CIS AND TRANS-REGULATION OF HUMAN PAPILLOMAVIRUS TYPES 16, 18, AND 11 GENE EXPRESSION

TRUDY MARSHALL

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BY

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ABSTRACT

A comparative study was made of the regulatory elements enhancers/promoters) of human papillomavirus (HPV) types 16, 18, and 11. This was accomplished by inserting the noncoding region (ncr) fragment of each viral type immediately upstream of the chloramphenicol acetyltransferase (CAT) gene in the pSV0-CAT plasmid to test for promoter function and into the enhancerless pA10 CAT plasmid to test for enhancer function. In this manner, the noncoding regions of HPV 16, 18, and 11 were shown to contain enhancer and/or promoter function. In addition, the constitutive enhancers of HPV 16, 18, and 11 were localized to 315 bp, 230 bp, and 213 bp fragments, respectively. Comparison of the DNA sequence homologies of the viral enhancers indicates that the HPV 16 and 18 enhancer elements are closer in sequence homology than either HPV 16 or 18 is to the HPV 11 enhancer. Enhancer activity of the ncr of these viruses was tested for expression in several cervical and noncervical cell lines. The sequence relatedness of these HPV ncrs corresponds to the differences in cell specificities observed among the HPV 16, 18, and 11 enhancer elements. HPV 16 and 18 enhancer activity is restricted to cervical epithelial cells, whereas the HPV 11 enhancer appears to be active in a wider range of cell lines. The conditional enhancer

activity of the ncr of these viruses is increased in trans by the E2 gene product of HPV 16 and is repressed by the E7 gene product of HPV 16., Trans-activation by E2 is mediated through the E2 binding motif on HPV enhancer plasmids with a heterologous but not with a homologous promoter.

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CHAPTER 1

INTRODUCTON

Papillomaviruses are small double-stranded DNA viruses which cause benign epithelial tumors or warts in humans and in a variety of animal species (zur Hausen, 1977; Lancaster and Olson, 1982). A subgroup of the human papillomaviruses (HPV types 6 and 11) are associated with benign lesions of external genitalia while the HPV types 16, 18, 31, 33, and 35 are known to be associated with cervical lesions which can progress to malignant carcinomas. This recent correlation between HPV types 16 and 18 and human cervical carcinomas has spurred a great deal of interest to study the molecular biology of these viruses. The initial step in studying the molecular genetics of these viruses was achieved by cloning and sequencing DNA isolated from various papillomatous tissues. Subsequent investigations have concentrated mainly on the function and regulation of viral genes. In this thesis, I have made a comparative study of the regulatory elements (enhancers/promoters) found in the noncoding regions of HPV 16, 18, and 11. Special emphasis has been placed on the cell type specificity of the HPV enhancer elements. This work also deals with the regulation of enhancer and promoter elements in HPV 16, 18, and 11 by certain viral (HPV 16) gene

products.

1.1 Genome organization of Papillomaviruses

Papillomaviruses are classified as members of the papovavirus family along with the mouse polyomavirus, the monkey simian vacuolating virus 40 (SV40), and the human BK and JC viruses (Melnick, 1962; Melnick et al, 1974). The genomes of papillomaviruses consist of closed circular double-stranded DNA molecules which are considerably larger than that of polyomaviruses (approximately 7900 bp versus approximately 5250 bp). Furthermore, the genomes of the papillomavirus and SV40/polyomavirus groups bear virtually no similarity in their genetic organization or sequence. In the past, papillomaviruses have received little attention in terms of molecular virology due to lack of host cells which will support the propagation of these viruses in culture. Using recombinant DNA technology, we are beginning to understand how the papillomavirus genome is organized, expressed, and regulated.

DNA sequence data of ten different papillomaviruses have shown a notably uniform organization of the viral genome. Most of the major open reading frames (ORFs; putative protein coding sequences) are located in similar positions and all are located on a single strand of viral DNA in the same direction. Until recently, functional analysis of papillomavirus

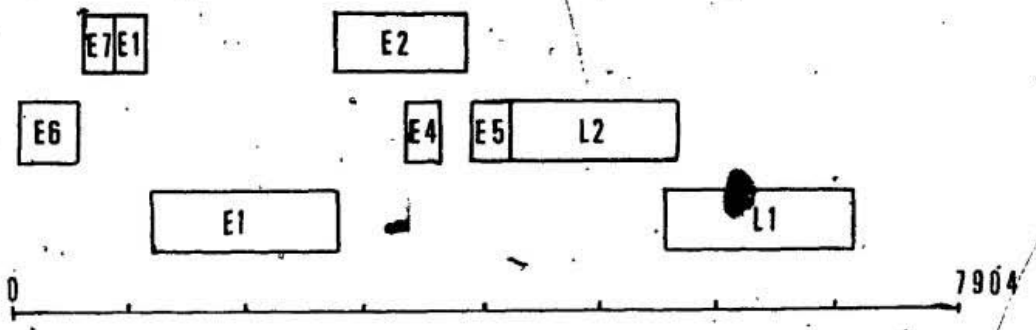
genes was largely restricted to the study of bovine papillomavirus type 1 (BPV 1)-encoded transcripts from transformed rodent cells and from productively infected cells of the viral-induced fibropapillomas in cattle (Baker and Howley, 1987; Engel et al, 1983; Heilman et al, 1982; Nakabayashi et al, 1983). Viral genes involved in cell transformation and plasmid replication have been mapped to a BPV 1 fragment encompassing 69 % of the viral genome. This early or E region of BPV 1 contains eight ORFs, namely E1 to E8 (Danos et al, 1983). The remaining 31 % of the viral genome, referred to as the late region, contain two large ORFs, L1 and L2, which code for the major structural polypeptides of the virions (Orth and Favre, 1985; Pilacinski et al, 1984). Five early ORFs (E1, E2, E4, E6, and E7) and the two late ORFs appear to have equivalent counterparts in all papillomaviruses sequenced to date.

On the basis of their sequence homologies and differences in genome organization, the papillomaviruses have been classified into three families displaying tropisms mostly for human genitals (eg. HPV 6, 11, 16, and 18), skin (eg. cottontail rabbit papillomavirus [CRPV] and, HPV 1 and 8), and animal fibroblasts (eg. BPV 1 and the deer papillomavirus; Cole and Danos, 1987). No gross differences in the overall genome organization were observed among the genital HPVs. Comparison of the genome organization (see Figure 1) of HPV 16 (Seedorf et al, 1985) to that of HPV 18 (Cole and Danos,

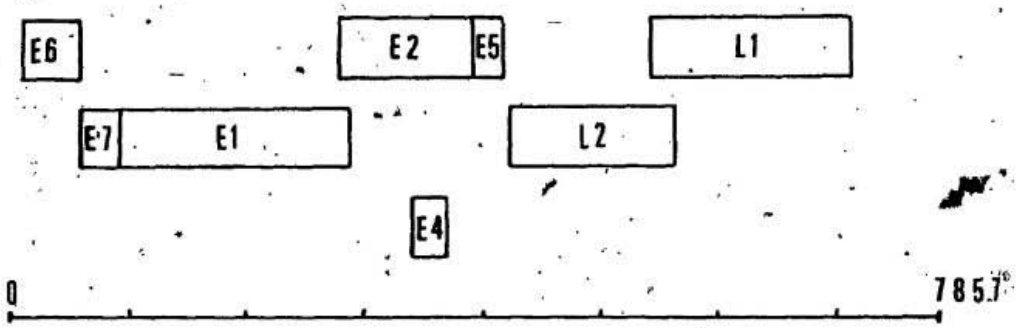
Figure 1. Comparison of the genome organization of HPV 16, 18, and 11.

This is a linear map of the circular HPV genome. The map is arranged in a standard format with the noncoding region on the right and the early region on the left. The early (E) and late (L) region open reading frames (ORFs; putative protein coding sequences), shown for each reading frame, are represented by open boxes. These ORFs were identified by comparing HPV 16, 18, and 11 sequences with other papillomavirus sequences (Cole and Danos, 1987; Dartmann et al, 1986; Seedorf et al, 1985). The scale, in kilobases, is indicated by marks on the genome maps and the total length (in base pairs) is shown on the right.

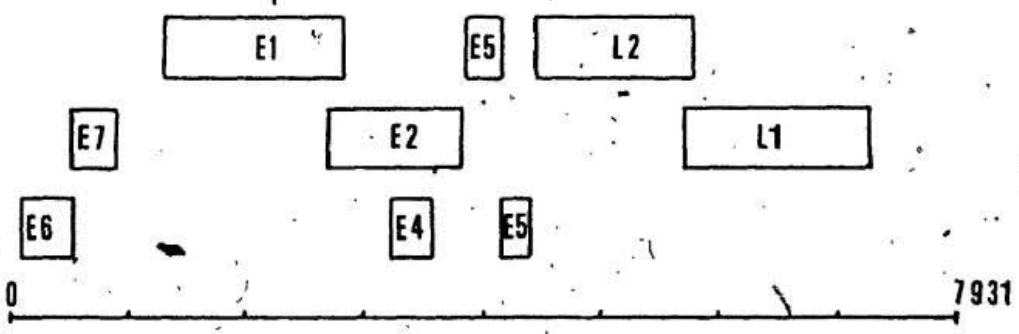
HPV 16
open reading frames



HPV 18
open reading frames



HPV 11
open reading frames



1987) and HPV 11 (Dartmann et al, 1986) reveals that the E1 ORF of HPV 16 is split into two ORFs, each of which has homology to portions of the E1 ORFs of the other papillomaviruses. This clone of HPV 16 was isolated from a genomic library of an invasive cervical carcinoma, thus indicating that this DNA may have been mutated before or during its integration into the host chromosome(s) (Durst et al, 1983, 1987b). Analysis of HPV 16 DNA isolated from other cervical tumor cells revealed an extra guanine at nucleotide 1138 indicating that in HPV 16 from most sources the E1 ORF is intact (Baker et al, 1987; Matsukura et al, 1986). This suggests that the genome organization of HPV 16 (i.e. the E1 ORF) is similar to the other human genital papillomaviruses. It would seem that differences in tissue tropism, pathogenicity, and host range of these genital papillomaviruses may involve changes in their regulatory sequences or subtle alteration in the coding region sequences; both of which may modify viral gene expression.

1.2 Control regions of Papillomaviruses

All of the ten papillomaviruses sequenced to date have a single (from 454 bp for HPV 8 to 979 bp for HPV 1) noncoding region (ncr) extending between the stop codon of L1 and the first ATG codon of E6. At the late region end of this noncoding region there is a purine + thymidine-rich

region (G + T-rich for genital papillomaviruses; A + G-rich for skin papillomaviruses and fibropapillomaviruses) of variable length (Cole and Danos, 1987). This segment contains, in all papillomaviruses, the polyadenylation signal for transcripts from the late region (Amtmann and Sauer, 1982; Chow et al, 1987a; 1987b; Nasseri and Wettstein, 1984). In BPV 1, this region has been shown to harbor an origin of replication referred to as the plasmid maintenance sequences or PMS-1 (Lusky and Botchan, 1984; Waldeck et al, 1984) and a promoter for the transcription of the late genes (Baker and Howley, 1987). The early region proximal segment of the noncoding region contains several repeats of the papillomavirus-specific sequence ($ACCGN_4CGGT$) positioned between (for genital papillomaviruses) and immediately upstream (for all papillomaviruses) of the typical polymerase promoter elements (TATA and CAAT-like boxes). In human genital papillomaviruses the middle segment of the ncr (upstream of $ACCGN_4CGGT$) contains a variable sequence of about 200 bp long (Cole and Danos, 1987). Cole and Streek (1986) have reported a 78 bp duplication in this region of HPV 33. Due to the size, position, and arrangement of the 78 bp repeats in the HPV 33 genome, these authors suggest that the repeats may function as enhancers of viral gene expression. Indeed, enhancer activity has been localized to this region in several human genital papillomaviruses (Chow et al, 1987c; Cripe et al, 1987; Rando et al, 1986a; Swift et al, 1987; Thierry and Yaniv,

1987; Thierry et al, 1987a). Immediately upstream of this enhancer region is another repeat of the papillomavirus-specific sequence, $ACCGN_4CGGT$; these highly conserved motifs are known to be involved in the transcriptional control of papillomaviruses (Haugen et al, 1987; Hirochika et al, 1988; Spalholz et al, 1987). Although the early region proximal portion of the noncoding region is highly conserved (especially among genital papillomaviruses), homology studies have shown that the ncr is one of the most variable regions even among related papillomaviruses. The possible association between tissue tropism and these cis-acting regulatory elements (i.e. enhancers) will be dicussed in later sections.

1.3 Papillomavirus protein function

The BPV 1 transformed mouse C127 and NIH 3T3 cells have been useful in defining the functional organization of the viral genome. For instance, cell transformation and plasmid replication functions have been mapped to certain early ORFs by in vitro dissection and mutagenesis of BPV 1 DNA. Two separate transforming genes, E5 and E6, were identified and their protein products characterized (Androphy et al, 1985; Burkhardt et al, 1987; DiMaio et al, 1986; Nakabayashi et al, 1983; Sarver et al, 1984; Schiller et al, 1984, 1986; Yang et al, 1985a). Similar studies have indicated that the E1 ORF of BPV 1 is essential for plasmid replication and maintenance (Lusky

and Botchan, 1985, 1986). In addition, the integrity of the E2 ORF was shown to be required for stable plasmid maintenance and for efficient transformation (Lusky and Botchan, 1985; Rabson et al, 1986; Sarver et al, 1984). Spalholz et al (1985) recently demonstrated that the full-length BPV 1 E2 ORF encodes a transcriptional trans-activator that is involved in viral gene expression. This indicates that the role of the E2 gene product in plasmid maintenance and transformation is indirect; activation of the conditional enhancer element ($ACCGN_4CGGT$) leads to increased expression of critical viral replication and transformation genes (Rabson et al, 1986; Yang et al, 1985b). Interestingly, the carboxyterminal portion of the E2 ORF product has been shown to possess a repressor function (Lambert et al, 1987; Cripe et al, 1987). A plasmid expressing the putative repressor region inhibits cellular transformation by BPV 1 DNA (Lambert et al, 1987). The authors suggest that this inhibition results from E2 repressor mediated down-regulation of viral gene expression. No identifiable function has been assigned to the E3, E4 or E8 ORFs using this in vitro transformation system (Neary et al, 1987 ; Schiller et al, 1986).

Similarities between papillomavirus genome size and organization have fostered the tendency to ascribe properties associated with a particular papillomavirus (i.e. BPV 1) to all viruses. In some situations the human and animal papillomavirus ORF products seem to share functional

homology. For instance, recent studies have assigned transforming and trans-regulating functions to products from the HPV 16 and 18 E6 and E2 ORFs (Bedell et al, 1987; Cripe et al, 1987; Matlashewski et al, 1987). These functions are similar to those identified for the analagous BPV 1 encoded proteins (Spalholz et al, 1985, 1987; Yang et al, 1985a). Alternatively, the E5 ORF, although important for transformation by BPV 1, is not homologous to the corresponding regions of other papillomaviruses except HPV 6b. Indeed, some papillomaviruses do not have an equivalent to the BPV 1 E5 ORF and for those that do it is not known if the E5 ORFs of these papillomaviruses encode protein products. Regulation of gene expression in both human and animal papillomaviruses appears to involve: differential splicing, selection of one of the two polyadenylation sites, and/or differential selection of transcriptional start sites (promoters). Preliminary studies suggest that this differential mRNA transcription and processing vary among these papillomaviruses. Therefore, further RNA and protein characterization studies are required to fully understand the life cycle of this medically important group of viruses.

1.4 Papillomaviruses and diseases

Papillomaviruses have been identified in warts from many animal species. Animal papillomaviruses induce three types of

lesions: (1) papillomas, which are benign tumors derived from the epithelium; (2) fibropapillomas, which contain in addition to epithelial-derived cells, fibrous connective tissue; and (3) fibromas, which consist mainly of fibrous connective tissue containing proliferating fibroblasts (Green, 1985; Lancaster and Olson, 1982). Three animal papillomaviruses have been shown to be oncogenic in their natural host. These include the above mentioned cottontail rabbit papillomavirus along with the bovine papillomavirus type 4 (BPV 4) and the multimammate mouse papillomavirus (MnPV). The cottontail rabbit (Shope) papillomavirus (CRPV) was the first oncogenic DNA virus to be isolated and characterized (Shope and Hurst, 1933). Shope's subsequent investigations provided a new and fascinating model of a naturally occurring virus-induced neoplasm (Shope, 1935) in which tumors either persisted as benign papillomas, regressed completely, or progressed to metastasizing carcinomas (see Kreider et al, 1980). Kidd and Rous (1940) also provided evidence for the development of carcinomas under natural conditions in cottontail rabbits. In 1978, Jarrett and his colleagues reported the development of BPV 4-induced alimentary tract and urinary bladder cancer in cattle. In addition, Muller and Gissmann (1978) reported that MnPV, a papillomavirus endemic in the multimammmate mouse is associated with skin tumor formation.

Few papillomaviruses can cross the host barrier. However, several papillomaviruses are capable of inducing fibroblastic

tumors in hamsters and of transforming the rodent tissue culture cell lines C127 and NIH 3T3. This subgroup includes the bovine papillomaviruses BPV 1 and BPV 2, the sheep papillomavirus, and the deer papillomavirus.

About 50 types of human papillomaviruses (HPVs) have been molecularly cloned from epithelial lesions. A new type is defined as a virus isolate exhibiting less than 50 % DNA homology with known HPV types, as determined by DNA-DNA hybridization under stringent conditions in liquid phase (Coggin and zur Hausen, 1979). Also, for many of these types a specific pattern of macroscopic and microscopic lesions has been established.

All human papillomaviruses described to date induce epithelial proliferations or warts. The clinical significance of warts is determined mostly by their location, the infecting virus type, and by factors relating to the host. A wart on the skin is most often a benign self-limiting tumor which regresses after a period of time. Warts may occur at any location on the skin, but certain sites are favored. Warts morphology is also variable. Although the correlation between wart morphology and infecting virus type is not clear, preferential association of HPV 1 with deep plantar warts, HPV 2 with common warts, and HPV 3 with flat skin warts has been demonstrated (Pfister et al, 1980; Shah, 1985).

It is generally believed that infection with some of the virus types, in combination with host and environmental

factors may lead to malignancy. Presence of HPV DNA, RNA, and/or protein in these lesions has lead to the belief that there is a correlation between papillomavirus infection and malignant carcinomas. One such clinical condition is the familial skin disorder epidermodysplasia verruciformis (EV). EV is a genetic disease characterized by disseminated, persistent skin warts arising during childhood and by a high risk for developing skin cancer later in life. The development of skin warts into cancerous lesions is known to require sunlight (ultraviolet light) as a cofactor. HPV types 5 and 8 and rarely other types have been reported to be associated with this squamous carcinoma of the skin, although patients with EV commonly reveal infections with up to 15 additional types of papillomaviruses (Kremsdorf et al, 1982, 1983; Orth et al, 1979, 1980; Ostrow et al, 1982; Pfister et al, 1981,1983; Yutsudo et al, 1985). Cancers arising from this condition represent an interesting example of interactions between a specific virus infection and physical carcinogens such as ultraviolet light (reviewed by zur Hausen, 1987).

HPV antigens and/or HPV DNA have been demonstrated in a high proportion of benign (condylomas), premalignant and malignant tumors of the cervix. This suggests that these viruses may play a pivotal role in the pathogenesis of cervical neoplasia (Boshart et al, 1984; Durst et al, 1983; Gissmann et al, 1983; Lancaster et al, 1983). Subsequently, it has been shown that specific HPV types were associated with benign

and malignant lesions, raising the possibility that the virus type may be an important factor in malignant conversion. HPV types 16 and 18 are present in 70 to 80 % of invasive squamous cancers of the cervix, vulva, and penis and in the higher grades of cervical intraepithelial neoplasias (CIN 3; Boshart et al, 1984; Durst et al, 1983; Pater et al, 1986). In contrast, HPV types 6 and 11 are more often associated with benign genital warts (Gissmann et al, 1983) and also with laryngeal (Gissmann et al, 1982, 1983) and nasal inverting papillomas (Respler et al, 1987). The condylomas usually remain static or regress whereas the higher grade CIN lesions virtually never regress and a certain proportion will evolve into invasive lesions.

Studies of cells that carry the HPV DNA are revealing how the viruses may contribute to cancer development. The physical state of HPV DNA in genital lesions has been examined by Southern blot hyridization. In condylomas, HPV 6 and 11 DNAs are present extrachromosomally, whereas, in carcinomas, HPV 16 and 18 DNA are frequently found covalently associated with the host cellular DNA. This suggests that viral DNA has been integrated into host DNA. HPV 16 and 18 DNAs have been found integrated into the chromosomal DNA from several cervical cell lines (Boshart et al, 1984; Pater and Pater, 1985; Schwarz et al, 1985; Yee et al, 1985). Integration may be one of the steps on the road to malignancy. This is supported by observations that HPV DNA

exists extrachromosomally in precancerous cervical cells showing early signs of abnormality (Crum et al, 1984; Durst et al, 1985; Pater et al, 1986).

Integration appears to occur at random sites in the host genome. However, in the cervical cell lines HeLa and C4-I, papillomavirus DNA is integrated in regions of the human genome that are near the cellular protooncogene c-myc (Durst et al, 1987a). In both of these cell lines, c-myc expression is elevated relative to that in SiHa and SW756 cells, which do not exhibit integration of papillomavirus DNA in the vacinity of c-myc. Furthermore, integration of the HPV enhancer into host chromosomes has the ability to alter the expression of the adjacent cellular gene pap-1 (Swift et al, 1987). The relationship of this elevated gene expression and progression of HPV-associated genital tumors to malignancy is unclear.

Examination of papillomavirus DNA integration patterns has consistently shown disruption of the early region thereby joining the ORFs E6, E7, and E1 to downstream host cell sequences (Schwarz et al, 1985). HPV 16 and 18 RNA transcripts have been detected in both tumors and tumor cell lines (Baker et al, 1987; Schneider-Gadicke and Schwarz, 1986; Schwarz et al, 1985; Smotkin and Wettstein, 1986; Yee et al, 1985). Schneider-Gadicke and Schwarz (1986) have reported that most or even all of the HPV 18-positive mRNAs transcribed in HPV 18-containing carcinoma cells are differentially spliced hybrid transcripts that are composed of

5'-terminal viral sequences (E6, E7, and/or E1) and 3'-terminal host cell sequences. The 3'-terminal host sequences are different for each cell line. This suggests that these particular host sequences may not play a role in tumorigenesis; instead their role may be to stabilize the mRNAs coding for the early region viral proteins. These viral proteins may be required for the maintenance of the malignant phenotype. For HPV 16 and 18, splicing generates a new ORF, designated E6* (Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986); HPV 6 and 11, however, do not contain the required splice donor and acceptor sites (Schneider-Gadicke and Schwarz, 1986). HPV 18 E6* polypeptides have been detected in HeLa cell extracts by monoclonal antibodies (Sneider-Gadicke, Kaul, Gausepohl, Schwarz, Frank, and Bastert, in abstracts of papers presented at the Sixth International Papillomavirus Workshop, 1987). It would seem that this protein may be at least partially responsible for the differential pattern of pathogenicity observed by the two genital papillomavirus groups (HPV 6/11 versus HPV 16/18).

Interestingly, the E7 gene product is the most abundant protein in cell lines containing HPV 16 and 18 DNA (Seedorf et al, 1987; Smotkin and Wettstein, 1986). Also reported by Seedorf et al (1987) was the presence of the E6 and E4 gene products in HPV 16-containing cells and the presence of the E1-encoded gene product in HPV 18-containing cells. The E7 protein of BPV 1 is involved in the maintenance of plasmids

at high copy number while the BPV 1 carboxyterminal E1 ORF product is required for episomal replication in transformed mouse cells (Lusky and Botchan, 1985, 1986); the BPV 1 amino terminal E1 ORF encodes a factor which acts as a modulator of viral DNA replication (Lusky and Botchan, 1986; Roberts and Weintraub, 1986). Some of these viral proteins may not be required for initiation or maintenance of transformation. However, the localization of the transforming function of HPV 16 and 18 to proteins from the E6/E7 region of the integrated papillomavirus genome (Bedell et al, 1987; Matlashewski et al, 1987), does indicate a role of the HPV E6 and E7 proteins in transformation.

The E6 and E7 protein sequences are poorly conserved among papillomaviruses. This may account for some or all of the observed variability in the oncogenic potential of the HPVs. However, the E6/E7 gene products do contain regularly spaced (Cys-X-X-Cys) cysteine doublets, similar to repeated units described in a number of eukaryotic nucleic acid binding proteins (Berg et al, 1986). Thus an attractive possibility is that the E6 and E7 ORFs encode nucleic acid binding proteins. Specific binding of the E6/E7 ORF gene products to chromosomal DNA may be a mechanism by which HPV mediated transformation occurs. Understanding the regulation of viral gene expression and the regulatory roles played by these viral proteins may shed light on the molecular mechanisms of carcinogenesis by these viruses.

