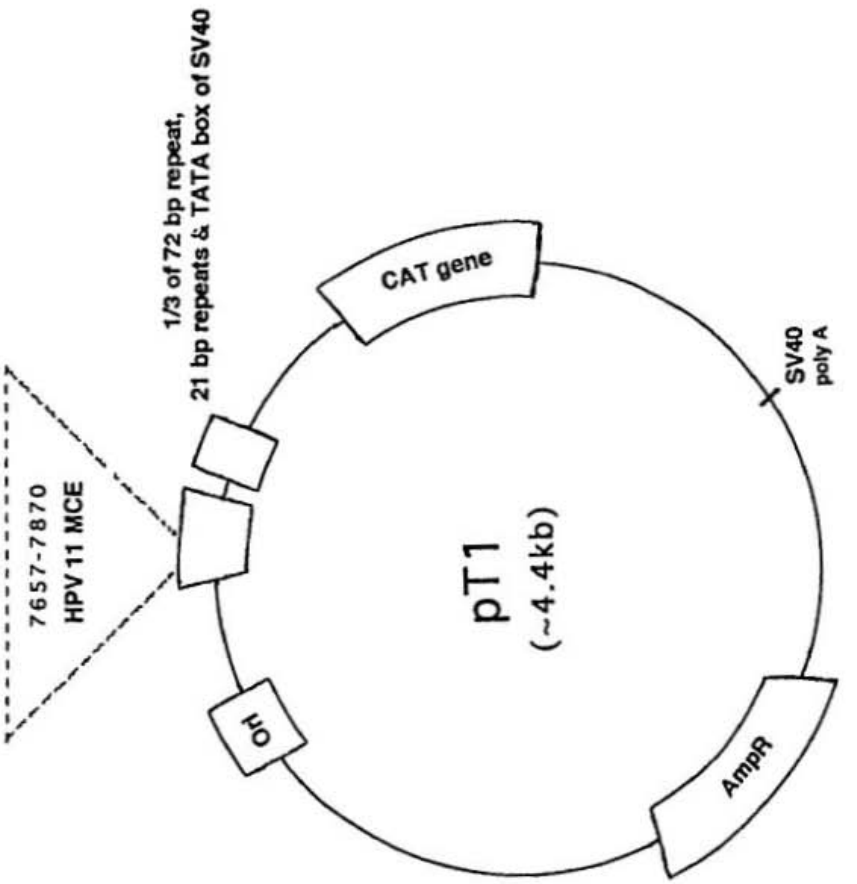
Fig.5. Physical map of the recombinant HPV11 enhancer-CAT plasmid construct, pT1.

The dashed lines of the plasmid indicate the location of minimal constitutive enhancer (MCE) sequences of HPV11 in the plasmid. The constitutive enhancers of HPV16 and HPV18 are present in a similar position in the other two plasmid constructs, pT3 and pT81, respectively which were used in the preliminary studies. Deletion enhancer derivatives were also cloned at the same site to make deletion mutant-CAT constructs, pKS1, pKS2 and pKS3.

CAT gene: Chloramphenicol acetyltransferase gene.

Ori: origin of replication.

AmpR: Ampicillin resistance gene.


7657-7870
HPV 11 MCE
1/3 of 72 bp repeat,
21 bp repeats & TATA box of SV40
CAT gene
SV40
poly A
pT1
(~4.4kb)
Ori
AmpR

and pT81 were as described by Marshall *et al.* (1989). The plasmid, pT1 is about 4.4kb HPV11 enhancer-CAT construct generated to have the 213 base pairs NdeI-SauI fragment (nucleotide 7657-7870) of HPV11 noncoding region (Dartmann *et al.* 1986) in the BglII site of pA10CAT (Fig.5). The HPV16 enhancer-CAT construct, pT3 has a 554 base pair SspI-HphI fragment (nucleotide 7224-7778) of HPV16 noncoding region cloned upstream of the 21 base pair repeats and TATA box of pA10CAT. The HPV18 enhancer-CAT construct, pT81 has the 230 base pair fragment (nucleotide 7508-7738) of HPV18 noncoding region cloned in the BglII site of pA10CAT.

2.2.1 Construction of deletion mutant plasmids:

The minimal constitutive enhancer sequences of the noncoding region of HPV11 (nucleotide 7657-7870) cloned into the XbaI site of pUC19 plasmid (Nakshatri, 1990). The deletions in the HPV11 MCE were generated as described previously (Henikoff, 1987). The HPV11 MCE cloned in pUC19 plasmid was linearized by BamHI and KpnI to generate 5′ and 3′ overhangs, respectively. Exonuclease III was used for making deletions as it does not attack a 4-base 3′ overhang obtained after KpnI digestion. At the same time exonuclease III digests the nucleotides uniformly from the other accessible 3′ end obtained due to BamHI digestion. After exonuclease III digestion, mungbean nuclease was preferred over S1 nuclease to

cleave the single stranded overhangs since mungbean nuclease does not attack any of the base-paired nucleotides.

The linearized DNA was suspended in 1X exonuclease III REact buffer (50mM Tris.Cl, pH 7.5, 5mM $MgCl_2$ and 5mM DTT) in 50 μl reaction volume and incubated at 25°C after the addition of 3 units of exonuclease III/1μg of DNA. At regular time intervals, aliquots of digested DNA were taken out and incubated at 70°C for 10 minutes. After heat inactivation, 1X mungbean REact buffer was added to each aliquot in a 25μl reaction volume. Mungbean nuclease was diluted with 1X mungbean nuclease REact buffer (30mM sodium acetate, pH 5, 50mM NaCl and 1mM zinc chloride) and 2 units of the diluted mungbean nuclease was added to each aliquot. The aliquots were incubated at 37°C for 20 minutes and the reaction was stopped by adding 1μl of 0.5M EDTA. The linearized exonuclease III digested DNA was recircularized by incubating overnight at 16°C after the addition of 15μl ligation premix and 1 unit of T4 DNA ligase.

The extent of digestion by exonuclease III was determined by dideoxy sequencing (Sanger *et al.* 1977) of the deletion derivatives of HPV11 MCE cloned in pUC19 plasmid. The fragments from nucleotide 7785-7870 and 7811-7870 of HPV11 MCE sequences derived after exonuclease III digestion were used to make deletion mutant-CAT constructs, pKS1 and pKS2. The vector

used to make deletion mutants was the linearized pA10CAT from which 1.7 kb AccI-BglII fragment was removed since the wild type HPV 11 MCE-CAT construct, pT1, does not contain this fragment. The linearized pA10CAT was blunt ended and the deletion derivative sequences of HPV11 MCE were cloned upstream of the SV40 promoter and CAT gene to generate the recombinant CAT plasmids, pKS1 and pKS2.

In order to make the deletion mutant, pKS3, the SalI-SmaI fragment of pUC19 having HPV11 MCE sequences was digested with AluI. The SmaI-AluI fragment was subjected to partial digestion with RsaI. BamHI linker was added to the RsaI-AluI fragment (nucleotide 7766-7814) on either side after blunt ending of the fragment. This linker added RsaI-AluI fragment was cloned into the BamHI site of Bluescript KS (+) plasmid. This Bluescript plasmid was digested with HindIII, blunt ended and digested with BamHI. This HindIII-BamHI fragment was cloned in the vector. The vector to make the mutant, pKS3 was similar to the one used for making the mutants, pKS1 and pKS2, except that the BglII site was left with a sticky end. The blunt ended HindIII-sticky end BamHI fragment was cloned in pA10CAT containing blunt ended AccI and sticky BglII end to generate the deletion mutant, pKS3.

2.3 Cell culture of P19 mouse embryonal carcinoma cells:

The P19 cells were cultured in α-MEM medium containing 10% heat inactivated fetal calf serum. The cells were differentiated by treatment with 300nM retinoic acid (RA) as previously described (Rudnicki and McBurney, 1987). Briefly, the undifferentiated cells were maintained in tissue culture plates. To differentiate P19 EC cells, first the cells were washed with PBS and trypsinized. The trypsinized cells were pelleted by centrifugation and resuspended in the α-MEM medium containing 300nM retinoic acid in bacterial dish plates. After two days of incubation at 37°C, the cell aggregates were seeded into fresh bacteriological plates at a ratio of 1:2 with fresh medium containing RA. Two days after second seeding, the cell aggregates were collected, washed and trypsinized. The cells were then plated in tissue culture plates with fresh α-MEM medium containing no retinoic acid. Both undifferentiated and RA-differentiated cells were used for transfection studies.

2.4 Transfection of undifferentiated and retinoic acid-differentiated cells:

Transfection was carried out 8 hours after plating the cells. The transfection was done using the calcium-phosphate precipitation method (Gorman *et al.* 1982). In short, the DNA precipitate was prepared by putting 10 μg plasmid DNA in 50μl

of 0.1X TE buffer and 62μl of 2M $CaCl_2$ in a final volume of 500μl and was mixed slowly with 500μl of 2X HBS (0.28M NaCl;0.05M HEPES; 2.8mM Na_2HPO4 adjusted to pH, 7.1) by constant bubbling under nitrogen. The white cloudy precipitate thus obtained was uniformly added to the cultured plates in duplicate. The plates were incubated in 3% CO_2 incubator at 37°C. The cells were subjected to glycerol shock 4 hours after transfection using 1.5ml of 15% glycerol made in 2X HBS. The transfected undifferentiated and RA-differentiated cells were subjected to glycerol shock for three and one minute, respectively. After the glycerol shock, the cells were washed three times with serum-free medium to remove traces of glycerol. The cells were fed with fresh α-MEM medium containing 10% FCS and incubated at 37°C. After 48 hours of incubation, the cells were harvested.

