POSTTRANSCRIPTIONAL REGULATION OF TRANSFORMATION BY HUMAN PAPILLOMAVIRUS TYPE 16 E7 AND EXPRESSION OF THIS ONCOGENE

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by

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ABSTRACT

Many epidemiological and experimental studies have strongly implicated human papillomavirus type 16 (HPV-16) in cervical neoplasia. The oncogenic potential of this virus can be demonstrated by transformation of primary baby rat kidney (BRK) cells by cotransfection of the viral genome with the activated EJ-ras oncogene. I performed a mutational analysis of the viral genome to map the regions essential for its transforming activity. For the SV40-based early region expression plasmids, the disruption of the E6, E2, E5 and the 3′ region of the E1 open reading frames (ORFs) did not affect the transforming activity of mutated plasmids, whereas the insertion of translation termination linkers within the E7 ORF abolished transformation. Additional sequences present in the 3′-flanking region of the E7 ORF were also required for efficient transformation.

The 3′ flanking region sequences were analyzed in detail in SV40-based E7 expression plasmids by progressive deletion analysis and site-directed mutagenesis. Disruption of the nucleotide (nt) 880 splice donor site within this 3′-flanking region abolished transformation. Regeneration of the wild-type sequence in a previously transformation incompetent splice site mutant restored transformation. Mutating the wild-type splice donor site to the consensus splice site

resulted in higher levels of transformation, whereas mutating the +2 position of the consensus sequence significantly reduced the frequency of transformation. It was shown with RNase protection assays that the transformation-deficient splice site mutants accumulated lower levels of E7 RNA, primarily because of rapid destabilization of E7 RNA.

The splice sites present within the E6 ORF were examined for their ability to substitute for the loss of nt 880 splice donor site function. The wild-type E6 splice sites, as well as the heterologous splice sites of the SV40 intron, were able to partially substitute for the nt 880 splice site function. These results indicated that the efficient accumulation of HPV-16 E7 RNA and transformation of BRK cells depend on the presence of an intron in the transcription unit.

Recent studies have indicated the presence of naturally occurring HPV-16 antisense (AS) RNA in cervical carcinomas. I have detected AS E7 and E7/E1 RNA in Cos-1 cells transiently transfected with SV40-based HPV-16 early region expression plasmids. By deletion mutation analysis, the AS promoter was localized to nt 4031-4338 of HPV-16. Primer extension analysis and RNase protection assays indicated that the AS RNA was initiated from multiple sites in an AT-rich region around nt 4100. The AS promoter was active when cloned downstream of the chloramphenicol acetyl transferase (Cat) gene, giving rise to AS Cat RNA.

AS E7 RNA was detected in both the nucleus and cytoplasm. The AS RNA formed a duplex with the sense (S) E7 RNA. The presence of AS RNA was correlated with reduced splicing of E7 RNA from the nt 880 splice site and the synthesis of E7 protein.

dedicated to

my grandparents

Smt Shankaramma

and

Sri Srikantaiah

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LIST OF ABBREVIATIONS

A	=	Adenosine
AA	=	Amino acid
AS RNA	=	Antisense RNA
ASF	=	Alternate splicing factor
ATP	=	Adenosine triphosphate
bp	=	Base pair
bFGF	=	Basic fibroblast growth factor
BP	=	Branch-point
BPV	=	Bovine papillomavirus
BRK	=	Baby rat kidney
BMK	=	Baby mouse kidney
C	=	Cytidine
Cat	=	Chloramphenicol acetyl transferase
cDNA	=	Complementary DNA
CF	=	Cleavage factor
CK II	=	Casein kinase II
CMV	=	Cytomegalovirus
CPE	=	Cytoplasmic polyadenylation element
cpm	=	Counts per minute
CR	=	Conserved region
CRPV	=	Cottontail rabbit papillomavirus
CstF	=	Cleavage specificity factor
DHFR	=	Dihydrofolate reductase
DNA	=	Deoxyribonucleic acid

DSE	=	Downstream sequence element
dsRNA	=	Double stranded RNA
EGF	=	Epidermal growth factor
EGFR	=	Epidermal growth factor receptor
G	=	Guanosine
GRE	=	Glucocorticoid response element
HIV	=	Human immunodeficiency virus
hnRNP	=	Heterogeneous nuclear ribonucleoprotein
HPV	=	Human papillomavirus
HSV	=	Herpes simplex virus
I	=	Inosine
IBP	=	Intron binding protein
ICP0	=	Infected cell polypeptide 0
kb	=	kilobase
kDa	=	kilodalton
LAT	=	Latency associated transcript
LCR	=	Long control region
MBP	=	Myelin basic protein
mld	=	Myelin deficient mutant
mRNA	=	Messenger RNA
NCR	=	Noncoding region
nt	=	Nucleotide
ODN	=	Oligodeoxyribonucleotide
ORF	=	Open reading frame
ORN	=	Oligoribonucleotide

PAP	=	Poly(A) polymerase
PBS	=	Phosphate buffered saline
PDGF	=	Platelet-derived growth factor
PDGFR	=	Platelet-derived growth factor receptor
PFA	=	Paraformaldehyde
PKR	=	dsRNA dependent protein kinase
Pol	=	Polymerase
PPT	=	Polypyrimidine tract
pre-mRNA	=	pre-messenger RNA
PTBP	=	Polypyrimidine tract-binding protein
PV	=	Papillomavirus
Rb	=	Retinoblastoma gene product
RNA	=	Ribonucleic acid
RNase	=	Ribonuclease
RSV	=	Rous sarcoma virus
S RNA	=	Sense RNA
SF	=	Splicing factor
snRNA	=	Small nuclear RNA
snRNP	=	Small nuclear ribonucleoprotein
SSC	=	Sodium chloride-sodium citrate buffer
SV40	=	Simian vacuolating virus 40
TGF	=	Transforming growth factor
TIMP	=	Tissue inhibitor of metalloprotease
tk	=	Thymidine kinase
TMG	=	Trimethyl guanosine

TPA	=	12-o-tetradecanoylphorbol-13-acetate
U	=	Uridine
U2AF	=	U2 snRNP auxillary factor
USE	=	Upstream sequence element
UV	=	Ultraviolet radiation

CHAPTER 1

INTRODUCTION

1.1 PAPILLOMAVIRUSES (PVs)

Human papillomaviruses (HPV) play an important etiological role in the development of neoplastic lesions of the epithelial tissue such as common cutaneous warts and genital, oral and laryngeal cancers. Of different types of HPVs, HPV type 16 (HPV-16) has been considered to be important, because several epidemiological and experimental studies have incriminated this virus as causing cervical cancer, the second most common cancer of women (zur Hausen, 1991). The in vitro transformation of rodent cells is a commonly used system to demonstrate the oncogenic potential of HPVs. Using this assay system, I have been studying the regions of HPV-16 required for its oncogenic activity. In addition, I have also studied the possible posttranscriptional mechanisms, such as splicing and the naturally occurring antisense (AS) RNA, by which the oncogenic activity of HPV-16 is regulated.

The family papovaviridae consists of two subfamilies, papovavirinae and papillomavirinae (Murphy and Kingsbury, 1990). Papillomaviruses (PVs) are members of the subfamily papillomavirinae. PVs induce proliferative lesions of cutaneous and mucosal epithelia in higher vertebrates, including humans. The cottontail rabbit papillomavirus (CRPV)

which causes cutaneous papillomatosis was the first member of this group to be identified (Shope and Hurst, 1933). Subsequently, bovine papillomavirus (BPV), which causes fibropapillomas in bovines was identified and since then, it has served as a model for the understanding of the molecular biology of PVs (Black et al., 1963; Lancaster and Olson, 1982).

HPVs are highly epitheliotropic viruses that cause proliferative disorders of their target tissue (Shah and Howley, 1990). Based on the cross hybridization of their genomes, HPVs have been grouped into different types (Coggin and zur Hausen, 1979). More than 60 different types have been identified to date (de Villiers, 1989). Of these, approximately 25 different types are associated with the lower anogenital tract mucosa. Based on the relative tendency of the lesions to progress to malignancy, genital HPVs are classified into high risk and low risk groups (de Villiers, 1989; Shah and Howley, 1990). High risk HPVs, such as HPV-16, -18, -31, and -33, are frequently found in high grade cervical intraepithelial neoplasia, which has a higher tendency for malignant conversion. In contrast, low risk HPVs, such as HPV-6 and -11, are commonly found in benign genital condylomas and nasal and laryngeal papillomas, that have a lower tendency for malignant conversion (Broker and Botchan, 1986; Galloway and McDougall, 1989; Shah and Howley, 1990).

1.1.1 Physical properties

PVs are small, naked, icosahedral viruses with covalently closed circular double stranded DNA genome of approximately 8,000 base pairs (bp). The viral genome is found in a chromatin-like complex in association with cellular proteins such as histones (Favre et al., 1975; Pfister et al., 1977). PV capsid is composed of 72 capsomeres with 12 pentons and 60 hexons (Salunke et al., 1989). Virions have a buoyant density of 1.34 g/ml and measure approximately 55 nm in diameter (Sterling et al., 1990; Zhou et al., 1991; Kirnbauer et al., 1992). The 55 kDa major capsid protein encoded by the L1 open reading frame (ORF) of the virus constitutes approximately 80% of the total virion protein. The 70 kDa minor capsid protein is encoded by the L2 ORF (Howley, 1990).

1.1.2 HPV life-cycle

Studies of the life-cycle of HPVs are hindered primarily by the lack of a suitable cell culture system for HPV cultivation and propagation, because the viral gene expression is closely coupled to differentiation of the host epithelial cells. The expression of late genes of the virus and productive viral infection are restricted to differentiated epithelial cells (Sterling et al., 1990; Dollard et al., 1992; Meyers et al., 1992). Keratinocytes, the natural target for HPV infection fail to support a productive infection of HPVs when optimal conditions for

differentiation of keratinocytes are not provided (Taichman and LaPorta, 1987; Compton et al., 1989). It has not been possible to achieve the terminal differentiation of keratinocytes in vitro. Recently, several groups have reported the successful cultivation and propagation of HPVs in different systems. HPV-11 has been successfully propagated by implanting infected neonatal tissue under the renal capsule of nude mice (Kreider et al., 1985; 1987; Stoler et al., 1990). Similarly, transplantation of an HPV-16-positive cell line, which maintains the viral genome in an episomal form into nude mice, resulted in the production of virus particles (Sterling et al., 1990). More recently, terminal differentiation of keratinocytes in organotypic (raft) cultures and the production of HPV-11 and -31b virions in these cultures have been achieved (Dollard et al., 1992; Meyers et al., 1992).

1.1.3 Genome organization

A number of PVs have been cloned and sequenced (de Villiers, 1989; Howley, 1990). A remarkable similarity in the genome organization has been noticed among all these PVs. All ORFs of PVs are encoded on the same strand of DNA and a few ORFs are overlapping. Many ORFs are well conserved with respect to their presence, size, position and function among different PVs. However, certain ORFs were not found in the genomes of all papillomaviruses. Compared with the genome of BPV-1, HPV-16 contained no equivalent of the E8 ORF (Howley,

1990). The HPV-16 genome consists of 6 early region (E6, E7, E1, E2, E4 and E5) and 2 late region (L1 and L2) ORFs. Fig. 1.1 depicts the genomic organization of HPV-16. All PVs have 0.6-1 kilobase pairs (kbp) of sequence which is devoid of significant ORFs and this region is variously termed as the long control region (LCR), noncoding region (NCR), and upstream regulatory region. This region provides binding sites for several cellular and viral transcription and replication factors (Broker and Botchan, 1986; Pfister, 1987; Galloway and McDougall, 1989; Howley, 1990).

1.1.4 PV proteins

1.1.4.1 E6

The E6 ORF of the high risk HPVs produce unspliced E6, and two internally spliced, E6* and E6**, mRNAs (Smotkin et al., 1989). In contrast, the low risk HPVs, which lack these E6 splice sites, can not produce E6* and E6** mRNAs (Smotkin et al., 1989; Roggenbuck et al., 1991). The ability of only the high risk HPVs to produce E6* and E6** mRNAs has suggested an important role for the products of these mRNAs in the process of oncogenesis. However, products of E6* and E6** mRNAs have not been detected to date in high risk HPV-transformed cells, except for a HPV-18 transformed cell line (Roggenbuck et al., 1991).

The full length E6 protein of HPV-16 is a 151 amino acid (AA), basic, Zn^{++}-binding protein (Androphy et al., 1987a;

Barbosa et al., 1989; Grossman and Laimins, 1989; Vousden, 1990; Munger et al., 1992). Four Cys-X-X-Cys motifs present in the carboxy terminus of the protein are known to coordinate Zn^{++} binding (Barbosa et al., 1989). The E6 protein binds double stranded DNA nonspecifically (Mallon et al., 1987; Grossman et al., 1989; Imai et al., 1989). Recent studies have indicated that the E6 proteins of both high risk and low risk HPVs can transcriptionally transactivate several viral and cellular promoters (Lamberti et al., 1990; Sedman et al., 1991; Desaintes et al., 1992; Munger et al., 1992; Smits et al., 1992).

The oncogenic potential of E6 has been demonstrated by several groups. The full length E6 of HPV-16 and -18 can transform NIH 3T3 cells to anchorage independence (Bedell et al., 1989; Sedman et al., 1991). The full length E6, but not the E6* or E6** can cooperate with E7 to immortalize primary human keratinocytes and fibroblasts (Hawley-Nelson et al., 1989; Munger et al., 1989b; Watanabe et al., 1989). It was reported recently that the E6 proteins of high risk HPVs but not low risk HPVs can immortalize baby mouse kidney (BMK) cells in EJ-ras cooperation assays (Storey and Banks, 1993).

One of the mechanisms by which HPVs induce tumors involves their interaction with cellular tumor suppressor proteins. The E6 proteins of both high and low risk HPVs bind the tumor suppressor protein, p53 (Scheffner et al., 1990;

Werness et al., 1990; Crook et al., 1991a; Munger et al., 1992). Unlike other DNA tumor virus oncoproteins, such as SV40 large T antigen and the adenovirus 55 kDa E1B, which bind and stabilize p53, the E6 proteins of high risk HPVs lead to selective degradation of p53 (Scheffner et al., 1990; Crook et al., 1991a; Levine et al., 1991; Levine, 1992). Very low levels of p53 in HPV-16-positive cervical carcinoma cell lines, HPV-16-immortalized human keratinocytes and high risk HPV E6-immortalized BMK cells suggest that p53 may be a physiological target for E6 (Matlashewski et al., 1986; Scheffner et al., 1991; Storey and Banks, 1993). Recent studies have indicated that the domains of E6 responsible for binding p53 and directing its degradation are separable (Crook et al., 1991a). The degradation of p53 by E6 is mediated by ubiquitin and requires an additional 100 kDa cellular protein (Scheffner et al., 1990; Huibregtse et al., 1991; 1993).

The high risk HPVs affect the function of p53 by more than one mechanism. Recently, it was shown that the HPV-16 E6 protein interferes with the transactivating activity of wild-type p53 (Mietz et al., 1992).

1.1.4.2 E7

The E7 protein of HPV-16 is a 19 kDa, Zn^{++}-binding, phosphoprotein (Smotkin and Wettstein, 1986; 1987; Seedorf et al., 1987; Barbosa et al., 1989; Sato et al., 1989a). The E7 protein was detected in both the cytoplasm and nucleus when

analyzed by cell fractionation (Smotkin and Wettstein, 1987; Sato et al., 1987a). A recent study has convincingly shown that E7 is a nuclear matrix associated protein (Greenfield et al., 1991).

A consistent feature of a majority of cervical cancers and cancer-derived cell lines is the expression of E7 ORF of the high risk HPVs that they contain (Pater and Pater, 1985; Schwarz et al., 1985; Yee et al., 1985; Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986; Seedorf et al., 1987; Shirasawa et al., 1988; Cone et al., 1992; Cooper et al., 1992). In agreement with this observation, several experimental studies have implicated E7 as the major oncogene of HPVs. The E7 ORF expressed from strong heterologous promoter-enhancer sequences can immortalize primary rodent cells and transform established rodent cells (Matlashewski et al., 1987a; Kanda et al., 1988a; 1988b; Phelps et al., 1988; Storey et al., 1988; Tanaka et al., 1989; Edmonds and Vousden, 1989; Chesters et al., 1990; Watanabe et al., 1990; Munger et al., 1992). Further, in cooperation with E6, E7 can immortalize primary human keratinocytes (Schlegel et al., 1988; Hawley-Nelson et al., 1989; Munger et al., 1989b; Barbosa et al., 1991; Halbert et al., 1991). The continued expression of E7 was also required for the maintenance of the transformed phenotype (Crook et al., 1989; Storey et al., 1991). The level of E7 expression correlated well with the

growth rate of transformed cells (von Knebel Doeberitz et al., 1988; 1991; 1992; Crook et al., 1989; Storey et al., 1991).

Recent studies have indicated that the E7 genes of the low risk HPVs have a weak immortalizing/transforming activity (Storey et al., 1990a; Halbert et al., 1992). The differences in the oncogenic potential of the E7 genes of high risk and low risk HPVs reside in the amino terminal 30 AAs of these proteins (Munger et al., 1992a; Pater et al., 1992a; Takami et al., 1992).

The E7 protein bears significant structural and functional similarity to the transforming genes of adenovirus, E1A and SV40 large T antigen. AAs 6 to 20 and 41 to 56 of E7 are similar to AAs 41 to 56 in the conserved region (CR) 1 and 121 to 139 in CR2, respectively, of adenovirus type 5 E1A (Phelps et al., 1988; Watanabe et al., 1990). Biological activities that these two proteins have in common are, immortalization of primary cells, transformation of rodent cells in cooperation with activated oncogenes, induction of host DNA synthesis and transcriptional regulation of viral and cellular promoters (Vousden, 1990; Nevins 1991; Shenk and Flint, 1991; Munger et al., 1992).

Similar to E1A, E7 interacts with several cellular proteins and some of these interactions are essential for the diverse biological activities of E7. The E7 proteins of both high and low risk HPVs bind the tumor suppressor gene product

pRb and the binding affinities corresponds to the oncogenic potential of these HPVs (Dyson et al., 1989; Munger et al., 1989; Barbosa et al., 1990; Gage et al., 1990; Heck et al., 1992; Sang and Barbosa, 1992a). The correlation between the transforming potential of HPVs and pRb binding was demonstrated by showing that the oncogenic potential of a low risk HPV, HPV-6, E7 can be increased by improving its binding affinity to pRb (Sang and Barbosa, 1992a). However, pRb binding was essential but not sufficient for transformation, because mutations in E7 that did not impair pRb binding severely affected transformation (Edmonds and Vousden, 1989; Banks et al., 1990; Chesters et al., 1990; Watanabe et al., 1990; Jewers et al., 1992; Phelps et al., 1992).

The outcome of E7 binding to pRb was clearly demonstrated in keratinocyte cell system. Inhibition of growth of keratinocytes by transforming growth factor (TGF)-β1 is associated with downregulation of c-myc (Pietenpol et al., 1990a). This growth inhibition was abrogated by HPV-16 E7 and the pRb binding ability was essential for this activity of E7 (Pietenpol et al., 1990b; Munger et al., 1991).

The E7 protein can also interact with other cellular proteins such as cyclin A, p107, a pRb related protein, and p130 (Dyson et al., 1992; Davies et al., 1993; Tommasino et al., 1993).

Immediately towards the C-terminus of the pRb binding region, between the AAs 30-37, is the casein kinase (CK) II

phosphorylation region. The serines, S^{31} and S^{32}, located within this region are phosphorylated by CK II (Firzlaff et al., 1989; Barbosa et al., 1990). Despite the proximity of the two regions, pRb binding and phosphorylation by CK II of E7 are independent of each other (Munger et al., 1989a; Barbosa et al., 1990; Firzlaff et al., 1991). The effects of mutations of the CK II phosphorylation region on transformation was dependent on whether primary or established cells were used for the assay and whether the mutations were deletions or substitutions. A moderate to severe reduction in transformation was noticed in various studies (Edmonds and Vousden, 1989; Chesters et al., 1990; Storey et al., 1990b; Watanabe et al., 1990; Firzlaff et al., 1991; Phelps et al., 1992).

The C-terminus half of E7 has two Cys-X-X-Cys motifs separated from each other by approximately 29 AAs (Watanabe et al., 1992b). The Zn^{++}-binding property of E7 has been shown to be dependent on the integrity of these motifs (Barbosa et al., 1989). Mutations altering these Cys residues and the spacing between the two motifs severely affected the stability of the protein and its transforming activity (Watanabe et al., 1990; 1992b; Phelps et al., 1992).

HPV-16 E7 has been shown to induce cellular DNA synthesis (Sato et al., 1989b; Banks et al., 1990a; 1990b; Rawls et al., 1990). The ability of E7 to induce cellular DNA synthesis was

enhanced by the growth factor insulin. The induction of DNA synthesis was dependent on the ability of E7 to bind pRb and release the transcriptionally active form of E2F from the E2F-pRb complex (Morris et al., 1993).

The E7 proteins of both the high risk and low risk HPVs have been shown to transactivate the adenovirus E2 early promoter (Phelps et al., 1988; Storey et al., 1990a; Munger et al., 1991; Watanabe et al., 1992a). The E2F binding sites present in the adenovirus E2 promoter were essential for the transactivation of this promoter by E7 (Phelps et al., 1991). Recent studies have shown that, similar to the E1A 12S mRNA product, the HPV-16 E7 can also dissociate the complexes of cellular transcription factor E2F and pRb (Phelps et al., 1991; Chellappan et al., 1992; Nevins, 1992; Pagano et al., 1992; Huang et al., 1993). The E7 protein of a low risk HPV, HPV-6, was less efficient at disrupting the E2F-pRb complex (Wu et al., 1993). Similar to transformation, the transactivating activity of E7 was severely affected by mutations affecting the pRb binding region (Edmonds and Vousden, 1989; Watanabe et al., 1990; Phelps et al., 1992; Watanabe et al., 1992a). Mutations affecting the phosphorylation of Ser^{31} and Ser^{32} of HPV-16 E7 by CK II, reduced, but did not totally eliminate the transactivating activity of E7 (Edmonds and Vousden, 1989; Storey et al., 1990; Watanabe et al., 1990; Phelps et al., 1992).

Transactivation was impaired by mutations affecting other regions of E7 (Edmonds and Vousden, 1989; Storey et al., 1990; Watanabe et al., 1990).

Extensive mutational analysis of HPV-16 E7 has clearly indicated that the transforming and transactivating activities of E7 are independent of each other (Edmonds and Vousden, 1989; Watanabe et al., 1990; Firzlaff et al., 1991; Phelps et al., 1992).

1.1.4.3 E1

Several studies have demonstrated an essential role for E1 in the process of replication and episomal maintenance of the BPV-1 genome (Groff and Lancaster, 1986; Rabson et al., 1986; Ustav and Stenlund, 1991; Chiang et al., 1992a, 1992b). The E1 ORF of BPV-1 encodes a nuclear phosphoprotein of 68-72 kDa (Santucci et al., 1990; Sun et al., 1990; Blitz and Laimins, 1991; Lusky and Fontane, 1991). The E1 protein of BPV-1 bears significant structural and functional similarity to the SV40 and polyomavirus large T antigens. The E1 protein binds ATP through its C-terminus ATP binding domain and mutations which affect ATP binding severely interfere with the activity of this E1 protein (Santucci et al., 1990; Sun et al., 1990; Blitz and Laimins, 1991). The E1 polypeptide binds the E2 transactivator protein and the C-terminal region of E1 is essential for this interaction (Mohr et al., 1990; Blitz and Laimins, 1991; Lusky and Fontane, 1991). Although earlier

studies suggested a nonspecific binding of DNA by E1 (Clertant and Seif, 1984; Santucci et al., 1990; Blitz and Laimins, 1991), several recent studies have clearly demonstrated the specific binding of E1 to the viral origin of replication (Wilson and Ludes-Meyer, 1991; Ustav et al., 1991; Yang et al., 1991). Further, the binding of E1 to the origin of replication was facilitated by its interaction with the E2 transactivator protein (Mohr et al., 1990; Blitz and Laimins, 1991; Yang et al., 1991).