1.5 Transcriptional regulation of gene expression.

Defining transcriptional regulatory signals represents an important step toward our understanding of eukaryotic gene expression. The transcriptional regulatory signals generally include (1) promoters, enhancers, and negative regulatory elements at the DNA level, and (2) trans-acting factors which interact with these DNA sequences to produce a negative and positive effect. A promoter is generally comprised of cis-acting sequences like the Goldberg/Hogness or TATA box ($TATA^{A}_{T}A$) and one or more upstream promoter element (UPE) such as the CAAT box ($GG^{C}_{T}CAATCT$) or the GC box (GGGCGG). The TATA box and the UPEs are generally required for accurate and efficient initiation of transcription (Sassone-Corsi and Borrelli, 1986).

Enhancers were originally identified in the DNA tumor virus SV40 (Banerji et al, 1981; Moreau et al, 1981). These cis-acting DNA elements dramatically stimulate transcription from either, homologous or heterologous promoters in an orientation-independent manner (reviewed by Sassone-Corsi and Borrelli, 1986; Serfling et al, 1985a). However, the distinction between enhancers and UPEs is not always clear-cut with examples of each type behaving interchangeably. For example, the immediate upstream region of the mouse metallothionein gene which, when detached from its TATA box and linked to a test gene, can act as an inducible enhancer

(Serfling et al, 1985b). In this thesis, a DNA fragment which stimulates transcription from a linked promoter is referred to as an enhancer.

Negative regulatory elements (or silencers) are cis-acting DNA sequences identified upstream of several cellular and viral genes (Brand et al, 1985; Gorman et al, 1985; Nelson et al, 1987; Rosen et al, 1985). Although not fully characterized, some of these negative regulatory elements have been reported to repress gene expression in an orientation- and distance-independent manner (Baniahmad et al, 1987; Laimins et al, 1986; Remmers et al, 1986).

Although enhancers were identified seven years ago (Banerji et al, 1981), the mechanism by which they activate transcription is still unclear. There is no extensive homology among these elements, however a number of short individual sequence motifs or core sequences have been identified (and often repeated) in a number of enhancers. A plausible belief is that the interaction of cellular trans-acting protein factors with the core sequences is central to enhancer function. In fact, several lines of evidence indicate such protein/DNA interaction. In vivo competition experiments performed with the SV40 enhancer, revealed that increasing amounts of the SV40 enhancer (pSV2-NEO; SV40 enhancer linked to the NEO gene) decreased transcription from pSV2-CAT (SV40 enhancer linked to the chloramphenicol acetyltransferase [CAT] gene) indicating the presence of a limiting amount of positive trans-

acting factors (Mercola et al, 1985; Sassone-Corsi et al, 1985; Scholer and Gruss, 1984; Wildeman et al, 1984). Similar in vivo experiments have indicated the presence of a negative regulatory factor(s) in undifferentiated EC cells which binds to viral enhancers (SV40 and MSV) and negatively regulates gene expression (Gorman et al, 1985). In addition, in vitro experiments such as DNaseI footprinting, gel retardation, dimethyl sulfate protection, and exonuclease protection assays have provided evidence for such interactions. Further results from competition experiments suggest that the SV40 enhancer (comprised of many core sequences) and factors that bind to it, seem to be involved in the establishment of stable transcriptional complexes (Wang and Calame, 1986). A requirement of stereospecific alignments between the enhancer, UPEs and the TATA box for initiation from the SV40 early promoter has been shown (Takahashi et al, 1986). This suggests that initiation of transcription may require the formation of protein/protein interactions involving the DNA sequences from the enhancer and promoter region (see below). Mark Ptashne, in an excellent review based on his work, attempted to explain several models put forth to elucidate the mechanism of enhancer function in transcriptional regulation. In one such model (looping) Ptashne maintains that proteins bound at widely separated sites act to regulate transcription by contacting one another, with the intervening DNA looping or bending to allow the protein/protein interaction. If looping

proves to be a general phenomenon (discussed mainly for λ repressor binding), positive and negative control might occur by proteins binding to the complex or loop, thereby increasing or decreasing the efficiency of polymerase II interaction with the promoter (Ptashne, 1986).

The SV40 enhancer has been shown to act in a wide variety of tissues and hosts. Many other viral and cellular enhancers show a strict host and cell-type specificity. The immunoglobulin heavy chain (IgH) enhancer was the first genetic element identified to be expressed in a tissue specific manner (Banerji et al, 1983; Gilles et al, 1983). Tissue-specific expression of cellular genes has been proposed to result from interactions between cell-specific trans-acting factors with the enhancer elements. For instance, a B cell-specific protein factor which binds to the octamer motif of the immunoglobulin heavy chain gene enhancer, has been implicated in the tissue-specific regulation of immunoglobulin genes (Rosales et al, 1987). Other studies suggest that immunoglobulin promoter and intragenic sequences are also capable of mediating cell-specific expression, independent of enhancer sequences (Calame, 1985; Foster et al, 1985; Grosschedl and Baltimore, 1985). In fact, a sequence identical to the enhancer - octamer sequence (ATGCAAT) is found upstream of the RNA start site of the immunoglobulin heavy and light chain genes. Earlier reports identified more species of factors that recognize these octamer motifs. One factor is present in all cells whereas the

other has been found only in B and T lymphocytes (Landolf et al, 1986; Staudt et al, 1986). Interestingly, Imler et al (1986) have shown that tissue-specific expression of the immunoglobulin gene is at least in part controlled by negative regulation in nonlymphoid cells. The situation observed for the regulation of immunoglobulin cell-specific expression supports the modular mechanism of tissue-specific gene expression. In this model, general and/or cell specific factors interact with ubiquitous core sequences or cell-specific motifs and in effect positively or negatively regulate gene expression leading to tissue-specific transcription (reviewed by Voss et al, 1986). The cell-specific factors mentioned above may be confined to cells of a specific tissue or developmental stage. Alternatively, as observed with the kappa chain gene enhancer, these factors may be present in many cell types in an inactive form and have to be modified in some way (e.g. phosphorylation) before they can bind specifically to the tissue-specific enhancer (reviewed by Dynan, 1987; Maniatis et al, 1987).

1.6 Statement on the research problem

Human papillomavirus types 16 and 18 are associated with the majority (70 to 80 %) of tumor and tumor cells of the cervix, whereas HPV 11 is associated predominantly with benign anogenital warts. This strongly suggests a differential role for these viruses in the development of malignancy.

However, because of the lack of a tissue culture system that can support the replication of the HPVs, it is not well understood how the genomes of these viruses are expressed or regulated. It is, however, important to study the gene regulation of HPVs that are associated with carcinomas of the cervix in order to increase our understanding of the molecular mechanisms of carcinogenesis by these viruses.

This investigation of HPV 16, 18, and 11 transcriptional regulation was possible due to the availability of the pSV-CAT (the bacterial chloramphenicol acetyltransferase gene driven by the SV40 early promoter) expression vector system (Gorman et al, 1982). This technology provided a quick and simple assay of enhancer and promoter function. Transfection of the HPV noncoding region/CAT plasmids into cell lines derived from cervical carcinoma cells (i.e. C33A, HeLa, and SiHa) generated an in vitro system capable of providing valuable information on HPV gene regulation.

The objectives of this investigation were:

1. To initiate a comparative study of and to characterize the sequences necessary for enhancer and promoter function of HPV 16, 18, and 11.
2. To determine whether HPV enhancers and/or promoters are expressed in a cell-specific manner.
3. To further characterize the regulatory function associated with the early gene products of HPV 16 by studying how these gene products regulate the enhancers and promoters of

HPV 16, 18, and 11.

CHAPTER 2

MATERALS AND METHODS

2.1 Materials

Restriction endonucleases were obtained from New England Biolabs, Bethesda Research Laboratories (BRL) or Boehringer Mannheim. T4 DNA Ligase was purchased from BRL, Calf intestinal phosphatase (CP) from Boehringer-Mannheim, reverse transcriptase from Life Sciences, and S1 nuclease from BRL. The radioactive [^{14}C] chloramphenicol was purchased from Du Pont while the acetyl coenzyme A, lithium was obtained from Pharmacia P-L Biochemicals. Flow Laboratories supplied the tissue culture medium (Dulbecco's Modified Media; DME), penicillin-streptomycin (Pen-Strep), phosphate buffered saline (PBS) and the trypsin-EDTA, while Bocknek Laboratories supplied the fetal bovine serum (FBS). The T4 ligase 5x premix and some of the restriction endonuclease (10x REact) buffers were obtained from BRL. The remaining restriction enzyme buffers and the reverse transcriptase premix were made according to the recipe from the company. The CIP 10x premix was made according to Maniatis (1982). The 10x S1 nuclease premix contained 50 mM NaOAc (pH 4.5), 150 mM NaCl, 3 mM $ZnCl_2$ (Hiroshi Hamada, personal communication).

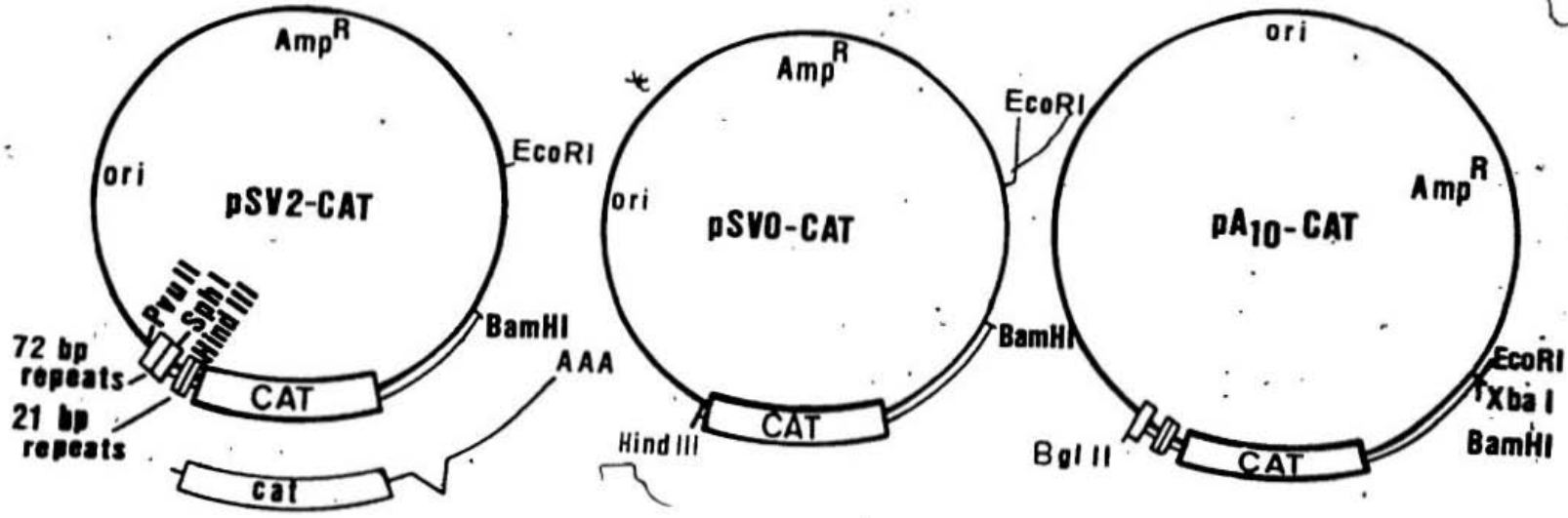
The plasmids pSV2-CAT,pSV0-CAT, and pA10-CAT were kindly provided by B. Howard while the HPV 11, 16, and 18 plasmids were obtained from L. Gissmann and H. zur Hausen.

2.2 Recombinant vectors

Plasmid pSV2-CAT (Gorman et al, 1982; Figure 2), containing SV40 regulatory sequences (promoter and enhancer) which control the expression of the linked bacterial chloramphenicol acetyltransferase [CAT] gene, was used as the positive control. The negative controls were pSV0-CAT and pA10-CAT; these plasmids are the vectors from which all HPV/CAT constructs were derived. The pSV0-CAT plasmid is derived from pSV2-CAT by the removal of the entire enhancer and promoter region (the PvuII/HindIII fragment of pSV2-CAT, Figure 2) of SV40 (Gorman et al, 1982); whereas, pA10-CAT is missing the SV40 enhancer sequences but retains the SV40 promoter (Figure 2). The plasmid, pA10-CAT, containing the 21-bp repeated sequences and Goldberg-Hogness box coupled to CAT was generated by joining the SphI/BamH (CAT-containing) fragment of a pBR322 derivative plasmid with the SphI /BamHI fragment of pSV2-CAT (Laimins et al, 1982). The SphI site was not regenerated, therefore a BglII linker was inserted in this site for cloning purposes (Figure 2). The CAT gene in pA10-CAT will be active only if the inserted HPV DNA fragment contains an enhancer element since the SV40

Figure 2. Physical map of the CAT recombinant plasmids: pSV2-CAT, pSV0-CAT, and pA10-CAT.

pSV2-CAT contains the following functional elements (counterclockwise from 12 o'clock on the circular map): the Amp^R cistron, the origin of replication from pBR322, the SV40 early promoter region, the chloramphenicol acetyltransferase (CAT) gene sequences, the SV40 small t antigen intron, and the SV40 early region polyadenylation addition site. The SV40 regulatory region (PvuII/HindIII) is indicated as two boxes representing the 21 bp repeats and two larger boxes representing the 72 bp repeats, while the downstream SV40 sequences are represented by a double line. The plasmid, pSV0-CAT was constructed by deleting the SV40 early region (the PvuII/HindIII fragment) from pSV2-CAT and religating the plasmid after the addition of HindIII linkers (Gorman et al, 1982). The plasmid pA10-CAT, carrying the 21 bp repeated sequences was generated by joining the SphI/BamHI fragment from a pBR322 derivative plasmid with the SphI/BamHI fragment from pSV2-CAT after the addition of BglII linkers (Laimins et al, 1982).


Amp^R
ori
pSV2-CAT
EcoRI
Pvu II
Sph I
Hind III
BamHI
72 bp repeats
21 bp repeats
CAT
AAA
cat
Amp^R
ori
pSV0-CAT
EcoRI
BamHI
Hind III
CAT
ori
Amp^R
pA10-CAT
EcoRI
Xba I
BamHI
Bgl II
CAT

promoter is enhancer dependent. For assaying HPV promoter activity, the HPV-pSV0-CAT plasmids in reverse orientation were used as negative controls since pSV0-CAT gave high background in some cell lines. This high background, which has also been observed by others, is probably due to cryptic promoter activity from pBR322 sequences.

2.3 HPV noncoding region constructs

To analyze the potential transcriptional enhancer elements contained within the noncoding region (ncr) of HPV 16, 18 and 11, a large fragment corresponding to the ncr of each virus was cloned into the plasmid pA10-CAT. This vector has a single BglII site for insertion of fragments to test for enhancer activity (Figure 2). To screen for the papillomavirus promoter (along with the enhancer) sequences, the ncr fragments were also cloned into pSV0-CAT, which contains a single HindIII site for insertion of putative promoter fragments. The HPV 16, 18, and 11 ncr fragment were separated from the viral coding region and pBR322 DNA by cleavage with an appropriate restriction endonuclease followed by reverse transcriptase or S1 nuclease treatment. The reverse transcriptase enzyme was used to fill in the termini of DNA fragments with protruding 5' ends. Cleavage by particular restriction enzymes (i.e. PstI) created recessed 3' ends; S1 nuclease was used in these instances to remove the single

strand tails from the DNA fragments thus producing blunt ends. Next, the blunt-ended fragments were subjected to electrophoresis together with a marker for size determination. The appropriate size fragments were excised from the agarose (or polyacrylamide) gels and subsequently isolated by electroelution. The electroeluted DNA was applied onto DE52 columns, washed with 1 x TE (Tris-EDTA), eluted by 5. M NaCl, and recovered by ethanol precipitation. Vectors were prepared for ligation of the inserts as follows: plasmid vectors pA10-CAT and pSV0-CAT were digested with BglII and HindIII, respectively, treated with reverse transcriptase and subsequently with calf intestinal phosphatase (CIP). CIP treatment was required to remove the terminal 5' phosphates from the ends of the vector DNA such that the level of self-ligation is diminished. Ligation of the vectors and inserts was, in most cases, performed in a 15 μL reaction overnight at 16 C. The ligated DNA was then transfected into bacterial HB101 competent cells and plated on Ampicillin LB plates (Maniatis et al, 1982). The desired recombinant clone was selected by using a modification of the Birnboim and Doly (1979) method for small scale DNA preparation. Screening was accomplished by restriction endonuclease digestion of this plasmid DNA followed by analysis on agarose or acrylamide gels. Recombinant plasmids were propagated in HB101 cells using M9 minimal media. After overnight amplification in the presence of 170 μg of chloramphenicol per mL, plasmid DNA was

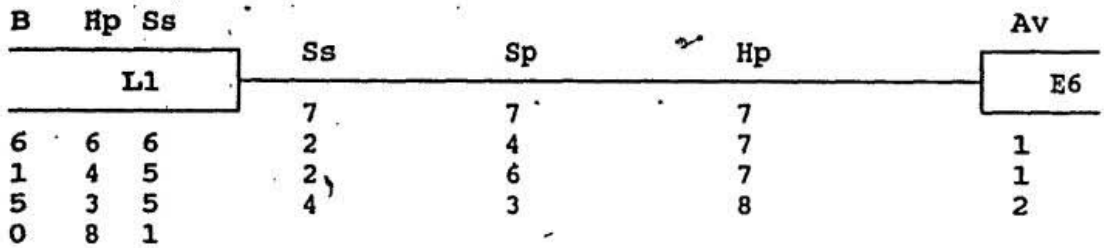
extracted using the lysis by alkali technique and purified by centrifigation to equilibrium in cesium chloride-ethidium bromide density gradients. These large scale plasmid preparation procedures are outlined in Maniatis et al (1982).

To screen for papillomavirus enhancer and promoter elements, large fragments containing the noncoding regions of HPV 16, 18, and 11 (Figure 3) were inserted into the CAT vectors, pA10-CAT and pSV0-CAT. The BamHI fragment (6929 to 119; Cole and Danos, 1987) of the HPV 18 noncoding region was S1 nuclease treated and inserted into the BglII site of pA10-CAT resulting in the constructs p8 (+; positive orientation relative to CAT) and p9 (-; negative orientation relative to CAT) and into the HindIII site of pSV0-CAT to generate p29 (+) and pT-13 (-). The HPV 11 ncr/CAT plasmids were constructed by inserting the S1 nuclease treated DraIII/SfaNI fragment (7486 to 114; Dartmann et al, 1986) into pA10-CAT to generate p6 (+) and p5 (-) and into pSV0-CAT to generate p2 (+) and p14 (-). The HPV 16 BamHI/AvaII fragment (6150 to 112; Seedorf et al, 1985) was treated with reverse transcriptase and ligated into pA10-CAT to produce plasmids p7 (+) and p4 (-) and into pSV0-CAT to give p15 (+) and p17 (-). The HPV-ncr-CAT constructs are outlined in Figure 9 (p 56).

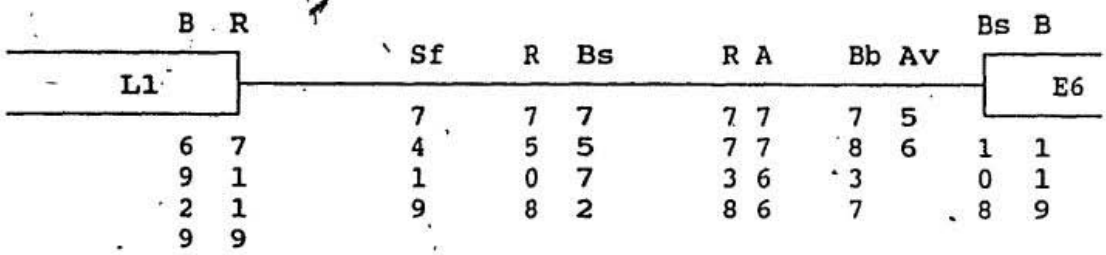
Figure 3. Restriction endonuclease maps of the noncoding regions of HPV 16, 18, and 11.

The diagram presents a linear restriction map of the noncoding region and surrounding L1 and E6 open reading frames. The restriction enzymes are: A, AccI; Av, AvaII; B, BamHI; Bb, BbvI; Bs, BssHII; D, DraIII; Hp, HphI; N, NdeI; R, RsaI; S, SauI; Sf, SfaNI; Sp, SphI; Ss, SspI. The numbers written vertically from top to bottom represent recognition sites (base pairs) for the various restriction enzymes (abbreviated above).

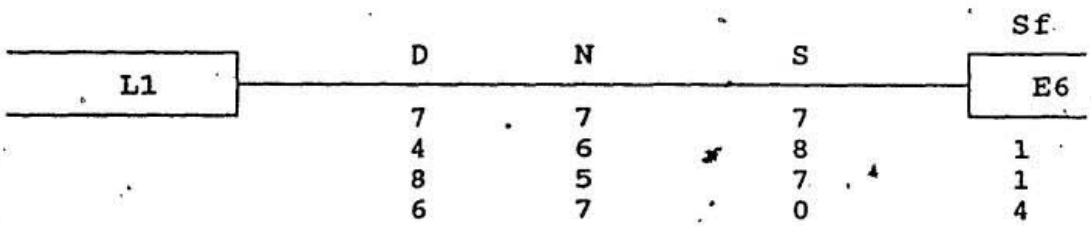
HPV 16



HPV 18



HPV 11



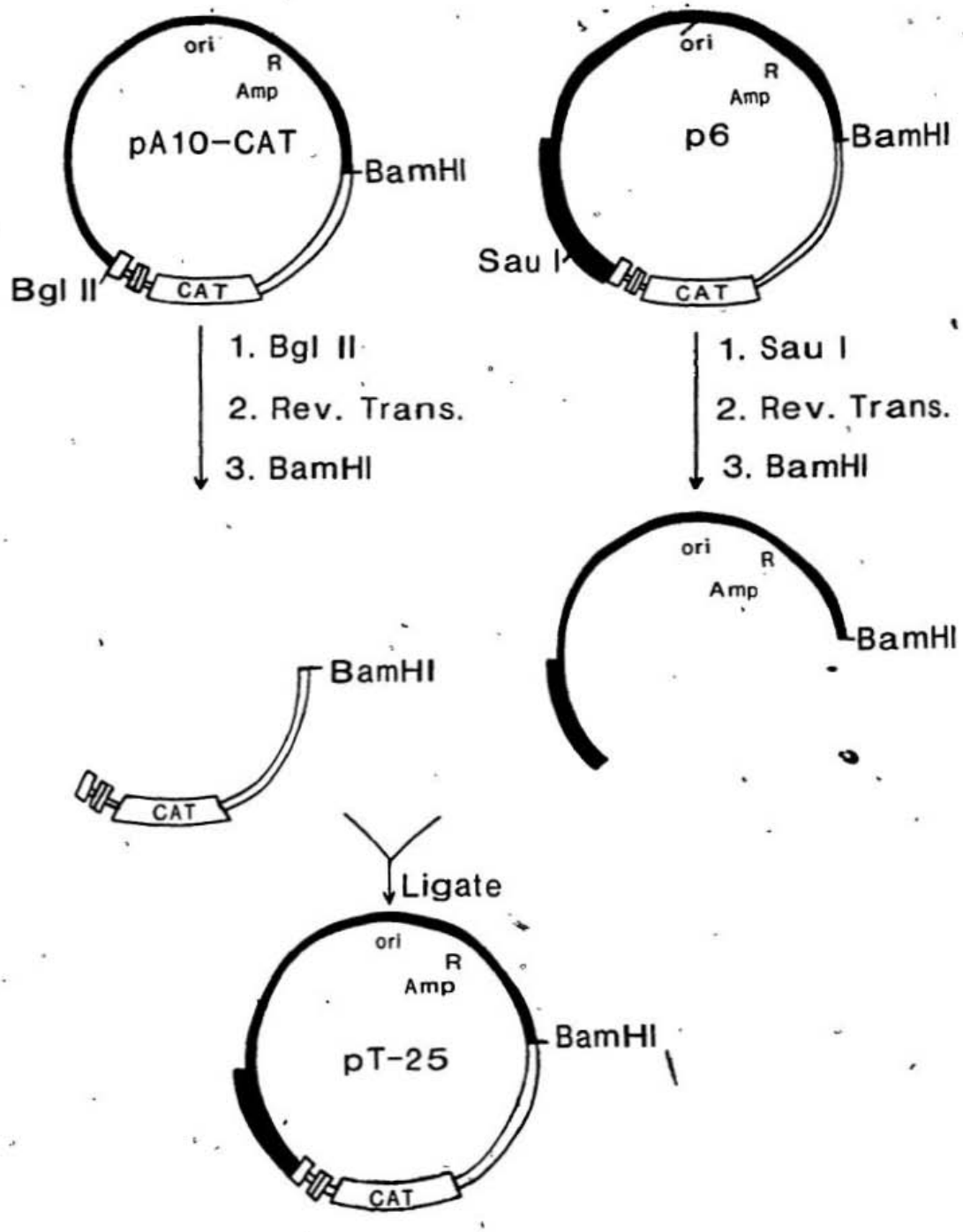
2.4 HPV enhancer constructs

The pA10-CAT vector was used to locate the smallest HPV DNA fragment with enhancer activity. The HPV 18 BamHI ncr fragment was digested with AccI at nucleotide 7766 to generate two fragments (BamH/AccI and AccI/BamH). The BamHI/AccI (837 bp) fragment was isolated, blunt-ended by reverse transcriptase and inserted into the BglII site of pA10-CAT to give the constructs pT-46 (+;Figure 10, p 61) and pT-4 (-). The HPV 18 plasmid DNA was digested with SfaNI and AccI and treated with S1 nuclease; a 347 bp fragment (7419 to 7766) which contains an E2 binding domain sequence was isolated and inserted into pA10-CAT to give pT-11 (+;Figure 6, p 42 and Figure 10, p 61) and pT-2 (-;Figure 6, p 42). The BamHI/AccI fragment mentioned above was digested to completion with RsaI to give four fragments. These blunt-ended fragments were inserted into pA10-CAT, and the plasmids pT-81 (+) and pT-18 (-) containing the 230 bp RsaI fragment (7508 to 7738) were isolated (Figure 10, p 61).