2.5 Harvesting the cells and preparation of cell lysates for CAT assays:

The transfected cells were harvested 48 hours after transfection as described (Gorman *et al.* 1982). The cells were washed thrice with 2ml of PBS and swirled occasionally for about 5 minutes at room temperature after the addition of Tris-EDTA-NaCl (0.04M Tris HCl pH 7.4; 1mM EDTA; 0.15M NaCl). After incubation for 5 minutes at room temperature, the cells

were scraped by rubber policeman and were collected in eppendorf tubes. The cells were pelleted by spinning for 30 seconds at 4°C in a microfuge. The supernatant was discarded carefully without disrupting the pellet and the cells were frozen at -70°C till they were used.

The frozen cells were thawed at 37°C for 5 minutes and resuspended in 100μl of 0.25M Tris.HCl (pH 7.8). The cell pellet was disrupted by vortexing. The cells were lysed by three cycles of freezing and thawing. The lysed cells were then spun in microfuge for 5 minutes at 4°C. The supernatant was collected in eppendorf tubes and assayed for CAT activity.

2.6 Chloramphenicol acetyltransferase (CAT) assays:

The *in vivo* activity of different enhancer-CAT constructs was determined by assaying the Chloramphenicol Acetyltransferase enzyme present in cell lysates as per the procedure described by Gorman *et al.* (1982). The assay reaction contained 20μl of the mixture of 14μl 1M Tris-HCl(pH 7.8), 1μl ^{14}[C]chloramphenicol, 1μl of distilled water, 4μl of acetyl coenzyme A mixed with 10μl of the cell lysate. The total assay mixture of 30μl was incubated at 37°C for 1 hour. After incubation, 0.5ml of ethyl acetate was added to stop the reaction. The assay mixture was vortexed for 30 seconds to extract chloramphenicol. The organic layer was taken out in

separate eppendorf tubes avoiding the interface and dried in a vacuum aspirator (Savant). The dried organic layer was dissolved in 15μl of ethyl acetate and vortexed for 1 minute and then spotted on silica gel thin layer chromatography (TLC) plates. The TLC plates were run with chloroform-methanol (95:5). The acetylated and non-acetylated forms were separated by this ascending type of TLC. The TLC plates were sprayed with Omnifluor EnHance to increase the signal. The TLC plates were exposed to Kodak X-ray film at -70°C. The quantitation of the acetylated and non-acetylated forms was done after autoradiography by cutting the spots and counting by liquid scintillation. Data were expressed as the percentage of chloramphenicol converted to acetylated form against the nonacetylated form. The data for CAT activities were expressed after subtracting the residual acetylated form counts of the SV40 promoter of pA10CAT from those of the deletion mutant and wild type CAT constructs.

2.7 Preparation of nuclear extracts:

2.7.1 Preparation of nuclei:

The nuclear extracts were prepared according to the procedure described by Hennighausen and Luban (1987). About twenty five 100mm plates of undifferentiated cells and forty 100mm plates of RA-differentiated cells were harvested for preparing nuclear extracts. The cells were scraped with rubber

policeman and harvested by centrifugation for 10 minutes at 1800 rpm at 4°C in a Sorvall HB-4 rotor. The pelleted cells were resuspended in 10 volumes of PBS and centrifuged once again. The pelleted cells were resuspended in 5 pellet volumes of 0.3 sucrose in Buffer A. Buffer A contains 10mM HEPES-KOH (pH7.9), 10mM KCl, 1.5mM $MgCl_2$, 0.1 mM EGTA, 0.5mM dithiothreitol (DTT), 0.5mM of protease inhibitor-phenylmethylsulfonylfluoride (PMSF) and 2μg/ml each of antipain, leupeptin and pepstatin A. The pelleted cells resuspended in Buffer A were lysed by 8-12 strokes with a B-pestle in a Dounce glass homogenizer and again 1-2 strokes in the presence of 0.3-0.4% nonidet P-40 (NP-40). Completion of lysis was monitered under microscope and the homogenate was pelleted by centrifugation for 10 minutes at 2700 rpm at 4°C. The pelleted nuclei were washed twice in 0.3M sucrose in Buffer A without NP-40.

2.7.2 Nuclear extract preparation:

The nuclear extracts were prepared as per the protocol of Hennighausen and Luban (1987). The nuclei were resuspended in all-glass Dounce homogenizer in 2.5 pelleted volumes of the buffer containing 400mM NaCl, 10mM HEPES-KOH (pH7.9), 1.5mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, 5% glycerol and 0.5mM PMSF. The nuclei were disrupted by 10 strokes with B-pestle and were

gently stirred for 30 minutes at 4°C followed by centrifugation for 60 minutes at 39000 rpm in 75Ti rotor. The supernatant was collected and dialysed for 2-4 hours against 50 volumes of buffer containing 20mM Hepes-KOH (pH 7.9), 75mM NaCl, 0.1mM EDTA, 0.5mM DTT, 20% glycerol and 0.5mM PMSF. The extract was cleared by centrifugation at 20000 rpm for 15 minutes to remove the precipitated material and some lipids. The extract was distributed in aliquots and frozen at -70°C. The protein concentrations in the nuclear extracts of both undifferentiated and RA-differentiated cells were by the method of Bradford (1976).

2.8 Preparation of radiolabeled DNA probes for *in vitro* assays:

The minimal constitutive enhancer fragment of HPV11 (nucleotide 7657-7870) cloned into the XbaI site of pUC19 plasmid was digested with SalI and SmaI. The SalI-SmaI fragment was electroeluted from 1.5% agarose gel. The SalI site of the fragment was end-labeled using reverse transcriptase and $\alpha[^{32}P]$dCTP. The end-labeled DNA was separated from the unincorporated nucleotides by passing through Sephadex G-50 columns. The separated radiolabeled DNA which was precipitated, washed, dried and dissolved in distilled water was used as a probe in the DNaseI protection assay.

The plasmid, Bluescript KS (+) having 49 base pairs of HPV11 MCE (nucleotide 7766-7814) was digested with PstI and BamHI. The PstI-BamHI fragment containing HPV11 enhancer sequences (nucleotide 7766-7814) was separated and electroeluted from 6% PAGE. The PstI-BamHI fragment was end labeled at BamHI site using reverse transcriptase and $\alpha[^{32}P]$dCTP. The radiolabeled DNA was precipitated at -70°C and washed thrice with 70% ethanol in order to remove the unincorporated nucleotides. The washed radiolabeled DNA was dried and used as a probe.

For UV cross-linking, a 19 base pair oligonucleotide corresponding to the imperfect NF1 palindrome (nucleotide 7776-7794) of HPV 11 was used. The two strands of the oligonucleotide were annealed as per Kadonaga and Tjian (1986) and nick-translated in the presence of $\alpha[^{32}P]$dCTP, 5-Bromodeoxyuridine (BUDR), DNaseI, polymeraseI and dNTPs. The radiolabeled DNA was precipitated at -70°C and washed thrice with 70% ethanol to remove the unincorporated nucleotides. The washed and dried radiolabeled DNA was dissolved in distilled water and used as a probe in the UV cross-linking experiments.

2.8.1 Competitors used in the gel retardation assay:

The 19 base pair oligonucleotide corresponding to HPV11 MCE sequences (nucleotide 7776-7794) which is part of the

protected region II was used as competitor. It has the imperfect NF1 palindrome present in the sequence, 5'-TGGATTGCAGCCAAAGGTT-3'. A 24 base pair oligonucleotide having the NF1 half site corresponding to the sequence, 5'-GTTAAAAGCATTTTTGGCTTCTAG-3' of the protected region II of HPV11 MCE (nucleotide 7793-7815) served as another competitor. The oligonucleotide corresponding to JC virus perfect NF1 palindrome 5'-TGGCTGCCAGCCAA-3' and the mutant oligonucleotide of the JC virus NF1 palindrome 5'-GTACTGCCAGACCA-3' were also used as competitors in the gel retardation assay. The complementary strands of the oligonucleotides were annealed according to Kadonaga and Tjian (1986). The oligonucleotides were used without adding any radiolabel.

2.9 Protocols for *in vitro* assays:

2.9.1 DNaseI protection assay:

The DNaseI protection assay was performed according to the procedure described by Hennighausen and Luban (1987). Two different protein concentrations (55μg and 65μg) of nuclear extracts were used in the DNaseI protection assay to map the protected sequences of the minimal constitutive enhancer (nucleotide 7657-7870) of HPV11. The nuclear extracts prepared from undifferentiated (UD) and RA-differentiated (RA) cells in a total volume of 50μl binding buffer (50mM NaCl, 0.1mM EDTA, 20mM HEPES-KOH-pH 7.5, 0.5mM DTT, 10% glycerol) along with 1μg

of poly (dI.dC) were incubated for 20 minutes on ice. About 40,000 cpm of the radiolabeled HPV11 MCE probe was added and incubated at room temperature for 10 minutes. Later on 2μl of a mixture of $MgCl_2$ and $Cacl_2$ was added to obtain a final concentration of 5mM and 1mM respectively. Afterwards, DNaseI stock solution (2μg/μl in 150mM NaCl and 50% glycerol) was diluted with buffer containing 25mM NaCl, 10mM HEPES-KOH at pH 7.5 and 0.5mM DTT and used at a concentration of 300 ng for the probes incubated with nuclear extracts. Two different concentrations, 16ng and 8ng (F1 and F2 in the figure.7) of DNaseI were used to digest naked probe with no protein added. The naked DNA probes F1 and F2 served as controls since no nuclear extracts were added to them. The DNaseI was allowed to act on the DNA probe for 30 seconds at room temperature. The reaction was stopped by adding 100μl stop buffer (0.375% SDS, 15mM EDTA, 100mM NaCl, 100mM Tris.HCl at pH 7.6) containing 50μg/ml sonicated salmon sperm DNA and 100μg/ml pronase. This was followed by incubation for 15 minutes at 37°C and 2 minutes at 90°C. The reaction products were subjected to phenol-chloroform-isoamylalcohol extraction and ethanol precipitation before loading in polyacrylamide-8M sequencing gel. The gel was dried and subjected to autoradiography.