1.1.4.4 E2

The E2 ORF encodes a DNA binding protein which can act as a transcriptional transactivator and a repressor, depending on the context of its binding sites in the LCR. The product of the E2 ORF binds as dimers to a palindromic sequence, $ACCGN_4CGGT$ (Androphy et al., 1987b; Dostani et al., 1988; Li et al., 1989; McBride et al., 1988). The C-terminal domain of E2 mediates DNA binding, and the amino-terminal domain is required for transcriptional regulation (Dostani et al., 1988; Giri and Yaniv, 1988; McBride et al., 1989). A truncated form of E2 encoded by BPV-1 E2 ORF, lacking the transcriptional activating domain acts as a transcriptional repressor, presumably by competing with the full-length E2 for DNA binding sites (Lambert et al., 1987). By sterically interfering with the assembly of transcription initiation complex and binding of essential transcription factor SP1, the

full-length E2 protein can also repress different HPV promoters (Thierry and Yaniv, 1987; Bernard et al., 1989; Chin et al., 1989; Romanczuk et al., 1990; Dostani et al., 1991; McBride et al., 1991; Thierry and Howley 1991). The E2 dimers can cooperate with each other and with other cellular transcription factors, such as AP1, glucocorticoid receptor and keratinocyte-specific NF1/K factor, to synergistically activate transcription (Gauthier et al., 1991; Ham et al., 1991; Monini et al., 1991).

In addition to its role in transcriptional regulation, the E2 protein has been shown to be required for replication of the viral genome (Lambert, 1991). The binding of E2 near the origin of replication causes a change in the local chromatin structure (Moskaluk and Bastia, 1988). This change in the conformation of DNA might be essential for the initiation of DNA replication. Another mechanism by which E2 participates in the process of virus genome replication is by interacting with and targeting the product of the E1 ORF to the origin of replication (Mohr et al., 1990; Blitz and Laimins, 1991; Lusky and Fontane, 1991). The amino-terminal transactivating domain of E2 was essential for its interaction with E1 (Mohr et al., 1990).

1.1.4.5 E4

Although the E4 ORFs of different PVs have undergone significant divergence, many physical characteristics of the

encoded proteins are conserved (Doorbar et al., 1986; 1989). The E4 ORF encodes an abundant cytoplasmic protein of 10-17 kDa (Doorbar et al., 1986; Breitburd et al., 1987; Neary et al., 1987; Brown et al., 1988; Crum et al., 1990; Doorbar et al., 1991). In some cutaneous warts containing HPV-1, the E4 protein may constitute up to 20% of the total cellular protein (Breitburd et al., 1987). The mRNAs capable of encoding E4 were readily detected in both parabasal and superficial layers of the differentiating epithelium (Crum et al., 1990). High levels of E4 protein was coexpressed in differentiating keratinocytes along with the late proteins, L1 and L2 (Doorbar et al., 1986; Breitburd et al., 1987; Doorbar et al., 1989; Crum et al., 1990). The abundance of E4 protein in superficial cells could be related to the replication of the viral DNA, resulting in amplified levels of DNA template for transcription of E4 message. Although, a role for E4 in the process of virion assembly was suggested by Doorbar et al., (1986; 1989), recent studies have ruled out this possibility (Zhou et al., 1991; Kirnbauer et al., 1992). More recently, Doorbar et al., (1991), have shown the specific interaction of E4 with the cytokeratins, that may result in the collapse of cellular intermediate filament network.

1.1.4.6 E5

Our knowledge of the E5 protein has primarily emerged from studies done with BPV-1. The BPV-1 E5 protein is a

small, highly hydrophobic, golgi membrane-associated, 8 kDa protein (Burkhardt et al., 1989). E5 is the major oncogene of BPV-1 and transforms established rodent cells, such as NIH 3T3 and C127, to anchorage independence (Schiller et al., 1986; Schlegel et al., 1986).

The ability of E5 to interact with growth factor receptors has been thought to be, at least partially, responsible for the transforming activity of E5. E5 has been shown to extend the half-life of the internalized epidermal growth factor receptor (EGFR), thus prolonging the duration of the mitogenic signal (Martin et al., 1989). The delayed turnover of EGFR could be related to the ability of E5 to interact with a 16 kDa protein component of the vacuolar H^+ ATPase (Goldstein and Schlegel, 1990; Goldstein et al., 1991). E5 can also provide the mitogenic signal by a totally different mechanism, by directly activating platelet derived growth factor receptor (PDGFR) [Petti et al., 1991].

The frequent deletions of the 3' early region ORFs, including the E5 ORF, in cervical carcinoma cell lines, has reduced the potential role of HPV-16 E5 in the process of cervical carcinogenesis. Despite considerable divergence in the AA sequence, the HPV-16 E5 is structurally similar to the BPV-1 E5 (Bubb et al., 1988; Halbert and Galloway, 1988). The HPV-16 E5 encodes a small, hydrophobic, 10 kDa protein (Halbert and Galloway, 1988). The HPV-16 E5 is only weakly

oncogenic. It can cooperate with the EGFR to transform established rodent cells and to enhance EGF-mediated mitogenic signal transduction to the nucleus (Leechanachai et al., 1992; Pim et al., 1992). The HPV-16 E5-transformed cells express higher levels of c-fos upon treatment with EGF (Leechanachai et al., 1992). c-fos is a member of a family of transcription factors called AP-1. The LCR of HPV-16 contains three AP-1 binding sites and AP-1 modulates HPV-16 gene expression from the promoter p97 (Chan et al., 1990). These observations have suggested a significant role for HPV-16 E5 in the early stages of the oncogenic process. In the early stages of infection, the viral genome remains episomal with all the ORFs intact, including E5. At this stage, E5 could be involved in initiating the early events of oncogenesis, such as induction of high levels of viral E6 and E7 oncoproteins, by upregulating c-fos.

1.1.4.7 L1

The L1 ORF is the most highly conserved ORF among the different HPVs (Cole and Streeck., 1986; Cole and Danos, 1987). This is further reflected by the cross reactivity of antibodies raised against the L1 protein of one type to the L1 proteins from a variety of HPVs (Firzlaff et al., 1988). Expression of L1 protein is restricted to terminally differentiated keratinocytes. Overexpression of L1 using heterologous promoters in a variety of cell types results in

self-assembly of this protein into virion-like particles (Zhou et al., 1991; Kirnbauer et al., 1992; Rose et al., 1993).

1.1.4.8 L2

The L2 ORF encodes a minor capsid protein. Comparison of the L2 ORF sequences from various PVs indicate that the L2 region is rather poorly conserved. Similar to L1, the expression of L2 is restricted to highly differentiated keratinocytes (Firzlaff et al., 1988).

1.1.5 Regulation of PV gene expression

Although the oncogenes of HPVs can immortalize and transform a variety of cell types when expressed from stronger heterologous promoters, they show strict specificity towards epithelial cells when expressed from homologous promoter-enhancer sequences. Several studies have suggested that the epithelial cell type specific expression of HPVs is related to cell type-restricted transcription factors or critically balanced levels of various ubiquitous transcription factors.

All genital HPVs exhibit a similar organization of the LCR. The genomes of HPVs contain a major promoter located upstream of the E6 ORF. Upstream of the HPV-16 promoter p97, at nt position -32 to -26 with respect to the transcription initiation site, is a TATA box. Various cellular and viral transcription factors are known to bind the LCR of HPV-16 and regulate transcription from the viral promoter p97 (Gloss et al., 1987; Phelps and Howley, 1987; Chan et al., 1989; Gloss

et al., 1989a; 1989b; Chan et al., 1990; Chong et al., 1990; Cripe et al., 1990; Gloss and Bernard, 1990; Nakshatri et al., 1990; Romanczuk et al., 1990; Sibbet and Campo, 1990; Chang et al., 1991; Gauthier et al., 1991; Ishiji et al., 1992; Tan et al., 1992; Morris et al., 1993).

Extensive mutational analysis of the HPV-16 LCR has mapped an epithelial cell type-specific enhancer (Cripe et al., 1987; Gloss et al., 1987; Marshall et al., 1989; Cripe et al., 1990; Ishiji et al., 1992). Elements responsive to the viral E2 gene product and the steroid hormone glucocorticoid and progesterone were present in the LCR of HPV-16 (Cripe et al., 1987; Gloss et al., 1987; Chan et al., 1989; Mittal et al., 1993). The viral E2 gene product is a sequence-specific DNA binding transcriptional modulator that can either activate or repress transcription from the viral promoter, depending on the proximity of its binding sites to the TATA box sequence (Cripe et al., 1987; Phelps and Howley, 1987; Romanczuk et al., 1990). Steric hindrance with the binding of transcription factors to the adjacent motifs or assembly of transcription initiation complex might underlie the basis of E2-mediated repression (Gloss and Bernard, 1990; Dostani et al., 1991; Tan et al., 1992).

Twenty-nine different protected regions corresponding to NF1, AP1, Oct-1 and several other transcription factor binding sites were observed in the LCR of HPV-16, by <u>in vitro</u> foot-

printing analysis (Gloss et al., 1989a; Morris et al., 1993). Cooperation between NF1 and AP1 has been shown to result in increased transcription from the viral promoter (Chong et al., 1990). Two AP1 binding sites present in the LCR mediate the response of HPV-16 to tumor promoter, TPA (Chan et al., 1990). It has also been suggested that the AP1 binding sites mediate the response of HPV-16 LCR to various growth modulatory factors. Of relevance is a recent study which indicated that one of the viral oncoproteins, E5, is involved in amplifying and transducing the mitogenic signal from EGF and PDGF receptors to c-fos, a component of the transcription factor AP1 (Leechanachai et al., 1992; Pim et al., 1992). The HPV-16 LCR responds to growth factors, such as EGF, transforming growth factor (TGF), and the morphogen, retinoic acid (Agarwal et al., 1991; Braun et al., 1990; Yasumoto et al., 1991).

In addition, HPV-16 gene expression is also modulated by steroid hormones, glucocorticoids and progesterone (Gloss et al., 1987; Chan et al., 1989; Lees et al., 1990; von Knebel Doeberitz et al., 1991; Mittal et al., 1993). Regulation of HPV-16 gene expression by steroid hormones is of great relevance, because this virus primarily infects epithelia of the genital tract. These epithelial cells possess receptors for sex steroid hormones, estrogen and progesterone and are subjected to profound effects of these hormones (Sanborn et al., 1976). Both glucocorticoids and progesterone upregulate

the HPV-16 promoter p97 (Gloss et al., 1987; Chan et al., 1989; Lees et al., 1990; von Knebel Doeberitz et al., 1991; Mittal et al., 1993). This upregulation of HPV-16 gene expression by glucocorticoid hormones and progesterone forms the basis of transforming ability of this virus.

The effect of glucocorticoids is mediated through the glucocorticoid receptor. Glucocorticoid receptor is a sequence-specific DNA binding transcription factor that is normally present in an inactive complex with the heat shock protein, HSP 90, in the cytoplasmic compartment of cells (Beato, 1989; Smith and Toft, 1993). Specific ligand-binding activates the translocation of and transcription by this receptor (Landers and Spelsberg, 1992). Several studies have identified glucocorticoid response elements (GRE) in the LCR of HPV-16 (Gloss et al., 1987; Chan et al., 1989; Mittal et al., 1993). Similar sequences have also been noticed in many other genital HPVs (Chan et al., 1989). One of the GREs of HPV-16 identified earlier overlaps an AP1 binding site and was responsive to both glucocorticoids and progesterone, and mutations within this sequence lowered the response to glucocorticoids (Chan et al., 1989; Chan et al., 1990). Furthermore, this sequence specifically binds the purified glucocorticoid receptor (Chan et al., 1989). The induction by glucocorticoids was inhibited by the progestin/glucocorticoid hormone antagonist, RU486 (Chan et al., 1989; Mittal et al., 1993).

1.1.6 Transcription pattern

PVs generate a wide variety of mRNAs by utilizing different promoters and following a complex splicing pattern of a number of splice sites. In the genome of BPV-1, seven different promoters have been identified (Ahola et al., 1987; Baker and Howley, 1987; Stenlund et al., 1987; Choe et al., 1989). The genomes of HPVs contain a single major promoter located immediately upstream of the E6 ORF, from which all the transcripts are initiated. In addition to the major promoter, an internal promoter is present within the E6 ORF of low risk HPVs, HPV-1, -6 and -11 (Chow et al., 1987a; 1987b; Smotkin et al., 1989).

A detailed analysis of HPV-16 transcripts has been performed by using various techniques. A majority of transcripts in HPV-16-positive cervical cancers and cancer-derived cell lines correspond to the E6-E7 region. In several different cell lines, the nt 226 splice donor site was spliced alternately to nt 409, 526 and 3357 splice acceptor sites, to generate E6*, E6** and E6*** mRNAs, respectively (Smotkin et al., 1989; Cornelissen et al., 1990; Doorbar et al., 1990; Johnson et al., 1990; Schneider-Maunoury et al., 1990; Nasseri et al., 1991; Rohlfs et al., 1991; Shirasawa et al., 1991; Sherman and Alloul, 1992; Sherman et al., 1992). One of the most commonly used splice donor sites in the genome of HPV-16 is the nt 880 site. This site is alternately spliced to nt

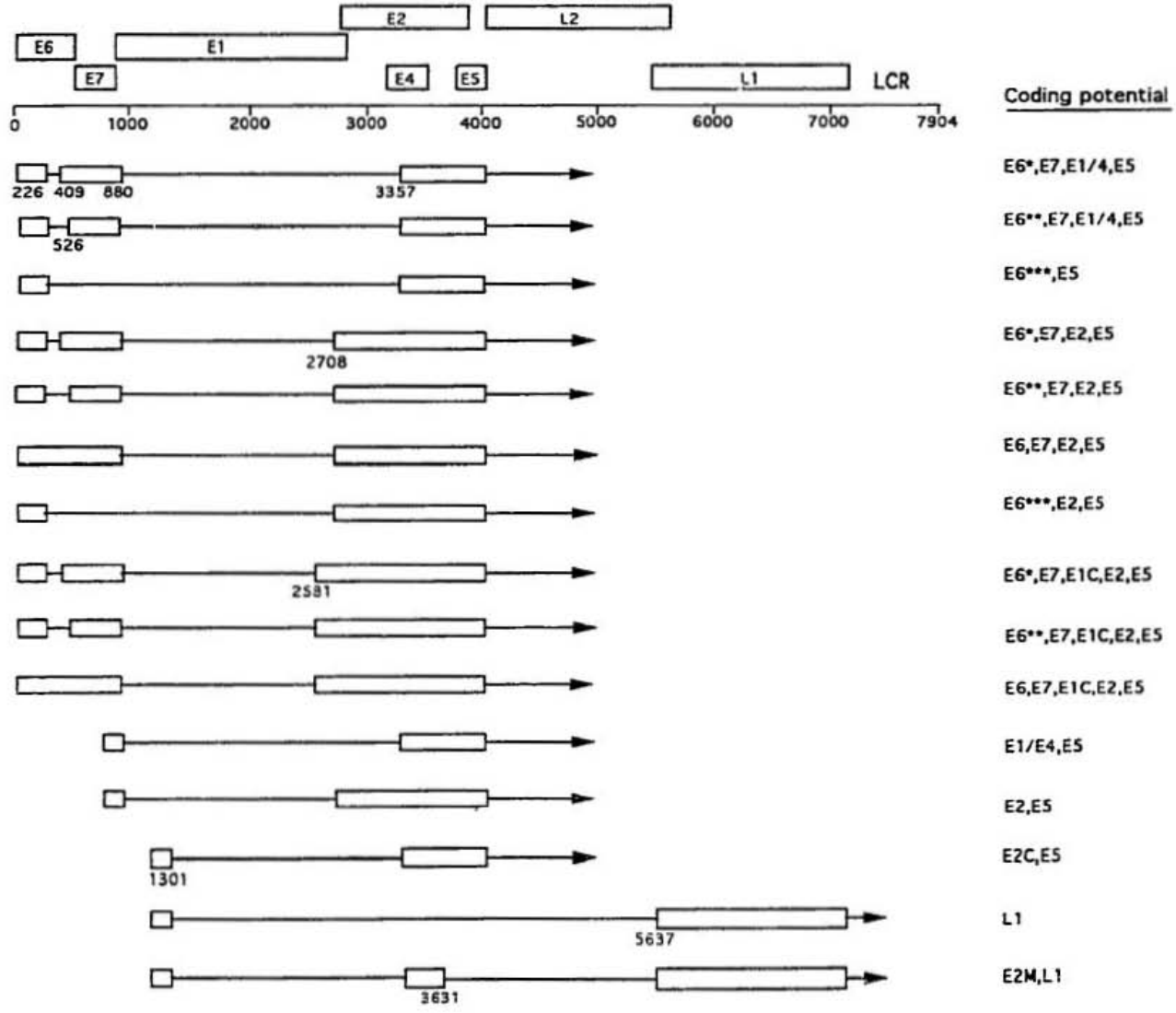
2708 and 3357 splice acceptor sites (Doorbar et al., 1990; Rohlfs et al., 1991; Sherman et al., 1992). An infrequently used splice donor site is the nt 1301 site, which is spliced to the nt 3357 and 5637 splice acceptor sites (Doorbar et al., 1990; Sherman et al., 1992). The mRNAs corresponding to the E2 and L1 regions have also been detected in a few cell lines (Doorbar et al., 1990; Nasseri et al., 1991; Rohlfs et al., 1991; Sherman and Alloul, 1992; Sherman et al 1992). Different species of mRNAs detected in several cell lines are depicted in the fig. 1.1.

The different species of mRNAs undergo 3' cleavage and polyadenylation at the viral or unknown cellular polyadenylation signals (Doorbar et al., 1990; Rohlfs et al., 1991; Smits et al., 1991; Sherman et al., 1992). A cellular polyadenylation signal is used in cells in which the HPV polyadenylation signal is deleted as a consequence of integration of the viral genome (Smits et al., 1991). In the genome of HPV-16, 1 early region (EP1) and 3 late region (LP1, LP2 and LP3) polyadenylation signals are present (Seedorf et al., 1985). Of the 3 late region polyadenylation signals, LP2 is the most commonly used (Kennedy et al., 1990). Alternate utilization of these polyadenylation signals may be important, because an AU rich instability element present upstream of LP2 and LP3 polyadenylation signals might regulate the stability of late region transcripts in different cell types (Kennedy et al., 1990).

Figure 1.1 Diagrammatic representation of HPV-16 transcripts.

The genomic organization of HPV-16 and the positions of ORFs indicated by open boxes, are depicted on top. Exons and introns are indicated by open boxes and lines, respectively. Numbers below boxes corresponds to HPV-16 nt. The 5′ ends of the last five transcripts were not precisely mapped. The continuity of transcripts are indicated by arrows. Because the 3′ early region ORFs including the early polyadenylation signal sequences are deleted in several cell lines as a consequence of integration of the viral genome, many transcripts utilize cellular polyadenylation signals. Coding potentials of transcripts are indicated on the right. Abbreviations: LCR, long control region.

The data for the figure is from Smotkin et al., 1989; Doorbar et al., 1990; Nasseri et al., 1991; Rohlfs et al., 1991; Sherman and Alloul, 1992; Sherman et al., 1992.


E2
L2
E6
E1
E7
E4
E5
L1
LCR
0
1000
2000
3000
4000
5000
6000
7000
7904
Coding potential
226 409 880
3357
E6*,E7,E1/4,E5
526
E6**,E7,E1/4,E5
E6***,E5
2708
E6*,E7,E2,E5
E6**,E7,E2,E5
E6,E7,E2,E5
E6***,E2,E5
2581
E6*,E7,E1C,E2,E5
E6**,E7,E1C,E2,E5
E6,E7,E1C,E2,E5
E1/E4,E5
E2,E5
1301
E2C,E5
5637
L1
3631
E2M,L1

1.1.7 Replication of the PV genome

Studies on BPV-1 have contributed significantly to understanding the PV replication. In transformed and transiently transfected cells, the BPV-1 genome undergoes regulated replication once per cell cycle and is stably maintained as a multicopy nuclear episome (Lambert, 1991). The replication of the genome was dependent on the presence of the viral origin of replication and the full-length E1 and E2 proteins (Sarver et al., 1984; Groff and Lancaster, 1986; Rabson et al., 1986; DiMaio and Settleman, 1988; Ustav and Stenlund, 1991; Yang et al., 1991). The viral origin of replication includes an A/T-rich region and the binding sites for the E1 and E2 proteins (Ustav and Stenlund, 1991; Ustav et al., 1991; Yang et al., 1991). The E1 ORF encodes an ATP binding, phosphoprotein with helicase activity (Santucci et al., 1990; Sun et al., 1990; Yang et al., 1991). The E1 protein has been shown to interact with the E2 transactivator protein, and this interaction facilitates the targeting of E1 protein to the viral origin of replication (Mohr et al., 1990; Blitz and Laimins, 1991; Lusky and Fontane, 1991; Yang et al., 1991).

Little information is available on the replication of HPVs, primarily because of the lack of a suitable cell system in which the viral genome can undergo replication. Several recent studies have demonstrated that the replication of the

genomes of HPV-6, -11, -16 and -18 can be achieved in a variety of cell systems (Chiang et al., 1992a; 1992b; Del Vecchio et al., 1992; Remm et al., 1992). The replication was strictly dependent on the presence of E1 and E2 gene products. A mixed combination of E1 and E2 proteins of different HPV types supported the replication of homologous as well as heterologous HPV origins, albeit with different efficiencies (Chiang et al., 1992a; Del Vecchio et al., 1992).

1.1.8 Oncogenesis by HPVs

1.1.8.1 Viral factors

HPVs are associated with a variety of proliferative disorders of epithelia. The role of cutaneous HPVs in the causation of common cutaneous warts has been very well established (Rapini et al., 1992).

Genital HPVs have received greater attention, because several experimental and epidemiological studies have suggested that these HPVs play an important role in genital cancers, especially cervical cancer that constitutes a high percentage of human cancers (zur Hausen, 1977; Koutsky et al., 1988; Schiffman, 1992). The low risk HPVs are the most frequent types associated with benign genital warts. Their genomes have also been detected in oral and laryngeal cancers (Gissmann et al., 1982; Mounts and Shah, 1984; de Villiers et al., 1985). Approaching 100% of cervical cancers contain high risk HPV genomes (Schwarz et al., 1985; Gergley et al.,

1987; de Villiers, 1989; van den Brule et al., 1989; Young et al., 1989). Of different types of high risk HPVs associated with this condition, HPV-16 and -18 are the most common types. Approximately 55% of invasive cervical cancers contain the HPV-16 genome, whereas 15% harbour the HPV-18 genome (Shah and Howley, 1990). Several laboratory studies have demonstrated the oncogenic potential of high risk HPVs. The high risk HPVs such as HPV-16, -18 and -33 have been shown to immortalize human cervical and foreskin keratinocytes, which are the natural target cells for these viruses (Durst et al., 1987b; Pirisi et al., 1987; Kaur and McDougall, 1988; Schlegel et al., 1988; Woodworth et al., 1988; Kaur et al., 1989; Pecoraro et al., 1989; Tsutsumi et al., 1992; Gilles et al., 1993). The histological abnormalities and the aberrant differentiation pattern exhibited by these immortalized keratinocytes resemble various abnormalities present in naturally infected cells (McCance et al., 1988; Hudson et al., 1990; Woodworth et al., 1990; Qi et al., 1992).

One of the mechanisms by which high risk HPVs induce immortalization is by deregulating the cell cycle. The tumor suppressor proteins, p53 and pRb, have been shown to negatively regulate the cell cycle (Ducommun, 1991; Cobrinik et al., 1992; Cowell, 1992; Meek and Street, 1992). Viral oncoproteins, E6 and E7, interfere with the normal functioning of these proteins. The E6 protein of high risk HPVs can bind and selectively target the degradation of p53 (Scheffner et

al., 1990; Werness et al., 1990; Crook et al., 1991a). The E7 protein binds and sequesters pRb in a functionally inactive complex (Dyson et al., 1989; Munger et al., 1989a; Barbosa et al., 1990; Gage et al., 1990; Heck et al., 1992; Sang and Barbosa, 1992). The interaction between pRb and E7 also results in the release of the cellular transcription factor, E2F, which activates genes concerned with DNA replication, from a functionally inactive E2F-pRb complex (Phelps et al., 1991; Pagano et al., 1992; Huang et al., 1993). Recently, the association of the HPV-16 E7 protein with the cell cycle regulatory protein $p33^{cdk2}$ was demonstrated (Tommasino et al., 1993).