The HPV 11 enhancer plasmid was derived from p6 (+). This DraIII/SfaNI-pA10-CAT plasmid (Figure 4) was digested with SauI, filled in by reverse transcriptase and then digested with BamHI to generate two fragments with sizes 2007 bp and 4183 bp. Similarly, pA10-CAT was digested with BglII, followed by treatment with reverse transcriptase and BamHI digestion to give two fragments with sizes 1832 bp and 3799 bp. The p6

Figure 4. Construction of a CAT/recombinant plasmid carrying the HPV 11 enhancer upstream of the SV40 promoter.

The plasmid pT-25 carrying the HPV 11 enhancer (represented by the thick solid line) upstream of the SV40 21 bp repeats and Goldberg/Hogness box was generated by joining the SauI/BamHI fragment of p6 (the HPV 11 ncr/pA10-CAT plasmid) with the BglII/BamHI fragment of pA10-CAT.

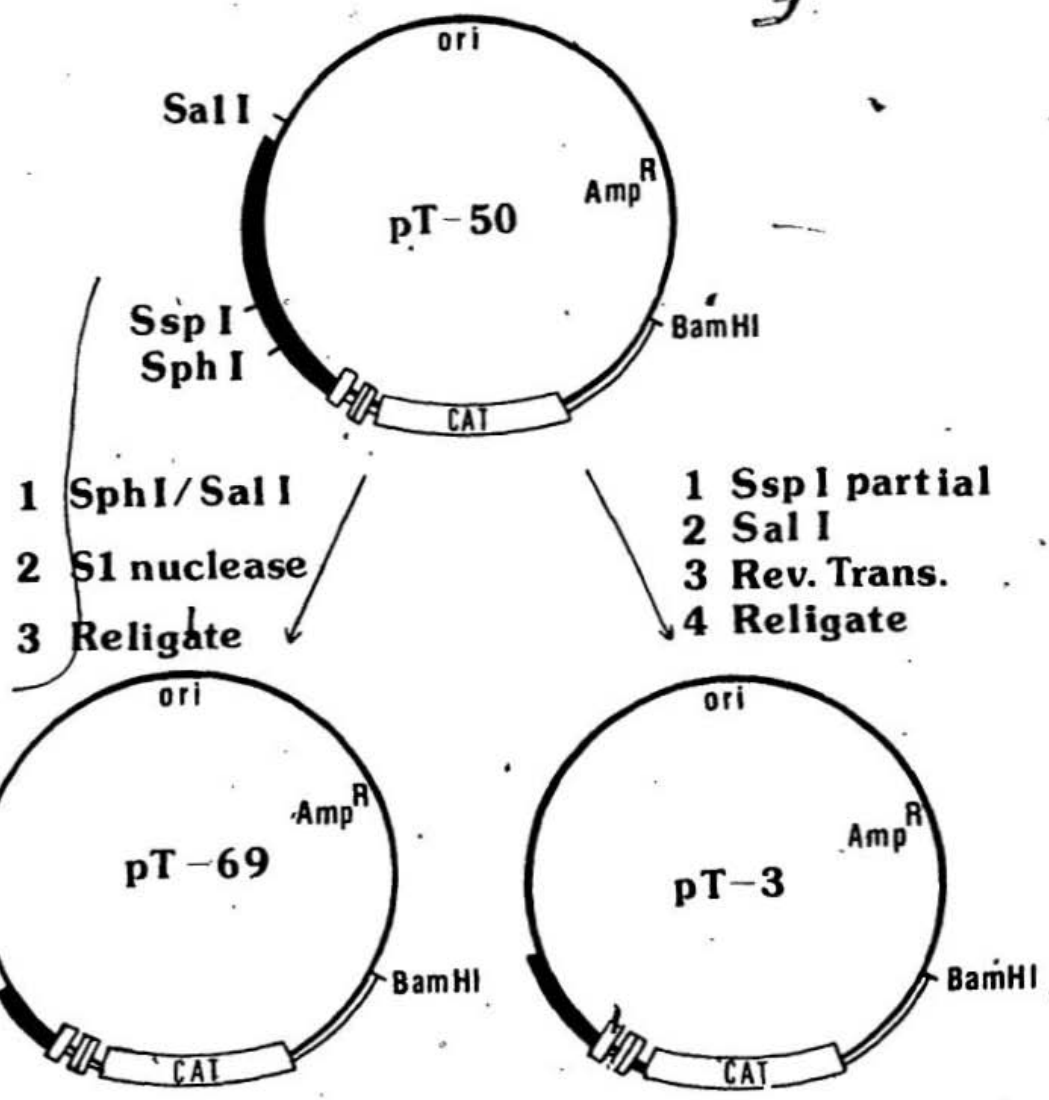

ori
R
Amp
pA10-CAT
BamHI
Bgl II
CAT
1. Bgl II
2. Rev. Trans.
3. BamHI
ori
R
Amp
p6
BamHI
Sau I
CAT
1. Sau I
2. Rev. Trans.
3. BamHI
ori
R
Amp
BamHI
BamHI
CAT
Ligate
ori
R
Amp
pT-25
BamHI
CAT

and pA10-CAT derived fragments were isolated and the 1832 bp pA10-CAT fragment (containing CAT sequences) was ligated to the 4183 bp p6 fragment (containing pBR322 and HPV 11 [DraIII/SauI] enhancer sequences) to generate the enhancer plasmid pT-25 (+). The plasmid pT-25 differs from p6 in that the SauI to SfaNI fragment containing the promoter sequences has been removed (Figure 4). Plasmid, pT-25 was digested to completion with NdeI and the 4110 bp fragment containing 213 bp (7657 to 7870) of the HPV 11 ncr and most of the pA10-CAT sequences was isolated and self-ligated to generate pT-1 (+; Figure 11, p 65).

The HPV 16 BamHI/AvaII fragment was digested to completion with HphI to give three fragments. The middle 1340 bp fragment was S1 nuclease treated and inserted into pA10-CAT to give the enhancer plasmid pT-50 (+) (Figure 12; p 67). Plasmids pT-3 and pT-69 are derivatives of pT-50 (Figure 5). Plasmid pT-3 (+) was obtained by subjecting pT-50 to partial digestion with SspI since there are two SspI sites in the HPV 16 HphI fragment (at 6551 and at 7224) and many sites in pA10-CAT. The DNA was then digested with SalI and flush-ended by reverse transcriptase. The fragment containing HPV 16 sequences from 7224 to 7778 along with most of the upstream sequences of pA10-CAT was isolated and self-ligated to produce pT-3. Similarly, the pT-69 (+) construct was obtained by digesting pT-50 with SphI and SalI. The fragment containing HPV 16 DNA from 7463 to 7778 (315 bp) and most

Figure 5. Schematic diagram of HPV 16 enhancer plasmid constructions.

HPV 16 enhancer subfragments were constructed by removing upstream noncoding region fragment (represented by thick solid lines) from the HPV 16 enhancer plasmid, pT-50. The resulting constructs, pT-69 and pT-3, contain the SphI/HphI and the SspI/HphI enhancer fragments of HPV 16, respectively, inserted in the BglII site of the enhancerless plasmid pA10-CAT.


ori
Sal I
Amp R
pT-50
Ssp I
Sph I
BamHI
CAT
1 Sph I / Sal I
2 S1 nuclease
3 Religate
1 Ssp I partial
2 Sal I
3 Rev. Trans.
4 Religate
ori
Amp R
pT-69
BamHI
CAT
ori
Amp R
pT-3
BamHI
CAT

of pA10-CAT was blunt-ended by S1 nuclease treatment and self-ligated to generate pT-69. A restriction enzyme map of the noncoding regions of HPV 16, 18, and 11 is given in Figure 3 (p 32).

2.5 Construction of plasmids to test the effect of progressive deletions on the enhancer activity of HPV 18

The plasmid pT-39 (+) was constructed by insertion of the BamHI/AvaII (6929 to 56) fragment (blunt-ended by reverse transcriptase) into the BglII site of pA10-CAT. Similarly, the BamHI/BbvI (6929 to 7837) fragment was treated with reverse transcriptase (resulting in an endpoint at 7854) and inserted into pA10-CAT to generate the plasmid pT-33 (+). The positions of AvaII and BbvI in the noncoding region of HPV 18 are shown in Figure 3 (p 32) and in Figure 16 (p 98).

2.6 Constructs containing the SV40 and HPV 18 enhancers upstream of and downstream from the CAT gene

The 342 bp PvuII/HindIII fragment of pSV2-CAT containing the enhancer and promoter of SV40 (Figure 2, p 27) was filled in by reverse transcriptase and inserted into the XbaI (blunt-ended by reverse transcriptase) site of pA10-CAT to give plasmids pT-51 (+; positive relative to the direction of CAT

transcription) and pT-42 (-;Figure 6). Similarly, the S1 nuclease treated 347 bp SfaNI/AccI fragment of HPV 18 was inserted into the XbaI site of pA10-CAT to generate pT-53 (+) and pT-52 (-;Figure 6). Plasmids pT-11 (+) and pT-2 (-), mentioned earlier, have this 347 bp fragment inserted into the BglII site of pA10-CAT (Figure 6).

2.7 Construction of plasmids to analyze sequences required for promoter function of HPV 18

The HPV-ncr-pSV0-CAT plasmid, p29, was subjected to partial BssHII digestion since there are two BssHII sites in the HPV 18 BamHI ncr fragment (at nucleotides 7572 and 108; Figure 3, p 32). The DNA was then digested to completion with NdeI generating four fragments of sizes 694 bp, 1084 bp, 4460 bp and 4853 bp. The ends were modified by treatment with reverse transcriptase and the largest fragment (4853 bp) was subsequently isolated and self-ligated to give pT-29 (Figure 7). Plasmid pT-29 has both enhancer and promoter (7572 to 119) in the same orientation. Plasmid pT-4, which contains a single BssHII site at nucleotide 7572 (HPV DNA), was digested to completion with this enzyme, blunt-ended with reverse transcriptase, and subsequently digested with BamHI. Similarly, p29 was digested with AccI, filled in by reverse transcriptase, and then digested with BamHI. The resulting fragments of pT-4 and p29 were run separately on agarose

Figure 6. Strategy for construction of plasmids containing the SV40 and HPV 18 enhancers adjacent to and distant from the SV40 promoter.

The HPV 18 347 bp (SfaNI/AccI) enhancer fragment was inserted upstream and downstream of the CAT gene at the BglII and XbaI sites, respectively. Similarly the SV40 PvuII/HindIII fragment was removed from the pSV2-CAT plasmid and inserted downstream of the CAT gene in the XbaI site (pT-42 and pT-51). The arrows represent the direction of the HPV and SV40 enhancer fragments. A, AccI; Hi, HindIII; Pv, PvuII; Sf, SfaNI.

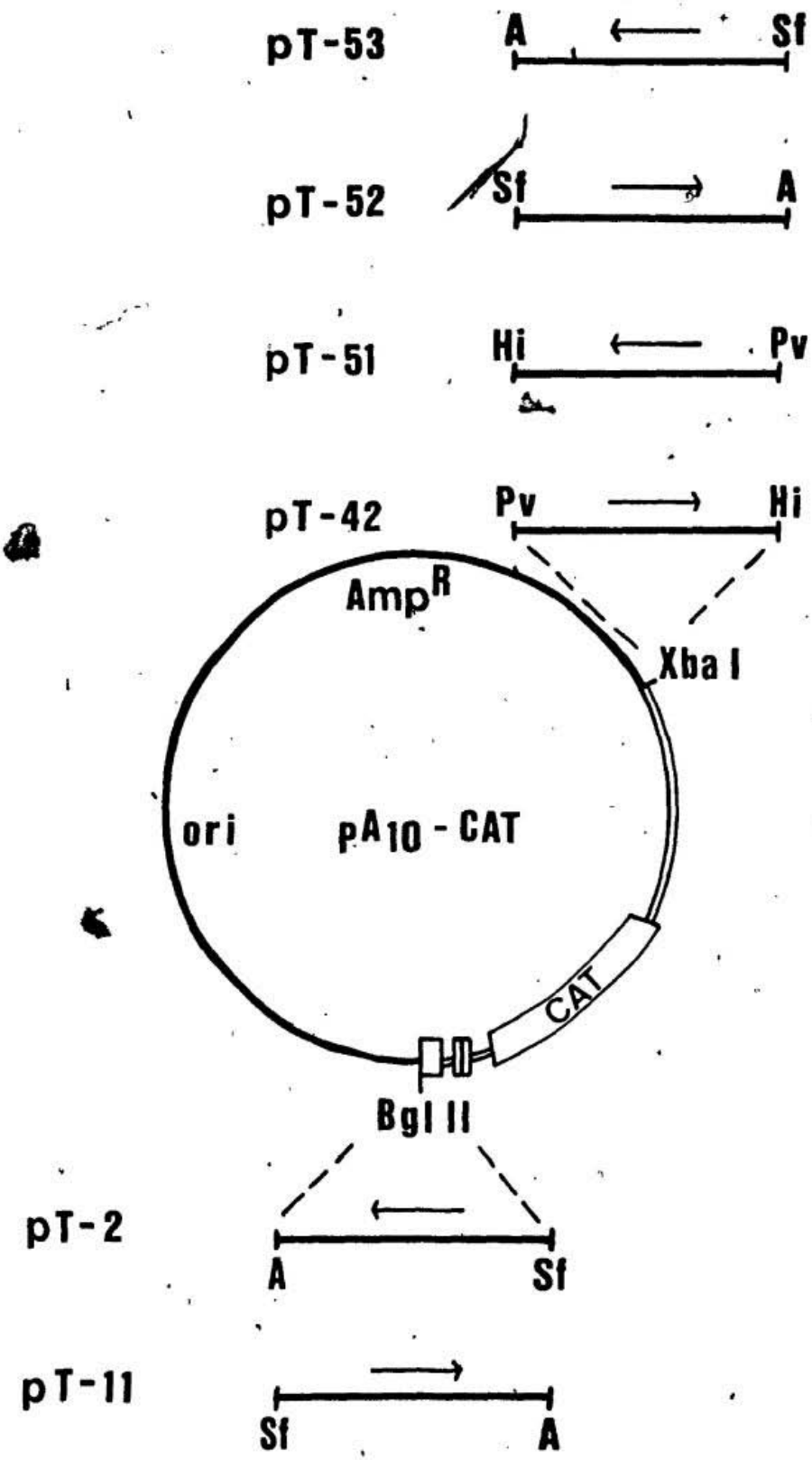
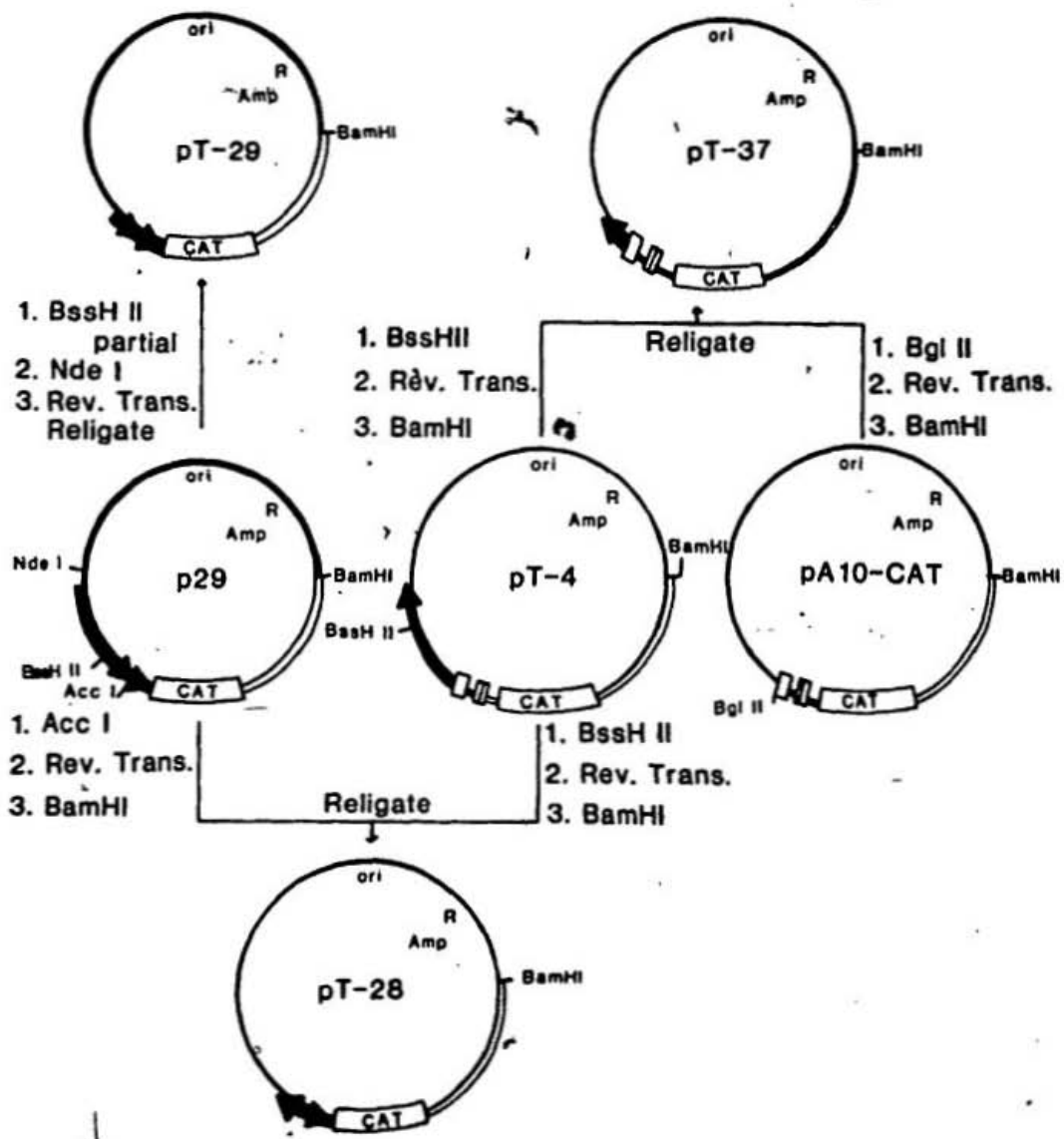

pT-53
A
Sf
pT-52
Sf
A
pT-51
Hi
Pv
pT-42
Pv
Hi
Amp^R
Xba I
ori
pA10 - CAT
CAT
Bgl II
pT-2
A
Sf
pT-11
Sf
A

Figure 7. Schematic diagram of plasmid constructions.

Plasmid p29 is a HPV 18 ncr/pSV0-CAT construct in which the HPV 18 enhancer and promoter are represented by two thick arrows. The HPV 18 enhancer and promoter are in the same orientation as the coupled CAT gene in p29 (BamHI; 6929 to 119) and in pT-29 (BssHII/BamHI; 7572 to 119). However, the pT-28 and pT-37 constructs have the HPV 18 enhancer (BssH/AccI; 7572 to 7766) in the opposite orientation relative to the HPV promoter and SV40 promoter, respectively. Plasmid pT-4 has the HPV 18 BamHI/AccI fragment (6929 to 119) inserted upstream of the CAT gene (in the opposite orientation).


ori
R
Amp
pT-29
BamHI
CAT
1. BssH II
partial
2. Nde I
3. Rev. Trans.
Religate
ori
R
Amp
pT-37
BamHI
CAT
1. BssHII
2. Rev. Trans.
3. BamHI
Religate
1. Bgl II
2. Rev. Trans.
3. BamHI
ori
R
Amp
Nde I
p29
BamHI
BssH II
Acc I
CAT
ori
R
Amp
pT-4
BamHI
BssH II
CAT
ori
R
Amp
pA10-CAT
BamHI
Bgl II
CAT
1. Acc I
2. Rev. Trans.
3. BamHI
1. BssH II
2. Rev. Trans.
3. BamHI
Religate
ori
R
Amp
pT-28
BamHI
CAT

gels. The pBR322 DNA segment together with HPV enhancer sequences of pT-4 (3993 bp) and the CAT DNA together with the HPV promoter sequences of p29 (1844 bp) were electroeluted and ligated to generate pT-28 (Figure 7). Also, the same pT-4 fragment was ligated to a pA10-CAT fragment to give pT-37 (Figure 7). This pA10-CAT fragment was produced from a BglI/BamHI digestion with the BglII ends (but not the BamHI ends) filled in by reverse transcriptase treatment.

2.8 Construction of plasmids expressing the HPV 16 E2 and E7 gene products

Plasmid pD-7 (501 to 4334; Figure 14, p 82) contains intact E7, E1, E2, E4, and E5 ORFs (pD-2 contains only part of E7; 501 to 700 are missing; Figure 14, p 82) driven by the SV40 early promoter and uses its own polyadenylation signal. These plasmids were constructed by subjecting HPV 16 plasmid DNA to partial digestion with HpaII. The DNA was then digested to completion with SauI. The HpaII/SauI 3833 bp (pD-7) and the 3634 bp (pD-2) fragments were isolated, blunt-ended with reverse transcriptase, and cloned into a pSV2-NEO derivative (similar to pSV2-CAT shown in Figure 2, p 27). This pSV2-NEO derivative was obtained by modifying pSV2-NEO (Southern and Berg, 1982) by replacing pBR322 sequences with pML (Nakshatri, Pater and Pater, unpublished). This plasmid,

pSV2-NEO-pML, was digested with HindIII and EcoRI, treated with reverse transcriptase and the fragment containing the SV40 early promoter (342 bp) plus pML (650 to 4360; Sutcliffe, 1979) was isolated and ligated with each of the above mentioned HPV 16 DNA fragments.

Deletion mutants of E2 were constructed as follows. Plasmids pT-57 and pT-48 (pD-7 and pD-2 derivatives, respectively; Figure 14, p 82) were constructed by digesting each of the pD-2 and pD-7 plasmids with NdeI which cleaves HPV 16 DNA at nucleotide 3126 and 3761 and pML DNA at 2296. The resulting fragments were filled in by reverse transcriptase and the largest fragments were religated together. The pT-57 and pT-48 constructs are missing 635 bp of the E2 ORF with the downstream sequences out of frame. These constructs are also missing the E4 ORF. The plasmid pT-20 contains a 4 bp deletion generated by S1 nuclease treatment of the BstXI site (2891). This 4 bp deletion places the remainder of E2 out of frame. These plasmids are outlined in Figure 14, p 82).

A brief description of the plasmids used in this study is given in Appendix A (p 154).

2.9 Cell culture and transfection

African green monkey kidney CV-1 cells were maintained in Dulbecco's modified Eagle's medium (DME) with 10 % fetal

bovine serum and supplemented with Pen-Strep (5000 IU/mL penicillin; 5000 MC9/mL streptomycin). The human cervical cell lines C33A, SiHa and HeLa and the rhabdomyosarcoma cell line, A204 were obtained from the American Type Culture Collection and grown in the same medium as described above. The cells were passed by aspirating the medium from the cells and washing the cells twice with 10 mL PBS (phosphate buffered saline without Ca or Mg). The cells were then incubated in 1.5 to 2.0 mL of trypsin-EDTA for approximately 15 minutes at 37 ^{o}C and then resuspended in DME plus 10 % FBS. The appropriate amount of cell-medium mixture was added to 10 mL of the same medium. For transfection experiments, cells were seeded at equal density such that all plates contained approximately the same number. Cells, (60 to 70 % confluency) were fed with fresh medium 3 hours prior to the addition of DNA. DNA transfections were performed by calcium phosphate coprecipitation (Gorman et al, 1982). Five hundred microlitres of a $CaCl_2$/DNA solution were bubbled slowly into 500 μL of 2 x HBS (0.28 M NaCl; 0.05 M HEPES; 2.8 mM Na_2HPO_2, pH 7.1). This fine, cloudy precipitate was then carefully added evenly to the cells. For most experiments a total of 20 μg of DNA was used per 100 mm dish (10 μg of test plasmid plus 10 μg of pUC 19 plasmid DNA). For experiments with plasmids expressing the early genes of HPV 16, a total of 25 μg was used (5 μg of test plasmid plus 20 μg of plasmid expressing E2 and/or E7 ORF or pUC 19 plasmid

DNA as control). Four hours after transfection the cells were carefully washed with serum-free medium and subsequently exposed to glycerol (1.5 ml of 15 % glycerol in 1 x HBS) for 2 minutes. The cells were then washed twice with serum-free medium and incubated in DME plus 10 % FBS for 48 hours.