2.9.2 Gel retardation assay:

The gel retardation assay was performed according to the procedures described previously (Hennighausen and Luban, 1987; Chodosh *et al.* 1988b) with a few modifications. The nuclear extracts having a protein concentration of 5μg were incubated with 5μg of poly (dI.dC) (double stranded) in a total volume of 15μl containing 12 mM HEPES-NaOH (pH 7.9), 4mM Tris.HCl (pH 7.9), 60mM KCl, 1mM EDTA, 10% glycerol, 0.6mM DTT and 300μg/ml bovine serum albumin for 10 minutes on ice. About 20,000 cpm of the end-labeled PstI-BamHI fragment of HPV11 enhancer sequences (nucleotide 7766-7814) was added and the mixture was incubated at 30°C for 30 minutes. For samples used in the competition studies, the different competitors were added at the time of adding probe. Naked DNA probe without nuclear extracts served as control. The reaction products were run on 4% polyacrylamide gel electrophoresis at 4°C with constant circulation of buffer (6.6mM Tris.HCl pH 7.5, 3.3mM sodium acetate and 1mM EDTA). The gel was dried and subjected to autoradiography to detect the DNA-protein complexes.

2.9.3 UV cross-linking:

This was done according to the procedure described by Chodosh *et al.* (1986) with slight modifications. Briefly, the binding buffer as described in 2.9.2. was used for the reaction. The nuclear extracts of both UD and RA-

differentiated extracts were added at a concentration of 60μg to the binding buffer along with 10μg of poly (dI.dC) in a total volume of 30 μl. About 100,000 cpm of the nick-translated and BUDR-incorporated 19 base pair oligonucleotide probe was added to the reaction and incubated at 30°C for 30 minutes. The naked DNA probe without nuclear extracts served as a control. After incubation, the reaction mixture was transferred to the NUNC vial and sealed with saran wrap. The mixture was irradiated under a Fotodyne UV lamp (maximum emission wave length, 310nM; maximum intensity, 70000μW/cm^2) for 10 minutes. The $CaCl_2$ concentration in the mixture was adjusted to 10mM. The DNA which was not bound by proteins in the reaction mixture, was digested into mononucleotides with 0.6μg of DNaseI (Sigma) and 1 unit of micrococcal nuclease by incubating at 37°C for 30 minutes. The reaction products in which the proteins binding the probe were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Before loading the reaction products into the gel, 2X loading buffer (25 ml 4X Tris.HCl/SDS pH 6.8; 20ml glycerol; 4g SDS, 3.1g DTT, 1mg bromophenol blue in 100ml) was added to the samples and the samples were boiled for 3 minutes. The prestained protein markers of high molecular weight range (myosin heavy chain-200kd, phosphorylase b 97.4 kd, bovine serum albumin-68kd, ovalbumin-43kd, carbonic

anhydrase-29kd, β-lactoglobulin-18.4kd and lysozyme-14.3 kd) were also loaded into the gel to determine the sizes of proteins interacting with DNA. After separating the reaction products for 16 hours at room temperature, the gel was dried and subjected to autoradiography.

CHAPTER 3

RESULTS

3.1 Activities of enhancers of HPV11, HPV16 and HPV18 in undifferentiated and retinoic acid-differentiated P19 cells:

The minimal constitutive enhancer fragments of HPV11 (nucleotide 7657-7870), HPV16 (nucleotide 7224-7778) and HPV18 (nucleotide 7508-7738) cloned in the CAT plasmids (pT1, pT3 and pT81, respectively) were used for the preliminary *in vivo* studies. The SV40 enhancerless-CAT plasmid pA10CAT and the SV40 enhancer plasmid pSV2CAT were used as negative and positive control plasmids, respectively.

P19 mouse embryonal carcinoma cells were differentiated with 300nM retinoic acid into a heterogeneous population consisting mostly of neuronal cells and a minor population of fibroblast-like cells (Jones-Villeneuve *et al.* 1982). The recombinant CAT plasmids were tested in both undifferentiated (UD) and RA-differentiated P19 cells in transfection assays. None of the HPV enhancers were active in UD cells. The positive control plasmid pSV2CAT was active at a low level in UD cells compared to its high activity in RA-differentiated cells. The enhancer elements of HPV11 and HPV16 were active in RA-differentiated cells whereas the HPV18 enhancer was active

Fig.6. Activities of the minimal constitutive enhancers of HPV11, HPV16 and HPV18 in undifferentiated and RA-differentiated P19 EC cells.

UD: Undifferentiated

RA: Retinoic acid-differentiated

pT1, pT3 and pT81: Enhancer-CAT constructs of HPV11, HPV16 and HPV18 respectively.

pA10CAT: SV40 enhancerless-CAT plasmid serving as negative control.

pSV2CAT: SV40 enhancer-CAT plasmid construct used as positive control.

The percentages of CAT activities of the plasmid constructs as averages of three experiments are shown in table 1.

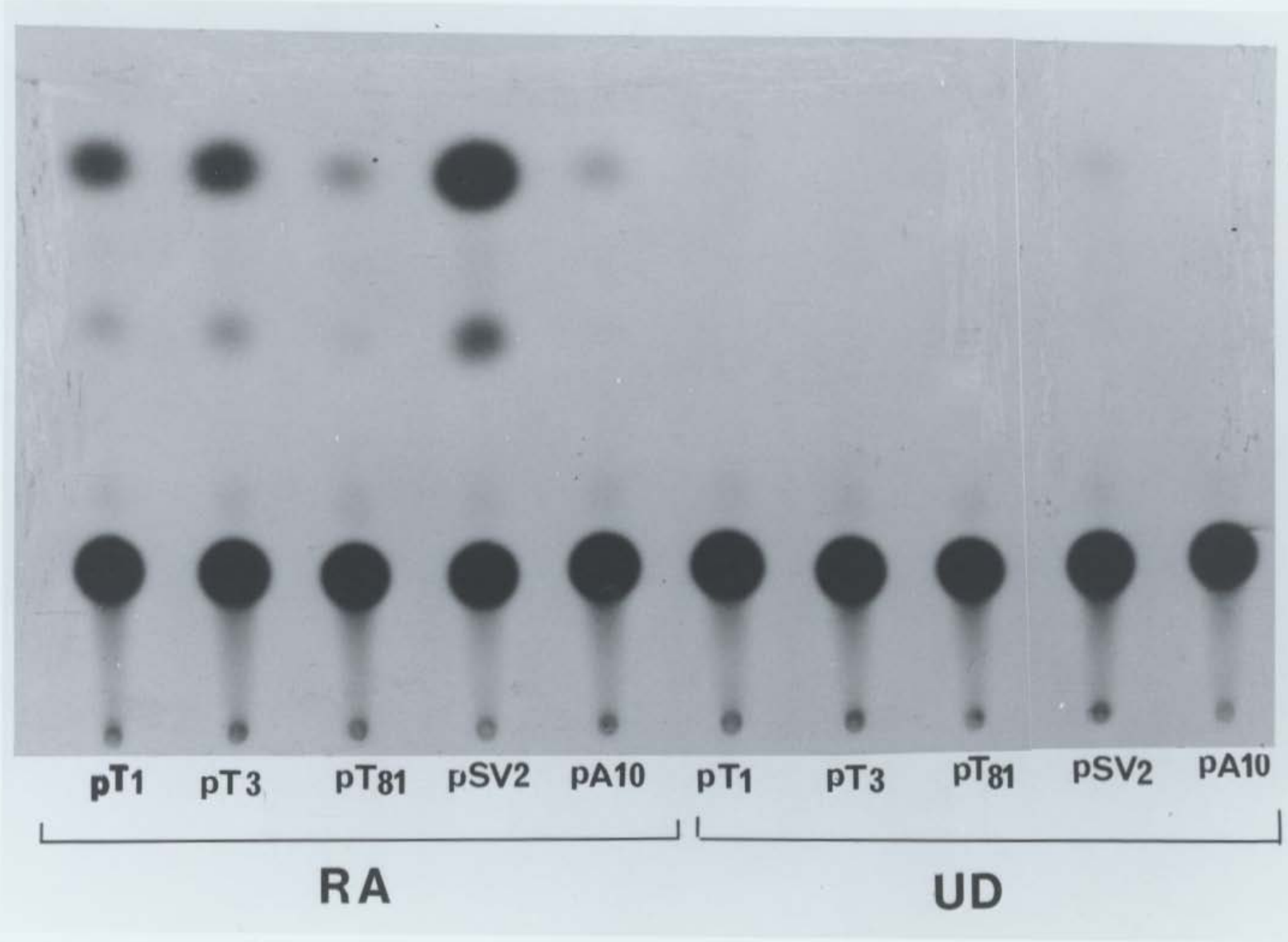

pT1
pT3
pT81
pSV2
pA10
pT1
pT3
pT81
pSV2
pA10
RA
UD

Table 1.