Several studies have suggested that the ability to interfere with the function of these tumor suppressor and cell cycle regulatory proteins is essential for the oncogenic activity of HPVs (Vousden, 1990; Munger et al., 1992). In several HPV-positive cervical carcinoma cell lines, only wild-type alleles of p53 or pRb were detected, whereas in HPV-negative cervical carcinoma cell lines, these genes were found to be inactivated by mutations (Crook et al., 1991b; Scheffner et al., 1991). Interference with the activity of these proteins by the expression of E6-E7 ORFs was sufficient for immortalization of keratinocytes (Schlegel et al., 1988; Hawley-Nelson et al., 1989; Munger et al., 1989; Inoue et al., 1991; Halbert et al., 1991). Maintenance of the transformed

phenotype of HPV-transformed cells was dependent on the continued expression of E7 (von Knebel Doeberitz et al., 1988; 1991; 1992; Crook et al., 1989; Storey et al., 1991). The oncogenic potential of HPV-16 has also been demonstrated at the organismal level. Transgenic animals expressing the E6-E7 region of HPV-16 showed a high incidence of germ cell and neuroepithelial cell tumors (Kondoh et al., 1991; Arbeit et al., 1993). Targeting the expression of these genes to the lens tissue of transgenic animals resulted in altered epithelial cell growth and differentiation and tumor formation (Griep et al., 1993).

The products of other genes, such as E1 and E2, may play indirect roles in the oncogenic process by affecting the expression of E6 and E7 oncoproteins. In premalignant lesions, malignant tumors and tumor-derived cell lines, the viral genome was found to be integrated with the host DNA. These integration events resulted in selective retention of E6 and E7 ORFs and disruptions of E1 and E2 ORFs and frequent deletions of the 3′ early region ORFs (Pater and Pater, 1985: Seedorf et al., 1985; Yee et al., 1985; Durst et al., 1986; Matsukura et al., 1986; Smotkin and Wettstein, 1986; Androphy et al., 1987a; Awady et al., 1987; Baker et al., 1987; Choo et al., 1987; 1988; Seedorf et al., 1987; Shirasawa et al., 1988; Wagatsuma et al., 1990). The E2 ORF encodes a transcriptional repressor protein which downregulates the expression of E6 and

E7 from the viral promoter (Ham et al., 1991; McBride et al., 1991). In keratinocyte immortalization assays, disruption of E1 or E2 ORFs resulted in a higher efficiency of immortalization (Romanczuk and Howley, 1992). Inactivation of E2, as a result of integration of the viral genome, may lead to enhanced expression of viral oncogenes E6 and E7 and augment the oncogenic potential of the HPVs (Sang and Barbosa, 1992b).

Recent studies have identified E5 as an oncogene. The E5 protein may play a significant role in the early stages of tumor induction by enhancing the mitogenic signal of growth factors such as EGF and PDGF (Leechanachai et al., 1992; Pim et al., 1992; Banks and Matlashewski, 1993).

1.1.8.2 Host factors

The incidence of HPV infection is worldwide (Durst et al., 1983; Fukushima et al., 1985) and high risk HPVs are found in both normal and dysplastic cervical tissues (Crum et al., 1985b; Gissmann and Schneider, 1986; Macnab et al., 1986; McCance et al., 1986). However, the high risk HPV-associated disease process follows a prolonged course and only a small proportion of HPV-16-positive dysplastic tissue progresses to become preneoplastic and neoplastic lesions. The long latency period and variability of the outcome of the infection has lead to the suggestion that several other genetic changes and cofactors may be required to precipitate the actual disease (Vousden, 1989; zur Hausen, 1989).

Several experimental studies are also in agreement with this suggestion. The genomes of high risk HPVs induced immortalization, but not transformation of primary human and rodent cells (Durst et al., 1987b; Pirisi et al., 1987; Kanda et al., 1988a; Schlegel et al., 1988; Woodworth et al., 1988; 1989; Pecoraro et al., 1989; Tsutsumi et al., 1992). Transformation of HPV-16-immortalized human and rodent cells was dependent on additional genetic changes, which occurred spontaneously or were induced exogenously. In contrast to primary cells, transformation of immortalized rodent cells, which had undergone certain genetic alterations to become immortal, was readily achieved by transfection of high risk HPV DNA (Tsunokawa et al., 1986; Yasumoto et al., 1986; Kanda et al., 1987; Matlashewski et al., 1987a; Kanda et al., 1988a, Vousden et al., 1988; Yutsudo et al., 1988; Bedell et al., 1989). Further, cotransfection with oncogenes, such as EJ-ras, which is found mutated in a wide variety of cancers, or v-fos, was sufficient to induce transformation of primary rodent cells, indicating the need for additional genetic alterations for transformation (Crook et al., 1988; Pater et al., 1988; Lees et al., 1990; Vousden, 1990; Munger et al., 1992). Similarly, transformation of immortalized human keratinocytes was achieved either by sequential transfection with activated EJ-ras or by maintaining the keratinocytes in culture for an extended period (DiPaolo et al., 1989; Durst et

al., 1989; Hurlin et al., 1991; Pecoraro et al., 1991). Spontaneous secondary mutations or deregulated expression of certain genes which arise during prolonged culture could contribute to transformation. Supporting this hypothesis is the observation that spontaneous transformation of immortalized BRK cells in culture was accompanied with increased expression of K-ras (Inoue et al., 1991).

The evidence for the presence of genetic changes in cervical cancers comes from several cytogenetic studies. These changes include deregulated expression and/or mutations in protooncogenes and antioncogenes and gross chromosomal abnormalities (Atkin and Baker, 1982; Crum et al., 1985a; Durst et al., 1987b; Pirisi et al., 1987; Atkin and Baker, 1988; Kaur and McDougall, 1988; Woodworth et al., 1990; Inoue et al., 1991; Smits et al., 1992; Klingelhutz et al., 1993). Recent studies have shown that the viral E7 oncoprotein enhances cellular genetic instability (Hashida and Yasumoto, 1991).

Integration of the viral genome into the cellular genome might affect the expression of flanking cellular genes. Although the sites of integration on the chromosome were random, disruption of cellular genes and their expression can occur for different integration events near or into these genes (Durst et al., 1987a; Popescu et al., 1987; Schneider-Maunouroy et al., 1987; Lazo, 1988; Lazo et al., 1992).

The resistance of primary cells as compared with the established cells, to transformation, could be due to extinction of the viral oncogene functions in primary cells (Bosch et al., 1990; Durst et al., 1991; Miyasaka et al., 1991; Rosl et al., 1991). The modulation of E6 and E7 by cellular factors may underlie the ability of primary cells to suppress the tumorigenic phenotype of cell hybrids, which were derived by fusing HPV-16- or -18-positive invasive cervical cancer cells with normal primary cells (Saxon et al., 1986; Srivatsan et al., 1986; Koi et al., 1989; zur Hausen, 1989; Stanbridge, 1990; Miyasaka et al., 1991).

1.1.8.3 Cofactors

Several cofactors such as hormones, ultraviolet (UV) radiation, environmental carcinogens and infection with other viruses may enhance the oncogenic potential of HPVs and/or required for progression of the HPV induced lesions to a more fully malignant state.

Epidermodysplasia verruciformis is a rare skin condition characterized by the presence of multiple warts caused by different types of HPVs. The warts present on areas frequently exposed to UV radiation show a higher tendency to become malignant (Galloway and McDougall, 1989; Shah and Howley, 1990).

It has been suggested that several other biological agents, such as herpes simplex virus (HSV), human

immunodeficiency virus (HIV), chlamydia and bacteria associated with vaginosis and gonorrhoeae may act as cofactors for the development of cervical cancer (Galloway and McDougall, 1989). One of these agents, HSV type 2, can transform HPV-immortalized human keratinocytes (DiPaolo et al., 1990; Hildesheim et al., 1991).

The chronic use of oral contraceptives has been established as a risk factor for cervical cancer (Hildesheim et al., 1990; Honore et al., 1991). In experimental studies, the immortalization/transformation efficiency of high risk HPVs was enhanced by steroid hormones dexamethasone and progesterone (Crook et al., 1988; Pater et al., 1988; Durst et al., 1989; Lees et al., 1990; Pater et al., 1990; Storey et al., 1992; Sexton et al., 1993).

Exposure to chemical carcinogens is a well established risk factor for malignant conversion of papillomas. In experimental systems, the presence of carcinogens facilitated transformation of HPV-immortalized keratinocytes (Li et al., 1992; Garrett et al., 1993; Klingelhutz et al., 1993). Smoking has been considered to be a risk factor for cervical cancer (Winkelstein, 1990). Accumulation of tobacco metabolites in vaginal secretions could contribute to the development of cervical cancer (Sasson et al., 1985; Holly et al., 1986; McCann et al., 1992). Convincing evidence for cooperation between HPVs and chemical carcinogens was provided

by an *in vivo* experiment (Sasagawa et al., 1992). The induction of invasive squamous cell carcinoma of cervix in mice locally injected with HPV-16 E6-E7-expressing retrovirus was dependent on the application of tumor promoters and carcinogens, such as TPA or MNNG.

Oncogenesis due to HPV is a process that probably requires decades after infection to develop, because genital infections occur earlier and cancer develops later in life (zur Hausen, 1991). Cofactors such as UV radiation and hormones may be prerequisites or corequisites for oncogenesis due to HPV, whereas cofactors such as HSV, tumor promoters and carcinogens, that have been shown to further oncogenically insult HPV-immortalized cells, may be factors in the progression from HPV-initiated premalignant cervical lesions to the malignant carcinomas occurring later in life.

1.2 RNA PROCESSING

In the process of becoming messenger RNA (mRNA), the pre-messenger RNA (pre-mRNA) transcribed by RNA polymerase II (pol II) undergoes several posttranscriptional processing events. These steps include capping, splicing, 3′ end cleavage and polyadenylation and transportation of mRNA to the cytoplasm.

Alternative splicing is one of the important posttranscriptional mechanisms for the regulation of HPV-16 gene expression. Several species of mRNAs with different coding potentials are generated from a limited number of primary transcripts by using a number of alternative splice sites and a complex splicing pattern. My studies indicate that the nt 880 splice donor site is essential for the accumulation of HPV-16 RNA encoding the viral oncoproteins. Studies with an emphasis on the nt 880 splice donor site constitute the major part of this thesis. Because of the close coupling of the processes of splicing and 3′ end formation of pre-mRNA, a brief introduction to the process of 3′ end formation of pre-mRNA is also included. In this section, the posttranscriptional processing of mammalian pre-mRNA is discussed.

1.2.1 CAPPING

The 5′ ends of all pre-mRNAs cotranscriptionally acquire a monomethylated, inverted guanosine nucleotide, $m^7G(5')ppp(5')N$, called the "cap" (Shatkin 1976). The cap

structure has been thought to protect the 5′ ends of mRNAs from exonucleolytic degradation (Green, 1986; Gallie, 1991). The cap structure serves various other functions, such as (i) providing a targeting signal for the export of mRNAs, (ii) increasing the efficiency of splicing and, (iii) enhancing the translatability of mRNA.

The monomethylated cap structure has been shown to facilitate the export of mature mRNAs from the nucleus to cytoplasm (Hamm and Mattaj, 1990). One of the targeting signals for the import of U snRNPs (uridine-rich small nuclear ribonucleoprotein particles) into the nucleus is their trimethylguanosine (TMG) cap (Fischer and Luhrmann, 1990; Hamm et al., 1990; Lamond, 1990; Anderson and Zieve, 1991).

The cap structure has been shown to influence the efficiency of splicing (Konarska et al., 1984; Krainer et al., 1984; Edery and Sonenberg, 1985; Ohno et al., 1987; Inoue et al., 1989). Splicing of the cap proximal, but not the distal, intron was enhanced in the presence of a cap at the 5′ end of the first exon (Ohno et al., 1987; Inoue et al., 1989).

The 5′ cap of mRNAs has an important role in the process of translation (Shatkin, 1976; Kozak, 1989). Binding of the translation initiation factor, eukaryotic initiation factor-(eIF)-4F α subunit (eIF-4E) to the 5′ cap of mRNA is an essential step for the initiation of translation (Rhoads, 1988; Sonenberg, 1988; Hershey, 1991). Apart from directly

facilitating translation, the 5′ cap cooperates with the poly(A) tail to further enhance translation (Gallie, 1991)

1.2.2 SPLICING OF PRE-mRNA

Splicing is a biochemical process by which the introns of a pre-mRNA are precisely excised and the exons are religated. Splicing can be broadly grouped into constitutive splicing and alternate splicing (Smith et al., 1989a). In constitutive splicing, all the introns present in a pre-mRNA are excised without preference for any intron. In alternate splicing, one or more introns are selectively retained or spliced out. Alternate splicing is one of the modes by which various isoforms of proteins with different functions are produced from the same gene (Andreadis et al., 1987; Breitbart et al., 1987; Bingham et al., 1988; Baker, 1989; Mattaj and Hamm, 1989; McKeown, 1990; Maniatis, 1991; Foulkes and Sassone-Corsi, 1992). Although, the yeast system has contributed to our knowledge of the process of splicing, the introduction in this section is confined mostly to mammalian systems.

Several cis-acting elements and trans-acting factors have been shown to be essential for the splicing of pre-mRNA (Maniatis and Reed, 1987; Guthrie and Patterson, 1988; Bindereif and Green, 1990; Green, 1991). These are described below in greater detail.

1.2.2.1 Cis-acting elements

1.2.2.1.1 5′ splice site

The boundaries of exons and introns are determined by a set of conserved sequences called 5′ (splice donor) and 3′ (splice acceptor) splice sites. The consensus 5′ splice site sequence, (C/A)AG/GURAGU, has been deduced by comparing several 5′ splice site sequences. The majority of mammalian splice sites do not conform to this consensus sequence. However, the first 2 nucleotides of the intron, GU, are invariant among all naturally-occurring splice sites (Senapathy et al., 1990). Mutation of the 5′ splice site affects the efficiency of splicing, but does not always prevent 5′ splice site cleavage (Aebi et al., 1986; 1987; Smith et al., 1989a). Often a cryptic splice site is activated by such mutations (Aebi et al., 1987). Mutation of the 5′ splice site of an internal exon has been shown to result in skipping of that exon (Robberson et al., 1990; Talerico and Berget, 1990; Niwa et al., 1992). The 5′ splice site sequence is complementary to the first 9 nt of the U1 snRNA (uridine-rich small nuclear RNA), and these two sequences interact by base-pairing (Zhuang and Weiner, 1986). The effect of 5′ splice site mutation on the efficiency of splicing can be suppressed by compensatory mutations in the U1 snRNA indicating that the degree of complementarity between the two determines the efficiency of splicing (Aebi et al.,

1986; Zhuang and Weiner, 1986; Zhuang et al., 1987; Mayeda and Oshima, 1988; Peterson and Perry, 1989; Nelson and Green, 1990).

1.2.2.1.2 3′ splice site

The 3′ splice site that determines the 3′ boundary of the intron, has the consensus sequence Y_nNCAG/G (Mount, 1982; Senapathy et al., 1990). The 3′splice site consists of two elements, the conserved AG dinucleotide and the polypyrimidine tract (PPT). Mutation of the 3′ splice site often results in activation of a nearby cryptic 3′ splice site (Aebi et al., 1986; Deshler and Rossi, 1991; Belaguli et al., 1992). Mutations which failed to activate a cryptic splice site lead to inhibition of the second step of splicing. However, their effects on the first step of splicing were variable (Ruskin et al., 1985; Aebi et al., 1986; Bindereif and Green, 1986; Lamond et al., 1987). Several heterogeneous nuclear ribonucleoproteins (hnRNPs) such as A1, C and D, have been shown to associate with the 3′ splice site. Mutations affecting the conserved AG dinucleotide impaired the binding of hnRNP protein A1 to the 3′ splice site (Swanson and Dreyfuss, 1988). It has been suggested that the selection of the 3′ spice site depends on the process of scanning and the first AG dinucleotide downstream of the branch point is selected as the 3′ splice site (Smith et al., 1989b).

The PPT is found a few nucleotides upstream of the conserved AG dinucleotide. This poorly conserved element is absent in a few introns (Shelley and Baralle, 1987). A repeating GU rich element can functionally substitute for the PPT (Shelley and Baralle, 1987). Non-snRNP splicing factors, such as U2 snRNP auxillary factor (U2AF) and the polypyrimidine tract-binding protein (PTB), specifically bind PPT (Ruskin et al., 1988; Zamore and Green, 1989). Disruption of the PPT has been shown to interfere with binding of U2 snRNP to the adjacent branch-point sequence (Frendeway and Keller, 1985; Ruskin et al., 1988; Zamore and Green, 1989). Several recent studies have indicated that the location and the extent of the PPT determines the strength of a 3′ splice site (Helfman and Ricci, 1989; Smith and Nadal-Ginard, 1989; Zamore et al., 1992).

1.2.2.1.3 Branch-point (BP) sequence

The BP sequence is generally located 20-40 nucleotides upstream of the 3′ splice site. Although the consensus sequence, YNYURAY, has been derived by comparing the BP sequences of several introns, this sequence is rather poorly conserved among mammalian introns (Senapathy et al., 1990). During the first step of splicing, the conserved nucleotide, A, of the BP sequence forms a 2′-5′ phosphodiester bond with the invariant nt G of the 5′ splice site. In some introns, nt such as C or U are used for branch formation (Hartmuth and

Barta, 1988; Nobel et al., 1988). The branch point sequence is specified independently of the 3′ splice site and the sequence context and the proximity to the PPT have been reported to influence the selection of the BP sequence (Smith et al., 1989b). The unusually long spacing between the 3′ splice site and the BP sequence has been suggested to play an important role in the tissue specific alternative splicing of tropomyosin mRNAs (Smith et al., 1989b; Helfman et al., 1990). The efficiency of splicing of an intron is not greatly affected by mutations at the BP sequence, because such mutations generally activate a nearby cryptic BP sequence (Padgett et al., 1985; Reed and Maniatis, 1985; Ruskin et al., 1985). The BP sequence interacts with the U2 snRNA by base-pair interaction (Wu and Manley, 1989; Zhuang et al., 1989). Several studies have suggested a similar interaction of the BP sequence with U5 snRNA (Hartmuth and Barta, 1988).

1.2.2.1.4 Other cis-acting elements

Primary transcripts from several genes contain multiple 5′ splice sites, 3′ splice sites and BP sequences. Presence of such multiple elements greatly facilitate alternate splicing. Several other cis-acting sequences present within introns and flanking exons are known to affect the choice of splice site and the efficiency of splicing. These elements act by changing the secondary structure of the RNA, by providing binding sites for specific trans-acting splicing

factors and by unknown mechanisms. Elements which alter the secondary structure of the pre-mRNA have been thought to affect splicing primarily by regulating the accessibility of splice sites to the trans-acting splicing factors (Solnick, 1985; Eperon et al., 1988; Balvay et al., 1993).

Sequence elements present within the introns of Rous sarcoma virus (RSV), HIV-1 and influenza virus pre-mRNAs regulate splicing and transportation of these RNAs (Chang and Sharp, 1989; Malim et al., 1989; Lu et al., 1990; Alonso-Caplen and Krug. 1991; McNally et al., 1991; McNally and Beemon, 1992). Similarly, cis-acting elements present within certain exons of human fibronectin, leukocyte common antigen, immunoglobulin heavy chain, adenovirus early region 3 and bovine growth hormone pre-mRNA affect the splicing of flanking introns (Mardon et al., 1987; Hampson et al., 1989; Streuli and Saito, 1989; Domenjoud et al., 1991; Watakabe et al., 1993).

The other cis-acting elements have been shown to play an important role in developmentally regulated and tissue specific alternate splicing of various pre-mRNAs. The neuron specific splicing of c-src pre-mRNA was dependent on the flanking intron sequences (Black, 1991; 1992). Similarly, the tissue specific alternative splicing of β-tropomyosin pre-mRNA required sequence elements present within the alternately spliced exon 7 and the adjacent upstream intron (Helfman et

al., 1990; Guo et al., 1991). The somatic inhibition of splicing of the third intron of the Drosophila P element was suggested to be related to the pseudo-5′ splice sites in the 5′ exon and the third intron (Siebel and Rio, 1990; Siebel et al., 1992). Sequences present in the female specific exon of the Drosophila double sex pre-mRNA were essential for the activation of the double sex female specific 3′ splice site by the products of transformer and transformer-2 genes (Hoshijima et al., 1991; Ryner and Baker, 1991; Tian and Maniatis, 1992).

1.2.2.2 Trans-acting factors

1.2.2.2.1 snRNPs

Splicing of pre-mRNA requires a variety of U snRNAs, such as U1, U2, U4, U5 and U6 snRNAs. The snRNAs associate with a common set of 6-10 core proteins and form ribonucleoprotein complexes called snRNPs. The core proteins specifically bind a highly conserved consensus sequence, $PuAU_{3-4}NUGPu$, present in snRNAs (Guthrie and Patterson, 1988). In addition to the core proteins, snRNA-specific proteins are also found in snRNPs (Smith et al., 1989a; Anderson and Zieve, 1991; Green, 1991).

U snRNAs have a complex secondary structure (Guthrie and Patterson, 1988; Green, 1991). Several variant forms of U snRNAs are known to exist and production of some of these variant snRNAs are developmentally regulated (Mattaj and Hamm, 1989). Temporally regulated alternate splicing of various transcripts during development could be related to the regulated production of U snRNA isoforms.

1.2.2.2.1.1 U1 snRNP

The U1 snRNP consists of U1 snRNA and three U1 snRNA-specific proteins. The mammalian U1 snRNA has 160 nt (Anderson and Zieve, 1991; Green, 1991). The U1 snRNP binds the 5′ splice site and the 5′ end of the U1 snRNA is essential for this interaction (Bindereif and Green, 1987; Zhuang and Weiner, 1986; Rosbash and Seraphin, 1991; Wassarman and Steitz, 1992; Cohen et al., 1993). One of the three U1 snRNP-specific proteins, U1C, potentiates this base-pair interaction between the U1 snRNA and the 5′ splice site of pre-mRNA (Heirichs et al., 1990). The differential binding of U1 snRNP to 5′ splice sites is one of the bases for alternative splicing (Kuo et al., 1991). The alternate splicing of the exon, E4, of the rat preprotachykinin pre-mRNA was related to the binding affinity of U1 snRNP to the 5′ splice site of the E4 exon. Increasing the affinity of binding of the E4 exon 5′ splice site to U1 snRNP resulted in inclusion of the exon, whereas reducing the affinity between the two by introducing mutations at the 5′ splice site resulted in skipping of the E4 exon (Kuo et al., 1991).

1.2.2.2.1.2 U2 snRNP

The mammalian U2 snRNA has 189 nt (Guthrie and patterson, 1988). The first 110 nt of U2 snRNA are highly conserved among different species of eukaryotes. Two U2 snRNA-specific proteins have been detected in the U2 snRNP (Anderson and

Zieve, 1991; Green, 1991). The U2 snRNP binds the BP sequence of an intron and this binding is dependent on the presence of U1 snRNP (Barabino et al., 1990). Non-snRNP proteins, such as U2AF, splicing factor (SF)1 and SF3 promote an efficient binding of U2 snRNP to the BP sequence (Kramer, 1988; Ruskin et al., 1988; Zamore and Green, 1989; 1991). Base-pair interaction occurs between the 5' end of U2 snRNA and the 3' end of U6 snRNA and this interaction is essential for efficient splicing (Datta and Weiner, 1991; Wu and Manley, 1991; Wassarman and Steitz, 1992).