2.10 CAT assays

Using recombinant DNA technology and molecular cloning it has been possible to ligate regulatory sequences upstream from a gene which is readily expressed and has an easily assayable product. Such a vector system, developed by Gorman and her colleagues (1982), utilizes the ability of SV40 regulatory and other regulatory sequences (such as HPV) to control the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene. The CAT vector system, used in this study to measure promoter and/or enhancer function in mammalian tissue culture cells, was chosen for several reasons. First, absence of endogenous CAT enzymatic activities from mammalian cells, ensures that the assay is easily interpreted; second, the assay is simple, rapid, sensitive, and reproducible. The transfected cells were harvested to assay the CAT activity by washing the plates carefully three times with 2 mLs of PBS (without Ca/Mg); the cells were then allowed to sit at room temperature for 5 minutes in 1 mL per plate of Tris-EDTA-NaCl (0.04 M Tris HCl, pH 7.4; 1 mM EDTA; 0.15 M

NaCl). The cells were scraped from the plate with a rubber policeman and pelleted by microfuging for 2 minutes at 4 ^{o}C. The pellet was then frozen at -70 ^{o}C until ready to assay. The frozen cells were thawed on ice and the pellet dispersed by vortexing in 100 μL (for C33A, ID14, C127 or 143B extracts) or in 50 μL (for SiHa, HeLa, A204, and CV-1 extracts) of 0.25 M Tris HCl, pH 7.8. Cells were disrupted by repeated freezing and thawing: eppendorf tubes containing the dispersed pellets were immersed in liquid N_2 for 5 minutes then thawed in a waterbath set at room temperature for five minutes. This cycle was repeated three times to disrupt the cells. The cell debris was pelleted (5 minutes in a microfuge at 4 ^{o}C) and the supernatant was transferred to a new tube. One-tenth (10 μL) of the C33A (ID14, C127 and 143B) extract or three-tenths (15 μL) of the CV-1, SiHa, HeLa and A204 extracts was added to 1 μL of [^{14}C] chloramphenicol (50 mCi/mMole) and 4 μL of 4 mM acetyl Coenzyme A. The tubes were incubated at 37 ^{o}C for 60 minutes before the addition of ethyl acetate, an organic solvent which extracts the chloramphenicol from the other reactants. The extract was dried in a speed vacuum and resuspended in 15 μL of ethyl acetate. The sample was spotted on silica gel thin layer chromatography plates. Acetylated and nonacetylated forms of chloramphenicol were separated ascending by thin layer chromatography (TLC) in chloroform:methanol (95:5). The TLC plate was treated with Omnifluor enhance spray to increase

sensitivity and was exposed overnight against XAR-5 Kodak film at -70 °C. After autoradiography, quantification of the enzyme reaction products was achieved by liquid scintillation counting of the acetylated and nonacetylated chloramphenicol excised from the chromatography plate. Data are expressed as the percent conversion of chloramphenicol to its acetylated form (% CAT activity).

CHAPTER 3

RESULTS

A transient expression system was used to screen the noncoding region of HPV 16, 18, and 11 for enhancer and promoter activity. The test plasmids were constructed by inserting the ncr fragments of interest from HPV 16, 18 or 11 upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene. These recombinant clones were transfected into cells and the amount of CAT activity produced by each plasmid was determined.

3.1 Analysis of HPV-ncr/CAT recombinant plasmids for enhancer and promoter activity

A computer search was executed and this search indicated the presence of a putative HPV promoter in the downstream region of the HPV 16, 18, and 11 ncr. Alignment of sequences located in this region of HPV 16, 18, and 11 revealed the presence of sequences similar to the promoter consensus sequences, TATA$^{A}_{T}$TA and GG$^{C}_{T}$CAATCT (Figure 8). Suspecting the presence of a promoter, I assayed for promoter activity in the noncoding region of HPV 16, 18, and 11. In HPV 11 experiments, the 559 bp DraIII/SfaNI (7486 to 114) ncr fragment was cloned into pSVO$_{\Delta}$CAT. The positive orientation

Figure 8. Alignment and comparison of the downstream noncoding region sequences of HPV 16, 18, and 11.

The following consensus sequences were written on the basis of the sequence homology between the noncoding regions of HPV 16, 18, and 11: (1) CAAT, potential CAAT box; (2) TATA, potential TATA box; (3) $ACCGN_4CGGT$, E2 binding site. These sequences of the HPV 16, 18, and 11 noncoding regions (nucleotides 1 to 100), obtained from previous reports, were entered into a computer file and aligned with one another using a NUCALN program.

HPV 16

VERSUS

HPV 18

VERSUS

HPV 11

```
                10        20        30        40        50
      ACTACAATAATTCATGTATAAAACTAAGGGCGTAACCGAAATCGGTTGA-ACC
        :::::   :  ::::: ::  ::::: :::::::::: :::: :  :::
AATACTTTTAACAAT---TGTAGTATATAAAAAAGGGAGTAACCGAAAACGGTCGGGACC
    :  :::::::     :::: :: :::::  ::::  ::::::::::::    :::
  CTTAATAACAAT---CTTAGTTTA-AAAAAGAGGAGGGACCGAAAACGGTTCA-ACC
```

CONSENSUS:

```
      ---ACAAT--------T-TA-AAAAA--------ACCGAAA-CGGT----ACC
```

```
       60        70        80        90
GAAACCGGTT-AGTATAAAAGCAGACATTTTATGCACCAAAAGAGAACT    HPV 16
:::: ::::    :::::::::  :  :         :  ::
GAAAACGGTG-TATATAAAAGATGTGAGAAACACACCACAA    HPV 18
::::::::::  :::::::::  :    : :::   : :: :
GAAAACGGTTATATATAAACCAGCCCAAAAAATTAGCAGACGAGGCAT    HPV 11
```

CONSENSUS:

```
GAAA-CGGT----TATAAA----------------------
```

pSV0-CAT construct, which measures HPV 11 promoter activity, gave 10-30 times more activity than the negative orientation construct (compare p2 and p14; Figure 9). Similarly in HPV 18, the CAT activity of the 1.1 Kb BamHI-pSV0-CAT positive orientation construct (p29) was significantly higher than the activity measured for the opposite orientation plasmid (pT-13; Figure 9A). However, with the 1.866 Kb BamHI/AvaII fragment (6150 to 112) of HPV 16, little if any promoter (enhancer) activity was detected in the transfected C33A (Table I) or SiHa cells (Figure 9).

Next, enhancer activity was examined by inserting the 1.1 Kb BamH (6929 to 119) fragment of HPV 18 upstream of the SV40 promoter (pA10-CAT) in both orientations. The negative orientation plasmid (p9) gave values near that of the negative control (pA10-CAT), whereas the positive orientation plasmid (p8) gave weak but reproducible enhancer activity (Figure 9B).

The HPV 11 noncoding region was also found to function as an enhancer when placed upstream of the heterologous (SV40) promoter. However, unlike the BamHI HPV 18 enhancer-containing fragment this 559 bp sequence was orientation-independent (compare p5 and p6; Figure 9B). On average, the HPV 11 promoter and enhancer gave significantly higher CAT enzymatic activities. The HPV 11 promoter had twice as much CAT activity as the HPV 18 promoter (compare p2 and p29; Figure 9A) and the enhancer of HPV 11 was six times stronger than the HPV 18 enhancer (compare p6 and p8;

Figure 9. Examination of the HPV 16, 18, and 11 noncoding regions for enhancer and promoter CAT activity.

(A) pSV0-CAT promoter/enhancer series. (B) pA10-CAT enhancer series. HPV 11 and 18 results are from transfected C33A cells while those for HPV 16 are from SiHa cells. The arrow indicates that the ncr fragment has been placed in the negative orientation relative to the CAT gene. The pA10-CAT and pSV2-CAT plasmids were used as negative and positive controls, respectively. Most extracts from pSV2-CAT transfected C33A cells were diluted to give a value within the linear range. The percent CAT activity was then corrected by multiplying by the dilution factor. The HPV 11 series of experiments had pA10-CAT and pSV2-CAT enzymatic levels of 0.9 and 361.0 percent, respectively for experiment 1 (1) and 1.1 and 330.6 percent, respectively for experiment 2 (2). The HPV 18 series had pA10-CAT and pSV2-CAT enzymatic levels 0.4 and 178.0 percent, respectively for experiment 1 and 0.6 and 296.0 percent, respectively for experiment 2. For the HPV 16 series pA10-CAT and pSV2-CAT values were 0.5 and 13.8 percent, respectively for experiment 1 and 0.4 and 16.0 percent, respectively for experiment 2. Transfections and CAT assays were as described in Materials and Methods. Data are expressed as the percent conversion of chloramphenicol to its acetylated form (% CAT activity). Av, AvaII; B, BamHI; D,DraIII; Sf, SfaNI.

A

pSV0-CAT PLASMIDS

% CAT ACTIVITY

		1	2
HPV 11			
p14	114 Sf — 7486 D	1.0	3.2
p2	7486 D — 114 Sf	27.4	39.1
HPV 18			
pT-13	119 B — 6929 B	3.3	0.1
p29	6929 B — 119 B	17.7	17.0
HPV16			
p17	112 Av — 6150 B	0.1	0.1
p15	6150 B — 112 Av	0.1	0.1

B

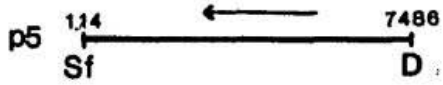
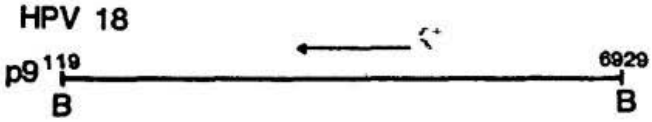
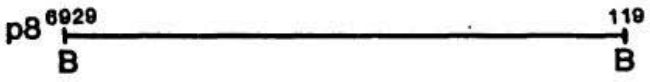
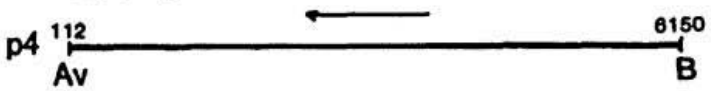
pA10-CAT PLASMIDS		% CAT ACTIVITY 1	2
HPV 11			
p5		38.0	30.9
p6		28.5	32.0
HPV 18			
p9		0.6	1.5
p8		6.0	4.3
HPV 16			
p4		0.4	0.7
p7		0.2	0.2

Table I. Testing of the HPV 16 promoter and enhancer in C33A

Plasmids	% CAT Activity	
	1	2
pA10-CAT	0.3	0.3
pSV2-CAT	145.4	148.5
p17[a]	0.1	0.1
p15[a]	0.9	1.0
p4[a]	0.4	0.4
p7[a]	0.8	0.9
pT-50[b]	1.3	1.5
pT-3[b]	1.4	1.7
pT-69[b]	0.5	0.4

[a] These plasmids are as described in Figure 9, p 56.

[b] These plasmids are as described in Figure 12 ,p 67.

Figure 9B).

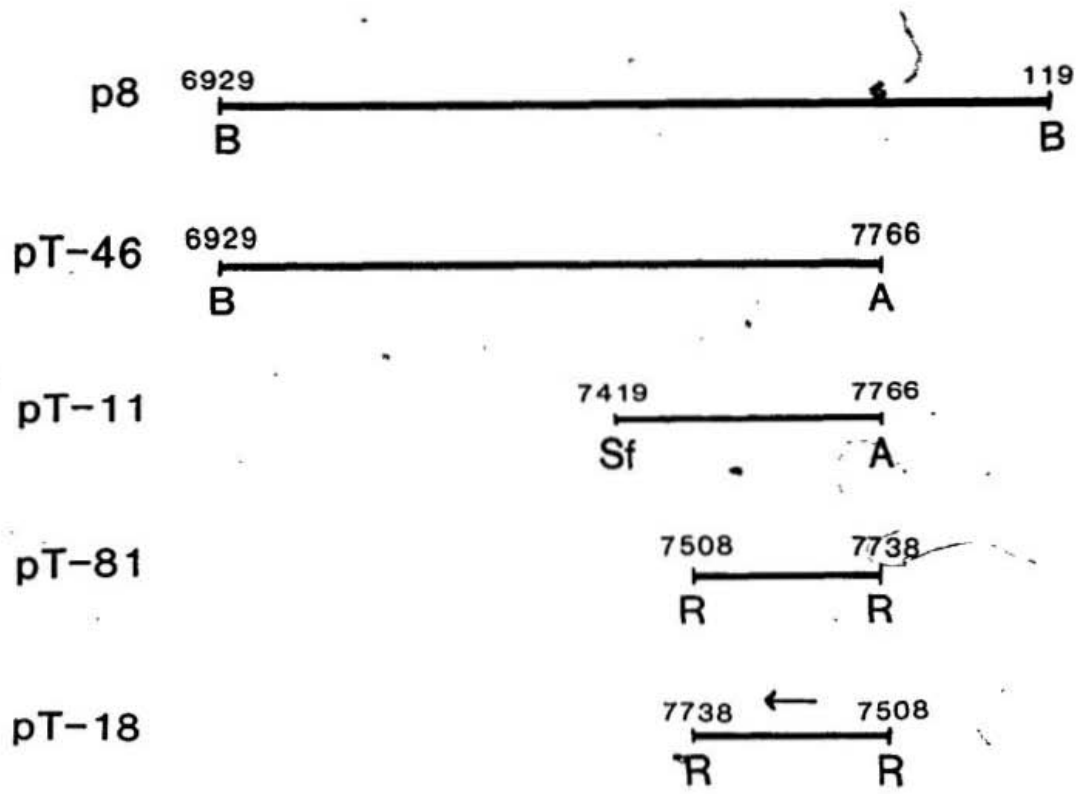
3.2 Characterization of enhancer elements in the regulatory region of HPV 16, 18, and 11

To localize the enhancer element of HPV 16, 18, and 11, CAT plasmids containing subfragments of the ncr region were constructed. The upstream 837 bp of the HPV 18 noncoding region (pT-46) gave about ten times more enhancer activity than the fragment (p8) which retained the early region proximal 210 bp sequence (Figure 10). In addition, deletion of sequences from the upstream portion of the noncoding region was found to slightly diminish CAT activity (compare pT-46 and pT-11; Figure 10). Interestingly, orientation dependence was observed when the 837 bp (pT-46) and 347 bp (pT-11) fragments of HPV 18 were cloned in the opposite orientation relative to the CAT gene (pT-4 and pT-2, respectively; Table II). However, the 230 bp RsaI fragment, generated enhancer activity in an orientation independent manner (compare pT-81 and pT-18; Figure 10); this is in agreement with a previous study by Swift et al, 1987.

The results from CAT assays given in Figure 9A and 9B indicate that the DraIII/SfaNI 559 bp fragment from the noncoding region of HPV 11 DNA contained all the elements necessary for promoter and enhancer function. Removal of 175 bp from the downstream portion of this 559 bp fragment

Figure 10. Localization of the HPV 18 enhancer.

Various restriction fragments of HPV 18 were cloned into the pA10-CAT vector, as described in the Materials and Methods. Plasmids pSV2-CAT and pA10-CAT were transfected as controls into C33A and gave 174.0 and 0.6 percent CAT activity, respectively for experiment 1. The CAT assay values for experiment 2 were 174.0 for pSV2-CAT and 0.5 percent for pA10-CAT. A, AccI; B, BamHI; R,RsaI; Sf, SfaNI.


% CAT ACTIVITY
1
2
p8
6929
119
B
B
4.4
7.1
pT-46
6929
7766
B
A
40.4
60.5
pT-11
7419
7766
Sf
A
19.3
43.5
pT-81
7508
7738
R
R
23.3
32.0
pT-18
7738
7508
R
R
19.5
34.0

62

Table II. Examination of the orientation-dependence of the HPV 18 noncoding region enhancer

Plasmid	HPV 18 Fragment	% CAT Activity
pA10-CAT	-	1.4
pSV2-CAT	-	89.8[a]
p9	BamHI (119 to 6929)	6.5
p8	BamHI (6929 to 119)	10.4
pT-4	AccI/BamHI (7766 to 6929)	4.4
pT-46	BamHI/AccI (6929 to 7766)	52.8[a]
pT-2	AccI/SfaNI (7766 to 7419)	11
pT-11	SfaNI/AccI (7419 to 7766)	52.2[a]

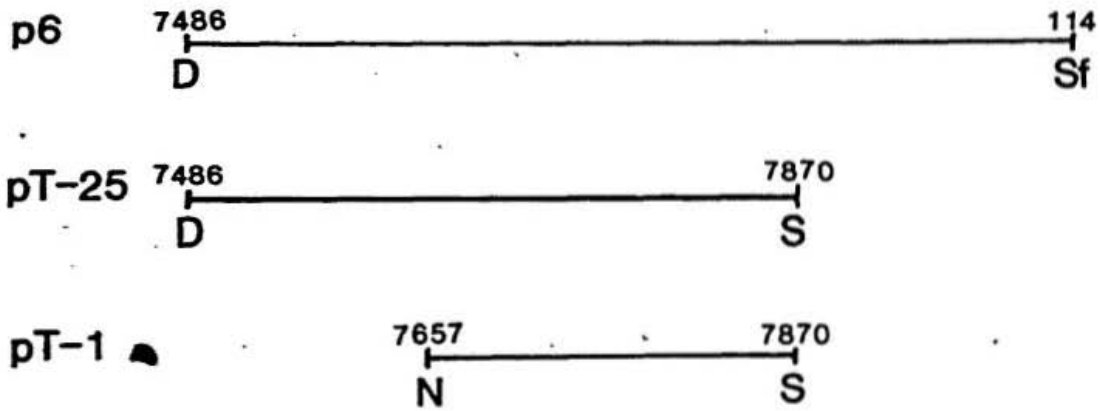
[a] The percent of CAT activity (in C33A) is above that of the linear range, resulting in an underestimation of relative CAT activity.

enhanced CAT activity (compare p6 and pT-25; Figure 11). By examining different subfragments for CAT activity, the enhancer of HPV 11 was localized to a 213 bp NdeI/SauI (7657 to 7870) fragment. Again, removal of sequences from the upstream portion of the HPV noncoding region resulted in a lowering of CAT activity.

Similarly, each of the HPV 16-ncr/CAT- recombinant plasmids were transfected into SiHa cells and assayed for CAT activity. No significant levels of CAT activity above that of the negative control, pA10-CAT, were detected in cells transfected with the HPV 16-ncr/CAT plasmids (Figure 9A and 9B). To test whether removal of 238 bp from the downstream portion of the BamHI/AvaII fragment would result in detectable enhancer activity, I constructed plasmid pT-50. This plasmid, also missing 288 bp from the upstream portion of the ncr, when transfected was found to enhance the CAT activity by up to 25 fold (compare p7 and pT-50; Figure 12). The enhancer activity of pT-50 and another plasmid retaining the 554 bp fragment (SspI/HphI; 7224 to 7778) of the HPV 16 ncr (pT-3) was relatively weak (compared to pSV2-CAT) but reproducible. The HPV 16 enhancer was further localized to a 315 bp SphI/HphI fragment (pT-69; Figure 12). The activity of the enhancer fragments seem to diminish as the upstream sequences are removed. This was similar to the situation observed in HPV 18 and 11 enhancer subfragments.

Figure 11. Localization of the HPV 11 enhancer element.

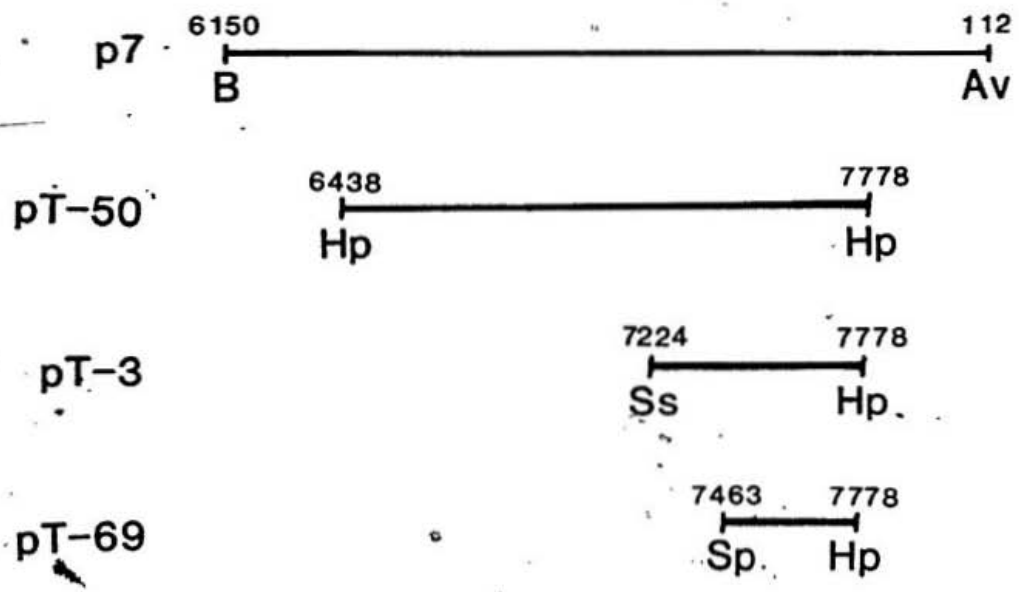
The HPV 11 enhancer pA10-CAT constructs (p6, pT-25, and pT-1 as described in Materials and Methods), the enhancerless pA10-CAT vector, and the enhancer-positive pSV2-CAT clones were transfected into C33A cells. CAT enzyme levels for pA10-CAT and pSV2-CAT were 0.3 and 716.0 percent, respectively for experiment 1 and 0.3 and 713.0 percent, respectively for experiment 2. D, DraIII; N, NdeI; S, SauI; Sf, SfaNI.



	% CAT ACTIVITY	
	1	2
p6	35.4	35.1
pT-25	56.3	58.7
pT-1	24.1	20.6

Figure 12. Localization of the HPV 16 enhancer.

The plasmids were constructed as described in Materials and Methods. The pSV2-CAT and pA10-CAT plasmids, when transfected into SiHa cells, gave CAT assay values of 44.6 and 0.8 percent, respectively for experiment 1 and 35.4 and 1.0 percent, respectively for experiment 2. Av, AvaII; B, BamHI; Hp, HphI; Sp, SphI; Ss, SspI.


% CAT ACTIVITY
1
2
p7
6150
112
B
Av
0.3
0.3
pT-50
6438
7778
Hp
Hp
7.5
6.7
pT-3
7224
7778
Ss
Hp
5.1
6.5
pT-69
7463
7778
Sp
Hp
2.7
3.2

3.3 The HPV 16, 18 and 11 enhancers are cell type specific

Papillomaviruses have a highly restricted host range and grow lytically only in differentiated keratinocytes of epithelia (Broker and Botchan, 1986). To check whether enhancer activity can be correlated with cell type specificity, the HPV 16, 18, and 11 ncr enhancer constructs were transfected into a range of host/cell types. The results shown in Table III indicate that the HPV 11 enhancer appears to function in a wider range of cell types as compared to the enhancers of HPV 16 and 18. The HPV 16 and 18 enhancers were restricted in activity to cell lines derived from human cervical carcinomas. The HPV 16 and 18 enhancer constructs were not active in the human fibroblast cell line, 143B or in the human rhabdomyosarcoma cell line, A204. The nonhuman cell lines, CV-1, a monkey kidney derivative, and the mouse fibroblast C127 also gave negative results for these regulatory elements. Interestingly, the HPV 16 and 18 enhancers were also inactive in the C127/BPV 1 transformed cell line, ID14. Therefore, it would appear that most of the epithelium-derived cervical cell lines contain the factors necessary to support HPV enhancer activity. This also correlates with the strict tissue tropism of these viruses. On the contrary, the HPV 11 enhancer appeared to be active in both cervical and non-cervical cells alike. Correlation of the cell-type specificity of the HPV 11 enhancer with its tissue tropism is discussed later.

Table III. Cell type specifity of the HPV 16, 18, and 11 enhancers

Cell lines	Ability to support the HPV enhancer		
	HPV 11	HPV 18	HPV 16
Cervical carcinoma			
HeLa (18)[a]	+	+[b]	+[b]
ME180 (18)	+	+	+[b]
MS751 (18)	+	+	+
SiHa (16)	+	+	+
CaSKi (16)	+	-	+
C33A (-)	+	+	+[b]
Human fibroblast			
143B (-)	ND[c]	-	-
Human rhabdomyosarcoma			
A204 (-)	+	-	-
Monkey kidney			
CV-1 (-)	+[b]	-	-
Mouse fibroblasts			
C127 (-)	ND[c]	-	-
ID14 (BPV 1)	ND[c]	-	-

[a] The numbers in the brackets indicate the HPV type integrated in the cell line; (-) indicates there is no known HPV DNA present in these cell lines. This information was taken from previous studies (Boshart et al, 1983; Pater and Pater, 1985; Yee et al, 1985).