CAT activities of HPV enhancer-CAT constructs in UD and RA-differentiated P19 cells

Status of transfected cells	pT1 (HPV11)	pT3 (HPV16)	pT81 (HPV18)	pSV2 CAT (SV40)	pA10 CAT
UD	1.33	0.99	1.29	13.95	1.0
RA-differentiated	8.74	9.57	2.76	127.98	1.0

pA10 CAT activity was normalized to 1.0 and numbers indicate fold activation above that level by different enhancers of human papillomaviruses 11, 16 and 18. pSV2 CAT was the positive control SV40 enhancer-CAT construct. pA10 CAT was the negative control SV40 enhancer less-CAT construct. The results were expressed as means of three experiments.

at a low level. The activities of different recombinant plasmids is shown in figure 6 and table 1. The necessary cellular factors for the activity of HPV11 and HPV16 enhancers are expressed in the RA-differentiated cells. The essential transactivating factors for the HPV18 enhancer may be absent in this cell-type. Another possibility is that other repressing factors might affect HPV18 enhancer function.

The HPV11 enhancer was chosen for further studies. This was based upon the importance of the HPV11 noncoding region in the rare cases of malignancy where it was found to be duplicated. Moreover, the HPV11 enhancer consists of different constitutive enhancer elements. Additionally HPV11 enhancer is relatively short when compared to that of HPV16 to be used for further characterization purposes.

3.2 Correlation of *in vivo* activity of HPV11 MCE with in *in vitro* DNA-protein interactions:

In order to determine whether RA induced the synthesis of cellular factors which were responsible for the activity of HPV11 enhancer in RA-differentiated cells and to correlate the *in vivo* function with the *in vitro* DNA-protein interactions, DNaseI protection assay was performed. The results of DNase I protection assay are presented in figure 7. The DNaseI protection assay revealed four protected regions, I-IV, with RA-differentiated nuclear extracts. This suggests that the

Fig.7. DNaseI protection assay of the minimal constitutive enhancer (MCE) of HPV11.

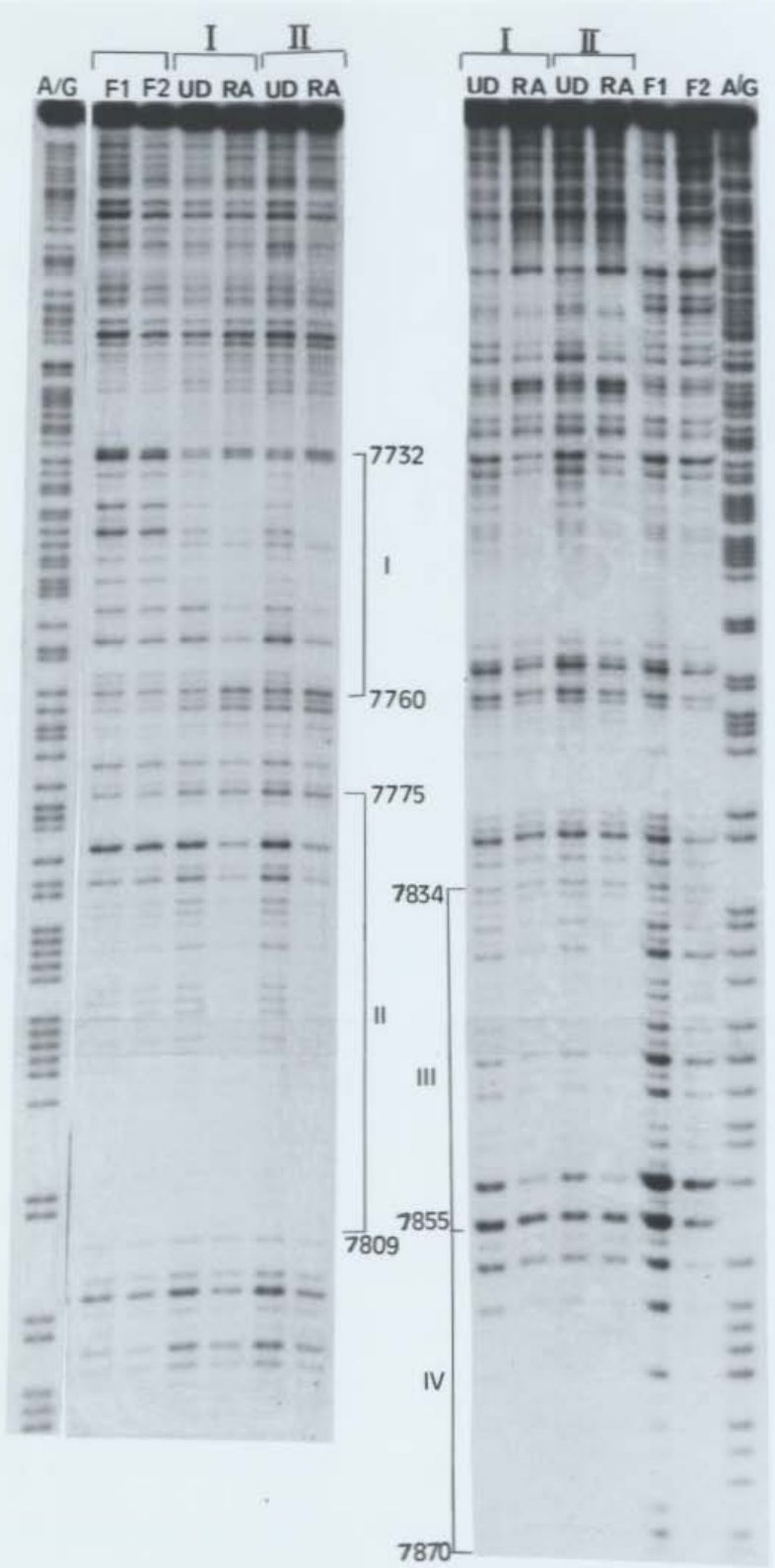
UD: Undifferentiated

RA: Retinoic acid (RA)-differentiated

F1 and F2: Naked DNA probe in the absence of nuclear extracts. DNaseI used was 16ng and 8ng, respectively.

I and II: Indicate 55μg and 65μg of protein concentrations of nuclear extracts, respectively and digested with 300ng of DNaseI.

The protected regions are bracketed and denoted as I-IV. The nucleotide positions of the protected regions are denoted. A/G indicates the chemical cleavage of purines.


I
II
A/G
F1
F2
UD
RA
UD
RA
7732
I
7760
7775
II
7809
7834
III
7855
IV
7870
I
II
UD
RA
UD
RA
F1
F2
A/G

cellular factors after RA-differentiation of P19 cells bind the enhancer regions at nucleotides from 7732-7760 (I), 7775-7809 (II), 7834-7855 (III) and 7855-7870 (IV).

The criterion for mapping the protected regions was comparison of the intensity of the bands in the presence and absence of protein (F1 and F2 in figure.7).

The protected regions I and II are interesting because the factors induced upon RA-differentiation potentially bind these two regions. The protected regions III and IV showed some protection with UD cell nuclear extracts. But as the protein concentration increased to 65μg, protected region IV appeared to be similar both with UD and RA-differentiated nuclear extracts.

The four protected regions have significant sequence motifs homologous to those seen in the known protein recognition sequences of other viral enhancers and cellular genes which are presented in table 2. Protected region I and IV have putative AP1 binding sites homologous to those observed in the HPV18 enhancer (Thierry *et al*. 1992) or SV40 P-motifs (Hirochika *et al*.1988). Homologies were observed for the sph-motifs of SV40 enhancer to which TEF-1 or TEF-2 bind. An homologous motif for HPV16 TEF-2 binding site was also observed (Hirochika *et al*.1988; Chong *et al*.1991). Protected region I has sequence motifs homologous to the binding sites observed with the CEIII element of HPV11 which was not

Table.2. Protected sequences of the HPV11 minimal constitutive enhancer using P19 RA-differentiated nuclear extracts and their homology to known transcription factor binding motifs.

The sequence motifs identified in the protected regions I-IV and their homologous protein recognition sequences of transcription factors of other viral and cellular origins are given.

NF1: Nuclear factor 1; AP1: Activator protein 1; TEF: Transcription enhancer factor.

The protein recognition sequences for AP1 were adapted from Hirochika *et al.* (1988) and Thierry *et al.* (1992); for NF1 from de Vries *et al.* (1987) and Chong *et al.* (1991); for TEF2 from Chong *et al.* (1991).

Underlined sequences indicate the homologous sph motifs of the SV40 enhancer to which TEF1 and TEF2 bind (adapted from Hirochika *et al.* 1988).

Wavy line indicates the homologous C/EBP binding site of the prototype motif ATTGG (adapted from Graves *et al.* 1986).

Dashed line indicates the homologous motif for the nuclear factor 1-associated factor (NFA) motif of HPV16 (Chong *et al.* 1991; Dollard *et al.* 1993). Motifs with superscripts 'a' and 'b' are the factor recognition sequences of CEIII of HPV11 (Auborn *et al.* 1989; Auborn and Steinberg, 1991).