1.2.2.2.1.3 U4 and U6 snRNPs

The mammalian U4 snRNA consists of 139 nt and the U6 snRNA is 107 nt long. The U6 snRNA differs from other spliceosomal snRNAs in that it is transcribed by RNA pol III and lacks the TMG cap structure and the core protein binding consensus sequence (Guthrie and Patterson, 1988). In addition, U6 snRNA contains an mRNA-type intron. It has been suggested that this mRNA-type intron was inserted into U6 snRNA erroneously by reverse splicing, because of the proximity of U6 snRNA to the catalytic core of the spliceosome (Tani and Ohshima, 1991). The U6 snRNA is highly conserved among different species of organisms (Brow and Guthrie, 1988). The high degree of conservation of both sequence and structure has suggested an important role in splicing for this snRNA (Guthrie and Patterson, 1988).

The U4 and U6 snRNPs are found in a U4/U6 snRNP complex. These two snRNPs are held together by base-pair interactions between U4 and U6 snRNAs (Bindereif et al., 1990). The U5 snRNP associates with the U4/U6 snRNP complex to form a multi-snRNP complex, which is incorporated into the spliceosome (Konarska and Sharp, 1987). In the spliceosome, the U4 and U6 snRNAs undergo unpairing in a process likely to be mediated by RNA helicases (Lamond et al., 1988; Green, 1991). Several studies have suggested a catalytic role for U6 snRNA in the process of splicing (Guthrie, 1991). Supporting this hypothesis, the U6 snRNA is present in close proximity to the 5′ splice site in a spliceosome, as shown by crosslinking to this region of the pre-mRNA (Sawa and Shimura, 1992; Wassarman and Steitz, 1992).

1.2.2.2.1.4 U5 snRNP

The U5 snRNP is the largest and the most complex of the snRNPs. It consists of a 117 nt long U5 snRNA and at least seven U5 snRNP-specific proteins (Bindereif and Green, 1990; Anderson and Zieve, 1991; Green, 1991). A fraction of U5 snRNP is also found in a triple snRNP complex with the U4/U6 snRNPs (Konarska and Sharp, 1987). This interaction with U4/U6 snRNP complex appears to be mediated by the triple snRNP associated proteins, but not the individual U4/U6 or U5, snRNP associated proteins (Bach et al., 1989; Utans et al., 1992). The U5 snRNA and the U5 snRNP protein, p220, were shown to

interact with the 5′ splice site sequence of pre-mRNA (Wassarman and Steitz, 1992; Wyatt et al., 1992). Recent studies have shown that the U5 snRNA interacts with both the 5′ and 3′ splice sites and determines the position of 5′ splice site cleavage (Newman and Norman, 1991; 1992).

1.2.2.2.2 Non-snRNP splicing factors

Several non-snRNP splicing factors have been detected by using different techniques, such as UV crosslinking, protein purification, inhibition of in vitro splicing by antibody/immunodepletion, complementation of splicing extracts with purified proteins and cloning of the corresponding cDNAs. Precise functions and the mechanisms of action of a majority of these factors remain to be elucidated.

A number of proteins have been identified which associate with the 5′ splice site region of pre-mRNA. The binding of two proteins, the 27 kDa SPP-1 and 37 kDa SPP-2, to the 5′ splice site was independent of U1 snRNP and occurred prior to the association of pre-mRNA with the U1 snRNP (Stolow and Berget, 1991). In addition, a 55 kDa protein which interacts with the 5′ splice site has been detected (Garcia-Blanco et al., 1989). These 5′ splice site binding proteins could either facilitate recognition of splice sites or stabilize the interaction of U1 snRNP with pre-mRNA.

The PPT/3′ splice site of introns is a target for several non-snRNP splicing factors. Only a few such proteins are

considered here. U2AF has been shown to play an important role in the assembly of spliceosome and the process of splicing. Depletion of U2AF from in vitro splicing extracts resulted in an inhibition of splicing (Zamore and Green, 1991). U2AF consists of 65 kDa and 35 kDa subunits (Zamore and Green, 1989). The 65 kDa subunit has three ribonucleoprotein-consensus domains which are essential for RNA binding. U2AF binds the PPT/3' splice site region and this interaction facilitates binding of U2 snRNP to the adjacent branch site sequence (Zamore and Green, 1989; 1991). The binding affinity of U2AF is determined by the pyrimidine content and the length of PPT (Zamore et al., 1992). The binding of U2AF to the PPT/3' splice site was shown to be facilitated by binding of U1 snRNP to the downstream 5' splice site region (Hoffman and Grabowski, 1992). Several recent studies have suggested that the modulation of the binding activity of U2AF is an important mechanism for alternative splicing of pre-mRNAs (Green, 1991; Hoffman and Grabowski, 1992; Valcarcel et al., 1993).

The 62 kDa PPT binding protein (PTBP) binds the PPT of introns. Mutation of the PPT, which affected the binding of the PTBP, resulted in a lower efficiency of pre-spliceosome complex assembly (Garcia-Blanco et al., 1989). PTBP is a component of spliceosome and its association with the PPT is independent of snRNPs and splicing factors (Paton et al.,

1991). Immunodepletion of PTBP and an associated protein, PTB-associated splicing factor, from splicing extracts inhibited splicing (Garcia-Blanco et al., 1989; Paton et al., 1991; 1993).

Intron binding protein (IBP) is a 70-100 kDa PPT binding protein mediating the interaction of U5 snRNP with the 3' splice site. Despite its interaction and copurification with U5 snRNP, IBP is distinct from other snRNP proteins (Bindereif and Green, 1990).

A recently identified protein of 88 kDa has been shown to be required for the assembly of the spliceosome. Immunodepletion of this protein from the splicing extract resulted in inhibition of splicing and supplementing the immunodepleted extract with affinity-purified p88 restored splicing (Ast et al., 1991).

Several studies have indicated an essential role for hnRNPs in splicing. Some hnRNPs, such as A1, C and D, have been shown to bind the PPT/3' splice site region (Kumar et al., 1987; Swanson and Dreyfuss, 1988; Garcia-Blanco et al., 1989; Stolow and Berget, 1990). Mutation of the conserved AG dinucleotide sequence abolished the binding of hnRNP A1 (Swanson and Dreyfuss, 1988). In vitro splicing of pre-mRNA was inhibited by the addition of anti hnRNP C antibodies to the splicing extract or immunodepletion of hnRNP C from the splicing extract (Bindereif and Green, 1990). The

concentration of the hnRNP A1 relative to the splicing factor (SF) 2 has been shown to modulate alternative splicing of pre-mRNA (Mayeda and Krainer, 1992; Mayeda et al., 1993).

By fractionation of nuclear extract, proteins with essential roles in the process of spliceosome assembly and splicing have been purified (Krainer and Maniatis, 1985; Kramer and Keller, 1985; Fu and Maniatis, 1990). Fractions containing SF1 and SF3 were required for the assembly of spliceosome and SF2 and SF4 were required for 5′ splice site cleavage and lariat formation (Kramer et al., 1987; Kramer, 1988). Subsequently, SF4 was shown to convert an assembled functionally inactive complex into a functional complex (Utans and Kramer, 1990).

There is also evidence for the presence of non-snRNP factors which affect the choice of splice sites. The factor alternate splicing factor (ASF)/SF2 was identified as an activity required for the assembly and stabilization of spliceosomes. ASF/SF2 consists of related 30-35 kDa polypeptides and selectively promote the splicing of the most proximal 5′ splice site to a 3′ splice site (Ge and Manley, 1990; Krainer et al., 1990a; 1990b; Ge et al., 1991; Krainer et al., 1991; Lamond, 1991; Mayeda and Krainer, 1992). Recently it was reported that the ASF/SF2 belongs to a family of proteins, called SR proteins, which are involved in constitutive and alternative splicing (Mayeda et al., 1992;

Zahler et al., 1992; 1993). Factors which promote the use of a distal 5′ splice site have also been identified. Distal splicing factor (DSF), SF5 and hnRNP A1 were shown to enhance the utilization of a distal 5′ splice site (Harper and Manley, 1991; Mayeda and Krainer, 1992).

1.2.2.3 Assembly of spliceosomes

Splicing of pre-mRNA occurs in a ribonucleoprotein complex called the spliceosome. Spliceosome, which consists of five snRNAs (U1, U2, U4/U6 and U5) and more than fifty different proteins, are assembled in four different stages (Reed, 1990; Green, 1991; Guthrie, 1991).

The binding of the U1 snRNP to the 5′ splice site constitutes the first stage of the assembly process and this binding is independent of requirement for other snRNPs, splicing factors and ATP hydrolysis (Rosbash and Seraphin, 1991).

In the second stage, the U2 snRNP binds the BP sequence. This binding is an ATP-dependent process and commits the pre-mRNA to the splicing pathway. Presence of U1 snRNP and other splicing factors such as U2AF, SF1 and SF3 promote stronger binding of U2 snRNP (Green, 1991; Hoffman and Grabowski, 1992).

Incorporation of the pre-assembled U4/U6/U5 multi-snRNP complex into the spliceosome marks the beginning of the third stage of the assembly process (Konarska and Sharp, 1987; Utans

et al., 1992). The U4/U6/U5 multi-snRNP complex associates with the pre-mRNA-bound U1 and U2 snRNPs (Bindereif and Green, 1987; Wassarman and Steitz, 1992; Wyatt et al., 1992).

In the fourth stage, which immediately precedes the 5′ splice site cleavage, the assembled spliceosome undergoes a conformational change. Although the base-pair interaction between the U4 and U6 snRNAs is destabilized, the U4 snRNP is not released from the complex (Blencowe et al., 1989).

1.2.2.4 Mechanism of splicing

Splicing involves two successive transesterification reactions and proceeds in two steps (Green, 1991; Guthrie, 1991). In the first step, cleavage occurs at the 5′ splice site resulting in a linear first exon and an intron-second exon lariat structure. Formation of 2′-5′ phosphodiester bond between the invariant first base, G, of the intron and the 2′-hydroxyl group of the conserved A of the BP sequence results in the lariat structure. In the second step, cleavage occurs at the 3′splice site with subsequent ligation of the second exon to the first exon. The intron is released as a lariat. The 2′-5′ phosphodiester bond in the lariat intron is cleaved by a debranching activity and the linearized intron undergoes degradation (Smith et al., 1989a; Green, 1991).

1.2.3 3′ END CLEAVAGE AND POLYADENYLATION

1.2.3.1 Nuclear polyadenylation

Eukaryotic mRNAs have poly(A) tails at their 3′ ends. The poly(A) tail has been shown to confer stability and facilitate translation of mRNA (Jackson and Standart, 1990). The 3′ ends of eukaryotic mRNAs, except for histone mRNAs, are generated by specific endonucleolytic cleavage of the 3′ end of pre-mRNAs and addition of 200-300 A residues by a process called polyadenylation. The endonucleolytic cleavage occurs while the RNA pol II is transcribing the template DNA downstream of the cleavage site. In vivo, the 3′ end cleavage and polyadenylation are closely coupled. However, these two processes can be uncoupled in in vitro systems. The 3′ end cleavage and polyadenylation are highly dependent on certain cis-acting elements and trans-acting factors which interact among themselves and with the cis-acting elements.

1.2.3.1.1 Cis-acting elements

1.2.3.1.1.1 Hexanucleotide AAUAAA sequence

One of the well defined and highly conserved cis-acting elements required for pre-mRNA 3′ end cleavage and polyadenylation, also called 3′ end formation, is the hexanucleotide consensus sequence, AAUAAA. This element is usually located 20-30 nt upstream of the cleavage site. The percentage frequency of nt in this consensus is $A_{98}A_{86}U_{98}A_{98}A_{95}A_{96}$ (Wickens, 1990a). The natural variant of this consensus

sequence, AUUAAA, is found in approximately 12% of mRNAs. The ability of this variant to direct 3′ end cleavage and polyadenylation is comparable to that of the consensus sequence (Manley, 1988). Recently, another naturally occurring variant of the consensus sequence, CAUAAA, was detected in the Xenopus α-tubulin mRNA (Rabbitts et al., 1992). Mutation of the AAUAAA sequence affected 3′ end cleavage, polyadenylation and mRNA accumulation (Manley, 1988). In in vitro assays in which the 3′ end cleavage and polyadenylation were uncoupled, the effect of mutation of the AAUAAA sequence on the efficiency of polyadenylation was dependent on the position and the nt substituted (Sheets et al., 1990). A mutation at the +2 position was better tolerated than mutations elsewhere in the hexanucleotide sequence. In contrast, modification by methylation of the 2′ OH group of ribose of the +2 position nt, but not the +3 position nt, affected polyadenylation (Bardwell et al., 1991). Apart from underscoring the importance of the AAUAAA haxanucleotide sequence, the above-mentioned study highlighted the nature of protein-RNA interactions.

1.2.3.1.1.2 Downstream GU/U rich elements (DSE)

Certain sequences located downstream of the cleavage site are essential for proper mRNA 3′ end formation. The DSE is involved in specifying a unique 3′ cleavage site. Deletion of this element resulted in the production of heterogeneous 3′

ends (Manley, 1988). These sequence elements are weakly conserved and generally GU or U rich. Several in vivo and in vitro studies have indicated the importance of DSE for efficient 3' end formation (Conway and Wickens, 1985; Hart et al., 1985; Gil and Proudfoot, 1987; Manley, 1988; Ryner et al., 1989a). The SV40 early polyadenylation signal has a GU-rich DSE, and the adenovirus E2A transcription unit has a U-rich DSE. Despite the difference in the primary sequences, these two elements were functionally interchangeable (Hart et al., 1985).

The approximately 40 nt spacing between the AAUAAA hexanucleotide element and the DSE is fairly well conserved. Alteration of the spacing, but not the sequence, between these two elements affected the activity of the polyadenylation signal (McDevitt et al., 1986; Gil and Proudfoot, 1987; Heath et al., 1990; Wingley et al., 1990). A systematic study on the effect of spacing between these two elements was carried out by Heath et al., (1990). A spacing of 19 nt was optimal for the activity of HSV-1 thymidine kinase (tk) polyadenylation signal. Increasing this spacing to 43 nt or reducing to 7-9 nt severely affected the function of the polyadenylation signal (Heath et al., 1990). The AAUAAA element and the DSE of human T cell leukemia virus (HTLV) are separated by 274 nt and the 3' end cleavage occurs approximately 250 nt downstream of the AAUAAA sequence.

Looping out and folding of the sequences located in between the AAUAAA sequence and the DSE brought these two elements into optimal spacing and facilitated 3' end formation (Ahmed et al., 1991). The 3' end formation of several other pre-mRNAs have been shown to depend on a similar looping mechanism (Brown et al., 1991).

1.2.3.1.1.3 Upstream elements (USE)

Recently, certain elements located upstream of the AAUAAA sequence were shown to enhance the activity of the polyadenylation signal. A 20 nt USE is present 29 bases upstream of the AAUAAA sequence of SV40 late polyadenylation signal (Carswell and Alwine, 1989). A similar USE is present upstream of the polyadenylation signal of the L1 major late transcript of adenovirus and this element has been suggested to promote the preferential utilization of the L1 polyadenylation signal during the early phase of infection (DeZazzo and Imperiale, 1989). USEs are also present upstream of the polyadenylation signals of several retroviruses and retroid viruses and these USEs regulate the activity of the polyadenylation signal by different mechanisms (Proudfoot, 1991). Although USEs of different pre-mRNAs bear little sequence similarity, they are functionally interchangeable. The USE of HIV-1 is U-rich and bipartite in nature. It can functionally substitute for the USE of SV40 late polyadenylation signal (Valsamakis et al., 1991). Similarly,

the USEs of retroid viruses, such as ground squirrel hepatitis virus and spleen necrosis virus, were also functionally interchangeable (Russnak and Ganem, 1990).

1.2.3.1.2 *Trans*-acting factors

The development of *in vitro* polyadenylation system which accurately reproduces mRNA 3' end formation has greatly facilitated identification of individual factors required for this process. Because of the different fractionation procedures followed by different laboratories, it is possible that the same factors may have been given different designations.

One of the factors required for 3' end formation is poly(A) polymerase (PAP). PAP has been purified from calf thymus and HeLa cells. It is a 40-50 kDa protein with an ATP polymerizing activity (Christofori and Keller, 1989; Gilmartin and Nevins, 1989; Ryner et al., 1989b). The gene encoding PAP has been cloned recently (Linger et al., 1991; Raabe et al., 1991). Purified PAP adds poly(A) tail nonspecifically to any RNA substrate. Addition of another factor to the *in vitro* polyadenylation reaction conferred AAUAAA sequence dependent specific activity to PAP (Takagaki et al., 1988). PAP was required for both 3' end cleavage and polyadenylation.

The factor that conferred specificity on PAP can be further fractionated into 4 factors; specificity factor (SF), cleavage factors I and II (CF I and CF II) and cleavage

specificity factor (CstF) [Takagaki et al., 1989]. SF was required for both 3' end cleavage and polyadenylation and bound the hexanucleotide sequence AAUAAA. SF may be functionally analogous to a factor called PF2 described by Gilmartin and Nevins, (1989). The cleavage factors, CF I and CF II, were required for the 3' end cleavage reaction. CF I was shown to associate with the DSE (Gilmartin and Nevins, 1989). CstF consists of 77 kDa, 64 kDa and 50 kDa subunits. The 64 kDa subunit of CstF can be specifically crosslinked to the AAUAAA sequence-containing RNA substrate (Wiluz and Shenk, 1988; Takagaki et al., 1989; Wiluz et al., 1990). An additional 155 kDa protein has also been crosslinked to AAUAAA sequence-containing RNA substrates (Moore et al, 1988). The functions of this protein has not been established.

HnRNP C has been shown to bind the DSE (Wiluz et al., 1988). Recently, Quin and Wiluz, (1991), have described interaction of a 50 kDa protein, DSEF-1, with the GU-rich DSE of late polyadenylation signal of SV40.

An earlier study had suggested the involvement of U snRNPs in the process of 3' end cleavage (Hashimoto and Steitz, 1986). However, several studies have clearly excluded the requirement for U snRNPs for mRNA 3' end formation (Ryner and Manley, 1987).

1.2.3.1.3 Poly(A)some assembly and mRNA 3′ end formation

The formation of the 3′ end of mRNA occurs in a large ribonucleoprotein complex called the poly(A)some. The assembly of poly(A)some and the process of mRNA 3′ end formation have been studied in in vitro polyadenylation systems using purified/partially purified fractions. The assembled poly(A)some protected the AAUAAA sequence, as well as those of USE and DSE, from RNase T1 digestion (Humphrey et al., 1987). SF, the first factor to bind the pre-mRNA substrate, binds the AAUAAA sequence. Because of the lower affinity of SF to RNA, this binary complex was unstable. However, the binding of CF I to DSE stabilized the interaction of SF with the AAUAAA sequence. A prerequisite for the binding of CF I was the prior association of SF with AAUAAA sequence. The AAUAAA sequence-bound SF interacted with CstF and facilitated its binding to pre-mRNA through the 64 kDa subunit. The binding of CstF to RNA further stabilized the interaction of SF with the AAUAAA sequence (Wahle and Keller, 1992). Subsequent addition of CF II and PAP resulted in specific endonucleolytic cleavage and poly(A) addition (Gilmartin and Nevins, 1989; Takagaki et al., 1989; 1990). The mechanism by which DSE-binding proteins, such as DSEF-1 and hnRNP C, contributed to the process of mRNA 3′ end formation was not clear.

The addition of a poly(A) tail to the cleaved 3′ end occurred in two phases, (i) the AAUAAA sequence dependent

addition of the first 10 A residues, and (ii) the AAUAAA sequence independent elongation of this oligo(A) primer (Wickens, 1990a). At the transition between the two phases, SF dissociated from the AAUAAA sequence. However, it remained associated with the elongating PAP (Bardwell and Wickens, 1990). The mechanism by which the activity of PAP is terminated is not known.

1.2.3.2 Cytoplasmic polyadenylation

Temporal expression of several genes during development is regulated by Cytoplasmic polyadenylation. In Xenopus oocytes, several maternal mRNAs are stored in a translationally repressed state. In mature oocytes, some of these maternal mRNAs are selectively activated and recruited into polysomes. Mobilization of these mRNAs into polysomes is accompanied with changes in the length of poly(A) tails. Alterations in the length of poly(A) tails occurs in the cytoplasm. The process of cytoplasmic poly(A) tail addition/elongation differs significantly from the nuclear polyadenylation. The 3' end cleavage is not necessary for cytoplasmic poly(A) tail elongation. The cytoplasmic poly(A) tail elongation is catalyzed by a PAP, which appeared to be distinct from the nuclear PAP (Wickens, 1990b).

Cytoplasmic poly(A) tail elongation of C12, D7 and G10 mRNAs required a conserved hexanucleotide sequence AAUAAA and an upstream U-rich element called the cytoplasmic polyadenylation element (CPE) [Fox et al., 1989; Lynn McGrew

et al., 1989; Simon et al., 1992]. Several other mRNAs, which are known to undergo cytoplasmic poly(A) tail elongation contained a similar U-rich element. The CPE of G10 mRNA was functional when placed downstream of the AAUAAA sequence (Lynn McGrew et al., 1989). For C12 mRNA, the spacing between the CPE and the AAUAAA sequence was found to be important for temporal regulation of translational activation of this mRNA (Simon et al., 1992).

Recently, several proteins, which may be involved in the process of cytoplasmic polyadenylation, were identified. A 58 kDa protein was found to associate with the CPE of B4 mRNA, as determined by UV crosslinking (Paris et al., 1991). A 82 kDa protein was found to interact with the CPE of G10 mRNA (Lynn McGrew and Richter, 1990). The CPE-binding proteins were present in both immature and mature oocytes, however, only the proteins from mature oocyte were functional in in vitro polyadenylation assays (Lynn McGrew and Richter, 1990; Paris et al., 1991). It was suggested that the CPE-binding activity of these proteins may be modulated by phosphorylation. In an in vitro polyadenylation assay, both 58 kDa and 82 kDa proteins underwent phosphorylation by p34 cdc-2 kinase and this modification was essential for the binding of these proteins to CPE (Lynn McGrew and Richter, 1990; Paris et al., 1991).

1.3 ANTISENSE (AS) RNA

In several systems, naturally occurring AS RNAs provide a means for posttranscriptional regulation of gene expression (Helen and Toulme, 1990; Takayama and Inouye, 1990; Nellen et al., 1992). I have detected naturally occurring AS RNA to HPV-16 and studied the possible mechanisms by which the AS RNA can regulate the expression of the E7 oncogene of HPV-16.

AS RNA contains sequences complementary to the transcript of a gene. Both naturally occurring AS RNA and AS RNA experimentally introduced into various systems have provided insights into complex processes, such as regulation of gene expression, DNA replication, signal transduction, cell growth, development and differentiation. AS RNA technology has profound implications in diverse fields, such as therapeutics and agriculture (Stein and Cohen, 1988; Toulme and Helene, 1988; Walder, 1988; Helene and Toulme, 1990; Takayama and Inouye, 1990: Calabretta, 1991; Helene, 1991).

1.3.1 Naturally occurring AS RNAs

Naturally occurring AS RNA has been detected in both prokaryotes and eukaryotes. The functional significance for the majority of the reported eukaryotic AS RNA is not known. Some of these naturally occurring AS RNA species posttranscriptionally regulate the expression of corresponding genes. This section of the introduction is confined to eukaryotic AS RNA.

An AS RNA complementary to a portion of the myelin basic protein (MBP) transcript was detected in the brain tissue of myelin deficient mutant (mld) mice (Okano et al., 1988; Popko et al., 1988; Tosic et al., 1990). The brain tissues of mld mice express very low levels of MBP, despite the presence of wild-type MBP gene. In mld mice, the MBP gene is tandemly duplicated and the sequences from exons 3 to 7 of the upstream allele are inverted (Akowitz et al., 1987). Transcription of the upstream allele results in AS MBP RNA complementary to sequences from exons 3 to 7 of sense (S) MBP RNA (Okano et al., 1988; Tosic et al., 1990). Formation of an AS RNA-S RNA duplex interferes with processing and transport of the S MBP RNA (Okano et al., 1991; Matthieu et al., 1992).