[b] These CAT activities are just above background.

[c] ND: not determined.

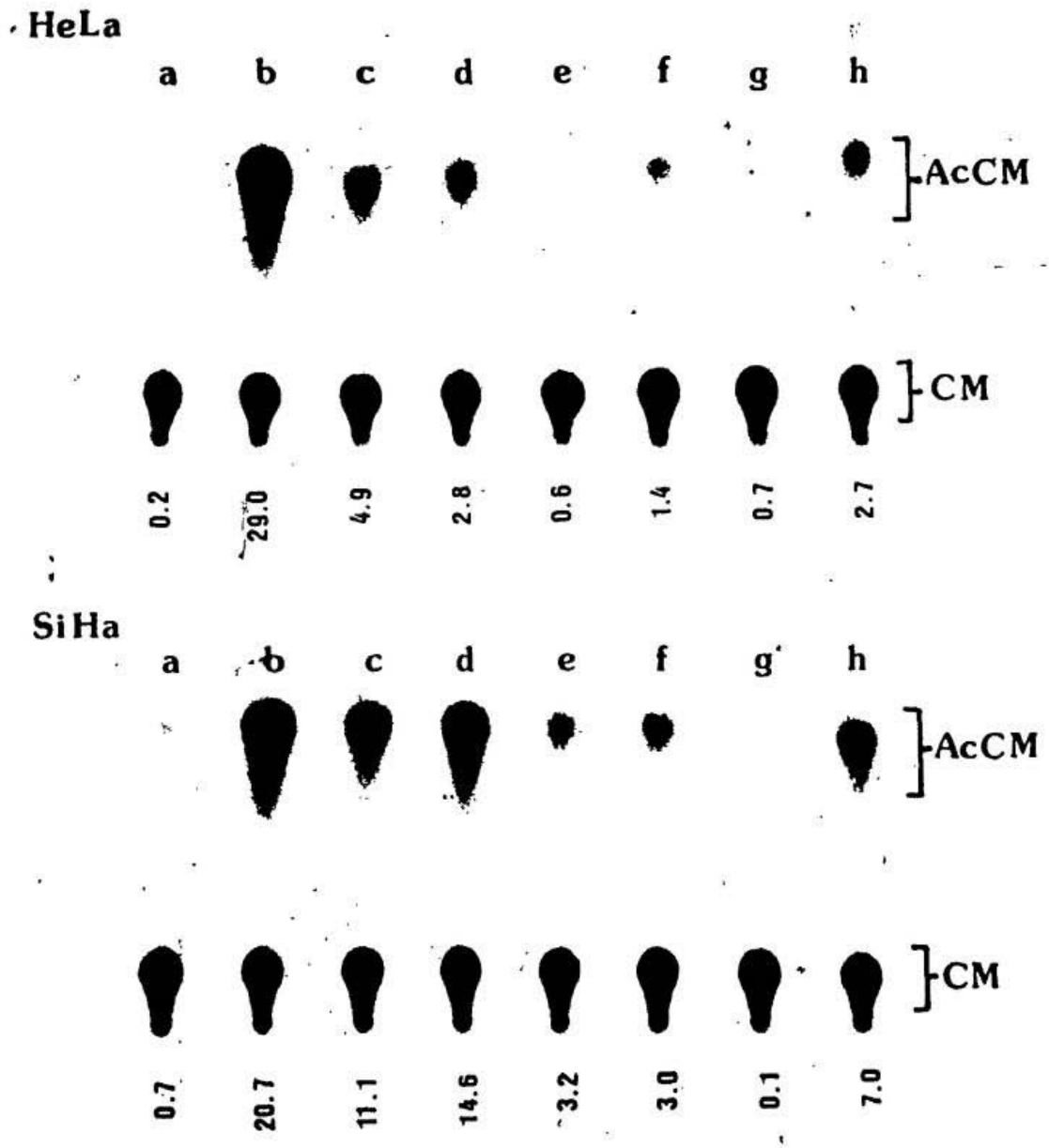
Further comparison of the HPV 16, 18, and 11 cell specific enhancer activities also involved transfecting the HPV/CAT constructs into three cervical carcinoma cell lines (HeLa, SiHa and C33A) and one noncervical cell line (A204). The HPV 11 enhancer was stronger than HPV 18 and 16 in all the cell lines examined. In, A204, a non-cervical cell line, the HPV 16 and 18 ncr/CAT constructs gave no enhancer activity; it was the same as that of pA10-CAT while the HPV 11/CAT plasmids gave CAT enzymatic activities much higher than that of the negative control (pA10-CAT). In fact, the enhancer activity of HPV 11 was 40 % of pSV2-CAT activity. The relative strengths of the enhancer elements from the oncogenic viruses, HPV 16 and 18, varied depending upon the cell type. HPV 18 enhancer activity was higher than HPV 16 in C33A cells, whereas in SiHa the reverse was true (lanes e and f versus lanes g and h; Figure 13A and 13B).

In those cell types for which the level of HPV 16 and/or 18 enhancer activity was above background, an increase in activity was observed with the removal of approximately 200 bp containing the putative promoter region. For instance, in C33A the CAT activity obtained with the HPV 18 ncr-pA10-CAT (p8) construct was 10.1 percent, whereas that obtained with pT-46 was 48.0 percent (Figure 13B). A similar tendency was found when the HPV 16/CAT recombinant plasmids were transfected into SiHa cells and HeLa cells (compare p7 and pT-50; Figure 13A) and to a lesser extent when the HPV

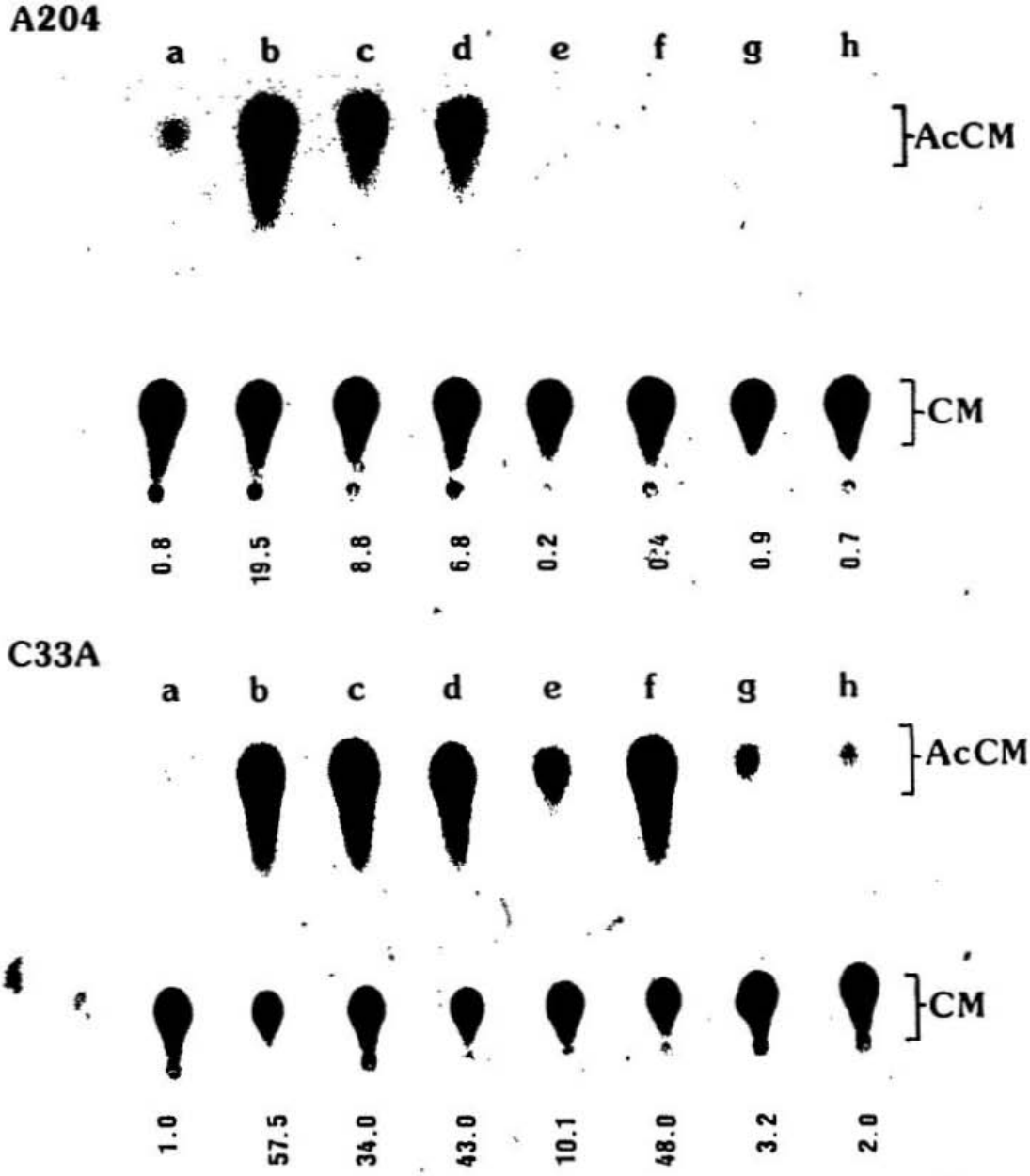
Figure 13. Cell type specificity of HPV 16, 18, and 11 enhancers.

Autoradiogram of CAT assays from transfection experiments in: (A) HeLa and SiHa cells; and in: (B) A204 and C33A cells. The extract from C33A cells transfected with pSV2-CAT was diluted by 10 fold. Plasmids were: a) pA10-CAT; b) pSV2-CAT; the HPV 11 constructs, c) p6 and d) pT-25; the HPV 18 constructs, e) p8 and f) pT-46; the HPV 16 constructs, g) p7 and h) pT-50. Numbers at the bottom of each autoradiogram are percent CAT activity. CM, chloramphenicol; AcCM, acetylated chloramphenicol.

A


HeLa
a
b
c
d
e
f
g
h
AcCM
CM
0.2
29.0
4.9
2.8
0.6
1.4
0.7
2.7
SiHa
a
b
c
d
e
f
g
h
AcCM
CM
0.7
20.7
11.1
14.6
3.2
3.0
0.1
7.0

B


A204
a
b
c
d
e
f
g
h
AcCM
CM
0.8
19.5
8.8
6.8
0.2
0.4
0.9
0.7
C33A
a
b
c
d
e
f
g
h
AcCM
CM
1.0
57.5
34.0
43.0
10.1
48.0
3.2
2.0

11/CAT constructs were tested in C33A (compare p6 and pT-25; Figure 13B). This difference in enhancer activity prompted me to check whether the removal of the promoter sequences (i.e. TATA box) alone can bring about this increase or if other upstream sequences act negatively to repress this activity.

This was determined by examining the effect of progressive removal of downstream sequences from the ncr of HPV 18 (Table IV). We used HPV 18 for these analyses since p8 had generated a significant increase in activity, with removal of the downstream 210 bp fragment (Figure 10, p 61). When a shorter HPV 18 fragment (BamHI/AvaII) was tested, the CAT activity increased slightly (compare p8 and pT-39; Table IV). The region removed from the BamHI/AvaII fragment contains a TATA box which might have interfered with the distal SV40 promoter (Wasylyk et al, 1983; Hirochika et al, 1987). Removal of a further 60 bp from the downstream portion of the BamHI/AvaII fragment also increased the CAT activity significantly (compare pT-39 and pT-33; Table IV). The fragment BamHI/AccI was also tested and the activity was found to increase further with removal of the 88 bp Acc/Bbv fragment (compare pT-33 and pT-46; Table IV). Data presented in Table IV suggests that a negative regulatory element may be present in this 88 bp fragment.

Table IV. Examination of the effect of progressive deletions on the enhancers of HPV 18 in C33A

Plasmids		% CAT Activity	
		1	2
pA10-CAT		0.3	0.3
pSV2-CAT		716.0	713.0
p8	(BamHI; 6929 to 119)	15.2	17.5
pT-39	(BamHI/AvaII; 6929 to 56)	22.2	24.4
pT-33	(BamHI/BbvI; 6929 to 7837)[a]	45.2	46.9
pT-46	(BamHI/AccI; 6929 to 7766)	50.3	57.0

[a] The BbvI site is at nucleotide 7837, however treatment with reverse transcriptase results in an endpoint at nucleotide 7854 (Figure 16, p 98).

3.4 Examination of the position and orientation dependence of the HPV 18 enhancer

Next, the position and orientation dependence of the HPV 18 enhancer was examined by placing the 347 bp ncr subfragment downstream of the CAT gene. Insertion of the 347 bp HPV 18 enhancer (SfaNI/AccI) fragment into the XbaI site of pA10-CAT in either orientation (Figure 6, p 42) dramatically decreased the ability of the 347 bp HPV 18 fragment to enhance transcription from the CAT gene (compare pT-52 and pT-11; Table V). Interestingly, placing of the SV40 regulatory sequences downstream of the CAT gene also greatly diminished its activity (see pSV2-CAT and pT-42, Table V). The orientation effect of the HPV 18/XbaI (downstream)/pA10-CAT constructs (compare pT-52 and pT-53; Table V) was similar to that observed with the HPV 18/BglII (upstream)/pA10-CAT constructs (compare pT-2 and pT-11; Table V); the greatest activity was observed when the enhancer (the AccI border) was placed nearest to the SV40 promoter (either upstream or downstream).

3.5 Analysis of sequences required for HPV 18 promoter function

Promoters are required unidirectionally for accurate and efficient transcription, whereas enhancers increase the rate of

Table V. Examination of the effect of position and orientation on the HPV 18 enhancer

Plasmid[a]	% CAT Activity	
	1	2
pA10-CAT	0.6	1.4
pSV2-CAT	344.0[c]	89.8[b]
pT-42	5.5	6.1
pT-51	1.1	1.6
pT-52	11.9	9.6
pT-53	1.5	1.5
pT-2	-	11.8
pT-11	78.0[b]	52.2[b]

a For a description of these plasmids see Figure 6, p 42.

b The percent of CAT activity (in C33A) is above that of the linear range, resulting in an underestimation of relative CAT activity.

c This pSV2-CAT extract was diluted to 1/10 relative to the others.

transcription from cis-linked promoters in an orientation-independent manner (reviewed by Maniatis et al, 1987). I. attempted to identify the minimal sequences required for HPV 18 promoter function. The enhancer/promoter activity of the HPV 18 1.1 Kb BamHI fragment (p29) was further localized to a 404 bp BssHII/BamHI (7572 to 119) fragment (pT-29; Figure 7, p 44). The BamHI and BssHII/BamHI fragments gave CAT activities of similar values (compare p29 and pT-29, Table VI). Since these plasmids have both the enhancer and the promoter in the same orientation, the enhancer fragment (BssHII/AccI) of HPV 18 was placed in the negative orientation relative to the HPV promoter (pT-28; Figure 7, p 44). This would indicate whether upstream enhancer sequences were required for promoter function. The results showed that sequences from the HPV 18 enhancer were required unidirectionally for HPV 18 promoter function (compare pT-29 and pT-28, Table VI). Insertion of this BssHII/AccI fragment into the BglII site of pA10-CAT generated a negative orientation construct (pT-37) which, when assayed for CAT activity, clearly indicated that this fragment by itself is capable of enhancer function in the negative orientation relative to a heterologous promoter (Table VI).

Table VI. Analysis of sequences required for HPV 18 promoter function

Plasmid	% CAT Activity	
	1	2
pA10-CAT	0.6	1.4
pSV2-CAT	296.0	89.8[a]
pT-13	0.1	1.0
p29[b]	17.0	14.0
pT-29[b]	17.0	21.0
pT-28[b]	1.0	0.9
pT-37[b]	-	14.9

a The pSV2-CAT (C33A) extract from experiment 2 was not diluted (1/10) as in experiment 1. The percent activity for pSV2-CAT (2) is above that of the linear range, resulting in an underestimation of relative activity.

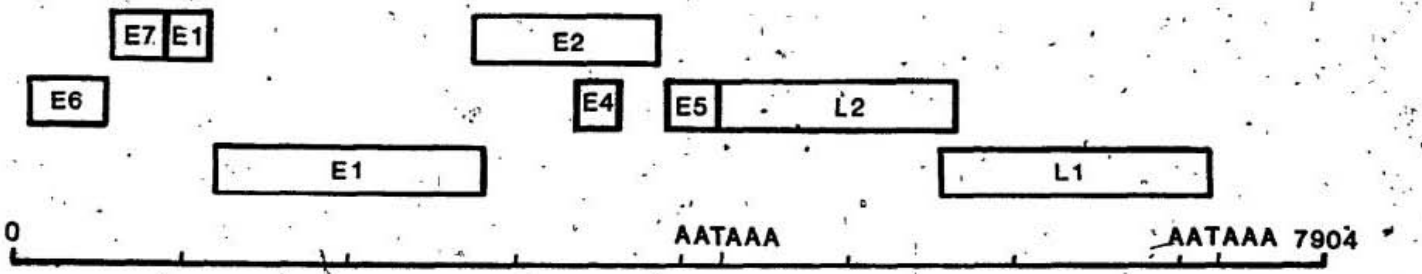
b For a description of these plasmids see Figure 7, p 44.

3.6 Trans-regulation of the HPV 16, 18, and 11 enhancers by the E2 and E7 genes of HPV 16

The control of viral gene expression is believed to involve a complex interaction of viral cis-acting elements with viral and cellular trans-acting factors. Cis-acting elements located in the noncoding regions of HPV 16, 18, and 11 have been shown to respond to trans-activating factors encoded by the BPV 1 and HPV E2 ORFs (Phelps and Howley, 1987; Hirochika et al, 1987; Thierry and Yaniv, 1987). A repressor function has also shown to be associated with the carboxyterminal region of the BPV 1 and HPV 16 E2 gene products (Lambert et al, 1987; Cripe et al, 1987). To further characterize the regulatory function associated with the early region of HPVs, most of the early genes (E7, E1, E2, E4, and E5) of HPV 16 were expressed under the control of the early promoter of SV40 (pD-7). Cotransfection of the HPV 16, 18, and 11 ncr/CAT constructs with this early region-expressing plasmid (pD-7) resulted in an increase in CAT activity. As shown in Figure 14, the HPV 16 enhancer was trans-activated by 7 fold, the HPV 11 enhancer by slightly less than 5 fold, while the HPV 18 enhancer was marginally stimulated from 0.48 percent to 0.77 percent. These results suggest that the relative amount of trans-activation by the HPV 16 early ORF product(s) is lower for the HPV 18 enhancer than for the HPV 16 and 11 enhancers. A deletion of 635 from the E2 gene (3126 to 3761)

Figure 14. HPV 16 E2 gene product trans-activation and E7 gene product trans-repression of HPV 16, 18, and 11 regulatory regions.

Cotransfections were in CV-1 cells with 5 μg of CAT plasmids along with 20 μg of each of the indicated HPV 16 early region plasmids or 20 μg of pUC 19 as control. Also, 5 μg of pSV2-CAT or pA10-CAT was transfected with 20 μg of pUC 19 into these CV-1 monkey kidney cells and gave the enzyme activities of 33.9 and 0.1 percent, respectively. CAT or HPV 16 early region expression plasmids are described in Materials and Methods. Plasmids used were: HPV 16, p7; HPV 11, p6; HPV 18, p8. The map of HPV 16 ORFs aligns with the solid lines which represent HPV 16 sequences present in the five HPV 16 early region expression plasmids. Dashed lines indicate the deletions and the triangle depicts a 4 bp deletion.



		HPV 16	HPV 11	HPV 18
		%CAT ACTIVITY		
pD-7	501 Hpa II – 4334 Sau I	1.15	4.00	0.77
pT-57	501 Hpa II – 3126 Nde I – 3761 Nde I – 4334 Sau I	0.20	0.33	0.20
pD-2	700 Hpa II – 4334 Sau I	5.40	18.86	2.63
pT-20	700 Hpa II – 4 bp deletion 2891 Bst XI – 4334 Sau I	0.32	0.30	0.28
pT-48	700 Hpa II – 3126 Nde I – 3761 Nde I – 4334 Sau I	0.38	1.18	0.24
pUC 19		0.17	0.86	0.48

abolished this trans-activation, indicating that the E2 gene product is responsible for this stimulation of the HPV enhancers (see pT-57, Figure 14). This has also been suggested by Phelps and Howley (1987) and by Cripe et al (1987). This deletion mutant (pT-57), when cotransfected with each of the enhancer plasmids gave a value at or near that observed for those cotransfected with pUC 19, indicating that the E7, E1, and E5 ORF products were insufficient for the above stimulation. Plasmid pD-2, containing a 200 bp deletion at the amino terminal end of E7 (501 to 700), stimulated the HPV enhancers to a level (of CAT activity) 3 to 5 fold higher than that observed in pD-7 cotransfections; this indicates the presence of a repressor in the E7 ORF (Figure 14). A plasmid containing deletions in both the E2 and E7 ORFs (pT-48) did not trans-activate the enhancer plasmids. Thus the remaining intact E1 and E5 ORF products were insufficient for the observed trans-activation. The pT-48 and pT-57 (mentioned above) plasmids are also missing the E4 ORF, therefore to further localize the trans-acting function to the E2 ORF, another deletion mutant plasmid was constructed. The plasmid pT-20 was generated by placing a frameshift mutation in the amino terminal portion of the E2 gene at the BstXI site (2891) of pD-2; this frameshift mutation should only affect the translation of the E2 ORF. Cotransfection experiments of the HPV enhancer/CAT constructs with this deletion mutant generated conclusive evidence that the HPV 16 E2 gene

product alone is responsible for the observed trans-activation.

Comparison of the trans-acting functions of pD-7 and pD-2 has designated a repressor function to the E7 ORF. To further verify trans-regulation by each of the E2 and E7 ORFs, the enhancer plasmids were cotransfected with two constructs, one expressing the E2 ORF (pD-2), the other expressing the E7 ORF (pT-57). The results presented in Table VII clearly show trans-activation of the enhancer by the E2 ORF and repression of this activity by the E7 ORF. These plasmids also contain the E1, E5, and in pD-2 the E4 ORFs. However, as demonstrated above, these ORFs do not encode products which are involved in trans-activation or trans-repression of viral gene function. Plasmid pT-48, containing deletions in both the E2 and E7 ORFs, was used as a control in these experiments to demonstrate that the negative effect of E7 from pT-57 is not due to other HPV 16 sequences. The data in Table VII also rule out the possibility that the results in Figure 14 might be due to cis effects from the E7 deletion. For example, the deletion might have altered the stability of or translation from a modified mRNA transcribed from the pD-2 plasmid.

The mechanism of transregulation was analyzed to determine whether the HPV 16 E2 and/or E7 involved the E2 binding motif, $ACCGN_4CGGT$. HPV 11 enhancer plasmids with four (p6; DraIII/SfaNI), one (pT-25; DraIII/SauI) or none (pT-1; NdeI/SauI) of these E2 binding motifs (Figure 17, p 101), were cotransfected with either the E2 expressing plasmid, pD-

Table VII. E2 trans-activation and E7 repression of the HPV 16, 18, and 11 enhancers

Plasmids	% CAT Activity		
	HPV 16	HPV 11	HPV 18
pD-2 + pT-48	5.4	30.4	4.7
pD-2 + pT-57	1.8	7.8	3.2
pUC 19	1.8	10.8	1.6

CAT assays were performed as described in the Results, on extracts from CV-1 cells transfected with 5 μg of HPV enhancer plasmid plus either 20 μg of pUC 19 or 10 μg of each of the HPV 16 early region expression plasmids. The CAT enzymatic levels were 461.8 percent for pSV2-CAT and 0.6 percent for pA10-CAT. The CAT constructs for HPV 16, 11, and 18 enhancers were p7, p6, and p8, respectively. The numbers are an average of duplicate transfection experiments.

2, or the E2 and E7 expressing plasmid, pD-7. The results (Table VIII) indicate that trans-activation by the E2 ORF requires the E2 binding motif (compare the CAT activity of p6 and pT-25 to that of pT-1 when cotransfected with pD-2). A similar observation has been made by others (Cripe et al, 1987; Haugen et al, 1987; Spalholz et al, 1987). Cotransfection of the pSV2-CAT plasmid with the E2 expressing plasmid results in little if any increase in CAT activity. However, less than half the activity is obtained with pSV2-CAT when cotransfected with the E7-expressing plasmid as compared to the activity of pSV2-CAT when transfected with pUC 19 alone or with the pT-48 deletion mutant plasmid (Table IX). It is important to note that repression of pSV2-CAT by the E7 gene product does not directly involve an E2 binding motif since such sequences are not present in pSV2-CAT.