Table 2

Protected sequences of HPV 11 minimal constitutive enhancer using P19 RA - differentiated nuclear extracts and homologies for known transcription factor binding motifs.

Protected region	Sequence protected	Nucleotide number	Motifs in the protected sequence	Known factor recognition sequence
I	TGCATGACTAAT GTACAATAAAC CTGTCG	7732-7760	TGACTAATGTA	TGACTAANCGA (putative AP1 site / SV-40 P- motif)
			CAATAAACCTG	CAATAAACAAT [a]
			CCTGTCG	CCNGTNAC [b]
II	GTGGATTGCAGCCAAA GGTTAAAAGCATTT TTGGC	7775-7809	TGGATTGCA GCCAA	TGGATTGAAGCCAA (Adeno virus NF1)
			TTGGC	TTGGA (Adeno virus NF1/ NF1 half-site)
III	TTAGTATATT ATGCACAATACC	7834-7855	ATGCACAATA	AGGCACATAT (TEF-2 site in HPV 16)
IV	CCACAAAATGAGTAAC	7855-7870	CCACAAAA	CCACACCC (TEF-2 site of globin genes)
			CCACAAATGA	TGACTAANCGA (putative AP1 site / SV-40 P- motif)
			TGAGTAAC	TGACTAANCGA (putative AP1 site / SV-40 P- motif)

included in the MCE (Auborn *et al.* 1989; Auborn and Steinberg, 1991).

In addition, sequences homologous to nuclear factor 1 (NF1) binding site were observed for the sequences of protected region II (Hirochika *et al.*1988; Dollard *et al.* 1993). There are an imperfect NF1 palindrome with the sequence 5'-TGGN$_6$GCCAA-3' and an NF1 half-site motif, TTGGC present in the protected region II (Table.2). The imperfect NF1 palindrome has a motif, ATTGC, homologous to the C/EBP binding site (Hirochika *et al.* 1988; Nakshatri, 1990; Graves *et al.* 1986; Dollard *et al.* 1993). There is a motif homologous to the nuclear factor 1-associated factor (NFA) motif of HPV16 (Chong *et al.* 1991; Dollard *et al.* 1993).

3.3 The role of protected regions in the *in vivo* function of the enhancer:

To study the functional importance of the protected regions, deletion mutant enhancer-CAT constructs were tested for their activity in UD and RA-differentiated cells. Mutants, pKS1 and pKS2 were not active in UD cells and were active in RA-differentiated cells. Their CAT activities are shown in figure 8.

The deletion mutant, pKS1 (nucleotide 7785-7870 of MCE) which lost protected region I and the TGGATTGCA (TGG N$_6$)

Fig.8. CAT activities of the deletion mutant CAT constructs of HPV11 MCE in RA-differentiated cells shown in relation to the protected regions observed for MCE.

I-IV: Protected regions of the wild type MCE.

pT1: Wild type MCE-CAT construct.

pKS1 and pKS2: Mutant enhancer-CAT constructs

pKS3: CAT construct of the protected region II.

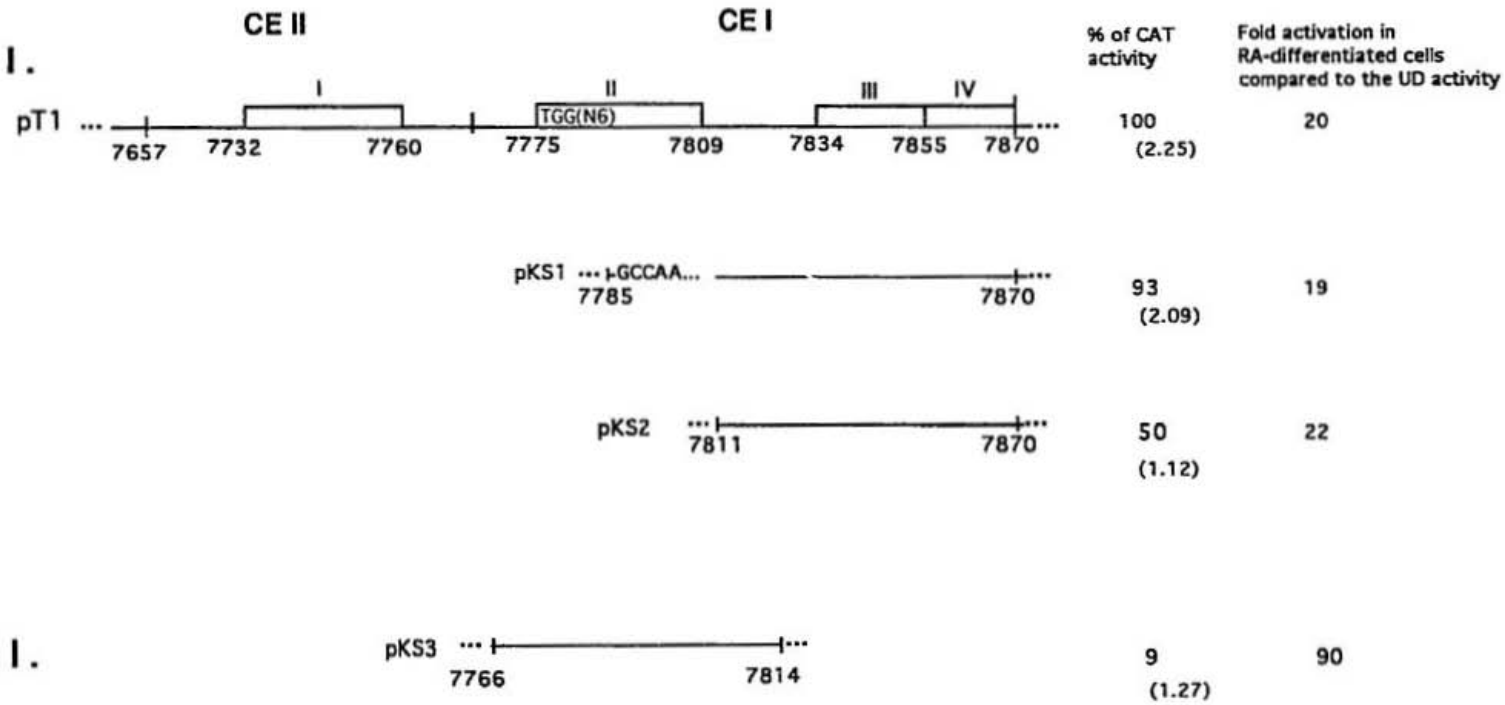
CEI and CEII: Designated by other researchers for epithelial cell-specific expression.

The wild type pT1 and mutants, pKS1, pKS2 and pKS3 have a CAT gene driven by the SV40 promoter and the respective enhancer elements. The CAT gene and promoter are not shown.

The CAT activities of pKS1 and pKS2 are averages of three different experiments and are shown relative to the wild type pT1 activity which was normalized to 100% in the first set of experiments. The CAT activity for pKS3 is an average of two different experiments and is shown relative to the wild type pT1 activity (13.5%) which was normalized (100%).

The percentage conversions of CAT substrate were determined after deducting residual promoter activity (acetylated form) of the negative control plasmid pA10CAT from those of wild type and mutant constructs.

The numbers in brackets indicate averages of actual *in vivo* activities. The residual activities of the wild type and mutant enhancers in UD cells were calculated to show fold increase of their activities in RA-differentiated cells.


I.
CE II
CE I
% of CAT activity
Fold activation in RA-differentiated cells compared to the UD activity
I
II
III
IV
pT1 ...
TGG(N6)
7657
7732
7760
7775
7809
7834
7855
7870
100
(2.25)
20
pKS1 ...GCCAA...
7785
7870
93
(2.09)
19
pKS2 ...
7811
7870
50
(1.12)
22
II.
pKS3 ...
7766
7814
9
(1.27)
90

sequence of the imperfect NF1 palindrome of protected region II gave 93% activity relative to wild type pT1 CAT activity (100%). It is not known from these results whether the $TGGN_6$ part of the imperfect NF1 palindrome which has the potential C/EBP site is necessary for high or low activity if included in the mutant, pKS1. The protected region I having homologous TEF and AP1 binding sequences appears to be not essential for the function of HPV11 MCE since the mutant pKS1 showed activity close to that of wild type.

The mutant, pKS1, even though it lost the $TGGN_6$ sequence, still showed high *in vivo* activity compared to wild type. Loss of protected region I resulted in slight reduction of activity with the mutant, pKS1. But loss of protected region I and II resulted in 50% reduction of wild type activity with the mutant, pKS2. These results suggest that the active minimal sequences necessary for the *in vivo* activity of MCE are located in protected region II. To understand the functional importance of the protected region II in detail, further work was carried out using this fragment.

In a separate set of experiments, the *in vivo* activities of the wild type plasmid, pT1 and the deletion mutant, pKS3 were tested. The protected region II sequences in the mutant, pKS3 gave 9% activity relative to wild type CAT activity (100%) in the second set of experiments (see panel II in

figure 8). Moreover, pKS3 gave 90-fold higher activity in RA-differentiated cells compared to its residual activity in UD cells. The activtiy of protected region II is 10-fold lower than that of MCE in RA-differentiated cells. The observation of 90-fold higher activity of the protected region II in RA-differentiated cells suggests that the induction of cellular factors and their interaction with protected region II is important for the *in vivo* activity of pKS3. Hence further *in vitro* studies were restricted to the protected region II.