Induction of differentiation of murine erythroleukemia and melanoma cell lines is associated with the downregulation of tumor suppressor p53 mRNA and accumulation of p53 pre-mRNA maturation intermediates. This downregulation of p53 mRNA is related to the accumulation of three different species of naturally occurring AS p53 RNA. One of these is a high molecular weight AS p53 RNA, which accumulates in the nucleus. Another species of 1.3 kb AS p53 RNA confined to the nuclear compartment, has sequences complementary to the B1 repetitive element present in the first intron of p53 pre-mRNA. A family of AS p53 RNA species related to B2 repetitive elements were also detected in the nucleus and cytoplasm of differentiating

cells. These AS RNAs were thought to downregulate p53 mRNA by interfering with the maturation of p53 pre-mRNA (Khochbin and Lawrence, 1989; Khochbin et al., 1992).

In Dictyostelium, accumulation of the EB4-PSV mRNA is developmentally regulated. The EB4-PSV locus remains transcriptionally active during all stages of development. However, the EB4-PSV mRNA does not accumulate until the cells begin aggregating to establish a prespore-prestalk structure. Disruption of aggregated cells results in rapid destabilization of EB4-PSV mRNA. This rapid destabilization is brought about by a cytoplasmic AS EB4-PSV RNA which forms an RNA hybrid with the EB4-PSV mRNA (Hildebrandt and Nellen, 1992).

Three maternal basic fibroblast growth factor (bFGF) transcripts of sizes 4.5 kb, 2.3 kb and 1.5 kb, have been detected in Xenopus oocytes (Kimelman et al., 1988). The 4.5 kb mRNA encodes the bFGF polypeptide. The most abundant, 1.5 kb transcript expressed at a level 20-fold higher than the 4.5 kb mRNA, is complementary to the third exon of the 4.5 kb transcript and forms a duplex with this RNA <u>in vivo</u>. This double stranded RNA (dsRNA) is a substrate for the dsRNA-specific enzymatic activity termed unwindase, which modifies A into I (Bass and Weintraub, 1987; 1988). It has been suggested that the rapid destabilization of the 4.5 kb bFGF transcript after oocyte maturation is related to the release

of unwindase from the nucleus and subsequent modification of this transcript (Kimelman and Kirschner, 1989). Unlike other AS RNA species, the AS bFGF RNA encodes a 25 kDa protein, which is highly conserved among different species of animals. The function of this protein is not clear.

Several early response genes, such as c-fos, c-myc, N-myc, L-myc and c-myb, for which expression is rapidly induced in response to mitogenic stimuli, were shown to express AS RNA (Bentley and Groudine, 1986; Nepveu and Marcu, 1986; Bender et al., 1987; Kindy et al., 1987; Krystal et al., 1988; Spicer and Sonenshein, 1992). However, the AS RNA could not be detected by commonly used techniques of Northern blotting and in situ hybridization, because the AS RNA was highly unstable (Bender et al., 1987; Krystal et al., 1988). Stable expression of AS c-myc RNA was detected in a murine plasmacytoma cell line, in which the c-myc locus had undergone translocation to the immunoglobulin alpha heavy chain constant region (Dean et al., 1983). The stability of the AS c-myc RNA in this cell line could have been a consequence of translocation. Using this cell line, the AS promoter was mapped to the second intron of the c-myc gene (Spicer and Sonenshein, 1992). It has been suggested that the AS c-myc RNA is involved in downregulating c-myc mRNA expression in pre-B cells in response to interleukin-3 deprivation (Chang et al., 1991).

In contrast to highly unstable AS c-myc and L-myc RNAs, the AS N-myc RNA was stable and accumulated in both the nucleus and cytoplasm. The promoter for the AS N-myc RNA was located within the first intron. The AS RNA was transcribed by RNA pol II and multiple initiation sites for the AS RNA were detected. The AS RNA underwent polyadenylation. Nonpolyadenylated cytoplasmic AS RNA was complementary to the first exon and intron sequences of N-myc RNA and formed an RNA-RNA duplex with the endogenous S N-myc RNA (Krystal et al., 1990). It was thought that the duplex formation could interfere with the splicing of N-myc pre-mRNA.

Recently, a 438 nt AS RNA with sequences complementary to the second intron of c-myc was cloned from the human colon cancer cell line (Celano et al., 1992). Unlike the previously described AS c-myc RNA (Spicer and Sonenshein, 1992), the AS RNA cloned from the human colon cancer cells was transcribed from a locus different than the c-myc locus. In addition, 120 nt of this AS RNA showed approximately 85% homology to the AS strands of growth-related genes, such as N-myc, p53 and thymidine kinase. This AS RNA was induced by the intracellular polyamine depleting compound, α-difluoromethylornithine, and the induction was accompanied by concomitant downregulation of the c-myc mRNA. The inverse correlation between the induction of the 438 nt AS c-myc RNA and the downregulation of c-myc mRNA and the complementarity

between this AS RNA and other growth related gene transcripts suggested a possible role for this naturally occurring AS RNA in the regulation of cell growth.

The proteins encoded by c-erbA, the cellular homolog of the viral oncogene, v-erbA, belong to the family of the thyroid/steroid hormone receptors and bind the hormone, triiodothyronine (Evans, 1988). Among the multiple forms of c-erbA, the c-erbAα is more closely related to the v-erbA. Two forms of c-erbAα, c-erbAα-1 and c-erbAα-2, have been detected in rats. Only the c-erbAα-1, but not the c-erbAα-2, binds the thyroid hormone, T3 (Izumo and Mahdavi, 1988; Lazar et al., 1988). A 3 kb AS RNA containing sequences complementary to the 269 nt of the last exon of the c-erbAα-2 was cloned from a rat cDNA library (Lazar et al., 1989). The AS RNA was polyadenylated and encoded a 56 kDa protein belonging to the steroid hormone receptor superfamily. A homolog of this AS RNA was also detected in human cells (Miyajima et al., 1989).

Small, nuclear, nonpolyadenylated AS RNAs complementary to portions of the major dihydrofolate reductase (DHFR) transcript were detected in murine cells. The AS RNA transcribed from the bidirectional DHFR promoter had heterogeneous 5′ ends that were complementary to the first 10 nt of DHFR mRNA (Farnham et al., 1985).

The naturally occurring AS RNA to the gonadotropin-releasing hormone transcripts was detected in the cardiac muscle of rats (Adelman et al., 1987). The AS RNA was alternately spliced and polyadenylated suggesting that it may code for protein(s). However, no protein(s) encoded by these AS RNAs have been detected.

The overlap between two transcription units transcribed in opposite orientations would result in RNAs with sequences complementary to each other. The transcription of two adjacent genes of a murine locus was shown to produce mRNAs which overlap by 133 nt at their 3′ untranslated regions (Williams and Fried, 1986). Similarly, an overlap between the dopa decarboxylase transcription unit and the adjacent transcription unit of unknown function of Drosophila resulted in mRNAs with 88 nt overlap at their 3′ ends (Spencer et al., 1986). An evolutionarily conserved AS RNA complementary to the 3′ regions of the DNA excision repair protein-encoding transcripts of human ERCC-1 and the yeast RAD10 mRNAs has been detected (van Duin et al., 1989). The complete 3′ untranslated region and the last two codons of the RAD10 mRNA were complementary to the AS RAD10 RNA.

Recently, the naturally occurring AS RNA to the human thymidylate synthase mRNA was detected in a variety of cell types and was cloned from the human oral epidermoid carcinoma cell line (Dolnick, 1993). The AS thymidylate synthase RNA

contained introns. The 1.8 kb mRNA for this AS RNA underwent polyadenylation and accumulated in the cytoplasm. Four large ORFs were detected in this AS RNA suggesting that it might encode protein(s). The possible functions and significance of this AS RNA remain to be investigated.

Several naturally occurring AS RNAs complementary to viral pre-mRNAs/mRNAs have been detected in infected cells. However, their significance to the life-cycle of the virus is not understood.

The latency-associated transcript (LAT) is a major HSV-1 immediate early region transcript present in the nuclei of latently infected neurons of mice and humans. Three species of nonpolyadenylated, colinear LATs of 2.0, 1.5 and 1.45 kb, have been detected (Rock et al., 1987; Stevens et al., 1987). The LATs are a part of a larger 8.3 kb transcription unit. A recent study has indicated that the 2.0 kb LAT represented a stable intron generated by splicing of a larger transcript (Farrell et al., 1991). The 750 nt at the 3' end of the 2.0 kb LAT were complementary to the immediate early region gene, infected cell polypeptide 0 (ICP0), which encodes a potent transcriptional transactivator. Although the 2.0 kb LAT interfered with the transactivating activity of ICP0 in an <u>in vitro</u> system, its role in the process of latent infection is not clearly understood (Farrell et al., 1991; Fraser et al., 1992).

AS RNA corresponding to different ORFs of HPV-16 has been detected in several HPV-16 positive squamous cell carcinomas. The AS RNA was detected by in situ hybridization and was complementary to the early, late and noncoding regions of the viral genome (Higgins et al., 1991; Vormwald-Dogan et al., 1992). HPV-16 AS RNA is discussed further in chapter 3.

1.3.2 Experimentally-produced and synthetic AS RNA

AS RNA has been used in several experimental systems to selectively block the expression of specific genes (Helene and Toulme, 1990; Takayama and Inouye, 1990). The AS RNA was generally produced in situ by transient or stable expression of the transfected AS expression plasmids. Inducible promoters, such as mouse mammary tumor virus, metallotheionine and heat shock promoters, provided a better control over the expression of AS RNA (Helene and Toulme, 1990; Takayama and Inouye, 1990). Viral vectors, such as retrovirus, adenovirus and adeno-associated virus vectors have provided a means for stable and efficient transduction of AS RNA (Helene and Toulme, 1990; Takayama and Inouye, 1990; Chatterjee et al., 1992). Another approach commonly used in the Xenopus system is the direct microinjection of AS RNA into the oocyte. Capped AS RNA for microinjection is produced *in vitro* using T3/T7/SP6 RNA polymerases.

Synthetic AS nucleic acids, such as oligoribonucleotide (ORN)/oligodeoxyribonucleotide (ODN), have been more widely

used in experimental systems (Stein and Cohen, 1988; Toulme and Helene, 1988; Walder, 1988; Calabretta, 1991; Helene, 1991; Leonetti et al., 1993). ODN has several advantages over ORN. ODN is easier to synthesize and process than ORN. ODN is also more stable. Further, ODN is more efficient at blocking the expression of genes, because it elicits RNaseH activity, which degrades and destabilizes the RNA portion of mRNA-DNA hybrid (Helene, 1991; Leonetti et al., 1993). ODN can be used to selectively degrade mRNA produced from mutant alleles, without affecting the wild-type mRNA (McManaway et al., 1990; Calabretta, 1991; Helene, 1991; Monia et al., 1992). The uptake, stability and affinity of ODN have been improved substantially by introducing several chemical modifications into the ODN (Stein and Cohen, 1988; Toulme and Helene, 1988; Helene, 1991; Leonetti et al., 1993). ODN can be used as a sequence-specific nuclease by coupling it to chelates, such as EDTA-Fe, phenanthroline-Cu and porphyrin-Fe, which induce strand breakage (Toulme and Helene, 1988; Helene and Toulme, 1990).

Ribozymes are a group of sequence-specific catalytic RNA molecules, which are known to catalyze a wide range of biological activities, such as the splicing of the group I type of intron, maturation of tRNA and replication of viroids (Cech, 1988a; Symons, 1989; 1992). The catalytic function is attributed to the active RNA core. Additional flanking

sequences are required for aligning ribozymes to specific sequences of the target/substrate RNA (Cech, 1988b). Ribozymes can be engineered to recognize and cleave specific sequences on RNA molecules. Such engineered ribozymes have been used as AS RNA to cleave specific RNA, both in vitro and in vivo (Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Cotten and Birnstiel, 1989; Eckner et al., 1991; Zaia et al., 1992).

1.3.3 Mechanism of action

AS RNA has been widely used to interfere with the expression of specific gene(s), and this interference generally depends on hybridization of the AS RNA to the pre-mRNA or mRNA. The specificity is provided by the sequence complementarity between the AS RNA and the target pre-mRNA or mRNA. The work done on AS RNA of prokaryotes has lead to a considerable understanding of the mechanism(s) by which AS RNA affects gene expression and DNA replication (Takayama and Inouye, 1990). Much of the information on the mechanism(s) of action of eukaryotic AS RNA has come from AS RNA used in various experimental systems (Denhardt, 1992). Although, the detailed mechanism(s) of action of eukaryotic AS RNA is not clear, generally, the AS RNA has been shown to act at the posttranscriptional and translational levels to affect gene expression.

1.3.3.1 Transcriptional regulation

AS RNA can also act at the level of transcription. An AS RNA targeted to sequences downstream of the c-myc promoter resulted in decreased transcription from this promoter by interfering with transcription (Yokoyama and Imamoto, 1987).

1.3.3.2 Posttranscriptional regulation

AS RNA may affect one or more posttranscriptional events, such as the splicing of pre-mRNA, transport of mRNA to the cytoplasm and turnover of mRNA. My studies of naturally occurring HPV-16 AS RNA have indicated that the HPV-16 AS RNA blocks the splicing of the HPV-16 S RNA at the nt 880 5′ splice site.

The generalized inhibition of splicing of pre-mRNAs could be achieved by targeting the U snRNA. AS RNA complementary to certain regions of U1, U2 and U4/U6 snRNA efficiently inhibited splicing (Blencowe et al., 1989). Inhibition of splicing of specific pre-mRNA species by AS RNA has been observed in both in vitro and in vivo systems. The splicing of exon 1 to exon 2 of β-globin pre-mRNA was inhibited in vitro by AS RNA complementary to 5′ and 3′ splice site regions (Munroe, 1988). AS tissue inhibitor of metalloprotease (TIMP) RNA, which was produced from a stably transfected plasmid, was shown to inhibit the splicing of TIMP pre-mRNA in vivo (Feng and Denhardt, 1992). Similarly, naturally occurring AS RNA to the gonadotropin-releasing hormone and erbAα2 transcripts

appeared to interfere with the processing of these two transcripts (Kelly et al., 1991; Munroe and Lazar, 1991). Naturally occurring AS N-myc RNA has been thought to inhibit the splicing of the first intron of the S N-myc RNA.

AS RNA can also interfere with the transport of mRNA to the cytoplasm. AS RNA targeted to the 3' end of HSV tk mRNA or to the complete coding region of c-myc mRNA resulted in the retention of respective mRNAs, in the nucleus (Kim and Wold, 1985; Prochownik et al., 1988). The naturally occurring AS MBP RNA of mld mice has been suggested to interfere with the export of MBP mRNA (Okano et al., 1991). In my studies, the naturally occurring AS HPV-16 RNA did not appear to affect the transportation of the S HPV-16 RNA from the nucleus to the cytoplasm.

AS RNA can downregulate gene expression by destabilizing its target mRNA. The downregulation of the HPV-16 E7 RNA by naturally occurring AS E7 RNA was not clearly evident in my studies. The naturally occurring AS RNA to the Dictyostelium prespore gene, EB4-PSV mRNA, induced the rapid degradation of this mRNA (Hildebrandt and Nellen, 1992). Similarly, in several experimental systems, AS RNA induced the rapid turnover of target pre-mRNA or mRNA (Helene and Toulme, 1990; Bradley et al., 1992). The induction of instability of S RNA by AS RNA could be due to dsRNA-specific RNases (Strickland et al., 1988). The instability of target mRNA induced by AS ODN

has been shown to be mediated by RNaseH, an activity which selectively hydrolyzes the RNA strand in a RNA-DNA hybrid (Helene and Toulme, 1990). Ribozymes and chelated ODN induce sequence-specific cleavage of target RNA. The cleaved RNA, which lacks the 5′ cap or the 3′ poly(A) tail, are subsequently degraded by RNases. In addition to these RNases, a dsRNA-specific unwindase activity present in several cell types is also known to affect the stability of dsRNA (Bass and Weintraub, 1987; Rebagliati and Melton, 1987; Wagner and Nishikura, 1988; Kimelman and Kirschner, 1989; Wagner et al., 1990). This activity has been shown to modify the A residues into I (Bass and Weintraub, 1988; Bass et al., 1989; Wagner et al., 1989; Wagner et al., 1990; Nishikura et al., 1991).

1.3.3.3 Translational regulation

Generally, AS RNA targeted to the 5′ end of mRNA efficiently downregulates gene expression, by interfering with the initiation of translation (Helene and Toulme, 1990; Takayama and Inouye, 1990). My studies of the naturally occurring HPV-16 AS RNA have indicated that the AS RNA interfered with the expression of the viral E7 gene. Because the AS RNA was complementary to the 5′ and 3′ and the coding regions of E7 RNA and formed an AS RNA-S RNA duplex with the S RNA, it is likely that the lowered E7 expression was due to interference with the translation of E7 S RNA by the AS RNA. AS RNA complementary to the coding region of mRNA is less

effective in blocking translational elongation, because helicase activity associated with the elongation complex can melt the AS RNA-mRNA duplex structure. However, translational elongation can be inhibited by modified ODN, which can be covalently crosslinked to mRNA (Helene, 1991). Recently developed peptide nucleic acids, in which the phosphodiester backbone of ODN is replaced with polyamide bonds, bind mRNA with a very high affinity and block translational elongation (Hanvey et al., 1992). Infrequently, AS RNA targeted to the 3′ coding and noncoding regions of mRNA can block translation in vivo (Ch'ng et al., 1989). The mechanism(s) by which this AS RNA downregulated translation of mRNA was not clear.

1.4 OBJECTIVES

The objectives of this study were:

(i) to delineate the sequences of HPV-16 required for transformation of BRK cells, and

(ii) to determine some of the possible posttranscriptional mechanisms, such as RNA splicing and AS RNA, for the regulation of HPV-16 E7 gene expression.

CHAPTER 2

SEQUENCES OF HPV-16 REQUIRED FOR TRANSFORMATION

2.1 INTRODUCTION

Several epidemiological and experimental studies have implicated HPV-16 in the etiology of cervical cancer (zur Hausen and Schneider, 1987; Galloway and McDougall, 1989; zur Hausen, 1989; 1991). In a majority of cervical cancers and cancer-derived cell lines, the HPV-16 genome was found to be integrated with the host cell genome resulting in disruption of E1 and/or E2 ORFs and deletion of the 3' early region ORFs (Matsukura et al., 1986; Durst et al., 1986; Baker et al., 1987; Wagatsuma et al., 1990). Occasional cases in which the viral genome occurs in an episomal form have also been noticed (Durst et al., 1985; Lehn et al., 1985; 1988; Matsukura et al., 1989; Stanley et al., 1989; Cullen et al., 1991). Irrespective of integration, part of the early region of the viral genome remained transcriptionally active and the early region genes, E6 and E7, were consistently expressed (Pater and Pater, 1985; Yee et al., 1985; Smotkin and Wettstein, 1986; Shirasawa et al., 1988).

Furthermore, the cloned genome of HPV-16 can immortalize, but not fully transform, primary human and rodent cells (Durst et al., 1987b; Pirisi et al., 1987; Kanda et al., 1988a; Schlegel et al., 1988; Woodworth et al., 1988; 1989; Pecoraro et al., 1989; Tsutsumi et al., 1992). Both E6 and E7 genes

were required for immortalization of human cells, whereas E7 was sufficient for rodent cells (Kanda et al., 1988a; Schlegel et al., 1988; Hawley-Nelson et al., 1989; Munger et al., 1989b; Watanabe et al., 1989; Inoue et al., 1991; Halbert et al., 1991). In addition to immortalization, HPV-16 can transform established rodent cell lines, such as NIH 3T3, 3Y1 and rat-1 cells, and E7 was essential for this activity (Tsunokawa et al., 1986; Yasumoto et al., 1986; Matlashewski et al., 1987a; Kanda et al., 1987; 1988b; Noda et al., 1988; Vousden et al., 1988; Edmonds and Vousden, 1989; Chesters et al., 1990; Watanabe et al., 1990).

HPV-16 can cooperate with activated oncogenes such as EJ-ras or v-fos to transform primary rodent cells in the presence of steroid hormone glucocorticoids or progesterone (Crook et al., 1988; Pater et al., 1988; 1990; Lees et al., 1990). The immortalization and transformation frequency of HPV-16 were enhanced by glucocorticoids (Durst et al., 1989; Storey et al., 1992; Sexton et al., 1993). The specific requirement for glucocorticoid hormones for transformation of BRK cells by HPV-16 was demonstrated by Pater and Pater (1991). RU486, an anti-glucocorticoid, interfered with transformation in a dose dependent manner (Pater and Pater, 1991).

The requirement for the steroid hormone can be overcome by expressing the E7 gene from strong heterologous promoter-enhancer sequences (Matlashewski et al., 1987b; Phelps et al.,

1988; Storey et al., 1988; Edmonds and Vousden, 1989; Watanabe et al., 1990; Tanaka et al., 1989; Chesters et al., 1990). Full transformation of immortalized human and rodent cells can be achieved by sequential transfection with activated EJ-ras or extensive passaging of immortalized cells or by exposure to chemical carcinogens (DiPaolo et al., 1989, Durst et al., 1989; Hurlin et al., 1991; Inoue et al 1991; Li et al., 1992; Garrett et al., 1993; Klingelhutz et al., 1993). The last two methods may be analogous to exogenous introduction of activated oncogenes, because extensive passaging of immortalized cells may result in selection of occasional cells with additional mutations conferring the distinct growth advantage required for transformation. Thus, the requirement for more than one oncogenic insult is consistent with the hypothesis of multistep carcinogenesis.

In this chapter, I have examined the sequences of HPV-16 required for transformation of BRK cells in the context of heterologous promoter-enhancer sequence and homologous message processing signal sequence-containing plasmids. Two separate regions of HPV-16, the E7 ORF and a cis-acting region flanking this ORF were essential for transformation. These studies highlighted the importance of splice sites present within the E6 (nt 226) and E1 (nt 880) ORFs for HPV-16 early gene expression and transformation.

2.2 MATERIALS AND METHODS

2.2.1 Construction of recombinant plasmids

For construction of recombinant plasmids, standard molecular biological techniques were used (Sambrook et al., 1989).

2.2.1.1 Expression plasmids for HPV-16 sequences

All plasmids in this group have the HindIII to EcoRI fragment (nt 0-2635) of pSV2Cat in common.

Plasmid pA1 was constructed by A. Mithal. It was derived from an intermediate plasmid constructed by ligating the blunt ended HpaII-SauI (nt 700-4338) fragment of HPV-16 to the common pSV2Cat fragment. Deletion of sequences between nt 2898 BstXI and nt 3763 NdeI sites, blunt ending of the termini and religation resulted in the plasmid pA1 (Fig. 2.2).

Plasmid pD71 was constructed by D. Hyslop and described earlier as pD7 (Marshall et al., 1990). It was constructed by ligating the blunt ended HpaII-SauI (nt 502-4338) fragment of HPV-16 to the blunt ended common pSV2Cat fragment (fig. 2.1). Plasmid pD72 was derived from pD71 by deleting sequences between NarI-HincII sites (nt 1312-3208) [Fig. 2.1]. Plasmids pY71, pY72, pY73, pY74 and pY75 were generated by deleting sequences from the common NdeI site (nt 3763) to the NarI (nt 1312), NcoI (nt 867), PstI (nt 875), KpnI (nt 880) and SspI (nt 1179) sites, respectively, from pD71 (Fig. 2.2). Deletion of the SspI fragment (nt 1179-3978) from pY71 resulted in pY76

(Fig. 2.1). Plasmids pY71PX, pY71SX and pY71RX1 were constructed by inserting translation termination linkers at the PvuII (nt 685), SspI (nt 720) and RsaI (nt 767) sites, respectively, in the plasmid pY71 (Fig. 2.1). Small deletions at the KpnI site (nt 880) of pY71 were introduced by limited Bal31 digestion (Fig. 2.4). The 4 bp deletion at the KpnI site in the plasmid pD7KB was introduced by blunt ending of the KpnI termini with T4 DNA polymerase (Fig. 2.4). Large deletions in the pY713'Δ series were created by Bal31 digestion of NarI-partially cleaved pD71. Deletion fragments containing E7 were gel-purified after complete digestion with PvuI and ligated to the smaller NdeI-PvuI fragment of pD71 (Fig. 2.3). All deletions were confirmed by either dideoxy or chemical sequencing.