3.7 Trans-activation by the E2 gene product is mediated through a heterologous but not a homologous promoter

The trans-activating function of the E2 gene, as described above, was examined using the HPV 16, 18, and 11 noncoding region in an enhancer configuration (i.e. the HPV enhancer fragments are under the control of the SV40 early promoter; see plasmids p7, p8, and p6, respectively, Figure 14). I next examined the effect of cotransfection with plasmid pD-2 on the activity of constructs containing the HPV ncrs in a

Table VIII. Requirement of the E2 binding motif for trans-activation by the E2 gene product

Plasmid	Number of E2 motifs	% CAT Activity		
		pUC 19	pD-2	pD-7
p6	4	5.2	21.7	10.1
pT-25	1	5.7	17.0	9.3
pT-1	0	7.4	9.3	5.1

Cotransfection into CV-1 cells was as described in Figure 14. The pSV2-CAT plasmids gave CAT activities of 96.0 (not diluted) and 0.3 percent, respectively.

Table IX. Effect of E2 and E7 gene products on the SV40 regulatory sequences

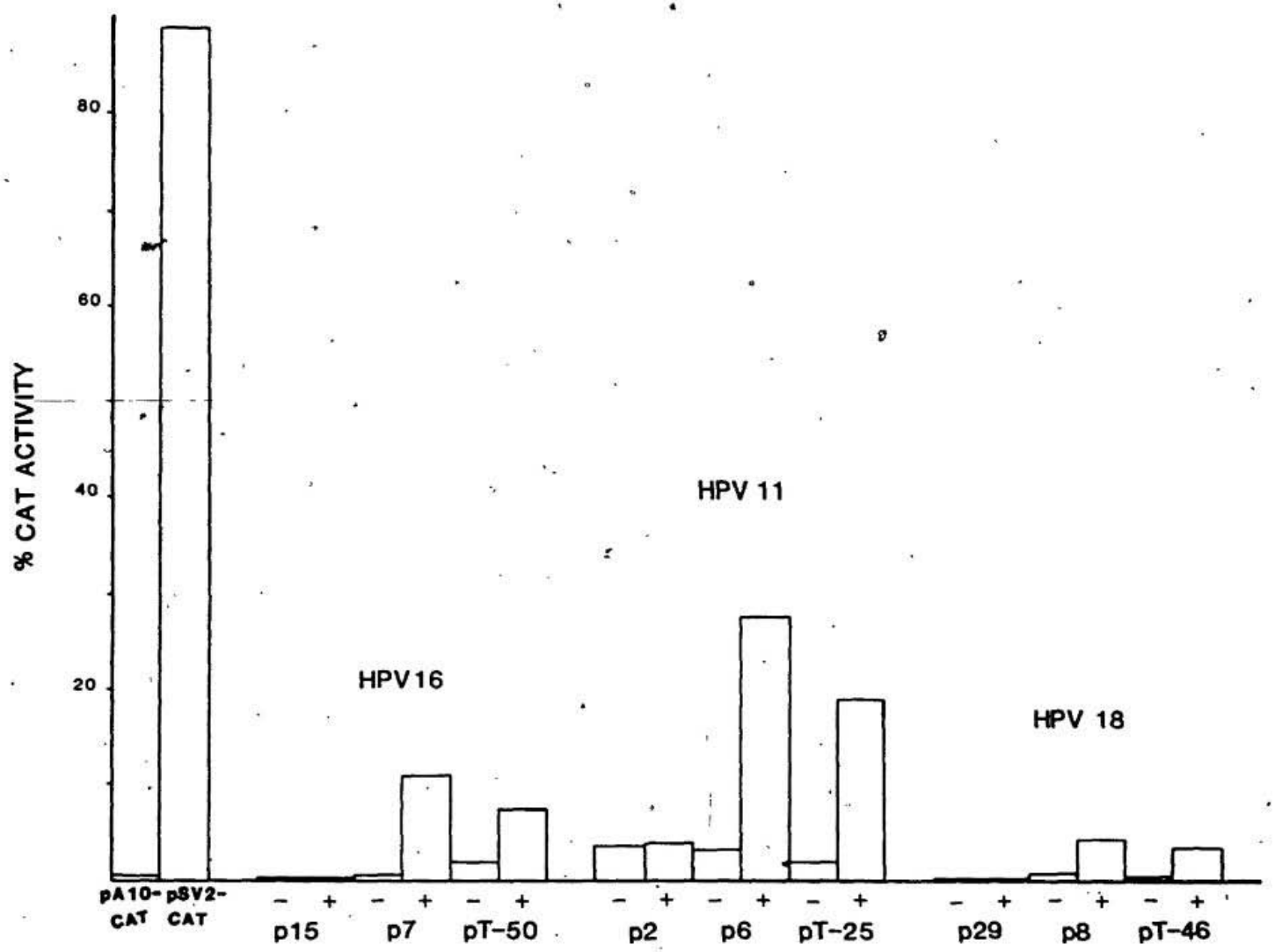
Plasmids	% CAT Activity	
	1	2
pSV2-CAT + pUC 19	25.6	20.6
pSV2-CAT + pT-48	25.4	20.7
pSV2-CAT + pT-57	11.0	7.6
pSV2-CAT + pD-2	26.2	-

Five micrograms of pA10-CAT was cotransfected along with 20 μg of pUC 19 plasmid DNA to give CAT activity values of 0.5 and 0.6 in CV-1 cells. Similarly, 5 μg of pSV2-CAT was cotransfected with 20 μg of pUC 19 or HPV 16 early region expression plasmids (listed above).

HPV 16, 18, and 11 promoters (plasmids p15, p29, and p2 respectively) was not trans-activated by cotransfection with pD-2 (Figure 15). Fragments containing the HPV enhancer and the upstream E2 binding motif, $ACCGN_4CGGT$, were inserted into the pA10-CAT plasmid (pT-50 for HPV 16, pT-46 for HPV 18, and pT-25 for HPV 11) and the resulting constructs when cotransfected were trans-activated by the E2 gene product (Figure 15). These results reaffirm the above observation that one E2 binding motif is sufficient for E2-mediated trans-activation. Similar activation ratios are obtained when the HPV ncr enhancer plasmids (p7, p8, and p6) and the HPV deletion mutant enhancer plasmids (pT-50, pT-46, and pT-25, respectively) are compared in cotransfection experiments.

Figure 15. The trans-activation of the enhancer plasmids of HPV 16, 18, and 11 by the HPV 16 E2 gene product.

The enhancer and promoter/enhancer plasmids of HPV 16, 18, and 11 were transfected into CV-1 cells in the absence (-) or presence (+) of pD-2 as described in Figure 14.


% CAT ACTIVITY
80
60
40
20
HPV16
HPV 11
HPV 18
pA10-CAT
pSV2-CAT
p15
p7
pT-50
p2
p6
pT-25
p29
p8
pT-46

CHAPTER 4

DISCUSSION

The studies described in this thesis were undertaken to further an understanding of the regulation of human genital papillomavirus (HPV 16, 18, and 11) gene expression. The objectives of this study were (1) to identify the cis-acting elements (i.e. enhancers/promoters) necessary for the HPV noncoding region promoter function and (2) to determine whether viral and cellular factors were involved in regulating expression from these sequences. The data presented in this study demonstrate that a transcriptional promoter and/or enhancer exists within the noncoding region of HPV 16, 18, and 11. These enhancer elements were also shown to be responsive to the negative and positive viral factors encoded by the HPV 16 E7 and E2 ORFs. The constitutive enhancers of HPV 16, 18, and 11 have been localized to 315, 230, and 213 bp fragments, respectively. Comparison of the DNA sequence homologies of the viral enhancers indicate that the HPV 16 and 18 enhancer elements are closer in sequence homology than either HPV 16 or 18 is to the HPV 11 enhancer (see below). The sequence relatedness of these HPV noncoding regions corresponds to the differences in cell specificities observed among the HPV 16, 18, and 11 enhancer elements. The restriction of HPV 16 and 18 enhancer function to

cervical epithelial cells is contrasted to the relatively broad cell-type expression of the HPV 11 enhancer. This allows for correlation of enhancer specificity to the differential tissue tropism and pathogenicity observed between the human nononcogenic (HPV 11) and oncogenic (HPV 16 and 18) genital papillomaviruses.

4.1 Identification of promoter and enhancer activity in the noncoding regions of HPV 16, 18, and 11

Promoters are defined as cis-acting elements which are required immediately upstream of and in the same orientation (positive), as linked genes to allow transcription, while enhancers are orientation-independent (Sassone-Corsi and Borrelli, 1986). Comparison of the noncoding region sequences of HPV 16, 18, and 11 indicated the presence of promoter-like sequences near the amino-terminal portion of E6 (Figure 8, p 53). Previous studies of SV40 and BPV 1 have localized enhancer and promoter functions upstream of the early genes of these viruses (Ahola et al, 1987; Benoist and Chambon, 1981; Spalholz et al, 1985). Initial identification of enhancer and promoter activity in the HPV noncoding region involved subcloning a fragment from the HPV 16 and 18 genome which have sequences that overlap with the L1 and E6 ORFs. Whereas, the enhancer and promoter of HPV 11 was initially localized to a relatively small 559 bp fragment which has its

upstream border at 7486 (the L1 stop codon is at 7274). The results demonstrated that the ncr fragments of HPV 11 and 18 contain enhancer and promoter function (see Figure 9, p 56). The HPV 11 and 18 positive orientation promoter constructs generated significant activity relative to that of the negative orientation constructs, indicating the presence of a functional promoter in these noncoding regions. The presence of enhancer elements upstream of the promoter was established by examining the ncr/pA10-CAT constructs of HPV 11 and 18.

The HPV 16 ncr BamHI/AvaII fragment generated little or no enhancer or promoter activity in all cell lines tested (i.e. in C33A and SiHa cervical cell lines). This result is consistent with previous studies. The major 5' cap site of HPV 16 E6-E7 transcripts has been mapped to nucleotide 97 in cervical carcinoma cells (Smotkin and Wettstein, 1986). When Cripe et al (1987) placed the HPV 16 promoter upstream of the HSV-1 thymidine kinase (tk) gene, transcripts were generated in both SiHa and HeLa cells. However, when this same group examined P97 promoter activity in the enzymatic CAT assay, the activity was very low in both cervical cell lines. Although the discrepancy observed in Cripe et al (1987) may be due to an out-of-frame translation initiation at the upstream AUG, the low level of HPV 16 promoter activity in this study ocurred in spite of an in-frame E6 ATG positioned upstream of the CAT gene. It is possible that HPV 16 enhancer/promoter activity is not favored in these systems.

4.2 Characterization of the constitutive enhancer elements of HPV 16, 18, and 11

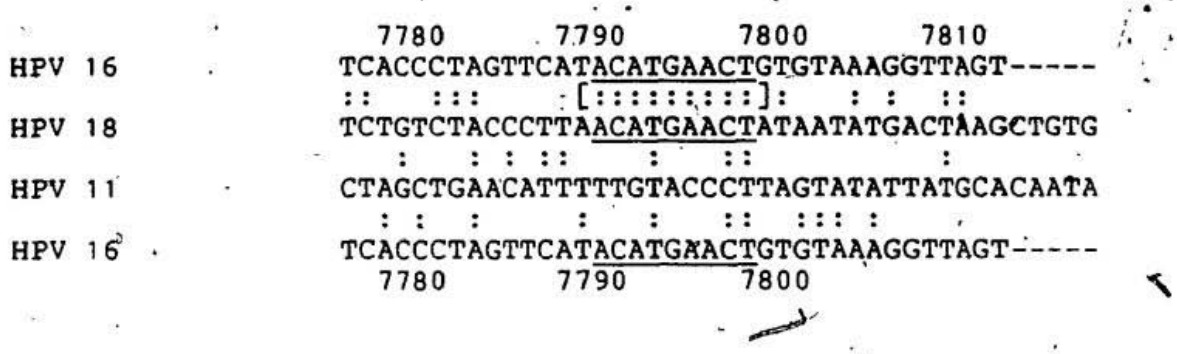
The noncoding region enhancer constructs of HPV 16 and 18 gave very low enhancer activity relative to that generated by the HPV 11 ncr construct. Interestingly, removal of approximately 200 bp from the downstream portion of the HPV 16 and 18 noncoding region fragment increased the enhancer activity by 10 fold for HPV 18 and by 20 to 25 fold for HPV 16. Although the HPV 11 enhancer activity increased by about 2 fold with removal of the E6 proximal 175 bp fragment, this increase in enhancer activity is likely a characteristic largely specific to the HPV 16 and 18 enhancers. In fact, it appears from the results of deletion mutagenesis of the HPV 16 (Cripe et al, 1987) and HPV 18 (Swift et al, 1987) enhancers that such a removal of early region proximal sequences leads to an increase in enhancer activity. A similar increase in activity with removal of promoter proximal sequences has been observed by Hirochika et al (1987) with the HPV 1 enhancer. One possible explanation is that this region contains promoter sequences (i.e. TATA box) which interfere with transcription from the distal SV40 promoter. Inhibition of enhancer activity, or "enhancer damping", has been reported to occur when promoter sequences are inserted between an enhancer and the transcriptional unit being monitored (Kadesh and Berg, 1986; Wasylyk et al, 1983). Another possibility is that there are

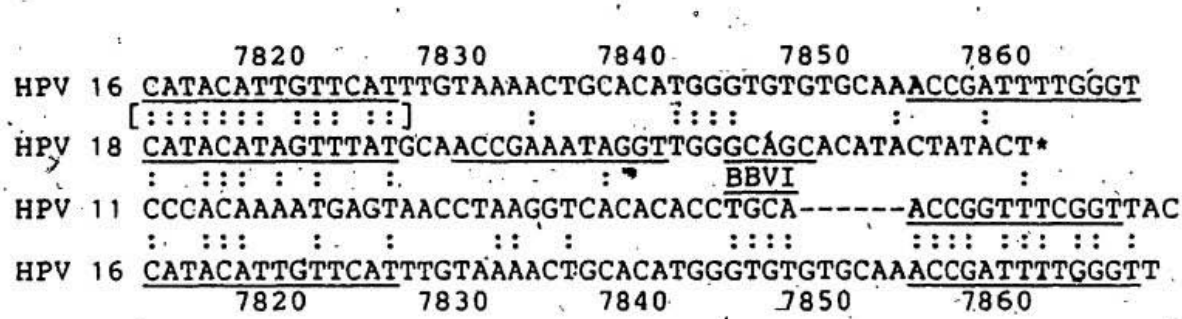
negative regulatory elements in the downstream portion of the noncoding region; removal of these elements increases enhancer activity. In case of HPV 16 and 18, the increase in activity was 10 to 25 fold with removal of E6 proximal sequences, whereas the increase in HPV 11 was only 2 fold. This slight increase in HPV 11 enhancer activity may be due to the removal of the TATA box from the downstream segment of the ncr fragment. However, the very large increase in HPV 16 and 18 enhancer activity would indicate that a mechanism such as suppression via a negative regulatory element is operating. There are two short stretches of homology found approximately 10 bp downstream of the enhancer in HPV 16 and 18 (Figure 16). However, in HPV 11 the homology was largely restricted to the upstream E2 binding motif, $ACCGN_4CGGT$ (Figure 16). Hence, the 88 bp fragment (AccI/BbvI) of HPV 18 along with the analogous HPV 16 ncr fragment may contain sequences that act as negative regulatory elements that function to negatively regulate HPV 16 and 18 enhancer activity.

Yet another possibility is that decreasing the distance between the HPV enhancer and the SV40 promoter somehow leads to an increase in activity. A progressive removal of E6 proximal sequences from the enhancer fragment of HPV 18, resulted in a progressive increase in activity. In a study by Swift et al (1987), the HPV 18 enhancer was shown to activate the SV40 early promoter from a distance of 20 Kb.

Figure 16. Alignment of the region located immediately downstream from the enhancers of HPV 16, 18, and 11.

The major regions of homology are the 9 and 14 bp elements of HPV 16 and 18 (underlined). The BbvI recognition site for HPV 18 is underlined, while the BbvI endpoint after treatment with reverse transcriptase is represented as an asterick [*], The E2 binding motifs, $ACCGN_4CGGT$, (degenerate in HPV 16 and 18) have been underlined. The HPV 18 region is from 7760 to 7854, whereas the sequence used for HPV 11 is from 7816 to 7903.


7780 7790 7800 7810
HPV 16 TCACCCTAGTTCATACATGAACTGTGTAAAGGTTAGT-----
HPV 18 TCTGTCTACCCTTAACATGAACTATAATATGACTAAGCTGTG
HPV 11 CTAGCTGAACATTTTTGTACCCTTAGTATATTATGCACAATA
HPV 16 TCACCCTAGTTCATACATGAACTGTGTAAAGGTTAGT-----
7780 7790 7800


7820 7830 7840 7850 7860
HPV 16 CATACATTGTTCATTTGTAAAACTGCACATGGGTGTGTGCAAACCGATTTTGGGT
HPV 18 CATACATAGTTTATGCAACCGAAATAGGTTGGGCAGCACATACTATACT*
BBVI
HPV 11 CCCACAAAATGAGTAACCTAAGGTCACACACCTGCA------ACCGGTTTCGGTTAC
HPV 16 CATACATTGTTCATTTGTAAAACTGCACATGGGTGTGTGCAAACCGATTTTGGGTT
7820 7830 7840 7850 7860

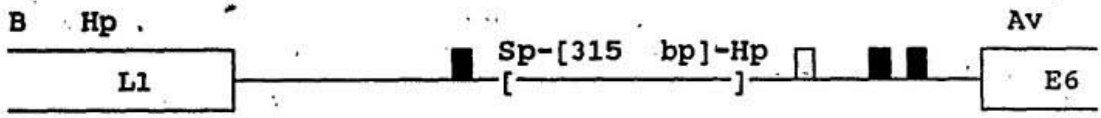
However, since the HPV 18 enhancer studied by this group was integrated into the host chromosomes, it is not directly comparable to the results of the present study. The current investigation suggests that the distance located between the HPV enhancer and SV40 promoter affects the efficiency of enhancer function. Further studies such as site directed or linker scanning mutagenesis are required to distinquish between above possibilities.

Localization of the constitutive enhancers of HPV 16, 18, and 11 to 315, 230, and 213 bp fragments, respectively, also involved the removal of late region proximal sequences from the noncoding region fragments (Figure 17). These upstream segments contain (1) the carboxyterminal portion of the L1 ORF (except for the HPV 11 fragment), (2) the GT-rich region found in all human genital papillomavirus noncoding regions, (3) putative polyadenylation signals for the late genes, and (4) one of the E2 binding motif sequences, $ACCGN_4CGGT$ (Cole and Danos, 1987; Dartmann et al, 1986; Seedorf et al, 1985). The results show that removal of this upstream segment consistently decreased the enhancer activity from the HPV noncoding region subfragments. In a previous study a second enhancer element was localized to the analagous upstream portion of the HPV 6vc ncr (a variant of HPV 6 isolated from a verrucous carcinoma; Rando et al, 1986a). However, no significant activity was detected when such regions of HPV 16 (6150 to 7463) and HPV 18 (7119 to 7508) were examined for

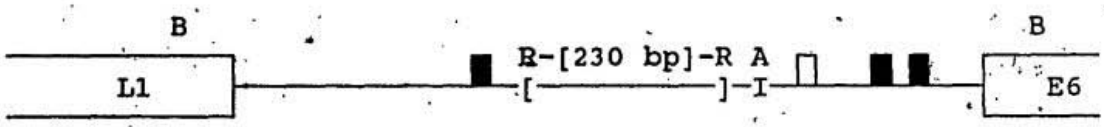
Figure 17. Organization of the noncoding regions of HPV 16, 18, and 11 showing the localized constitutive enhancers.

The diagram represents a linear restriction map of the noncoding region and the surrounding L1 and E6 open reading frames. The constitutive enhancers of HPV 16, 18, and 11 are shown within the brackets [] as 315 bp, 230 bp, and 213 bp. Filled boxes show the positions of the E2 motif; open boxes correspond to degenerate versions. The restriction enzymes are: A, AccI; Av, AvaII; B, BamHI; D, DraIII; H, HphI; N, NdeI; R, RsaI; S, SauI; Sp, SphI.

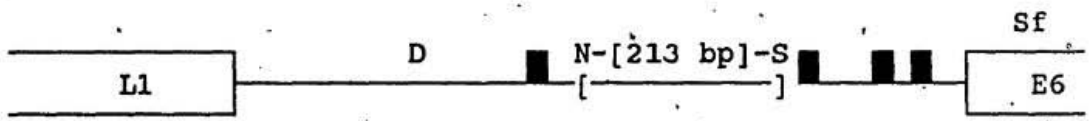
HPV 16



HPV 18



HPV 11



enhancer function (Cripe et al, 1987; Swift et al, 1987).

This decrease in enhancer activity may result from removal of upstream ncr sequences. It is possible that the HPV enhancer is made up of the several modular elements, some of which are not able to function independently as enhancer elements. In this situation the upstream region would contain sequences that function to augment enhancer activity only when placed upstream of the constitutive enhancer elements. In fact, two sequence motifs having complete or almost complete homology with the SV40 core sequence, GTGG$^{A}_{T}$$^{A}_{T}$$^{A}_{T}$G, are found upstream of the HPV 18 enhancer at nucleotide 7490 and 7350. Removal of upstream ncr sequences from the enhancer of HPV 18 does not result in a decrease in enhancer activity when the enhancer is placed in front of the homologous (HPV 18) promoter (Table VI, p 78). These data suggest that the upstream ncr sequences may be important for HPV enhancer (but not promoter) function. However, at least for HPV 16 and 18, independent constitutive enhancer elements are not present in this region. Gius and his colleagues (1988) have recently located an enhancer (IE6) approximately 500 bp upstream of the E6 cap site of HPV 18. Since this enhancer is dependent upon the viral E6 gene product for function, I can predict that at least for HPV 11 and 18 (examined in HPV negative C33A cells) the enhancer activity observed in the present study is not due to E6-mediated activation.

In the present study the HPV 18 constitutive 230 bp

enhancer was shown to be orientation-independent (see also, Swift et al, 1987). However, the ncr 1.1 Kb (BamHI) and the 837 bp (BamHI/AccI) and the 347 bp (SfaNI/AccI) fragments of HPV 18 activated the SV40 enhancerless promoter in an orientation-dependent manner (Table II, p 61). This phenomenon may be due to the surrounding viral sequences somehow causing orientation-dependent regulation of transcriptional activity. This orientation-dependence may be explained by a distance-related effect.—The location of the 230 bp enhancer towards the downstream portion of the inserted fragment indicates that there may be a "distance" effect operating in the 1.1 Kb, 837 bp, and 347 bp fragments but not the 230 bp enhancer fragment (see diagram below). Futhermore, the central positioning of the 213 bp constitutive enhancer in the 559 bp HPV 11 ncr fragment seems to have allowed orientation-independent activity without interference from any distance-related parameters. It is, therefore, probable that the length of the viral sequence between the HPV enhancer and the SV40 promoter may be able to influence orientation independence of the enhancer. This possibility is favored when examining the diagram shown below with the constitutive enhancer of HPV 11/18 represented by ([#]/[*]) and the surrounding viral sequences indicated by the dotted line (---).

FRAGMENT		ORIENTATION INDEPENDENCE
	HPV 11	
559 bp	------[######]------	YES
213 bp	[######]	
	HPV 18	
1.1 Kb	----------[*******]--------	NO
837 bp	----------[*******]	NO
347 bp	------[*******]	NO
230 bp	[*******]	YES

The results presented in Table V (p 78) indicate that the HPV 18 347 bp enhancer can function when located downstream from the CAT gene. However, the level of enhancer activity was less than in the constructs in which the 347 bp enhancer fragment was placed upstream to the CAT gene. The SV40 340 bp enhancer and promoter region fragment (PvuII/HindIII; Figure 2, p 27) was also placed downstream of the CAT gene in an attempt to produce an internal control for this experiment. The ability of the SV40 enhancer to stimulate CAT activity was drastically reduced (by greater than 60 fold) when placed downstream from the gene (Table V, p 78). Earlier studies have reported the ability of the SV40

enhancer (72 bp repeats) to activate plasmid pBR322 "substitute" start sites (Moreau et al, 1981; Wasylyk et al, 1983). This effect is referred to as "enhancer damping" (Kadesh and Berg, 1786; Wasylyk, 1983). The decrease observed in activity when the HPV 18 enhancer fragment was placed downstream from the gene may also be due to "enhancer damping". Alternatively, this decrease in activity may be due to the larger distance between the enhancer and the upstream promoter increasing as the enhancer is placed downstream. In fact, the constructs with the enhancer placed nearest to the downstream portion of the gene (pT-52) do seem to generate the highest enzymatic CAT activity.