3.4 Identification of factor(s) binding to the protected region II determined by gel retardation assay:

The protected region II (nucleotide 7775-7809 of MCE) with some additional flanking base pairs making a 49 base pair region (nucleotide 7766-7814), was used as the probe to identify the interaction of factor(s). Since protected region II has known NF1 binding sites (TGGATTGCAGCCAA and TTGGC), oligonucleotides corresponding to both sites (nucleotide 7776-7794 and 7793-7815 of MCE) were used as specific competitors. In addition, the polyoma JC virus perfect NF1 palindrome and its mutant were also used as competitors.

Protected region II gave rise to an extra DNA-protein complex with RA-differentiated nuclear extracts. This extra complex formation was abolished by the imperfect NF1 palindrome of HPV11, whereas the NF1 half-site of HPV11 could

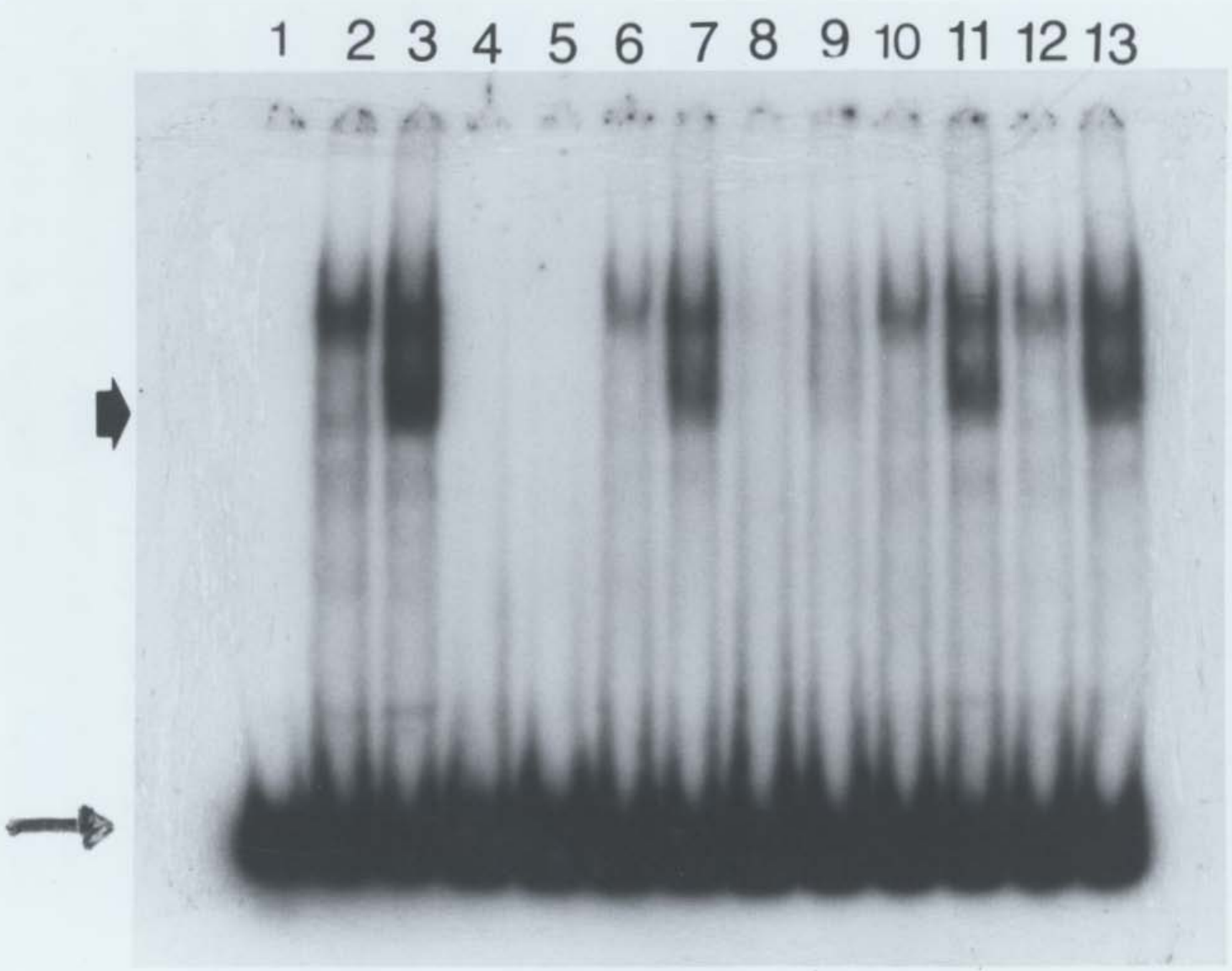
Fig.9. Interactions of cellular factors with the protected region II shown by gel retardation assay:

The 49 base pair protected region II in the (nucleotide 7766-7814) PstI-BamHI fragment of Bluescript KS (+) was end labeled and used as probe.

Lane 1: Naked DNA probe without nuclear extract and competitor DNA. Lanes 2-13: nuclear extracts were incubated at a protein concentration of 5μg with the probe. Even numbered lanes indicate undifferentiated nuclear extracts. Odd numbered lanes indicate RA-differentiated nuclear extracts.

Short dark arrow indicates the extra DNA-protein complex observed with RA-differentiated nuclear extracts. Long arrow indicates the unbound free probe.

Competitors used: Lanes 2 and 3: without competitor.
Lanes 4 and 5: Homologous unlabeled DNA (PstI-BamHI fragment). Lanes 6 and 7: Oligonucleotide 5′-GTTAAAAGCATTTTTGGCTTCTAG-3′ corresponding to the sequences of protected region II (nucleotide 7793-7815). Lanes 8 and 9: Oligonucleotide 5′-TGGATTGCAGCCAAAGGTT-3′ corresponding to the imperfect NF1 palindrome (nucleotide 7776-7794) of protected region II. Lanes 10 and 11: Oligonucleotide for the JC virus perfect NF1 palindrome 5′-TGGCTGCCAGCCAA-3′ and its mutant oligonucleotide with 5′-GTACTGCCAGACCA-3′ in the lanes 12 and 13. The complementary oligonucleotides were annealed and the cold competitors were used 400 fold excess of the DNA probe.


1 2 3 4 5 6 7 8 9 10 11 12 13

not compete for the extra complex. Similar to the NF1 half-site competition, the NF1 palindrome of JC virus and its mutant sequence could not abolish the formation of the complex (Fig.9). This suggests that the extra DNA-protein complex observed for protected region II with RA-differentiated nuclear extracts was due to the effective binding of the complex to the HPV11 imperfect NF1 palindrome (nucleotide 7776-7794) but not to the sequences (nucleotide 7793-7815) having sph, NFA and NF1 half-sites. The results also indicate that the RA-induced P19 cellular factor(s) that bind the NF1 sites of HPV11 MCE do not recognise the perfect NF1 palindromic sequences of JC virus. The extra DNA-protein complex noticed with RA-differentiated nuclear extracts was a specific complex as it was abolished only by the homologous sequence, but not by unrelated mutant JC virus NF1 sequence, in the competition studies.

Overall, the results suggest that the factor(s) interacting with the imperfect NF1 palindrome appear to be responsible for the *in vivo* activity of the deletion mutant, pKS3. UV cross-linking was performed to further identify the proteins interacting with the imperfect NF1 palindrome.

3.5 Proteins interacting with the imperfect NF1 palindromic sequences as shown by UV cross-linking:

Since the RA-induced factor(s) forming extra DNA-protein

Fig.10. Proteins of undifferentiated and RA-differentiated nuclear extracts interacting with the imperfect NF1 palindrome shown by UV cross-linking.

The oligonucleotide corresponding to the imperfect NF1 palindrome was nick-translated in the presence of $\alpha[^{32}P]$dCTP and BUDR and was used as probe.

F: naked DNA probe without nuclear extracts. The free end of the probe ran into the buffer and can not be seen.