Plasmid pBSSK II(+) 0528 was constructed by ligating the blunt ended HpaII-NcoI (nt 57-867) fragment of HPV-16 at the blunt ended BamHI-XbaI sites of Bluescript, pBSSK II(+) [Stratagene]. Plasmid pSV2E6E7 was derived from pBSSK II(+) 0528 by ligating the HindIII-SstII fragment of pBSSK II(+) 0528 to the HindIII-BglII site of DHFR deleted pSV2DHFR plasmid. Plasmids pBSSK II(+) 0528 and pSV2E6E7 were constructed by Qi Sun. Blunt ended StuI (nt 5190 of SV40)-SstI fragment containing E6-E7 ORFs were cloned between the blunt ended StuI-KpnI sites (nt 5190 of SV40-nt 880 of HPV-16) of pY71 to derive the plasmid, pD73I (Fig. 2.10). Replacing

the 452 bp BbsI fragment (nt 806-1258) of pD73I with that of pD71 resulted in pD73 (Fig. 2.10).

Plasmid pCMVE7 was derived from an intermediate construct, pBSKS(+)SN, which contains the blunt-ended StuI-NcoI fragment (nt 5190 of SV40-nt 867 of HPV-16) of pY71 cloned at the SmaI site of pBSKS(+). The HindIII-XbaI fragment from this intermediate plasmid was cloned at the corresponding sites of pRc/CMV (Invitrogen) to generate pCMVE7(Fig. 2.11). Plasmid pCMVE6E7 was constructed by R. Mittal by ligating the HindIII-SstI fragment of pBSSK II(+) 0528 at the HindIII-blunt ended XbaI site of pRc/CMV vector (Fig. 2.11).

Plasmids with the suffix pY72t were constructed by cloning the blunt ended XhoII fragment (nt 4099-4769) of SV40, upstream of E7 at the StuI site of pY72 in either sense (pY72t4) or antisense orientation (pY72t2) [Fig. 2.11].

2.2.1.2 HPV-16 promoter-enhancer based plasmids

The wild type HPV-16 plasmid has the complete HPV-16 genome cloned at the BamHI site of the vector pBR322. Small deletions around the nt 880 in pHKB series were introduced by limited Bal31 digestion of pHPV-16, partially-cleaved with KpnI and religation (Fig. 2.5). All deletions were confirmed by sequencing.

2.2.1.3 Site directed mutagenesis

Site directed mutagenesis was performed by the uracil incorporation method of Kunkel, (1985), using the Mutagene kit (BioRad). The SspI site (nt 1179) of pY71 and pY7KB1 was modified into an EcoRI site by the insertion of an EcoRI linker. Similarly, the SmaI site of pBSKS(+) was modified into NcoI site by inserting a NcoI linker. The 312 bp NcoI-EcoRI fragment (nt 867-1179) was cloned into the modified pBSKS(+). Mutations were introduced by using either 19-mer or 29-mer oligonucleotides. The nt 880 splice donor site was disrupted in the plasmid pY71SM1.5 by substituting TAA for the wild-type AGG (nt 879-881). In the construct pY71SM3.2, the wild-type splice donor site CCA (nt 884-886) was mutated to AGT, to convert this splice site into a consensus splice donor site. In the plasmid, pY71SM3.9, +2 position of the consensus splice site, T was mutated to G. The wild-type splice site was regenerated in the deletion plasmid, pY7KB1, to obtain pY71SM2.1. The mutated NcoI-EcoRI fragments were cloned into pY71 or pY7KB1 at the appropriate sites (Table 2.1).

A 31-mer oligonucleotide was used to introduce mutations at nt positions 222, 226 and 227. The nucleotide "G" at these positions was mutated to "T". Single stranded DNA rescued from the plasmid PBSSK II(+) 0528 served as the template for these mutations. A HindIII-SstI fragment containing these mutations was cloned into pRc/CMV vector as described earlier.

The 452 bp BbsI fragments from pY71SM1.5, pY71SM3.2 and pY71SM3.9 were cloned at the appropriate site of pD73I to derive plasmids pD73SM1.5, pD73SM3.2 and pD73SM3.9, respectively (Fig. 2.10).

All mutations were confirmed by dideoxy sequencing.

2.2.2 Cell culture and transformation assays

All cells were maintained in Dulbecco's modified Eagle medium with 10% fetal calf serum, unless otherwise specified. Subconfluent BRK cells (in 60-mm plates) prepared from 5 days old rats were transfected with 5 μg each of the indicated plasmids and activated EJ-ras according to Chen and Okayama (1987). Two days after transfection, the media was changed to 2% fetal calf serum-containing medium. For HPV-16 promoter-enhancer based plasmids, this medium contained 10^{-6} M dexamethasone. Selection was carried out in 2% fetal calf serum-containing medium for 4 weeks. The total number of colonies in each plate was counted at the end of 4 weeks. Random individual colonies were selected and expanded if further analysis was required.

2.2.3 RNA preparation and analysis

Cos-1 cells in 100-mm plates were transfected with 10 μg of the indicated plasmids and harvested 48 hours after transfection. Total cellular RNA was prepared by the method of Chirgwin et al., (1979). Five μg of this total RNA was analyzed by RNase protection assays as described by Kreig and

Melton, (1987). Hybridization was performed at 56°C for 14 hours with 100,000 cpm each of E7 and large T antigen (or CAT) probes and RNase digestion was done at 30°C for 90 minutes, with 700 U of RNase T1 per ml. RNase resistant hybrids were resolved on 5% denaturing polyacrylamide-urea sequencing gels. Laser densitometry was used for quantitating the signals.

The RNA probes used in these experiments were generated from the corresponding DNA fragments that were cloned into the vector Bluescript KS/SK (+/-). A fragment of pY71 from the StuI site of SV40 (nt 5190) to the SspI site of HPV-16 (nt 720) was used for the E7 probe. The E6 probe was derived from HpaII-PvuII (nt 57-553) fragment of HPV-16. A 153 bp RsaI fragment (nt 3072-3225) of SV40 was used to generate large T antigen probe. The Cat probe corresponded to the HindIII-EcoRI (nt 5018-4768) fragment of pSV2Cat.

2.2.4 Actinomycin D chase experiments

Forty eight hours after transfection with the indicated plasmids, actinomycin D was added at a concentration of 5 μg per ml to the culture media and the total cellular RNA was harvested after 0, 0.5, 1, 2, 3, 4, 5 and 6 hours. Five μg of total RNA was analyzed by RNase protection assays with the E7 probe. To determine the stability of E7 RNA in the presence of steroid hormones, actinomycin D was added to the pD71 plasmid transformed BRK cells 12 hours after the addition of the indicated hormones. Twenty μg of total RNA harvested

after 0, 1, 2, 4, 6 and 8 hours was analyzed by dot blot assays with nick translated E7 probe as described by Sambrook et al., (1989). The E7 probe for dot blot assays was the smaller StuI-KpnI fragment of the plasmid pY71. The blots were stripped and reprobed with the BamHI fragment containing γ-actin probe after nick translation.

2.2.5 Nuclear runoff assays

Nuclear runoff experiments were performed as described by Ausubel et al., (1990). Approximately $5x10^6$ Cos-1 cells were transiently transfected with either pY71 or pY72. Nuclei from these cells were isolated 48 hours after transfection by using a Dounce homogenizer. Nuclei were incubated with 100 μCi of [α-^{32}P]UTP for 30 minutes at 30°C and treated with protease K. Approximately 10^6 cpm of labelled RNA extracted from these nuclei were hybridized for 36 hours at 65°C with 5 μg of genomic HPV-16 and SV40 DNA immobilized on nitrocellulose filters. Filters were washed thrice in 2xSSC at 65°C and treated with 80 μg of RNase A. Filters were dried and autoradiographed.

2.3 RESULTS

2.3.1 Transformation by SV40 promoter-enhancer based deletion plasmids

It had been shown earlier that the HPV-16 can cooperate with the activated EJ-ras oncogene to transform primary BRK cells in the presence of the glucocorticoid hormone,

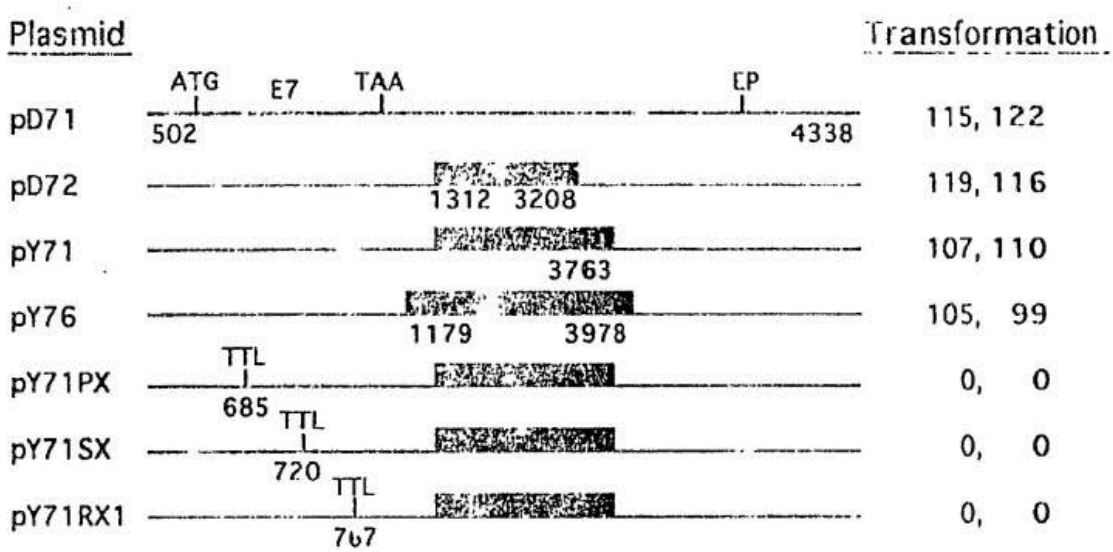
dexamethasone (Crook et al., 1988; Pater et al., 1988; Lees et al., 1990). The lower transformation efficiency of the whole HPV-16 plasmids prevented reliable evaluation of the contribution of different sequences of the viral genome for transformation. This deficiency can be overcome by expressing the HPV-16 sequences from a strong heterologous promoter-enhancer. The SV40 early promoter-enhancer, which is active in a wide variety of cell types, was chosen for this purpose. The expression from this promoter was independent of glucocorticoid hormone requirement. To map the regions of HPV-16 essential for transformation, a number of plasmids with deletions and translation termination linker insertions were constructed and tested for their ability to transform BRK cells.

The plasmid pD73, which expressed only the early region ORFs, was transformation competent, suggesting that the early region sequences of the viral genome are sufficient for transformation (Fig. 2.10).

Deletion of the complete E6 ORF sequences in the plasmid pD71 resulted in a slight increase in transformation (Fig. 2.1, 2.10). The possible reasons for the increased transformation for this plasmid are discussed later. The commonly used nt 226 splice donor site is present within the E6 ORF. In addition, a putative cryptic splice donor site is present immediately upstream of the nt 226 splice donor site

Figure 2.1 Transformation of BRK cells by HPV-16 expression plasmids.

All of the plasmids are in the pBR322 vector and expressed from the SV40 early promoter-enhancer. The thin lines represent the HPV-16 genome and the numbers below lines indicate the HPV-16 nt sequence. The black boxes represent deletions. The vertical lines within the E7 ORF sequences indicate translation termination linker insertions. The names of plasmids are shown to the left. The total number of colonies in eight 60-mm plates are presented to the right for duplicate experiments. The abbreviations used are: ATG, E7 start codon; TAA, E7 stop codon; EP, early polyadenylation signal; TTL, translation termination linker.


Plasmid
Transformation
ATG
E7
TAA
EP
pD71
502
4338
115, 122
pD72
1312
3208
119, 116
pY71
3763
107, 110
pY76
1179
3978
105, 99
TTL
pY71PX
685
0, 0
TTL
pY71SX
720
0, 0
TTL
pY71RX1
767
0, 0

(Sedman et al., 1991). Alternate splicing of the nt 226 splice donor site to the nt 409 and 526 splice acceptor sites generates E6* and E6** mRNA species (Smotkin and Wettstein, 1986; Smotkin et al., 1989; Cornellisen et al., 1990; Doorbar et al., 1990; Johnson et al., 1990; Schneider-Maunoury et al., 1990; Snijders et al., 1990; Rohlfs et al., 1991; Sedman et al., 1991; Sherman and Alloul, 1992; Sherman et al., 1992). Results of transformation experiments for the plasmid pD71 indicated that neither E6*, E6** nor intact E6 were required for the transformation of BRK cells. Further, they also suggested that the integrity of the nt 226 splice donor site was not essential for transformation. Several other studies have indicated that the E6 ORF sequences are dispensable for transformation of BRK cells (Vousden, 1990; Munger et al., 1992).

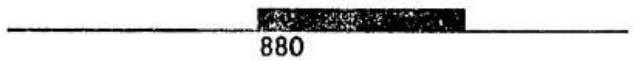
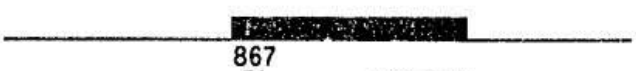
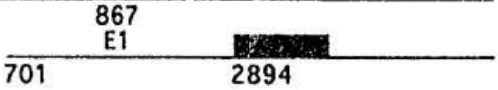
To determine the requirement for the other early region sequences for transformation of BRK cells, plasmids mutated in these sequences were used. The large deletion between nt 1312-3208 affecting the E1 and E2 ORF sequences in the plasmid pD72 did not affect transformation (Fig. 2.1). Transformation was not affected by a further deletion of 555 bp of E2 ORF sequences (nt 1312-3763) in the plasmid pY71 (Fig. 2.1). The plasmid pY71 is also deleted for the E4 ORF sequences. Insertion of translation termination linkers within the E7 ORF of the plasmid pY71, at nt 685 or 720 or 767, totally

abolished transformation (Fig. 2.1). Although these translation termination linker insertion plasmid clearly demonstrated the requirement for E7 ORF sequences for transformation, they did not eliminate the possible requirements for the E5 ORF and the 5' end sequences of the E1 ORF for transformation, because these sequences were also present in the plasmid pY71. Several recent studies have identified E5 as an oncoprotein for rodent cells (Leptak et al., 1991; Leechanachai et al., 1992; Pim et al., 1992; Banks and Matlashewski, 1993). The requirement for the E5 ORF sequences for transformation was analyzed by deleting the E5 ORF sequences in the plasmid pY76. The deletion extending to nt 3978 in the E5 ORF did not affect transformation significantly (Fig. 2.1).

The requirement for transformation of the 5' region sequences of E1 ORF present in the plasmid pY71 was investigated by deletion mutagenesis of this region. The additional deletion between nt 1179 and 1312 in the plasmid pY75 did not affect transformation (Fig. 2.2). A further deletion of the 312 bp between nt 867-1179 in the plasmid pY72 totally abolished transformation (Fig. 2.2). The plasmids, pY74 and pY73, with deletions extending to nt 875 or 880, respectively, were also transformation incompetent (Fig. 2.2). In the context of the whole HPV-16 genome, similar deletions of the 5' region sequences of E1 were reported to eliminate

Figure 2.2 Transformation by HPV-16 expression plasmids with deletions in the E7 3′ flanking region.

All of the plasmids are in the pBR322 vector and expressed from the SV40 early promoter-enhancer. The thin lines represent the HPV-16 genome and the numbers below lines indicate the HPV-16 nt sequence. The black boxes represent deletions. The names of plasmids are shown to the left. The total number of colonies in eight 60-mm plates are presented to the right for triplicate experiments. The abbreviations used are: ATG, E7 start codon; TAA, E7 stop codon; EP, early polyadenylation signal.

Plasmid		Transformation
pY71		112, 115, 128
pY75		103, 116, 118
pY73		0, 0, 0
pY74		0, 0, 0
pY72		0, 0, 0
pA1		0, 0, 0
pA1+pY72		0, 0, 0

transformation (Pater et al., 1992b). It was unlikely that the truncated E1 gene product encoded in the plasmid pY71 was essential for transformation, because several studies have shown that the E7 ORF sequences are necessary and sufficient for transformation of BRK cells in EJ-ras cooperation assays (Vousden, 1990; Munger et al., 1992). In those studies heterologous promoter-enhancers and message processing signals, such as splice sites and polyadenylation signal sequences, were used for the expression of HPV-16 sequences. Because the expression plasmids used in my studies did not possess heterologous splicing signals, it was possible that this function was provided by the E1 ORF sequences present in the plasmid pY71. The loss of transformation for the plasmids, pY72, pY73 and pY74, could be related to deletion of such cis-acting E1 ORF sequences. The evidence for the cis-acting nature of the E7 3' flanking sequence was obtained by cotransfecting pY72 with the E1-encoding plasmid, pA1, and EJ-ras. If the product of the E1 ORF was required for transformation in addition to E7, cotransfection of plasmids, pY72 and pA1, would be expected to restore transformation. Plasmid, pA1, failed to complement pY72 in cotransformation experiments, indicating that these E1 ORF sequences are cis-acting (Fig. 2.2). These results clearly indicated that in addition to the E7 ORF sequences, the E7 3' flanking sequences between the nt 880 and 1179 are required for transformation (Belaguli et al., 1992).

2.3.2 The E7 3′ flanking sequences required for transformation

To determine the minimum size of the E7 3′ flanking region required for transformation, various constructs with progressive unilateral deletions towards the E7 ORF were used in transformation assays. The exonuclease Bal31 was used to introduce deletions into the plasmid pY71. The progressive deletions extending from nt 1312 to 954, in pY713′Δ7, Δ15, Δ17, Δ1, Δ29, Δ16 and Δ1.2, did not affect the transforming activity (Fig. 2.3). No change in the frequency of transformation attributable to progressive deletions was observed, implicating the requirement of a narrowly defined region. A further deletion extending to nt 876 in the plasmid pY713′Δ2 totally abolished transformation (Fig. 2.3). To identify precisely the nature of this sequence, smaller deletions were introduced into the plasmid pY71 around the nt 880. Deletions of 4 bp in pY7KB1, 9 bp in pY7KB5 and 18 bp in pY7KB7 totally abolished transformation (Fig. 2.4). Deletions in these plasmids overlap a previously-identified splice donor site at nt 880 (Smotkin et al., 1989). An additional splice donor site located at nt 1301 was present in these plasmids. However, the function of this less frequently utilized splice donor site might have been impaired by the deletion between nt 1312 and 3763.

Figure 2.3 Transformation by E7 expression plasmids with progressive unilateral deletions in their E7-3′ flanking region.

All of the plasmids are in the pBR322 vector and expressed from the SV40 early promoter-enhancer. The thin lines represent the HPV-16 genome and the numbers below lines indicate the HPV-16 nt sequence. The black boxes represent deletions. The names of plasmids are shown to the left. The total number of colonies in eight 60-mm plates are presented to the right for triplicate experiments. The abbreviations used are: ATG, E7 start codon; TAA, E7 stop codon; EP, early polyadenylation signal.

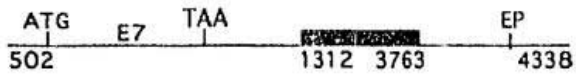
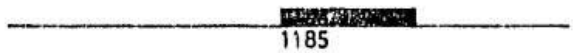
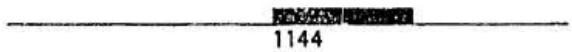
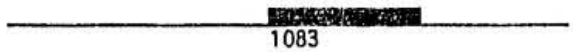
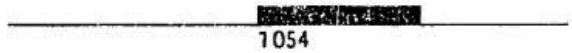
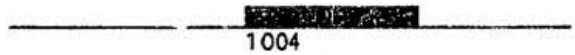
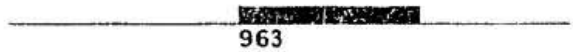
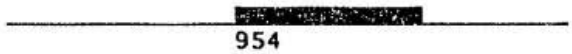
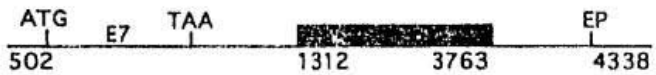
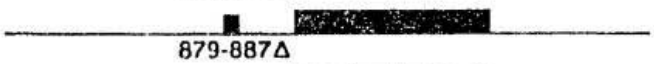
Plasmid		Transformation
pY71		100, 107, 112
pY713'Δ7		107, 109, 115
pY713'Δ15		111, 103, 114
pY713'Δ17		101, 109, 121
pY713'Δ1		99, 112, 115
pY713'Δ29		93, 99, 121
pY713'Δ16		96, 113, 106
pY713'Δ1.2		103, 115, 120
pY713'Δ2		0, 0, 0

Figure 2.4 Transformation by E7 expression plasmids with small deletions around the nt 880 splice donor site.

All of the plasmids are in the pBR322 vector and expressed from the SV40 early promoter-enhancer. The thin lines represent the HPV-16 genome and the numbers below lines indicate the HPV-16 nt sequence. The black boxes represent deletions. The names of plasmids are shown to the left. The total number of colonies in eight 60-mm plates are presented to the right for triplicate experiments. The abbreviations used are: ATG, E7 start codon; TAA, E7 stop codon; EP, early polyadenylation signal.

Plasmid		Transformation
pY71		104, 113, 96
pY7KB1		0, 0, 0
pY7KB5		0, 0, 0
pY7KB7		0, 0, 0
pD71		112, 116, 105
pD7KB		0, 0, 0

2.3.3 Effect of nucleotide 880 splice donor site mutations on transformation

Site directed mutagenesis of the nt 880 splice donor site was carried out to examine the role of this splice site in transformation. The mutation that disrupted this splice site by substituting taa in the wild-type sequence (pY71SM1.5) also eliminated transformation (table 2.1). Mutating the wild-type splice site into a consensus splice site (pY71SM3.2) resulted in higher frequency of transformation with 1.5-2 fold more colonies appearing at 2 to 3 weeks after transfection (data not shown). However, this difference was not apparent at 4 weeks after transfection when the number of colonies were counted, because of the appearance of secondary colonies for the wild-type plasmid (table 2.1). A single base substitution at the +2 position of the consensus splice donor site (plasmid pY71SM3.9) markedly reduced the transformation efficiency of pY71SM3.2 (table 2.1). In addition, regeneration of the wild-type splice site by inserting the taac sequence into a previously transformation incompetent splice site deletion mutant, pY7KB1 (Fig. 2.4) to derive pY71SM2.1, restored wild-type transforming activity (table 2.1). These results confirm that the integrity of the nt 880 splice donor site is essential for efficient transformation.

Table 2.1 Transformation by nt 880 splice site-specific mutants.

Wild-type and splice site mutated plasmids are indicated in the first column. In the second column, the splice site sequences from nt 878-886 for these plasmids are shown. Total number of colonies for each experiment is from eight 60-mm plates for triplicate experiments counted 4 weeks after transfection.

Plasmid	5' Splice Site Sequence	Transformation		
		Expt. 1	Expt. 2	Expt. 3
pY71	5' CAG GTACCA 3'	144	104	122
pY71SM1.5	CTA ATACCA	0	0	0
pY71SM3.2	CAG GTAAGT	156	121	135
pY71SM3.9	CAG GGAAGT	28	13	16
pY71SM2.1	CAG GTACCA	133	115	113

2.3.4 Effect of splice acceptor sites on transformation

Analysis of E7 mRNA from several premalignant and malignant cervical carcinoma cell lines, immortalized human cell lines and transformed rodent cell lines has indicated alternate splicing of the nt 880 splice donor site to nt 2708 or 3357 splice acceptor sites (Smotkin and Wettstein, 1986; Tanaka et al., 1989; Doorbar et al., 1990; Rohlfs et al., 1991; Sherman et al., 1992). Deletion of the nt 2708 and the nt 2708 and 3357 splice acceptor sites from the plasmids, pD72 and pY71, respectively, did not affect the transformation efficiency of these plasmids (Fig. 2.1). These results indicate that neither of the two reported authentic splice acceptor sites of the early region are required for transformation. This data is also suggestive of the presence of a cryptic splice acceptor site within the early region of HPV-16. Deletion of all the authentic splice acceptor sites may result in activation of this cryptic splice site. This cryptic splice site may be located between nt 3978 and 4338, because neither of the authentic splice acceptor sites was present in the plasmid pY71.