4.3 Upstream enhancer sequences are required unidirectionally for HPV 18 promoter function

By definition, promoter sequences are required immediately upstream of and in the same orientation as the linked gene, while enhancers activate transcription in an orientation-independent manner.The 1.1 Kb (BamHI) fragment of HPV 18 was shown to function as an active promoter when placed in the same orientation as that of the linked gene (p29). The minimal sequences required for HPV 18 promoter function were localized to the 404 bp (BssHII/BamHI) downstream portion of the 1.1 Kb ncr fragment (pT-29). The CAT activity generated by both HPV 18 promoter constructs, p29 and pT-29, were

identical, suggesting that regions upstream of the BssHII (7572) site were not required for transcription from the homologous HPV 18 promoter. To identify the smallest sequence required for promoter function, the BssHII/AccI 164 bp enhancer fragment was inserted in the opposite orientation, relative to that of the downstream promoter sequences (210 bp) to generate pT-28 (Figure 7, p 44). The CAT activity of this construct (pT-28) was negligible (close to that of the negative control) suggesting that some or all of the upstream 164 bp enhancer sequences are required in the positive orientation for HPV 18 promoter function. As stated above, the 164 bp enhancer fragment and the HPV 18 promoter do not function when the (164 bp) fragment is in the opposite orientation, whereas this 164 bp enhancer fragment activates a heterologous promoter even in the negative orientation (Table VI, p 80) This indicated that the promoter sequences overlap with the enhancer sequences.

A recent investigation has also localized the HPV 18 promoter/enhancer function to a 400 bp fragment comprising the early region proximal portion of the ncr (Thierry and Yaniv, 1987). When this same group cloned the E6 proximal 240 bp promoter fragment (RsaI/BamHI) of HPV 18 (Figure 17, p 101) downstream of the SV40 enhancer they obtained activity. This suggests that the 240 bp downstream segment of the HPV 18 ncr is sufficient to act as a promoter when activated by a heterologous enhancer. It is possible that the

extra 28 bp RsaI/AccI (7738 to 7766) included in the 240 bp promoter fragment (RsaI/BamHI; see Figure 17, p 101) contain important sequences required for promoter function; this 28 bp region is absent from the 210 bp (AccI/BamHI; see Figure 17, p 101) promoter fragment used in this study. Therefore, the HPV 18 promoter did not function, perhaps due to the requirement of this extra 28 bp upstream of and in the same orientation as the 210 bp AccI/BamHI promoter fragment. Another possibility is that the SV40 (heterologous) enhancer used by Thierry and Yaniv (1987) does not require promoter sequences upstream of this RsaI (7738) site. However, it is possible that activation by the homologous HPV 18 enhancer requires promoter sequences from the enhancer region (in the same orientation) to enable promoter function.

4.4 Cell type specificity and sequence relatedness of the HPV 16, 18, and 11 enhancers

As mentioned earlier, HPV types 16 and 18 are associated with cervical neoplasia, whereas HPV 11 has been largely isolated from papillomas of the head and neck region and also from benign lesions of the external genitalia. It was of interest to determine whether the pathology of the lesion or the tissue tropism of the infecting virus might be related to the regulatory elements (enhancers/promoters) which control early gene expression. The identification of duplications in the

regulatory regions of HPV 11 and 6b variants isolated from highly aggressive lesions would seem to support this possibility. These variants were isolated from a nasal inverting papilloma (HPV 11r; Respler et al, 1987), from a highly aggressive verrucous carcinoma (HPV 6vc, Rando et al, 1986b), and from a Buschke-Lowenstein tumor (HPV 6d, Boshart and zur Hausen, 1986). These duplications resulted in a much higher expression of the CAT gene driven by the regulatory regions from the variants HPV 11r and HPV 6vc (Pater et al, 1988; Rando et al, 1987a). Additional enhancer elements may be advantageous for the expression of specific early viral genes and could possibly contribute to the aggressiveness of the lesion or to its carcinogenic progression. Further, a recent report by Byrne et al (1987) indicates that malignant conversion of lung papillomatous lesions and subsequent metastasis into lymph nodes involves the duplication of the HPV 11 ncr. Thus it appears that changes in oncogenicity might be correlated with changes in the regulatory elements affecting gene expression. One goal of the present study has been to determine whether the tissue tropism exhibited by the genital HPVs may be associated with the (sequence and functional) relatedness of the regulatory elements of the prototype genital HPVs (16, 18, and 11).

I searched for homology among the enhancer sequences of these viruses. Since HPV 16 and 18 are oncogenic and show somewhat similar tissue tropism, while HPV 11 is nononcogenic

and shows different tissue tropism, I asked whether HPV 16 and 18 share a common sequence in their regulatory region which is different in HPV 11. Comparative analysis of the sequences of HPV 16 and 18 revealed a 60 bp region near the downstream end of the enhancer fragment having approximately 83 % homology (Figure 18; see also Swift et al, 1987). Between the oncogenic HPV 16 and 18 and the nononcogenic HPV 11 the only homology was a 11 bp stretch, ATTTTTGGCTT, found within this 60 bp sequence (see Figures 19 and 20; for HPV 18 the sequence was ATTTTAGTTTG). This 11 bp sequence is also found in HPV 6b (Schwarz et al, 1983), and with a single change in HPV 33 (Cole and Streeck, 1986), whereas seven of the 11 bp, TTTTTGG, are also found in the noncoding region of BPV 1. Cripe et al (1987) have also localized the enhancer of HPV 16 to 224 bp which includes the abbreviated octamer motif, TTTGGCTT. These authors note that the presence of this octamer motif in all sequenced genital HPVs and postulate that the keratinocyte-dependent expression of genital papillomaviruese may be attributed to this sequence. Indeed, the inverted consensus sequence, AAPuCCAAA, is found in the vacinity of the TATA box of several bovine, human, and murine epidermal cytokeratin genes and of the human involucrin gene (Blessing et al, 1987).

This sequence alone does not appear to regulate tissue tropism. Eight or nine of the 11 bp are found in the

Figure 18. Nucleotide alignment of the HPV 16 and 18 enhancer regions.

The region found between the brackets [] IS a 59 bp stretch of 83 % homology between HPV 16 and 18. The HPV-specific element of HPV 16, ATTTTTGGCTT, is underlined as is the closely related sequence found in HPV 18 (ATTTTAGTTTG). The 230 bp region of the HPV 18 enhancer has been indicated by the presence of two RsaI sites at 7508 and 7738. The enhancer region of HPV 16 and 18 is preceded by the E2 binding motif (underlined).

HPV 16

VERSUS

HPV 18

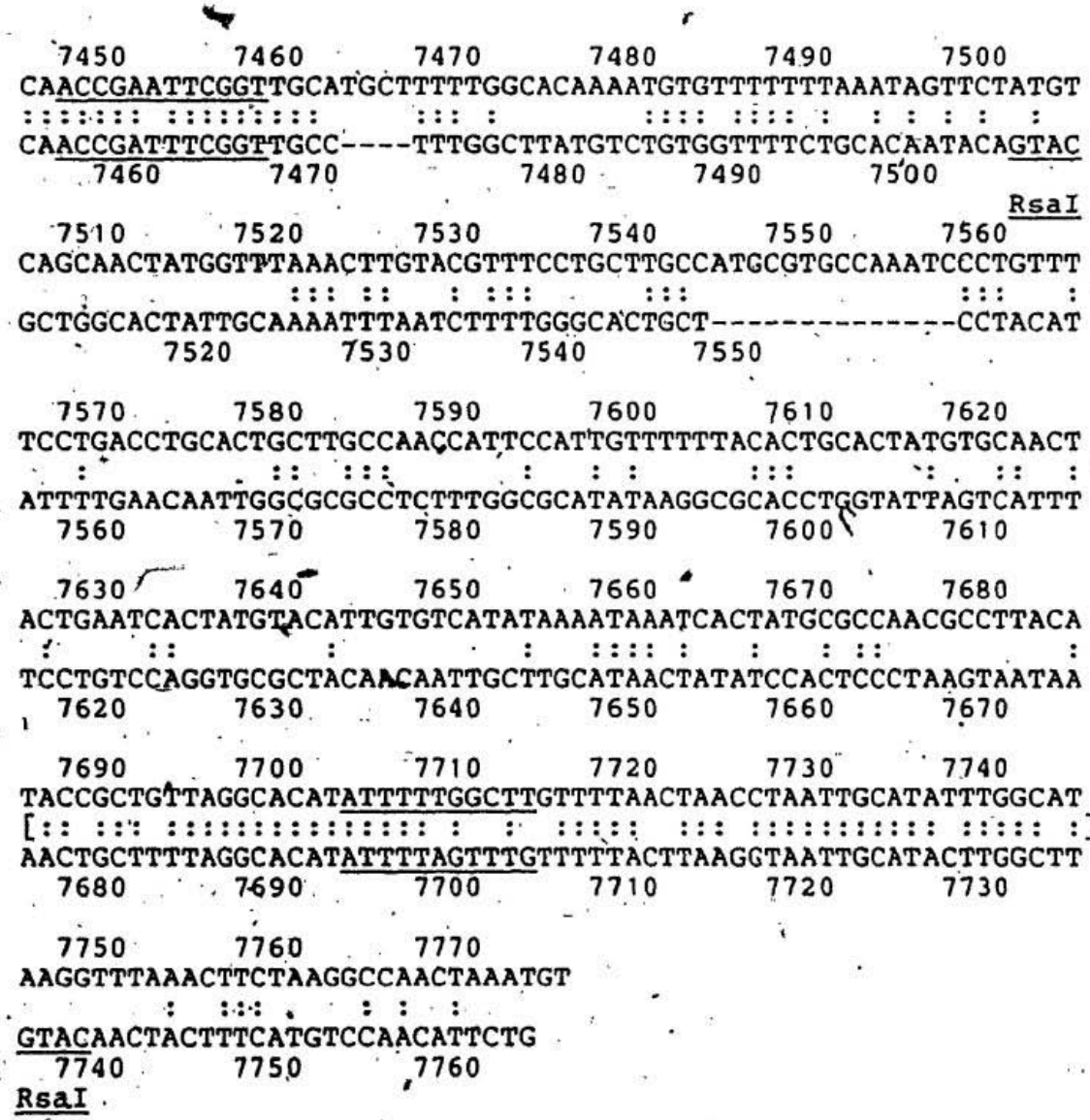


Figure 19. Nucleotide alignment of the enhancer sequences of HPV 16 and 11.

The E2 binding motif, $ACCGN_4CGGT$, is underlined, as is the HPV-specific motif, ATTTTTGGCTT.

HPV 11

VERSUS

HPV 16

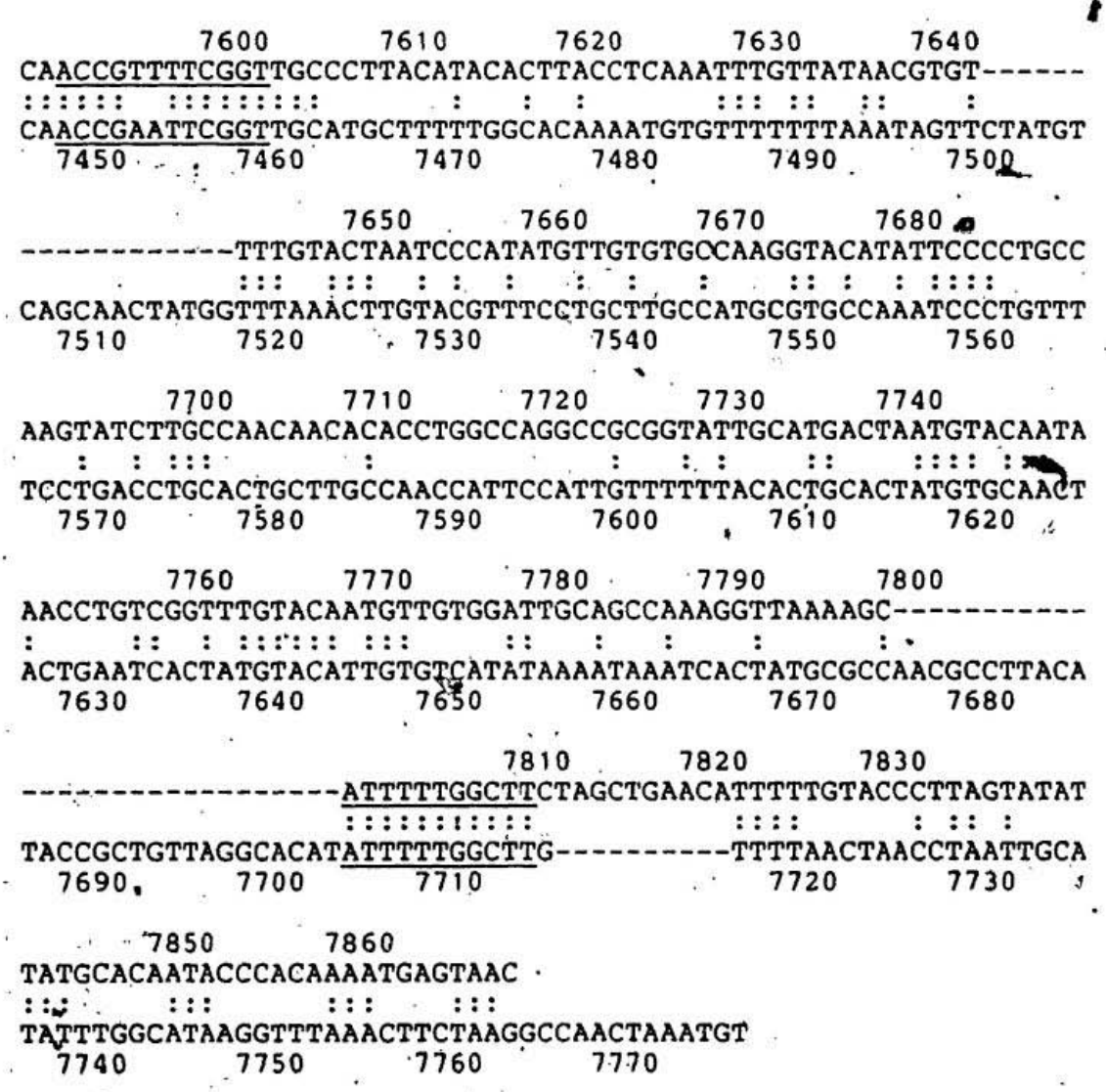


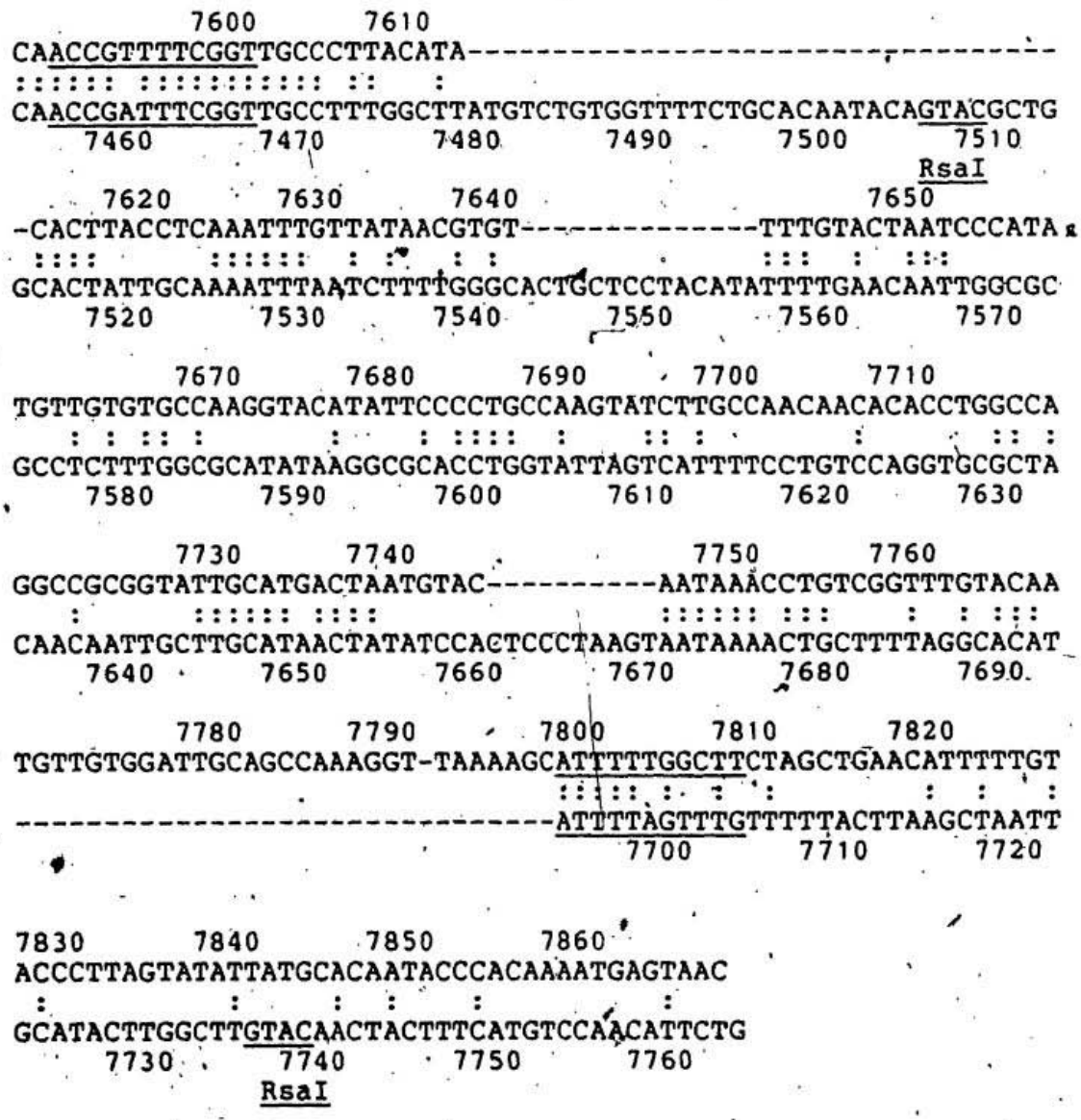
Figure 20. Alignment of the HPV 11 and 18 enhancer sequences.

The E2 binding motif, ACCGN_4CGGT, is underlined as is the HPV-specific element, ATTTTTGGCTT. This motif is represented by ATTTTAGTTTG in HPV 18.

HPV 11

VERSUS

HPV 18



regulatory and late regions of the papovavirus, BK which has a different tropism than that of papillomavirus (Seif et al, 1979). This 11 bp sequence is also found in pBR322 (Sutcliffe, 1979). Swift et al (1987) observed two functional domains in the enhancer regions of HPV 18 both having full enhancer activity in HeLa cells. However, only one of the domains contains the 11 bp sequence.

As mentioned above, the HPV 16, 18, and 11 enhancer activity exhibited cell-type specificity (Table III, p 70; Figure 13, p 72). The HPV 11 enhancer had consistently higher activity and appeared to be active in a wider range of cell types than the HPV 16 and 18 enhancers (see Table III, p 70; Figure 13, p 72). This is probably due to transcriptional control which is less stringent in HPV 11 than in HPV 16 and 18. The high level of variability observed between the sequences of the HPV 11 enhancer and the HPV 16 and 18 enhancers may be associated with the observed differences in cell-type specificities and enhancer strength (Figures 13, p 72; Figures 19 and 20). Recently Chow et al (1987c) located the enhancer region of HPV 11 to a 140 bp sequence (7730 to 7870). Interestingly, sequences resembling the GT, Sph, and P motifs (Zenke et al, 1986) and the recognition sequence for CTF (NF-1) were found in the HPV 11 enhancer (Chow et al, 1987c; Hirochika et al, 1988). Comparison of these sequences in HPV 11 (i.e. their presence and their position) with the HPV 16 and 18 enhancer sequences reveals no significant

homology. The presence or absence of these various motifs in a specific pattern may allow expression of the HPV 11 enhancer in a wider range of cell types.

HPV 16 and 18 are generally restricted to one particular type of lesion in the genital area, while HPV 11 shows pathology distinct from that of HPV 16 and 18 in the genital area and is also unique in its ability to grow in the upper respiratory tract. Investigation of HPV 16 and 18 enhancers indicated that the activity is generally restricted to human epithelial cells derived from cervical carcinomas (Table III, p 70; Figure 13, p 72; see also Cripe et al, 1987; Swift et al, 1987; Thierry et al, 1987b). However, the relative strengths of the HPV 16 and 18 enhancers vary depending upon cell type (Figure 13, p 72). This means that even though the early region proximal segment of the HPV 16 and 18 ncrs share significant homology (see Figure 8, p 53), other enhancer elements present in the upstream segment of both HPV 16 and 18 could contribute to the differential activity of these enhancers. Computer alignment programs were used in this study to identify HPV sequences having homology with a number of consensus sequences; these sequences are shared among different sets of enhancers. The HPV 16 enhancer (7450 to 7778) contains sequences resembling the SV40 "core" element, GTGG$^{A}_{T}{}^{A}_{T}{}^{A}_{T}$G, the Sph I motif (AAGCATGCA) and the recognition sequence for AP1 (TGACTCA; TPA-responsive

element) and IGNFA (ATTTGCAT) factors (Figure 21; Angel et al, 1987; Lee et al, 1987a, 1987b; Sen and Baltimore, 1986; Zenke et al, 1986). This region also has sequences similar to elements found in the polyoma A-2 (TCCACCCA) and polyoma alpha (GTCAGTTA) enhancers. In contrast, the HPV 18 enhancer was found to contain sequences that resemble the P (TCAATTAGTCA) and TC (TCCCCAT) motifs and the recognition sequence for AP3 (TGTGGTTG) (Figure 22; Zenke et al, 1986; Johnson et al, 1987). Sequences similar to elements found within the SV40 and HPRT enhancers (CCAGGCTCC; Melton et al, 1984) and to the recognition sequence for the IgNFA factor (Sen and Baltimore, 1986) were also present in the HPV 18 enhancer. However, there were no similarities between the HPV 16 and 18 enhancer motifs as revealed by this study except for the IgNFA recognition sequence which is found within the 60 bp region of the HPV 16 and 18 enhancers. Recent investigations have indicated that multiple copies of the GT- or Sph- motifs can function as enhancers with cell-type specificity (Ondek et al, 1987; Schirm et al, 1987). Results from these studies along with the present investigation indicate that enhancers of differing specificities/activities can be assembled from many individual sequence motifs by combining them in different patterns (Schirm et al, 1987). The sequence relatedness between the HPV 16 and 18 enhancers do seem to correlate with the observed epithelial-specificity. However, the differential cell-

Figure 21. Sequence of the HPV 16 enhancer showing homology to various SV40 and polyoma motifs.

The AP1 and IgNFA recognition sequences are homologous to the HPV 16 enhancer. Sequences similar to the Sph motif and "core" element of SV40 and to the polyoma alpha and A-2 elements are indicated. The E2 binding motif and HPV-specific elements of HPV 16 are underlined.

HPV 16

VERSUS

ENHANCER ELEMENTS

```
7450       7460       7470       7480       7490       7500
ACCGAATTCGGTTGCATGCTTTTTGGCACAAAATGTGTTTTTTTAAATAGTTCTATGTCA
             ::::::                                      ::::
          AAGCATGCA                                      GTCA
E2-binding    Sph
motif         motif

7510       7520       7530       7540       7550       7560
GCAACTATGGTTTAAACTTGTACGTTTCCTGCTTGCCATGCGTGCCAAATCCCTGTTTTC
:  :
GTTA
polyoma
alpha
element
7570       7580       7590       7600       7610       7620
CTGACCTGCACTGCTTGCCAACCATTCCATTGTTTTTTACACTGCACTATGTGCAACTAC
                 :::: :::                                   :
                TCCACCCA                                    C
                 polyoma
                   A-2
                 element
7630       7640       7650       7660       7670       7680
TGAATCACTATGTACATTGTGTCATATAAAATAAATCACTATGCGCCAACGCCTTACATA
::: :::
TGACTCA
AP1
recognition
sequence
7690       7700       7710       7720       7730       7740
CCGCTGTTAGGCACATATTTTTGGCTTGTTTTAACTAACCTAATTGCATATTTGGCATAA
                      ::: :::                 : ::::::
                     GTGGATTG                 ATTTGCAT
   HPV-specific       "core"                    IgNFA
   motif               element                  recognition
                                                sequence

7750       7760       7770
GGTTTAAACTTCTAAGGCCAACTAAATGTC
```

Figure 22. Sequence of the HPV 18 enhancer showing homology to various SV40 enhancer elements.

The AP3 and IgNFA recognition sequences are similar to motifs found in the HPV 18 enhancer. In addition, sequences similar to the PC and TC motifs of SV40 and the CCAGGCTCC motif of SV40 and of the hypoxanthine phosphoribosyltransferase (HPRT) genes are displayed underneath the HPV 18 enhancer sequence. The E2 binding motif and the HPV-specific element of HPV 18 are underlined.

HPV 18

VERSUS

ENHANCER ELEMENTS

```
  7460       7470       7480       7490       7500       7510
ACCGATTTCGGTTGCCTTTGGCTTATGTCTGTGGTTTTCTGCACAATACAGTACGCTGGC
                                  ::::::::
E2-binding                        TGTGGTTTG                 RsaI
motif                               AP3
                                  recognition
                                   sequence
  7520       7530       7540       7550       7560       7570
ACTATTGCAAAATTTAATCTTTTGGGCACTGCTCCTACATATTTTGAACAATTGGCGCGC

  7580       7590       7600       7610       7620       7630
CTCTTTGGCGCATATAAGGCGCACCTGGTATTAGTCATTTTCCTGTCCAGGTGCGCTACA
                                  ::::::::            :::::   :
                                  TCAATTAGTCA         CCAGGCTCC
                                  P motif             SV40/HPRT
                                                        element

  7640       7650       7660       7670       7680       7690
ACAATTGCTTGCATAACTATATCCACTCCCTAAGTAATAAAACTGCTTTTAGGCACATAT
                                  :::: :
                                  TCCCCAT
                                  TC motif

  7700       7710       7720       7730       7740
TTTAGTTTGTTTTTACTTAAGCTAATTGCATACTTGGCTTGTAC
                                  : ::::::
HPV-specific                      ATTTGCAT                  RsaI
  motif                             IgNFA
                                  recognition
                                   sequence
```

type specificity of the HPV enhancers observed for similar cervical cell lines could be associated with the presence of different motifs in the enhancer regions of these viruses.