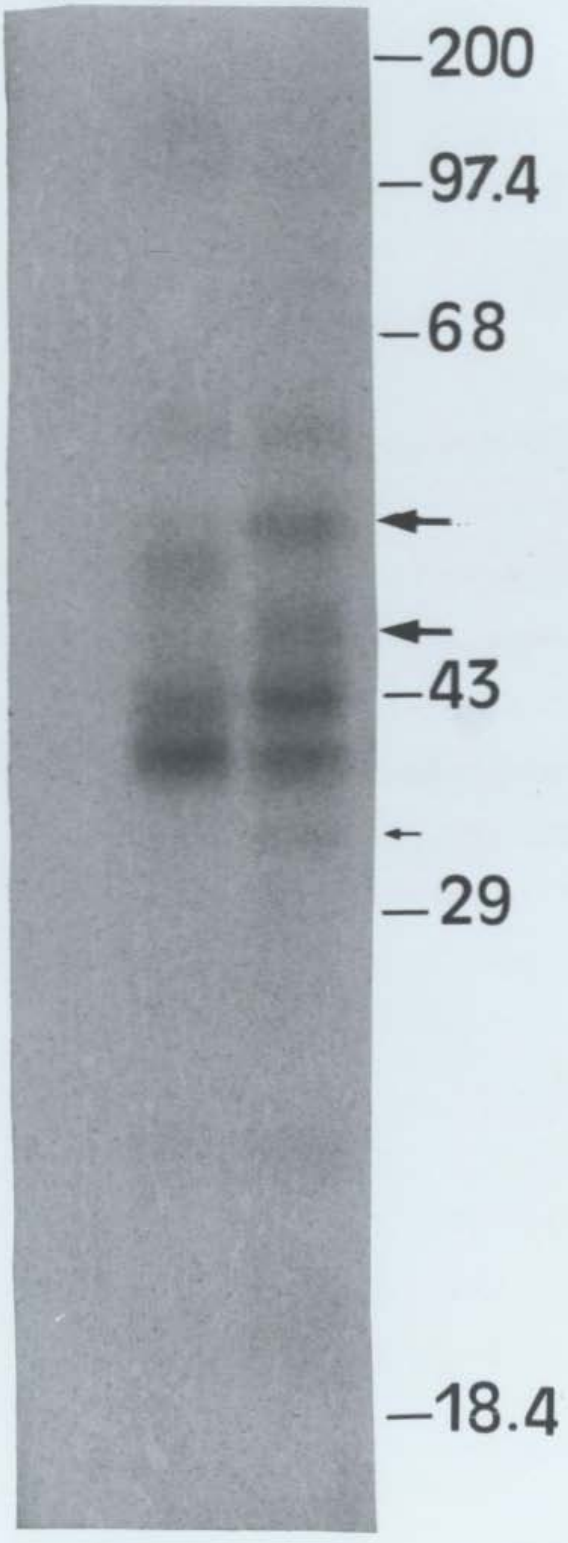
UD: Undifferentiated nuclear extracts.

RA: RA-differentiated nuclear extracts.

F, UD and RA samples were exposed to UV light, digested with DNaseI and micrococcal nuclease and run on 10% SDS-PAGE.

The numbers on the right indicate the prestained protein markers of high molecular weight range loaded into the gel. The 14.3 kd marker ran into the buffer.

Arrows indicate the different sizes of proteins observed only with RA-differentiated nuclear extracts.


F UD RA
200
97.4
68
43
29
18.4

complex are specific for HPV11 imperfect NF1 palindrome, UV cross-linking was undertaken to further confirm this result. At least six different proteins of 60, 56, 46, 43, 40 and 35 kd interacted with the 19 base pair NF1 oligonucleotide. Three of these proteins, with molecular weights of 56, 46 and 35 kd (Fig.10), were specific for RA-differentiated nuclear extracts.

CHAPTER 4

DISCUSSION

This study was undertaken to investigate the *in vivo* acitivity of the HPV11 minimal constitutive enhancer (MCE) in P19 EC cells and its correlation with *in vitro* DNA-protein interactions was fulfilled. The identification of minimal sequences that retain the activity comparable to that of wild type sequences was facilitated by the mutants used in this study. A 9 base pair sequence of protected region II was not included in the minimal sequences that showed activity with the mutant, pKS1. Hence further studies are needed to examine whether its inclusion would really elevate the activity of the identified minimal sequences of this study. The 9 base pair sequence (TGG N_6), along with the active NF1 recognition sequences of the identified minimal sequences which form the protected region II, was tested and found to be active by itself. The correlation of *in vivo* activity of protected region II sequences with the *in vitro* DNA-protein interactions was done. The identification of the type of proteins and their respective binding sites within the protected region II sequences require additional studies. These were not the objectives of this study.

4.1 Differences observed for the enhancer activities of HPVs in RA-differentiated cells:

The three HPV enhancers used in this study were not active in undifferentiated cells. The constitutive enhancers of HPV11 and HPV16 were active in RA-differentiated cells but the HPV18 enhancer was active only at a low level. This result was somewhat surprising because the HPV18 enhancer was found to be active in a neuroblastoma cell line (Swift *et al*. 1987). The HPV18 enhancer is known to show narrow cell type specificity since it can be active only in some cervical carcinoma cell lines but inactive in others (Swift *et al*. 1987). This suggests that certain transactivating factors required to support the HPV18 enhancer activity in P19 EC cells may be limiting or other repressing factors may dominate the scenario. Another possibility is that the RA-differentiated heterogeneous cell population contain limited number of cells which can support the HPV18 enhancer function.

In HeLa cells the ubiquitous transcription factor, Oct-1 represses the enhancer function of HPV18 (Hoppe-Seyler *et al*. 1991). Hence Oct-1 may repress the HPV18 enhancer activity in P19 RA-differentiated cells analogous to HeLa cells. Alternatively, the potential NF1 binding sites having GCCAA sequence are absent in the minimal constitutive enhancer fragment of HPV18 used in this study. Hence it is likely that this factor might be important for the enhancer function of

HPV18 in P19-RA differentiated cells. It has been well documented that the function of HPV18 enhancer mostly depends on the AP1 family members (Thierry *et al.* 1992). It is possible that retinoic acid receptors in the RA-differentiated cells might interfere with the AP1-dependent activity, a fact well documented using different cell types (Schule *et al.* 1991; Nicholson *et al.* 1990).

The HPV11 and HPV16 enhancers were almost equally active in the RA-differentiated cells. HPV11 exhibits certain features, for example, duplication of the HPV11 NCR in rare cases of malignancy (Byrne *et al.* 1987). Moreover, tandem duplication of part of NCR of HPV11 was also observed in cases of nasal inverting papilloma (Respler *et al.* 1987, Pater *et al.* 1988). The NCR part that was found duplicated in the above cases include the MCE used in this study. Additionally, the functional patterns of the constitutive enhancer elements, CEI and CEII in cells of epithelial origin were different (Hirochika *et al.* 1988; Chin *et al.* 1989). These features suggest that the minimal constitutive enhancer (MCE) consisting of both CEI and CEII might be functionally important for transcriptional regulation in the context of other cell types also. These characteristics of HPV11 prompted me to undertake further studies with HPV11 minimal constitutive enhancer (MCE).

4.2 Correlation of *in vivo* activity of HPV11 MCE with *in vitro* DNA-protein interactions:

The minimal constitutive enhancer of HPV11 required for RA differentiation-dependent activity in P19 EC cells was found to be protected at four different regions (I-IV) in a DNaseI protection assay. Regions I and II were exclusively protected with RA-differentiated nuclear extracts but not with UD nuclear extracts. Regions III and IV were also protected, albeit to a lesser extent, with undifferentiated nuclear extracts. Since the factors induced by retinoic acid specifically bind regions I and II, it was thought that either or both of these two regions might play a major role in the RA differentiation-dependent function of the HPV11 MCE. The protection improved for regions III and IV when increased protein concentration of RA-differentiated nuclear extracts was used. One can speculate that the factors binding these regions might undergo some modifications like phosphorylation or that extra factors are induced by RA-differentiation.

A number of protein binding motifs are present for the known transcription factors in all four protected regions (Table.2). The possible roles of each of these binding sites and functional cooperation among them in the context of minimal constitutive enhancer and mutant enhancers is discussed in 4.3.

4.3 Functional importance of the protected regions:

The functional importance of the protected regions which have different protein recognition motifs was confirmed by testing the *in vivo* activities of the deletion mutants of the minimal constitutive enhancer of HPV11. The deletion mutant pKS1, without protected region I and the TGGATTGCA part of the imperfect NF1 palindrome of protected region II, displayed activity close to that of wild type MCE plasmid, pT1. Deletion of protected region II also reduced the wild type activity to 50% as observed with the mutant, pKS2. This suggests that a lot of MCE activity is contributed by the sequences located in protected region II.

Protected region II alone was not responsible for the activities of both pT1 and pKS1 because the downstream protected regions III and IV in pKS2 also exhibited 50% of the wild type activity. Moreover, there was marginal loss of activity with the deletion mutant, pKS1 as compared to that of wild type. This can be attributed to either protected region I or to the NF1 half-site TGG or the potential C/EBP motif of the deleted imperfect NF1 palindromic sequences of protected region II. In contrast, drastic loss of activity of the CEI element in primary keratinocytes was observed when the C/EBP motif of the imperfect NF1 palindrome was disrupted (Dollard *et al.* 1993).

The functional role of region I in this study appears to

be negligible although it was protected exclusively with RA-differentiated nuclear extracts. In a similar fashion, sequences corresponding to the protected region I of MCE of this study were found interacting with factors of other cell types and such interactions displayed no functional role (Dollard *et al.* 1993) analogous to this study. Hence cellular factors binding to the different protected regions especially regions II, III and IV appear to functionally cooperate with each other to give the activities of pT1 and pKS1. The functional cooperation among factors binding to regions III and IV might be responsible for the *in vivo* activity of pKS2. Functional cooperation was suggested to be essential for the activation of a 91 base pair epithelial cell-specific enhancer of HPV16. Such a transcription factor synergism has been proposed for other HPV enhancers as well (Chong *et al.* 1990; Chong *et al.* 1991; Apt *et al.* 1993). Similarly, transcription factor synergism can be attributed to the factors binding the protected regions to give full MCE activity and the activities of mutants as well.

Different factors such as CTF/NF1, AP1 and TEF are possible candidate transcription factors to interact with their respective recognition sequences observed in the protected regions. There were homologies observed for CTF/NF1 for some of the sequences of protected region I. Similar sequences in the CEIII element of HPV11 were found to interact

with unidentified factors (superscript 'a' in table 2) as previously shown (Auborn *et al.* 1989; Auborn and Steinberg, 1991). The functional importance of such cellular factor interactions needs further investigation.