2.3.5 Effect of nt 226 and 1301 splice donor sites on transformation

Disruption of the nt 880 splice site in pY71 resulted in total loss of transformation. The effect of disruption of this splice site could be different for plasmids which have

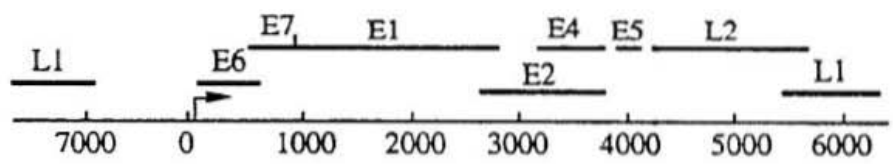
other authentic splice donor and acceptor and potential cryptic splice sites. To address this possibility, the nt 880 splice donor site was mutated in the early region expression plasmid, pD7KB, and the whole HPV-16 plasmids, pHKB1, pHKB5 and pHKB7. Deletion of 4 bp in the plasmid pD7KB totally abolished transformation (Fig. 2.4). Similar deletions in the context of whole HPV-16 genome plasmids, pHKB1, pHKB5 and pHKB7, resulted in a significant reduction, but not total abolition, of glucocorticoid dependent transformation (Fig. 2.5). Taken together, these results indicate the specific requirement for the nt 880 splice donor site for efficient transformation.

2.3.6 Effect of nucleotide 880 splice donor site mutations on E7 RNA levels

To investigate the molecular mechanism by which the integrity of the nt 880 splice site affects transformation function of E7, the levels of E7 message generated by various transforming and nontransforming plasmids were compared by RNase protection assays. Since BRK cells were not transformed by the splice site mutants, assays used total RNA from transiently transfected Cos-1 cells. Transformation competent plasmids, pY71 and pY75, generated 8-fold higher levels of E7 message than the nontransforming plasmids, pY72 and pY73. This was demonstrated in densitometric scans of autoradiograms from three independent experiments. A typical autoradiogram

Figure 2.5 Transformation by whole HPV-16 plasmids with small deletions around the nt 880 splice donor site.

All of the plasmids are in the pBR322 vector and expressed from the homologous HPV-16 promoter-enhancer. The genome and the ORFs of HPV-16 are shown in the diagram at the top. The arrow indicates the transcription initiation site. The thick lines represent the HPV-16 genome and numbers below lines correspond to HPV-16 nt sequence. The vertical black boxes represent deletions. The names of plasmids are shown to the left. The total number of colonies in eight 60-mm plates are presented to the right for duplicate experiments.



	Transformation	
	Expt.1	Expt.2
pHPV16	28	23
pHKB1	4	5
pHKB5	3	4
pHKB7	3	3

881-884

879-887

877-893

is shown (Fig. 2.6A). Plasmid pY75, which had a deletion of 136 bp in the E1 ORF, produced 1.4-fold higher levels of E7 RNA, compared with the wild-type pY71 (Fig. 2.6A). The nontransforming plasmid pY72, which had a large deletion in the E7 3′ flanking region, produced levels of E7 RNA comparable to that of another nontransforming plasmid, pY7KB1, which has only 4 bp deletion at the splice site (Fig. 2.6B). This indicates that the smaller size of the E7 RNA generated by pY72 is not the reason this plasmid is nontransforming. The splice donor site mutant pY71SM1.5 generated 10-fold lower levels of E7 RNA than the parental plasmid pY71 (Fig. 2.6C). This mutant precisely defined the sequence essential for both transformation and E7 RNA accumulation. Verifying that potential second site mutations are not involved, the plasmid in which the wild-type splice site was regenerated (pY71SM2.1), produced near-wild-type level of E7 RNA (Fig. 2.6C). In addition, the mutant containing the consensus splice donor site sequence had a 1.5-fold higher level of E7 RNA (fig. 2.6C). These results clearly indicate that the nt 880 splice site is indispensable for transformation and efficient E7 RNA accumulation.

That the presence of other authentic splice donor site at nt 1301 and acceptor sites and potential cryptic splice sites could not functionally substitute for the nt 880 splice site, was shown by the plasmid pD7KB. Deletion of 4 bp from

Figure 2.6 A, B and C RNase protection assays for pY7 series of plasmid.

Five μg of total RNA from CaSki cells or Cos-1 cells transfected with the pY7 series of plasmids was analyzed by RNase protection assays for E7 RNA. The E7 probe corresponds to the StuI (nt 5190 of SV40) to SspI (nt 720 of HPV-16) fragment of pY71. Constitutively expressed SV40 T antigen was used as an internal control and the T antigen probe was derived from the RsaI fragment (nt 3072-3286) of SV40. Control lanes and lanes for RNA from transfected Cos-1 cells are indicated on top: probe, undigested E7 and T antigen probes; CaSki, RNA from CaSki cervical carcinoma cells; Cos-1, RNA from untransfected Cos-1 cells; tRNA, E. coli tRNA. Molecular size markers are shown on the right. Protected E7 and T antigen fragments are indicated by arrow heads. CaSki E7 RNA fragment is smaller since it lacks SV40 sequences present in the probe and pY7 series of plasmids.

A
T
E7
E7
T
E7
Probe
CaSki
Y71
Y72
Y73
Y75
Cos-1
tRNA
160
190
242
309
B
T
E7
E7
T
E7
Probe
CaSki
Y71
Y72
Y7KB1
Cos-1
tRNA
160
190
242
309
C
T
E7
E7
T
E7
Probe
CaSki
Y71
Y71SM1-5
Y71SM2-1
Y71SM3-2
Cos-1
tRNA
160
190
242
309

the splice site in the plasmid pD7KB, that resulted in loss of transformation, also lead to a substantial reduction in the levels of E7 RNA (Fig. 2.7). This observation highlighted the specific requirement of the nt 880 splice site for transformation and E7 RNA accumulation.

Essentially, similar results were obtained in experiments in which the cotransfected pRSVCat was used as an internal control, instead of the constitutively expressed T-antigen (data not shown).

2.3.7 Requirement for the nucleotide 880 splice donor site for the stability of E7 RNA

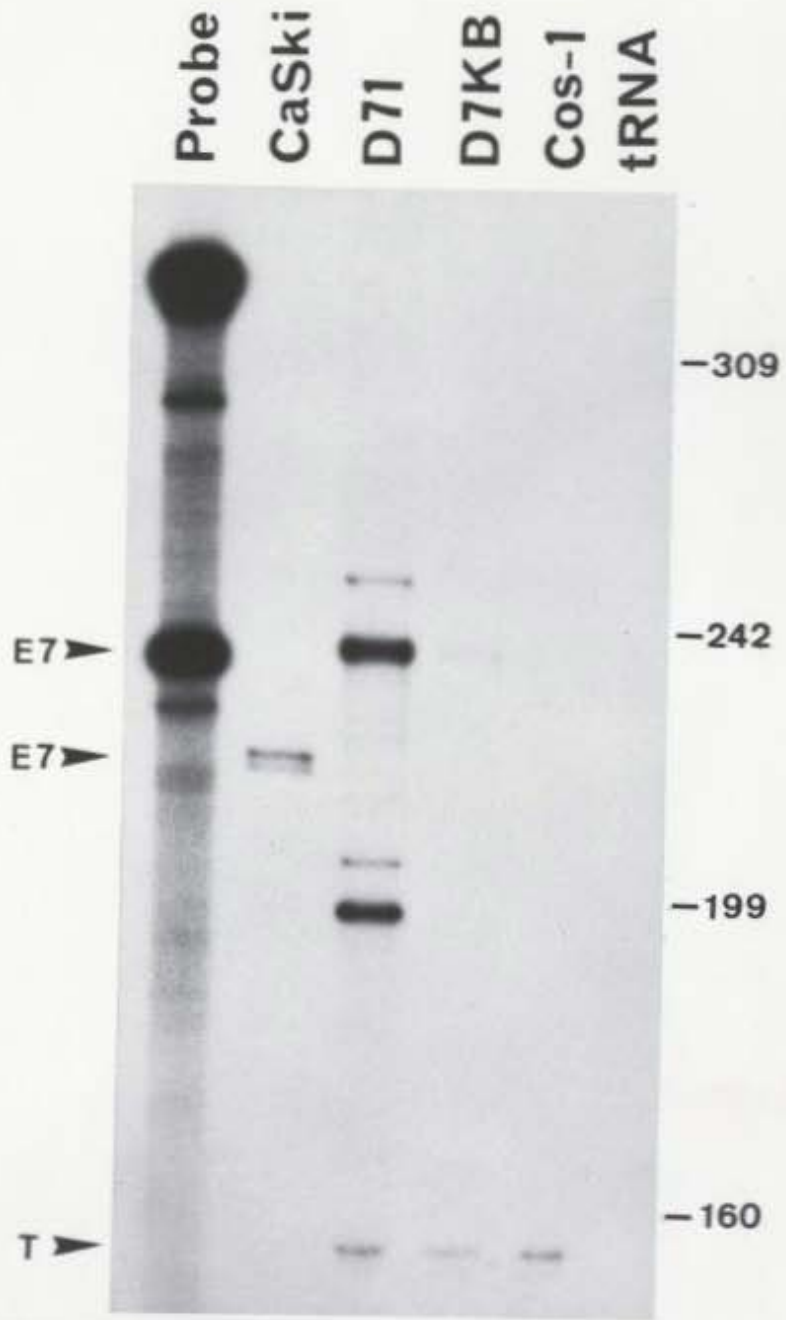
Splicing is an essential process for the production of many mature mRNAs. The lower levels of E7 RNA accumulation for splice site mutants could be due to either lower levels of synthesis or higher rates of degradation of this RNA. It is more likely that the mutations at the splice site affect the stability of E7 RNA. Actinomycin D chase experiments were performed to compare the half-life of E7 RNA produced by the wild-type plasmid pY71 and the splice site deletion mutant pY72. Constitutively expressed T antigen of SV40 was used as an internal control. The E7 RNA produced by the wild-type plasmid pY71 was stable in the 6 hour chase period with a half-life of approximately 4.0-4.5 hours. In contrast, the E7 RNA produced by the splice site deletion mutant pY72 was highly unstable, and no signal could be detected beyond 0 hour of the 6 hour chase period (data not shown).

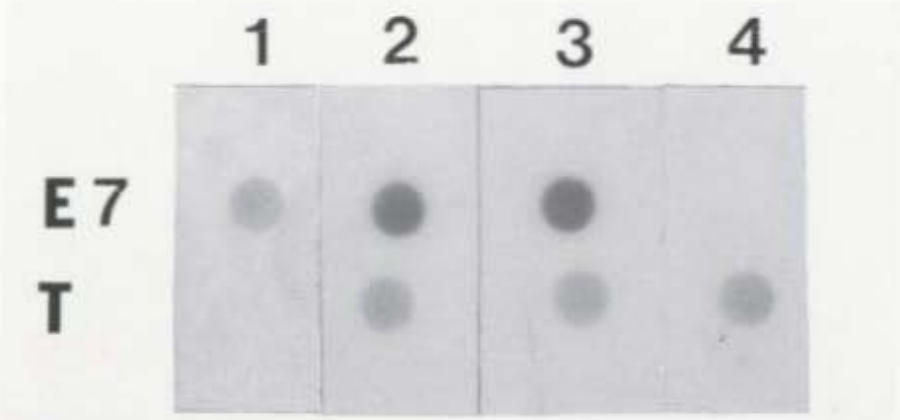
Figure 2.7 RNase protection assays for pD71 and pD7KB plasmids.

Five μg of total RNA from CaSki cells or Cos-1 cells transfected with the pD71 (D71) and pD7KB (D7KB) plasmids was analyzed by RNase protection assays for E7 RNA. The E7 probe corresponds to the StuI (nt 5190 of SV40) to SspI (nt 720 of HPV-16) fragment of pY71. Constitutively expressed SV40 T antigen was used as an internal control and the T antigen probe was derived from the RsaI fragment (nt 3072-3286) of SV40. Control lanes and lanes for RNA from transfected Cos-1 cells are indicated on top: probe, a mixture of undigested E7 (upper band) and T antigen (lower band) probes; CaSki, RNA from CaSki cervical carcinoma cells; Cos-1, RNA from untransfected Cos-1 cells; tRNA, E. coli tRNA. Molecular size markers are shown on the right. Protected E7 and T antigen fragments are indicated by arrow heads. CaSki E7 RNA fragment is smaller since it lacks SV40 sequences present in the probe and pY7 series of plasmids.

Figure 2.8 Nuclear runoff assays for wild-type and splice site deletion mutant E7 expression plasmids.

Immobilized HPV-16 (E7) and SV40 (T) DNA were hybridized with labeled RNA extracted from the nuclei of SiHa cells (lane 1), Cos-1 cells transfected with pY71 (lane 2), pY72 (lane 3) and untransfected Cos-1 cells (lane 4).


Probe
CaSki
D71
D7KB
Cos-1
tRNA
-309
-242
-199
-160
E7
E7
T


1
2
3
4
E7
T

To show that the deletion of the E7 3' flanking region including the nt 880 splice site did not affect the rate of synthesis of E7 RNA, nuclear runoff analysis was performed in Cos-1 cells transiently transfected with either pY71 or pY72. Relative to the internal control T antigen, synthesis of E7 was comparable in both pY71 and pY72 transfected nuclei (Fig. 2.8). Taken together, the results of actinomycin D chase and nuclear runoff experiments indicate that the lower levels of E7 RNA accumulated by splice site mutants is due to destabilization and higher rates of degradation of E7 RNA.

2.3.8 Effect of steroid hormones on the stability of E7 RNA

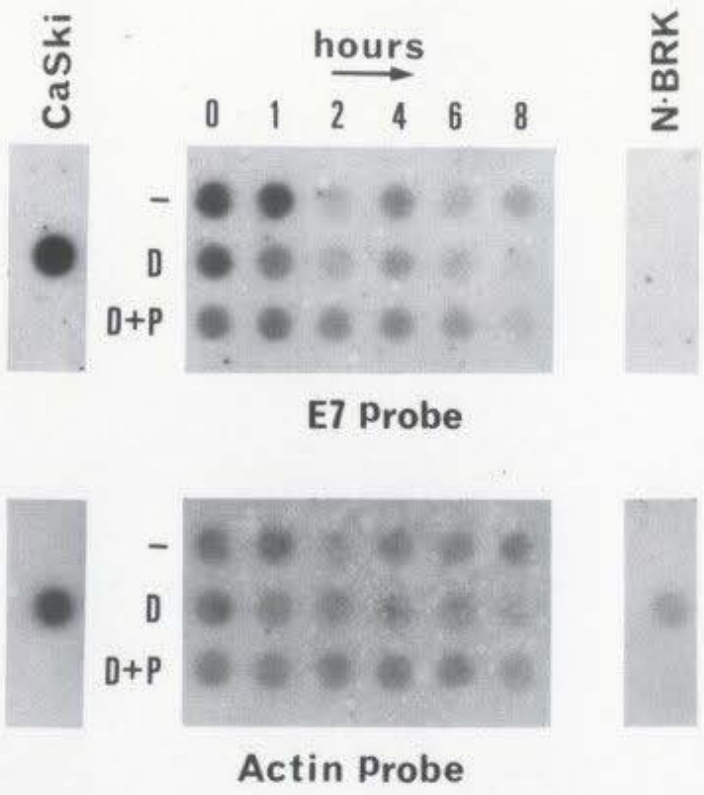
The disruption of the nt 880 splice donor site in the context of SV40 promoter-enhancer based E7 expression plasmids totally abolished transformation, whereas similar mutations in the context of whole HPV-16 plasmids significantly reduced, but did not abrogate, transformation. There was a definite correlation between the ability of the SV40 promoter-enhancer based E7 expression plasmids to transform BRK cells and generate stable E7 RNA (Belaguli et al 1992; Pater et al., 1992b). The whole HPV-16 plasmids with mutations at their nt 880 splice donor site may still be able to produce E7 RNA at levels sufficient for a low frequency of transformation. Although, the transformation assays were very similar for both types of plasmids, they differed with respect to the requirement for the steroid hormone dexamethasone for

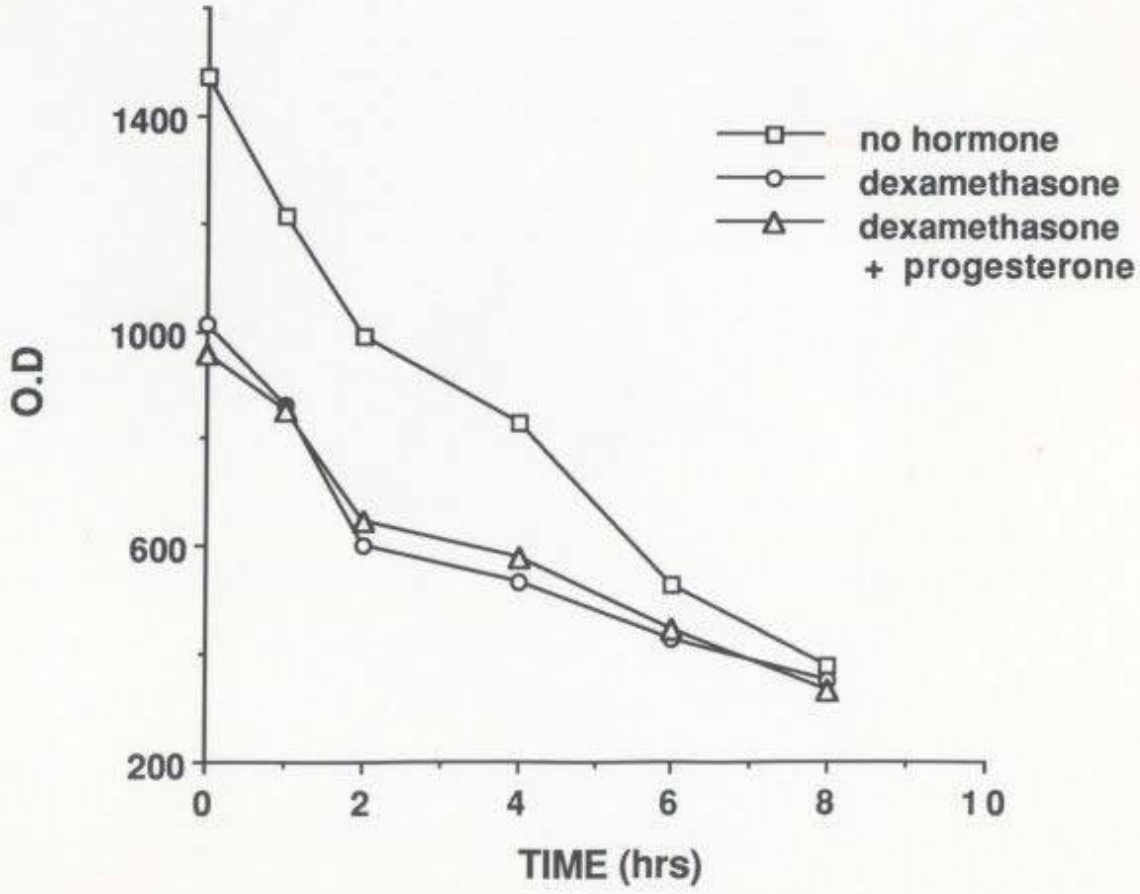
transformation. Dexamethasone was used only for assays with whole HPV-16 plasmids. The E7 RNA generated by the splice site mutant HPV-16 plasmids may be stabilized to a certain extent under the influence of steroid hormones used in transformation assays. Alternately, splice sites located within the E6 and E1 ORFs could compensate for the loss of nt 880 splice donor site. Steroid hormones have been shown previously, to stabilize many mRNAs posttranscriptionally (Nielsen and Shapiro, 1990). To address this possibility, a number of cell lines stably expressing the early regions of HPV-16, except the E6 ORF sequences, were derived by cotransfecting the plasmids pD71 and EJ-ras into BRK cells. After blocking transcription with actinomycin D, the half-life of E7 RNA expressed in pooled cell lines was measured by dot blot assays with nick translated E7 probe. Actinomycin D chase experiments were performed in the presence and absence of dexamethasone alone or in combination with progesterone. The E7 signals were quantitated by densitometric scanning and normalized to the internal control γ-actin. Addition of hormones resulted in slightly lower levels of E7 RNA. Irrespective of the presence or absence of hormones, the E7 RNA decayed with an approximate half-life of 3.5 hours and no apparent differences in the half-life of E7 RNA was observed (Fig. 2.9). Results of these experiments indicated that the steroid hormone dexamethasone, alone and in combination with

Figure 2.9 Actinomycin D chase experiments.

Total RNA from pD71 transformed BRK cells treated with and without the indicated hormones were harvested at 0, 1, 2, 4, 6 and 8 hours after the addition of actinomycin D. Twenty μg of RNA samples were analyzed for E7 RNA by dot blot assays. The E7 probe for dot blot assays was the smaller StuI-KpnI fragment of the plasmid pY71. The blots were stripped and reprobed with the BamHI fragment containing γ-actin probe after nick translation. Time in hours is shown on the top. The abbreviations used: CaSki, total RNA from CaSki cervical carcinoma cells; N.BRK, total RNA from normal baby rat kidney cells; E7 probe, the dot blots probed with the E7 probe; Actin probe, the dot blots reprobed with the γ probe; -, no hormone added; D, dexamethasone; D+P, combination of dexamethasone and progesterone.

The dot blots were scanned by laser densitometry and the results for duplicate experiments corrected for RNA loading were plotted for graphical representation of results on the bottom panel. Time in hours were plotted along the X-axis and the optical density (O.D) values were plotted along the Y-axis.


CaSki
hours
0 1 2 4 6 8
N·BRK
–
D
D+P
E7 Probe
–
D
D+P
Actin Probe


1400
1000
600
200
O.D
0
2
4
6
8
10
TIME (hrs)
no hormone
dexamethasone
dexamethasone
+ progesterone

progesterone does not contribute to the posttranscriptional stabilization of E7 RNA. Thus, it is unlikely that posttranscriptional stabilization of E7 RNA by steroid hormones was responsible for the transforming ability of HPV-16 splice site mutants.

2.3.9 Effect of E6 splice sites on transformation

Although the deletion analysis had made any role for E6 splice sites in transformation unlikely (Pater et al., 1992b), the presence of these splice sites in the E6-E7 mRNA produced from pHKB constructs, pHKB7, pHKB5 and pHKB1, might have contributed to the stabilization of these mRNAs and the residual transforming activity of these plasmids (Fig. 2.5). To study the role of E6 splice sites in transformation, the plasmids of the pD73 series were used (Fig. 2.10). The pD73 series of plasmids mutated for the nt 880 splice donor site were constructed by transferring the restriction fragments containing point mutations at this splice donor site from the previously-described pY71SM series of plasmids (Belaguli et al., 1992). The plasmid pD73I has a deletion of 25 bp (nt 863-887), spanning this splice site, whereas constructs pD73SM1.5, pD73SM3.2 and pD73SM3.9 have similar mutations as pY71SM1.5, pY71SM3.2 and pY71SM3.9, respectively (Fig. 2.10). All these plasmids were tested for their transforming activity. Construct pD73 was less efficient in transformation compared with the plasmid pD71 (Fig. 2.10). The efficiency of

Figure 2.10 Transformation of BRK cells by pD73 series of plasmids.

All of the plasmids are in the pBR322 vector and expressed from the SV40 early promoter-enhancer. HPV-16 sequences are indicated as lines and the HPV-16 nt numbers are shown below the lines. Wild-type and mutated nucleotide 880 splice site sequences are shown above the lines with lower case letters for mutated nt. Abbreviations used are: EP, early polyadenylation signal; 863-887Δ, deletion between nt 863 and 887. Total number of colonies in each duplicate experiment are from eight 60-mm plates. The percentage transformation frequencies for plasmids compared with the pD73 plasmid are indicated on the right.