4.5 Trans-regulation by the HPV 16 E2 gene product

As stated earlier, gene expression by many viruses is controlled not only by cis-acting elements, but also in trans by the gene products of the same virus. Results from the present study demonstrate that the HPV 16, 18, and 11 enhancers are trans-activated by the HPV 16 E2 gene product. This is in agreement with previous observations that trans-activation by the E2 gene product of either HPV 16 (Cripe et al, 1987; Phelps and Howley, 1987), HPV 1, HPV 11, HPV 18 (Hirochika et al, 1987), or BPV 1 (Spalholz et al, 1985, 1987) was required to detect HPV enhancer function in CV-1 and cervical carcinoma cells (Cripe et al, 1987; Thierry and Yaniv, 1987). CV-1 cells were chosen for the present studies because the constitutive activity of the HPV enhancers is relatively low in this cell line. The level of activation seems to be greater for the HPV 11 and HPV 16 enhancers compared to that of HPV 18 (Figure 14, p 82). This is consistent with the trend observed by Hirochika et al (1987) who observed that the E2 gene products of HPV 1, HPV 11, and BPV 1 activate the HPV 18 enhancer to a small extent or not at all in CV-1 cells and that the HPV 11 E2 gene product stimulates both

HPV 11 and HPV 16 enhancers to a greater extent. These enhancer regions of papillomaviruses contain multiple copies of the palindrome, $ACCGN_4CGGT$, which has been shown to be required for E2 trans-activation and for E2 protein binding in vitro (Androphy et al, 1987; Haugen et al, 1987; Hirochika et al, 1988; Moskaluk and Bastia, 1987; Spalholz et al, 1987). Comparison of the HPV noncoding region sequence indicates the presence of three of these motifs in HPV 16 and 18 whereas there are four copies in HPV 11. The fourth copy found in HPV 11, immediately downstream from the enhancer, has been replaced in the HPV 16 and 18 noncoding region with degenerate versions (see Figure 16, p 98). Recent evidence suggests that the degenerate version present in HPV 16, $ACCGN_5CGGT$, is not a target for E2 binding (Hirochika et al, 1988); this is due to the stringent requirement of 6 nucleotides (instead of 7) between the two halves of the palindrome, ACC and GGT, for in vitro binding to occur. The report by Hirochika et al (1988) also suggests that the sequence of these six central nucleotides may influence the strength of association with the E2 protein. Thus, the HPV 18 degenerate motif, $ACCGN_4AGGT$, probably has a lower affinity than the $ACCGN_4CGGT$ motifs. Positions of the other three motifs, one upstream of the enhancer and the other two repeats between the CAAT-like box and TATA box of the E6 promoter, are conserved among the three viral types. When the four central nucleotides of the E2 binding motifs of HPV

16, 18, and 11 are compared, patterns that may explain the high E2 gene product activation of HPV 16 and 11 relative to that of HPV 18 were not found. In fact, the binding motifs of HPV 11 and 18 are slightly more similar than those of HPV 16 are to 11. This suggests that activation of the HPV enhancer by the E2 gene product involves more than the interaction of the E2 protein with the $ACCGN_4CGGT$ motif. It is possible that the sequence of the nucleotides immediately outside of and/or the distance between the various E2 motifs may influence the affinity of E2 protein binding and subsequently E2-mediated trans-activation.

While results of the current study show that the presence of only one E2 binding motif in addition to viral enhancer sequences is sufficient for trans-activation, whereas the E2-dependent activation of transcription from the BPV 1 ncr has been reported to require at least one (but preferably two) motifs at each end of the element (Spalholz et al, 1987). This suggests that some cooperation between the two ends is involved in the activation mechanism. It is possible that the higher level of activation observed with the ncr plasmids (p6, p7, and p8) may be due to cooperation among the three (or four in HPV 11) E2 binding motifs. Indeed, insertion of one copy of the $ACCGN_4CGGT$ motif (without viral enhancer sequences) upstream of the SV40 promoter was insufficient to confer E2-dependent activation. Significant activation does occur when multiple copies ($5 > 2$) of the E2 motif are

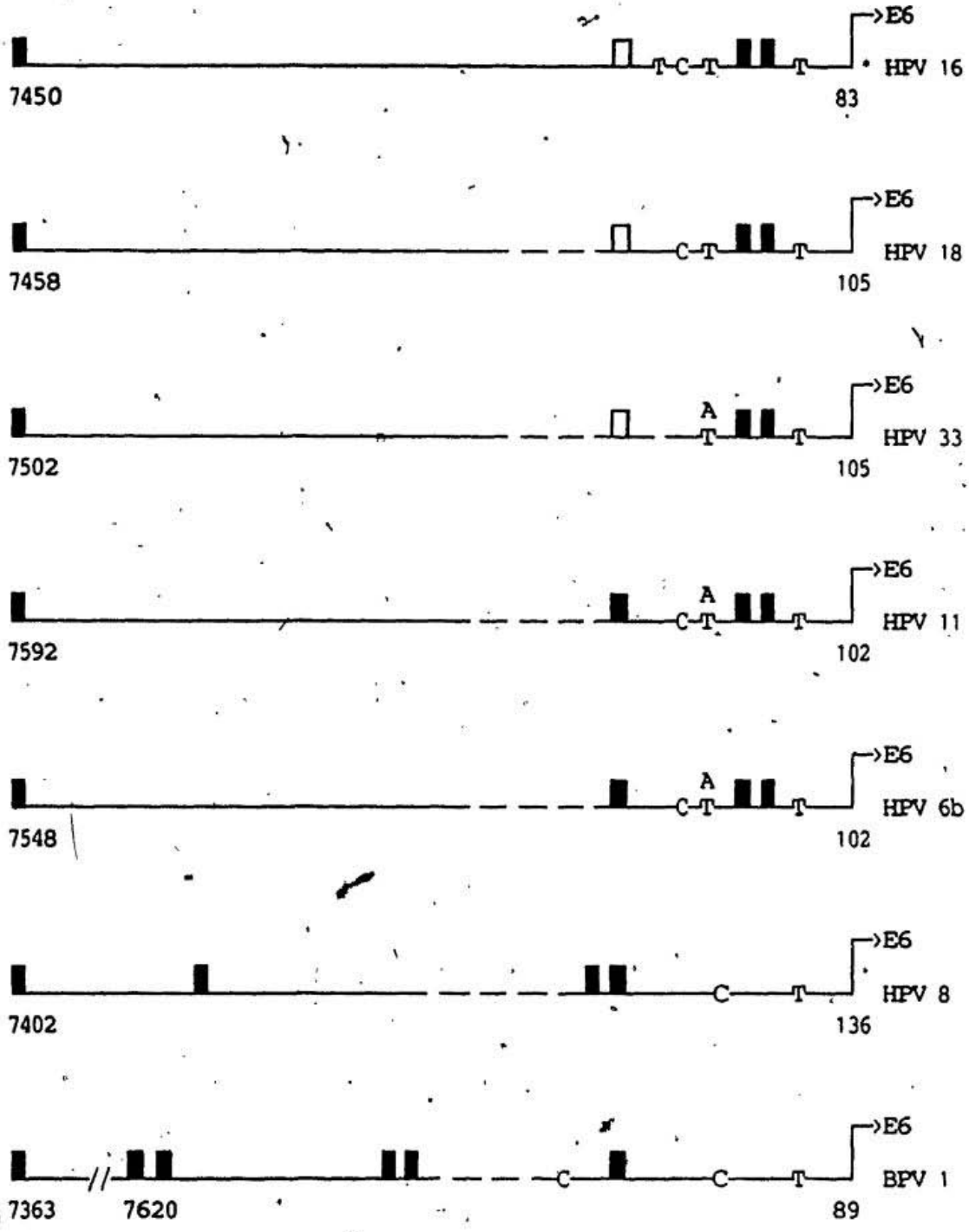
present (Hirochika et al, 1988). Fragments containing one or multiple E2 binding motif(s) along with viral enhancer sequences are activated by the E2 gene product. These results suggest that this E2-dependent trans-regulation may be influenced by the constitutive enhancer. Indeed, recent reports indicate that one copy of the E2 binding site has E2-dependent enhancer activity only when it is in the presence of the constitutive enhancer (Hirochika et al, 1988). The present investigation and others (Cripe et al, 1987; Hirochika et al, 1988) show that the constitutive enhancer alone is insufficient to confer E2-dependent activation. Collectively, the results suggest that the differential E2-dependent activation of the HPV 16, 18, and 11 regulatory regions may involve cooperative interaction of the E2 binding protein(s) with other transcriptional factors which bind to elements present in the constitutive enhancer.

The results show that the E2 gene product can trans-activate when the promoter is heterologous (i.e. SV40 early promoter) but not homologous (i.e. HPV promoters). This could be due to repression of homologous promoters by the E2 product. There is evidence from studies by others to support this. For instance, Lambert et al (1987) have identified a repressor function to the carboxyterminal portion of the BPV 1 E2 expressing plasmid. Furthermore, Thierry and Yaniv (1987) have reported the repression of transcription from the HPV 18 P105 promoter by cotransfecting with a BPV 1 E2

expressing plasmid. The same BPV 1 E2 expressing plasmid was found to trans-activate expression from the BPV 1 P89 promoter and from the enhancer of HPV 18 placed upstream of the SV40 promoter (Spalholz et al, 1987; Thierry and Yaniv, 1987). It is difficult from the present study to pinpoint a repressor function to the E2 gene product due to the low constitutive enhancer activity in CV-1 cells. The results certainly support the differential function of HPV 16 E2 gene product when HPV 16, 18, and 11 enhancers are expressed from homologous promoters as opposed to heterologous promoters. Comparative analysis of the sequences upstream the BPV 1 E6 ORF with those of the genital HPVs revealed that in BPV 1 the E2 binding motifs are found greater than 130 bp upstream of the major promoter (i.e. E6 promoter; Figure 23). On the contrary, in HPV 16, 18, and 11 two E2 binding sites are present between the TATA and CAAT-like consensus sequences that precede the major start site for the early transcripts of these viruses (see Figure 23; Pater et al, 1988; Thierry et al, 1987b; Smotkin and Wettstein, 1986). As speculated by Thierry and Yaniv (1987), the binding of multiple E2 protein molecules might sterically interfere with the formation of an active transcriptional complex. It is not yet known which of the E2 gene products (full length or the C-terminal products) are responsible for this repression. Both the full length and the C-terminal E2 products of BPV 1, HPV 16, and HPV 11 have been shown to require E2 binding motifs

Figure 23. General organization of the papillomavirus control region.

The dotted lines represent sequences missing from particular viruses. The potential ATG for the E6 ORF is represented by the arrow. The letters T and A/T indicate the positions of TATA boxes and A/T rich regions, respectively; the CAAT-like boxes are represented by the letter C. Filled boxes show the positions of the E2 binding motifs; open boxes correspond to degenerate versions. The sequences for BPV 1 (Chen et al, 1982), HPV 8 (Fuchs et al, 1986), HPV 6b (Schwarz et al, 1983), HPV 11 (Dartmann et al, 1986) and HPV 33 (Cole and Streek, 1987), HPV 18 (Cole and Danos, 1987) and HPV 16 (Seedorf et al, 1985) were obtained from previous reports.


E6
HPV 16
7450
83
E6
HPV 18
7458
105
E6
HPV 33
7502
105
E6
HPV 11
7592
102
E6
HPV 6b
7548
102
E6
HPV 8
7402
136
E6
BPV 1
7363
7620
89

for their action. Thus, it is possible that DNA binding by the intact and/or the C-terminal E2 gene products are sufficient to repress HPV expression by steric hindrance.

4.6 Trans-repression by the HPV 16 E7 gene product

The E7 ORF of HPV 16 encodes a trans-regulating product which was found to repress trans-activation mediated by the E2 gene product (Figure 14, p 82; Table VII, p 86). While it is difficult from this present study to determine the exact mechanism of trans-repression by the E7 gene product, the repression of pSV2-CAT by the E7 gene product (see Table IX, p 89) suggests that repression by this gene may not be mediated through the E2 binding motif. The E7 gene product may be a general inhibitor of transcription. This would account for the negative regulation of the heterologous SV40 early promoter present in pSV2-CAT. However, it is possible that the E7 gene product may have two separate functions, one which generally represses enhancer activity, and another which represses E2-mediated transcription (HPV-specific activity). To this end, the E7 gene product may interact directly or indirectly with the E2 gene product and interfere with the trans-activation domain of the protein. Alternatively, the E7 protein may interfere with the binding properties of the E2 gene product. Further deletion analysis studies are required (1) to determine which cis-acting sequences (i.e. the

E2 binding motif) if any, are required for this trans-repression and (2) to determine if direct repression of E2 activation or binding functions are involved in the trans-repression by the E7 gene product. In particular, experiments on cells expressing higher HPV constitutive enhancer activity are necessary to examine the mechanism(s) of the E7 gene product repressor function.

4.7 Viral gene regulation in HPV infection and cancer

Collectively, the results suggest a possible explanation for the status and expression of integrated HPV DNA in cervical carcinomas and of the extrachromosomal status of HPV DNA in benign warts or precancerous lesions. It has been suggested that transcription of the potential transforming gene(s) of the HPV 16 E6/E7 region (Bedell et al, 1987; Matlashewski et al, 1987) is tightly regulated in benign warts or precancerous lesions where HPV DNAs are replicating episomally (Cripe et al, 1987). This regulation may be mediated by the binding of the full-length E2 trans-activator and/or the short E2 repressor gene products to the repeated E2 binding motifs ($ACCGN_4CGGT$) located between the CAAT-like box and TATA box of the E6 promoter (Figure 23). As discussed previously, this binding could function to repress HPV E6/E7 expression by steric hindrance. This E2 gene product(s) along with cellular factors (which bind to HPV enhancer motifs) may also

function to regulate other HPV promoters. In addition, any expression of the E7 ORF would result in a protein whose function is to repress E2-activation. This repression may or may not involve interference with the binding of the full length E2 gene product to the upstream E2 motifs.

Examination of the physical state of integrated HPV DNA reveals that although different regions of the HPV genome were integrated in cervical carcinoma cell lines, the early region including the E6 and E7 ORFs/ncr are conserved in all/most cell lines (Baker et al, 1987; Pater and Pater, 1985; Schwarz et al, 1985; Shirasawa et al, 1988; Yee et al, 1985). These reports also indicate that integration usually occurs within the E1 ORF, the E2 ORF, or both. The disruption of the E2 ORF by integration should interfere with trans-regulation by the E2 ORF product(s). Therefore, it would seem that disruption of this gene may play a role in malignant progression because of the deregulation of E6/E7 ORF expression. Indeed, viral transcripts in cervical carcinoma cells appear to be primarily from the E6 and E7 ORFs (Baker et al, 1987; Sneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986). Thus, due to the deregulation of E2-mediated transcriptional control in cervical cells, integrated viral promoters are presumably activated by cellular transcriptional factors alone (implicated in this study) or are enhanced by adjacent cellular cis-acting regulatory elements. Furthermore, the agressiveness of lesions containing duplications of (or

within) the viral transcriptional enhancers may be explained by the increased affinity for cell factors leading to an increase in expression from the E6/E7 ORFs. In addition, splicing of the HPV sequences to adjacent human DNA and the use of cellular polyadenylation sites may function to increase the stability of E6/E7-encoding mRNA transcripts. Thus, it would seem that integration of HPV DNA is pivotal in producing a switch in the viral transcriptional pattern which may be an important aspect in progression of cervical lesions to malignancy. In fact, studies on HPV-mediated transformation suggest that transformation of Rat-1 cells by an E6/E7 expressing construct is in most cases more efficient than that observed with the entire viral genome (Bedell et al, 1987). The integration of the HPV genome may also play a role in cevical carcinogenesis via the HPV enhancer-induced activation of adjacent oncogenes (Durst et al, 1987a; Swift et al, 1987).

The recently reported homology between the oncogene c-jun and the transcriptional factor AP-1 has provided the strongest basis to date for the belief that transcriptional factors can function directly as oncoproteins (Bohmann et al, 1987; Varmus, 1987; Short, 1987). Furthermore, the repressor function of the E1a gene product of adenovirus is tightly associated with the transforming ability of this gene (Lillie et al, 1986). Similarly, it is possible that viral-induced transcriptional repression and/or activation plays a central

role in E6/E7-mediated transformation of HPV infected cervical cells. Several pieces of evidence support this proposal. First, the proteins encoded by the E6 and E7 ORFs contain a common cysteine doublet (Cys-X-X-Cys; X= any amino acid) repeated at regular intervals along the sequence (Danos et al, 1984; Danos and Yaniv, 1987). Sequences of the form Cys-X-X-Cys-X(13)-Cys-X-X-Cys have also been found in the adenovirus E1a gene products (Berg, 1986). A number of eukaryotic nucleic acid binding proteins have recently been shown to contain similar repeated units which are believed to sequester an atom of heavy metal, thereby forming a loop or "finger" with the ability to interact with DNA or RNA (Berg, 1986). Interestingly, the association of the "finger" structure with transcriptional regulation is so well accepted that the presence of this motif is considered diagnostic of a new transcriptional factor (Evans and Hollenberg, 1988). Second, the E7 gene product of HPV 16 and the adenovirus E1a gene product(s) share considerable functional homology. For instance, the E1a gene product of adenovirus can repress the enhancer function of HPV 18 (Swift et al, 1987; Thierry et al, 1987b), while as reported here, the E7 gene product can repress HPV-specific transcription. In addition, the E7 gene product is known to stimulate expression from the adenovirus E2 gene (Phelps, Yee and Howley, in abstracts of papers presented at the Sixth International Workshop, 1987). Similarly, the E1a protein(s) function to activate the rate of

transcription from several adenovirus early genes (Berk et al, 1979; Jones and Shenk, 1979). In addition, results of the present study indicate that the early promoter of SV40 is also repressed by the E7 ORF of HPV 16. Repression of the early promoter of SV40 by the E1a gene product is also well documented (Borrelli et al, 1984; Velcick and Ziff, 1985). Interestingly, a sequence closely related to the consensus sequence (GTGGTATG) in the SV40 enhancer elements is also found in the enhancers of HPV 16, 18, and 11. It remains to be seen whether the repressor function of the E7 ORF of HPV 16 is mediated through this consensus sequence.

Localization of a trans-acting function to the E6 gene product of HPV 18 and an E6-inducible enhancer to the HPV 18 noncoding region (Gius et al, 1988) provides additional evidence for the involvement of transcriptional regulation in cervical carcinogenesis. The gene products from the E6/E7 region of HPV 16 and 18 are sufficient to induce transformation of rat cells (Bedell et al, 1987; Matlashewski et al, 1987). These data, taken together, suggest that the oncogenic effect of the HPV E6/E7 proteins may work through transcriptional repression/activation of cellular genes involved in growth regulation.

4.8 Future studies

Results presented in this thesis suggest further studies that

may shed light on the molecular mechanism(s) of viral (HPV) transcriptional regulation.

(1) Site and deletion mutagenesis of the HPV ncr should be performed to determine:

(a) whether or not there is a negative regulatory element downstream of the HPV 16 and 18 constitutive enhancer.

(b) the enhancer sequences which are required for HPV promoter function.

(c) the enhancer motifs (short DNA sequences) required for constitutive enhancer function.

(2) Cell-specific enhancer activity is known to involve the interaction of cellular transcriptional factors with enhancer motifs. Future studies should attempt to determine whether there are ubiquitous and cell-specific factors binding to the HPV enhancer an/or the putative negative regulatory element.

(3) The mechanism of E7-mediated repression may involve binding of the E7 gene product to a sequence present in the HPV ncr. Alternatively, this repression may act via an indirect pathway. To investigate whether or not the E7 gene product binds to a sequence(s) within the HPV noncoding region, experiments using techniques such as gel retardation and DNase I footprinting should be considered.

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APPENDIX A

A brief description of the plasmids constructed in this study:

PLASMID	VECTOR (SITE)	HPV TYPE	FRAGMENT[a]	
p2	pSV0-CAT (HindIII)	11	DraIII/SfaNI (7486 to 114)	(+)
p4	pA10-CAT (BglII)	16	BamHI/AvaII (6150 to 112)	(-)
p5	pA10-CAT (BglII)	11	DraIII/SfaNI	(-)
p6	pA10-CAT (BglII)	11	DraIII/SfaNI	(+)
p7	pA10-CAT (BglII)	16	BamHI/AvaII	(+)
p8	pA10-CAT (BglII)	18	BamHI/BamHI (6929 to 119)	(+)
p9	pA10-CAT (BglII)	18	BamHI/BamHI	(-)
p14	pSV0-CAT (HindIII)	11	DraIII/SfaNI	(-)
p15	pSV0-CAT (HindIII)	16	BamHI/AvaII	(+)
p17	pSV0-CAT (HindIII)	16	BamHI/AvaII	(-)
p29	pSV0-CAT (HindIII)	18	BamHI/BamHI	(+)
pD-2	pSV2- pML[b]	16	HpaII/SauI (700 to 4334)	(+)
pD-7	pSV2- pML[b]	16	HpaII/SauI (501 to 4334)	(+)
pT-1	pA10-CAT (BglII)	11	NdeI/SauI (7657 to 7870)	(+)
pT-2	pA10-CAT (BglII)	18	SfaNI/AccI (7419 to 7766)	(-)
pT-3	pA10-CAT (BglII)	16	SspI/HphI (7224 to 7778)	(+)
pT-4	pA10-CAT (BglII)	18	BamHI/AccI (6929 to 7766)	(-)
pT-11	pA10-CAT (BglII)	18	SfaNI/AccI	(+)

PLASMID	VECTOR (SITE)	HPV TYPE	FRAGMENT[a]	
pT-13	pSV0-CAT (HindIII)	18	BamHI/BamHI	(-)
pT-18	pA10-CAT (BglII)	18	RsaI/RsaI (7508 to 7738)	(-)
pT-20	pSV2- pML[b]	16	HpaII/SauI[c] (700 to 4334)	(+)
pT-25	pA10-CAT (BglII)	11	DraIII/SauI (7486 to 7870)	(+)
pT-28	pSV0-CAT (HindIII)	18	BssHII/BamHI (7572 to 119)	(-/+)[d]
pT-29	pSV0-CAT (HindIII)	18	BssHII/BamHI	(+)
pT-33	pA10-CAT (BglII)	18	BamHI/BbvI (6929 to 7837)	(+)
pT-37	pA10-CAT (BglII)	18	BssHII/AccI (7572 to 7766)	(-)
pT-39	pA10-CAT (BglII)	18	BamHI/AvaII (6929 to 56)	(+)
pT-42	pA10-CAT (XbaI)	[SV40]	PvuII/HindIII (see Figure 2, p 27).	(-)
pT-46	pA10-CAT (BglII)	18	BamHI/AccI	(+)
pT-48	pSV2- pML[b]	16	HpaII/SauI[e] (700 to 4334)	(+)
pT-50	pA10-CAT (BglII)	16	HphI/HphI (6438 to 7778)	(+)
pT-51	pA10-CAT (XbaI)	[SV40]	PvuII/HindIII	(+)
pT-52	pA10-CAT (XbaI)	18	SfaNI/AccI	(-)
pT-53	pA10-CAT (XbaI)	18	SfaNI/AccI	(+)
pT-57	pSV2- pML[b]	16	HpaII/SfaNI[e] (501 to 4334)	(+)
pT-69	pA10-CAT (BglII)	16	SphI/HphI (7463 to 7778)	(+)
pT-81	pA10-CAT (BglII)	18	RsaI/RsaI	(+)

a
The HPV 16, 18, and 11 restriction enzyme sites are given in Figure 3, p 32; (+) and (-) represent the orientation of the fragment.

b
The vector pSV2-pML was obtained by digesting pSV2-NEO-pML with HindIII and EcoRI (found in approximately the same positions as those found in pSV2-CAT [Figure 2, p 27]). The HindIII/EcoRI sites were filled in by treatment with reverse transcriptase. The HpaII/SauI fragments were then cloned into this vector with the SV40 regulatory region and the HPV 16 ORFs in the same orientation.

c
There is a 4 bp deletion in the HPV 16 sequences (HpaII/SauI fragment) at the BstXI site (2891).

d
The BssHII/AccI (7572 to 7766) fragment is in the opposite orientation relative to CAT, whereas the AccI/BamHI fragment is in the positive.

e
There is a deletion of 635 bp in the HPV 16 sequences (HpaII/SauI fragment) at the NdeI sites 3126 and 3761.