If AP1 is predicted to bind the putative motifs in protected regions I, II and IV, retinoic acid receptors might interfere with the AP1 activity as observed with other cell types (Schlue *et al.* 1991; Nicholson *et al.* 1990). But some RA-induced AP1-like factors, for example, PEA-1, were observed to bind the 'P' motifs of the polyoma enhancers in F9 embryonal carcinoma cells (Kryszeke *et al.* 1987). There is a possibility that AP1-like factors in RA-differentiated P19 cells can bind the homologous 'P' motifs or putative AP1 motifs of the protected regions.

The other possible transcription factors that bind to different protected regions include TEF1 and TEF2. The sequences homologous to TEFs are located in the protected regions I, III and IV. The role of NF1 binding sites located in the protected region II in the transcriptional activation of HPV11 can not be excluded. The NF1 proteins binding to the protected region II may cooperate with other transcription factors such as TEF1 or TEF2 to confer maximal *in vivo* activity of HPV11 MCE.

The activities of pT1 and pKS1 appear to depend upon functional cooperation among the possible transcription

factors as discussed above. There is a possibility that AP1-like factors and TEFs bind to their respective motifs in protected regions I, III and IV. This leads to the functional cooperation with NF1 factors binding the protected region II to give full MCE activity. Similarly, either TEF or AP1-like factors or a combination of both may bind the protected regions III and IV which, in turn, can cooperate with the NF1 factors binding the protected region II leading to pKS1 activity. Additionally, possible functional synergism can be attributed to the activity of pKS2. It might depend upon the interactions of TEF and AP1-like factors or TEF1-TEF2 with the protected regions III and IV.

A TEF1-dependent coactivator was found to be important for transcriptional activation from HPV16 enhancer (Ishiji *et al.* 1992). There is a possibility that such a coactivator may exist in RA-differentiated P19 cells supporting the possible TEF-mediated activation for the mutant, pKS2 in particular. TEF1 was found to activate by means of a unique TFIID complex (Brou et al. 1993). Hence it is quite possible that such unique subsets of TFIID that interact with NF1 or TEF might be present or induced in P19 cells leading to the activation of wild type and mutant HPV11 enhancers.

4.4 Correlation of *in vivo* activity of protected region II with *in vitro* DNA-protein interactions:

The mutant pKS1, consisting of part of the protected region II, exhibited *in vivo* activity close to that of wild type. Since a 9 base pair sequence of the protected region II was not included in pKS2, its functional role cannot be deduced from this study. Since three-fourths of protected region II was responsible for the *in vivo* activity (pKS1) the functional importance of the complete protected region II alone was examined using its deletion mutant-CAT plasmid, pKS3. The protected region II having NF1 sequences gave 9% activity relative to that of wild type MCE (100%) in a different set of experiments.

The activity of protected region II was 10-fold lower than that of wild type MCE. This result was not surprising since analogous observations for the activities of HPV16 NF1 sites or HPV11 NF1 sites in other cell types were reported in previous studies. The wild type DraI-DraIII enhancer fragment having two NF1 motifs of HPV16 gave 10.2% CAT activity relative to that of parent vector alone (Apt *et al.* 1993). Moreover, the activity given by a similar type of enhancer fragment of HPV16 (nucleotide 7675-7755) having two NF1 sites, TEF and NFA motifs, was only 2.2% relative to that of parent vector in HeLa cells (Chong *et al.* 1990). In this study, the activity of the protected region II having an imperfect NF1

palindrome, homologous NFA motif and an NF1 half-site was 9% after subtracting the activity of negative control (parent vector).

Moreover, the activity exhibited by the protected region II in RA-differentiated cells can be indirectly compared with the activity of same sequences of HPV11 in keratinocytes as shown by Dollard *et al.* (1993). A tetramerized fragment corresponding to the sequences of protected region II displayed 60% CAT activity relative to that of wild type CEI (approximately 30% CAT conversion) in primary keratinocytes (Dollard *et al.* 1993). The activity shown in primary keratinocytes might be still less if a monomer was used.

The activity given by protected region II in RA-differentiated cells was 90-fold higher compared to its residual activity in UD cells. Hence it is possible that the cellular factors responsible for the *in vivo* activity of protected region II (pKS3) were induced by retinoic acid in P19 cells. The RA differentiation-dependent activity of protected region II sequences was further correlated with the binding of RA-induced cellular factor(s).

The sequences of protected region II were found to interact with specific cellular factor(s) induced by RA-differentiation. This was detected as an extra DNA-protein complex in the gel retardation assay. Hence the RA differentiation-dependent activity of the deletion mutant pKS3

appears to be due to the interaction of RA-induced factors in a sequence-specific manner.

The possibility of interactions of cellular factors with the sequence containing NF1 half-site TTGGC was ruled out since the oligonucleotide corresponding to this motif of HPV11 MCE did not compete for the extra DNA-protein complex formed by the protected region II. Since the imperfect NF1 palindrome located in the protected region II only abolished complex formation, the factors induced upon RA-differentiation appear to bind strongly to the imperfect NF1 palindrome.

The factors that interact with the imperfect NF1 palindrome are specific for HPV11 since JC virus perfect NF1 palindrome and its mutant could not compete for the extra DNA-protein complex formed with the RA-differentiated nuclear extracts. This suggests that the cellular factors induced by RA-differentiation bind the perfect and imperfect NF1 palindromic sequences differentially. This is quite informative because factors binding the NF1 sequences show different binding specificities (Chodosh *et al.* 1988a; Chodosh *et al.* 1988b). Moreover, recent studies have shown that the factors in RA-differentiated P19 cells, binding the imperfect and perfect NF1 palindromes of JC virus, are probably different (Kumar *et al.* 1993). This further confirms the existence of a family of NF1 proteins that exhibit different binding specificities.

The oligonucleotide having the NF1 half-site and homologous NFA motif did not abolish the formation of the extra DNA-protein complex when RA-differentiated P19 EC cell nuclear extracts were used in the gel retardation assay (Fig.9). But some of the nucleotides corresponding to the NFA motif were found protected in the DNaseI protection assay (Fig.7). These different findings regarding the interactions of cellular factors with the NFA motif in the *in vitro* assays can be explained as follows. DNaseI protection assay is a more direct way of showing sequence-specific interactions (Hennighausen and Luban, 1987). Moreover the cellular factors interacting with the NFA motif may be relatively low in abundance and whose binding may be detectable by DNaseI protection assay rather than by the gel retardation assay. This could be due to a higher concentration of DNA and protein used in the DNaseI protection assay compared to the gel retardation assay. Alternatively, cooperative binding of factors to the imperfect NF1 palindrome and NFA is possible and this cannot be addressed by the type of competitions performed in this gel retardation assay.

As the RA-differentiation-specific complex was effectively competed by the imperfect NF1 palindrome, UV cross-linking was carried out in order to confirm this observation and to find out the size of the protein(s). Six different proteins with molecular weights ranging from 35-60kd

were crosslinked with the NF1 oligonucleotide. Three of them of 56, 46 and 35 kd were observed exclusively with RA-differentiated nuclear extracts. Hence further studies are required to determine the functional importance of these proteins compared to the common proteins of UD and RA-differentiated cells. Moreover, further studies should be done to find out which of the three proteins bind to the NF1 motif, GCCAA and its 3′ flanking nucleotides and how important this is in terms of the *in vivo* activity of the deletion mutant, pKS1.

Further studies are to be done to investigate the roles of the different identified proteins for the expression of protected region II in P19 EC cells.

FUTURE STUDIES

The results of this study suggest the importance of imperfect NF1 palidromic sequences in the activation of the protected region II in P19 RA-differentiated cells. It has to be precisely studied whether NFA motif has a role in the activity of protected region II. The functional significance of the motifs, TGG, C/EBP motif, NF1 motif GCCAA and NFA motif will be clearly established only after mutating them by site-directed mutagenesis and testing the mutants for their *in vivo* activity. Mutating the NFA motif would definitely confirm whether the protein interacting with GCCAA motif extends its contacts into the nucleotides of the NFA motif or a different factor interacts with NFA motif.

The precise binding site among the observed factor binding motifs of the imperfect NF1 palindrome important for functional activity can be determined only after testing the site-directed mutants for their activities. Using UV-cross-linking, it will be known which one of the proteins out of the three identified RA-differentiated proteins, binds the functional protein binding site. Once it is clearly established, the isolation of protein and subsequent cloning of the factor can be achieved.

Using the factor binding site, the cDNA encoding that factor can be isolated from RA-differentiated cDNA library

employing southwestern blotting assay. If the factor interacting with NFA to be cloned is of interest, the isolated factor can be compared with known proteins of Oct family since NFA shares homologies with the octamer binding sequences. If the factor to be isolated is the one which interacts with the GCCAA motif, then the isolated factor has to be compared with known members of NF1 family. If it is a member of NF1 family, using the cDNA encoding NF1, complementation assays can be performed in order to know whether it can transactivate not only HPV11 NF1 binding sites but also NF1 sites of other HPVs in RA-differentiated and undifferentiated P19 cells. In a similar fashion, one can study whether the cloned NF1 can complement the activity of HPV11 enhancer in epithelial cells, particularly of primary nature.

Using squelching experiments with the cloned cDNA, one can study the existence of a coactivator bridging the factors binding the NF1 sequences of protected region II in P19 RA-differentiated cells.

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