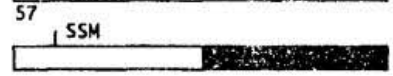
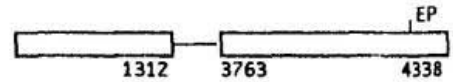
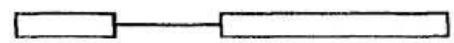
Plasmid		Transformation	
		Colonies	% of pD73
pD71		120, 114	118
pD73		102, 96	100
pD73I		22, 30	24
pD73SM1.5		28, 24	26
pD73SM3.2		122, 108	116
pD73SM3.9		38, 35	37

transformation of the plasmid, pD73SM3.2, was slightly higher than the pD73 construct and comparable to that of the pD71 plasmid (Fig. 2.10). The plasmids pD73I and pD73SM1.5 with their nt 880 splice sites disrupted and the plasmid pD73SM3.9 with a suboptimal nt 880 splice site, were transformation competent. However, the efficiencies of transformation were greatly reduced for these plasmids (Fig. 2.10).

To confirm these observations regarding the E6 splice sites, the E7 ORF and E6-E7 ORFs were expressed from the CMV immediate early promoter-enhancer. The CMV vector used for this purpose did not have any splice signals upstream of the bovine growth hormone polyadenylation signal. Only pCMVE6E7, which has sequences encoding both E6 and E7, was transformation competent, whereas pCMVE7 was transformation incompetent (Fig. 2.11). The E6 ORF present in the plasmid pCMVE6E7 could have acted by providing either the E6 gene product or the functional E6 splice signals. However, the possibility that the E6 gene product might have conferred the transformation phenotype on this plasmid was unlikely. Disruption of the nt 226 splice donor site and a potential cryptic splice donor site immediately upstream of it, in the plasmid pCMVE6ME7, resulted in the total loss of transformation, highlighting the requirement for the functional E6 splice sites, rather than the E6 gene product for transformation (Fig. 2.11).

Figure 2.11 Transformation of BRK cells by pCMV and pY72 series of plasmids.

The pY72 series of plasmids are in the pBR322 vector and expressed from the SV40 early promoter-enhancer. HPV-16 sequences are diagrammed as open boxes with the HPV-16 nt numbers shown below. Black boxes in the pCMV series of plasmids represents the bovine growth hormone gene polyadenylation sequences. In pY72 series of plasmids, boxed arrows indicates the presence and orientation of SV40 small t intron containing sequences. The deletions are indicated by thin lines. Abbreviations used are: E7, E7 ORF; E6, E6 ORF; SSM, E6 splice site mutation. Total number of colonies in each duplicate experiment are from eight 60-mm plates.

Plasmid		Transformation
pCMVE7		0, 0
pCMVE6E7		32, 29
pCMVE6ME7		0, 0
pY71		89, 80
pY72		0, 0
pY72t.1		0, 0
pY72t.4		46, 52

2.3.10 Effect of heterologous splice sites on transformation

To examine whether heterologous splice signals could substitute for the E6 splice sites, the SV40 small t intron was cloned in both S and AS orientations upstream of E7 in the transformation deficient plasmid, pY72. This heterologous intron restored transformation, only when present in S orientation in the plasmid pY72t4, but not in AS orientation in the plasmid pY72t1 (Fig. 2.11). However, the transformation efficiency of pY72t4 was lower compared with the wild-type plasmid pY71.

Considered together, these results indicate that transformation by HPV-16 E7 is dependent on the presence of functional splice sites. The presence of functional E6 splice sites in the pHKB series of plasmids could have been responsible for their residual transforming activity.

2.3.11 Effect of E6 and heterologous splice sites on E7 RNA accumulation

The presence of either E6 or heterologous splice sites upstream of E7 was sufficient to confer transforming ability to plasmids with mutations at their nt 880 splice sites. Since a good correlation had existed between the presence of functional splice sites and the stability of E7 RNA, it appeared likely that the E6 or heterologous splice sites present in these plasmids also contributed to the

stabilization and accumulation of E7 RNA. To investigate this possibility, total RNA from Cos-1 cells transiently transfected with E7 or E6-E7 expression plasmids was analyzed by RNase protection assays. As was reported earlier (Pater et al., 1992b), mutations at the nt 880 splice donor site in the plasmid pD7KB, which lacks the upstream E6 ORF, resulted in a large reduction in the steady state E7 RNA level, as compared with the wild-type plasmid pD71 (Fig. 2.7). The nt 880 splice site mutants, pD73I and pD73SM1.5 generated lower levels of E7 RNA when compared with pD73 and pD73SM3.2 (Fig. 2.12A). The plasmid pD73SM3.2, with a consensus nt 880 splice donor site, produced slightly higher level of E7 RNA as compared with the plasmid pD73 (Fig. 2.12A). The plasmid with a suboptimal nt 880 splice site, pD73SM3.9, gave rise to intermediate levels of E7 RNA (Fig. 2.12A).

Similarly, the transformation competent plasmid, pCMVE6E7 accumulated higher levels of E7 RNA than that of the nontransforming pCMVE7 plasmid (Fig. 2.12B). Disruption of the nt 226 splice site that eliminated transformation for pCMVE6ME7, resulted in lower levels of E7 RNA, compared with pCMVE6E7 (Fig. 2.12B).

Presence of SV40 small t intron upstream of E7, in the S orientation, but not in the AS orientation, stabilized the E7 RNA. There was a high level of E7 RNA for the transformation competent plasmid, pY72t.4, which has the SV40 small t intron

Figure 2.12A RNase protection assays for pD73 series of plasmids with the E7 probe.

Five μg of total RNA from CaSki cells and Cos-1 cells transfected with the indicated plasmids were analyzed by RNase protection assays with the E7 probe. The E7 probe corresponds to the StuI (nt 5190 of SV40) to SspI (nt 720 of HPV-16) fragment of pY71. Cotransfected plasmid, pRSVCat was used as an internal control and the Cat probe corresponds to nt 5018-4768 of pSV2Cat. Control lanes and lanes for RNA from transfected Cos-1 cells are shown on top: probe, a mixture of undigested Cat (upper band) and E7 (lower band); CaSki, RNA from CaSki cells; pSV2CAT, RNA from Cos-1 cells transfected with the plasmid pSV2Cat; tRNA, E. coli tRNA; M, HpaII-digested pBR322 molecular size markers. Protected CAT and E7 fragments are indicated by arrowheads.

Figure 2.12B RNase protection assays for pCMV series of plasmids with the E7 probe.

Twenty μg of total RNA was used for assays. Other conditions and labels are as in figure 2.12A.

Figure 2.12C RNase protection assays for pY72t series of plasmids.

Constitutively expressed SV40 T antigen was used as an internal control and the T antigen probe was derived from the RsaI fragment (nt 3072-3286) of SV40. E7 and T antigen signals are denoted by arrow heads. T antigen signals were clearly visible in darker exposures. Other conditions and labels are as in figure 2.12A.

A
E7
CAT
Probe
CaSki
D73
D73I
D73SM1-5
D73SM3-2
D73SM3-9
PSV2CAT
tRNA
M
199
217
242
309
B
E7
CAT
Probe
CaSki
CMVE7
CMVE6E7
CMVE6ME7
PSV2CAT
tRNA
M
199
217
242
309
C
T
E7
E7
Probe
CaSki
Y72t1
Y72t4
Cos-1
M
160
180
217
242
309

containing sequences in the S orientation. The transformation incompetent plasmid, pY72t.1, with the same SV40 sequences in the AS orientation, had undetectable levels of E7 RNA (Fig. 2.12C).

2.3.12 Splicing of E6 introns

As the presence of the E6 splice sites might have compensated for the loss of nt 880 splice donor site that was required for E7 RNA accumulation and transformation, it was of interest to determine whether splicing involving the E6 splice sites was occurring. For the nt 880 splice site mutations resulting in reduced transformation efficiency (Fig. 2.10), the deletion mutation in the plasmid pD73I and the taa mutation in the plasmid pD73SM1.5 lead to a small reduction of the RNA for the promoter-proximal, E6EX1 exon and a substantial reduction for the 3', more E7 ORF-proximal, E6EX2 exon (Fig. 2.13A). The CMV construct, pCMVE6ME7, that was completely defective for transformation due to a mutated nt 226 splice site, was completely defective for the stable production of spliced and unspliced E6 RNA (Fig. 2.13B).

2.4 DISCUSSION

Several epidemiological studies have implicated HPV-16 as one of the important etiological agents for cervical cancer. Supporting these observations is the experimental evidence that this virus can immortalize it's natural target cells,

Figure 2.13 RNase protection assays for pD73 and pCMV series of plasmids with the E6 probe.

Five μg of total RNA from CaSki cells and Cos-1 cells transfected with the indicated plasmids were analyzed by RNase protection assays with the E6 probe. The E6 probe was derived from the HpaII-PvuII (nt 57-553) fragment of HPV-16. Cotransfected plasmid, pRSVCat or pSV2Cat was used as an internal control and the Cat probe corresponds to nt 5018-4768 of pSV2Cat. Control lanes and lanes for RNA from transfected Cos-1 cells are shown on top: Probe, a mixture of undigested E6 (upper band) and Cat (lower band); CaSki, RNA from CaSki cells; PSV2CAT and RSVCat, RNA from Cos-1 cells transfected with the plasmids, pSV2Cat and pRSVCat, respectively; tRNA, E. Coli tRNA. Labels are: E6 EX1, E6 exon 1; E6 EX2, E6 exon 2; US, unspliced E6 RNA. The US and E6 EX1 fragments for CaSki are smaller because the transcription is initiated from the p97 promoter and the transcripts lack sequences between nt 57 and 97.

A. pD73 series of plasmids.

B. pCMV series of plasmids.

A
E6 EX1
E6 EX2
E6 EX1
CAT
US
Probe
CaSki
D73
D73I
D73SM1-5
D73SM3-2
D73SM3-9
RSVCat
tRNA
M
147
180
217
242
309
429

B
E6 EX1
E6 EX2
E6 EX1
CAT
US
US
Probe
CaSki
CMVE6E7
CMVE6ME7
PSV2Cat
tRNA
147
160
180
217
242
404
527

primary human keratinocytes, in culture (Durst et al., 1987b; Pirisi et al., 1987; Schlegel et al., 1988; Pecoraro et al., 1989; Woodworth et al., 1989). In addition, HPV-16 can fully transform established rodent cells (Tsunokawa et al., 1986; Yasumoto et al., 1986; Matlashewski et al., 1987a). HPV-16 can also cooperate with activated oncogenes such as EJ-*ras* and v-*fos* to transform primary rodent cells in the presence of the glucocorticoid hormone, dexamethasone (Crook et al., 1988; Pater et al., 1988; Lees et al., 1990). In human cell systems, the immortalizing activity of HPV-16 was localized to E6 and E7 ORFs (Schlegel et al., 1988; Hawley-Nelson et al., 1989; Munger et al., 1989b; Halbert et al., 1991). Several studies have established that E7 is the major transforming gene for rodent cells, but these studies utilized strong heterologous promoter-enhancer sequences and message processing signals for the expression of HPV-16 genes (Kanda et al., 1987; Kanda et al., 1988b; Phelps et al., 1988; Vousden et al., 1988; Tanaka et al., 1989; Chesters et al., 1990). However, the requirement for the homologous message processing signals, especially the splice site sequences, for the efficient expression of HPV-16 oncogenes, has not been determined to date. I have examined the requirement for the early region sequences of HPV-16 for transformation in the context of E7 expression plasmids, with an emphasis on the nt 880 splice donor site.

Deletion of the E6 ORF sequences did not affect transformation for the plasmid pD71 and several other SV40 promoter-enhancer based plasmids. In contrast to the results obtained for the SV40 promoter-enhancer based plasmid, pD71, for the CMV based E7 expression plasmid, pCMVE7, the E6 ORF sequences were essential for transformation. It has been reported earlier that the E6 ORF sequences of HPV-16 can transform established rodent cells (Sedman et al., 1991) and immortalize BMK cells in EJ-ras cooperation assays (Storey and Banks, 1993). Although the E6 ORF sequences are required for efficient immortalization of primary human keratinocytes, which are the natural targets for this virus, (Schlegel et al., 1988; Hawley-Nelson et al., 1989; Munger et al., 1989b; Watanabe et al., 1989; Halbert et al., 1991), several studies have established that the E6 ORF sequences are dispensable for transformation of BRK cells in EJ-ras cooperation assays (Vousden, 1990; Munger et al., 1992). Further studies for the CMV based E7 expression plasmids clearly indicated that the splice sites present within the E6 ORF sequences, but not the E6 gene product per se, were required for transformation. The high risk group of HPVs, including HPV-16, produces low levels of full length E6 and comparatively higher levels of a variety of spliced mRNAs referred to as E6* and E6** (Schneider-Gadicke and Schwarz, 1986; Smotkin et al., 1989). Ability of only high risk HPVs to produce such spliced transcripts

suggested an important role for their products in the process of tumorigenesis (Smotkin et al., 1989). Two recent studies have indicated that the E6* and E6** are not required for immortalization of primary human keratinocytes (Halbert et al., 1991; Sedman et al., 1991).

The E2 ORF of HPV-16 encodes a DNA binding transcriptional modulator protein, which can either downregulate or upregulate the viral promoter, depending on the context of E2 binding sites (Ham et al., 1991; McBride et al., 1991). Similar to the E2 protein of BPV-1, the HPV-16 E2 protein is also required for replication of the viral DNA (Lambert, 1991; Del Vecchio et al., 1992; Chiang et al., 1992a). In addition, several recent studies have assigned a significant role for E2 in the process of immortalization and transformation. Disruption of the E2 ORF sequences in the context of the whole HPV-16 plasmids, resulted in enhanced transformation of human fibroblasts and immortalization of primary human keratinocytes (Smits et al., 1988; Romanczuk and Howley, 1992). In contrast, mutations in the whole HPV-16 plasmids did not affect the glucocorticoid dependent transformation of BRK cells in EJ-ras cooperation assays (Pater et al., 1992b). The E2 ORF sequences were shown to be essential for immortalization of primary human keratinocytes but not for transformation of BRK cells (Storey et al., 1992). Recently, it was shown that the coexpression of E2 from a

strong heterologous promoter and the whole HPV-16 sequences results in an increase in the frequency of transformation of BRK cells (Lees et al., 1990). The discrepancy regarding the role of E2 in immortalization/transformation is not clear. It could be related to the differences in the assay systems and the level of expression of E2 protein. In the context of SV40 promoter-enhancer based HPV-16 early region plasmids, E2 ORF sequences were not required for transformation of BRK cells. Similar results have been reported from several laboratories (Vousden, 1990; Munger et al., 1992).

Plasmids pY71PX, pY71SX and pY71RX implicated E7 as the major transforming gene of HPV-16 (Fig. 2.1). Several other studies in which E7 ORF sequences were expressed from strong heterologous promoters have also indicated that E7 is necessary and sufficient for transformation (Phelps et al., 1988; Storey et al., 1988; Vousden et al., 1988; Edmonds and Vousden, 1989; Tanaka et al., 1989; Watanabe et al., 1990). The oncogenic potential of HPV-16 E7 protein has been shown to be closely related to its ability to complex with and interfere with the activities of the tumor suppressor protein, pRb, and various other cellular proteins such as cyclin A, p107 and p130 (Edmonds and Vousden, 1989; Watanabe et al., 1990; Dyson et al., 1992; Munger et al., 1992; Phelps et al., 1992; Sang and Barbosa, 1992a; Davies et al., 1993; Tommasino et al., 1993). The loss of transformation for the plasmids

pY71PX, pY71SX and pY71RX could be related to the instability of the truncated E7 proteins encoded by these E7 mutated plasmids, because similarly truncated E7 proteins have been shown to be unstable (Watanabe et al., 1990).

Several recent studies have assigned a role for E5 in the transformation of established rodent cells (Leechanachai et al., 1992; Pim et al., 1992; Banks and Matlashewski, 1993). Similar to the E5 protein of BPV-1, the oncogenic potential of the HPV-16 E5 protein is likely to be related to its ability to potentiate the EGFR and PDGFR-mediated signal transduction to the nucleus (Banks and Matlashewski, 1993). Comparison of the transforming activities of the plasmids pY71 and pY76 suggested that the contribution of E5 ORF sequences for transformation was not significant (Fig. 2.1). This could be related to the weak oncogenic potential of HPV-16 E5 protein. The other possibility is that the E5 ORF sequences present in the plasmid pY71 may not be expressed efficiently, because of the lack of proper message processing signals for the E5 RNA.

Recently Romanczuk and Howley, (1992), have correlated disruption of E1 ORF in the context of the whole HPV-16 genome with an increase in the frequency of immortalization of primary keratinocytes. Various studies have shown that the product of this highly conserved ORF is required for replication and maintenance of the viral genome (Lambert, 1991). The E1 gene product can interact with and form a

complex with the E2 transactivator protein and this interaction facilitates targeting of E1 to the viral origin of replication (Lambert, 1991). Because many of the cis-acting elements present in the LCR of HPV-16 are required for the regulation of transcription and replication and some of these sequences overlap with each other, it is possible that E1 participates in transcription of viral genes essential for transformation, in presently undefined ways. This could be responsible for the observed increase in the frequency of immortalization for E1 mutated plasmids (Romanczuk and Howley, 1992). The E1 ORF sequences were not required for the glucocorticoid dependent transformation of BRK cells by whole HPV-16 plasmids. However, the integrity of the nt 880 splice donor site present within the E1 ORF sequences was essential for efficient transformation (Pater et al., 1992b). The requirement for the nt 880 splice donor site for transformation is discussed later. In BRK cell system, in the context of SV40 promoter-enhancer based expression plasmids, the majority of E1 ORF sequences were not required for transformation (Fig. 2.1). The E1 ORF sequences present in the plasmid pY71 were required in cis-configuration for transformation and deletion of this sequence resulted in loss of transformation. The evidence for the cis-acting nature of this sequence was provided by the plasmid pA1 (Fig. 2.2).

That the deletion of this cis-acting 3' flanking region resulted in lower levels of E7 RNA and complete loss of transformation, suggested the presence of either a transcriptional enhancer or an mRNA stabilizing element(s) within the 3' flanking region. The possible presence of a transcriptional enhancer for E7 expression was clearly eliminated, since comparable levels of E7 RNA synthesis were found for the transforming construct pY71 and the nontransforming construct pY72 by nuclear runoff experiments (Fig. 2.8). Further, there was no evidence for the presence of an mRNA stabilizing element within this region, based on an analysis for homology to known structural or sequence elements. A previously mapped splice donor site was present within this region (Smotkin et al., 1989).

The results obtained from various constructs demonstrated that it is the integrity of this splice donor site that determines the level of E7 RNA and the transformation competency. This interpretation is supported by the following observations. (i) Deletion of as few as 4 bp in pY7KB1 or mutating the conserved 3 bp of the splice donor site in the plasmid pY71SM1.5 completely eliminated transformation and greatly reduced E7 RNA levels (Table 2.1, Fig. 2.4, 2.6B,C). The splice site sequence in these mutant plasmids does not conform to the GT-AG rule observed for 100% of naturally occurring splice junctions (Senapathy et al., 1990; Jackson,

1991). Similar substitutions in other splice donor sites have been shown to inactivate the splice site. (ii) Regeneration of the wild-type sequence in a previously transformation incompetent deletion plasmid pY7KB1 restored both near wild-type levels of transformation and E7 RNA (Table 2.1, Fig. 6B,C). (iii) Mutating the wild-type splice donor site to a consensus splice site resulted in a 1.5-fold higher level of E7 RNA, a slightly higher frequency of transformation and the early appearance of transformed colonies (Table 2.1, Fig. 2.6C). The consensus splice donor sequence is fully complementary to the first 9 nt of U1 snRNA and the degree of complementarity between the splice donor site sequence and the U1 snRNA has been shown to determine the efficiency of splicing (Nelson and Green, 1990). Thus, the higher frequency of transformation and earlier appearance of transformed colonies for the consensus splice site sequence plasmid, pY71SM3.2, could be related to a higher efficiency of E7 RNA processing, resulting in greater accumulation of E7 mRNA. (iv) A single base substitution at the +2 position of the consensus splice donor site significantly reduced the efficiency of transformation (Table 2.1). (v) The plasmid pY72 could support transformation when the heterologous SV40 small t intron was provided in the transcription unit in the sense orientation (Fig. 2.11). Taken together, this data indicates the crucial role of the nt 880 splice donor site for efficient E7 RNA accumulation and transformation.

The pY7 and pD7 series of plasmids mutated for the nt 880 splice site were transformation deficient. In contrast, whole HPV-16 plasmids containing similar mutations retained a low transforming activity (Belaguli et al., 1992; Pater et al., 1992b). Disruption of either the nt 226 or 1301 splice donor sites did not affect transformation in the presence of wild-type nt 880 splice site (Pater et al., 1992b). Considering the requirement for the steroid hormone dexamethasone for transformation by HPV-16 plasmids, a hypothesis suggesting a role for dexamethasone in posttranscriptional stabilization of E7 mRNA was considered. This hypothesis was consistent with several other studies, in which glucocorticoid hormone was shown to stabilize various mRNAs (Paek and Axel, 1987; Petersen et al., 1989; Nielsen and Shapiro, 1990; Mendelson and Boggaram, 1991; Pilkis and Granner, 1992). My experimental results failed to provide support for this hypothesis, because no apparent difference in the half-life of E7 RNA was noticed in the presence of dexamethasone, alone and in combination with progesterone (Fig. 2.9).

The alternate explanation that the nt 226 or 1301 splice sites present in the nt 880 splice site mutated HPV-16 plasmids functionally compensated to a certain extent for the loss of the nt 880 splice site appeared unlikely, because the disruption of either the nt 226 or 1301 splice sites did not affect transformation (Pater et al., 1992b). However, it was

possible that the presence of a dominant nt 880 splice site screened the effects of disruption of other splice donor sites. The ability of E6 splice sites to compensate for the loss of nt 880 splice site was demonstrated by the plasmids pD73I and pD73SM1.5. The presence of E6 splice sites in those plasmids which were mutated for the nt 880 splice site was sufficient to partially restore transformation (Fig. 2.10).

As was previously observed, a good correlation between the ability to produce stable E7 RNA and transform BRK cells was also observed for the pD73 series plasmids (Fig. 2.12A). Plasmids, pD73I, pD73SM1.5 and pD73SM3.9, which accumulated lower levels of E7 RNA compared with the wild-type plasmid, pD73, showed a lower frequency of transformation (Fig. 2.10, 2.12A). Similar plasmids, pY7SM1.5 and pD7KB, lacking the upstream E6 splice sites, were unable to generate stable E7 RNA and transform BRK cells (Belaguli et al., 1992; Pater et al., 1992b). Irrespective of the presence of sequences for two spliceable introns, the transformation efficiency of the plasmid pD73 was slightly lower compared with the plasmid pD71, which has sequences for only one spliceable intron (Fig. 2.10). A likely explanation is that the E7 RNA produced from the pY71 and pD71 plasmids are translated only into E7 protein, whereas the E6-E7 polycistronic RNA produced from pD73 and pD73SM3.2 is used for translating the E6*, E6**, E6 and E7 proteins. In addition, the alternate splicing of the

nt 226 splice donor site to splice acceptor sites present downstream of the E7 ORF sequences, resulting in E7 exon skipping, may lead to lower levels of E7 RNA and E7 protein for the pD73 plasmid. A slightly higher level of E6-E7 RNA produced from the plasmid pD73SM3.2 may compensate for the E7 exon skipping and a lower level of E7 synthesis.

For the pD73I and pD73SM1.5 plasmids compared with the pD73 plasmid, the levels of E7 RNA detected with the E7 probe were substantially lower (Fig. 2.12A). However, with the E6 probe, only a slight reduction in the promoter-proximal E6 exon (E6 EX1) was observed for these nt 880 splice site mutated plasmids (Fig. 2.13A). The splicing of this promoter proximal exon to the nt 409 splice acceptor site (and probably to the nt 526 splice acceptor site) of the second exon was severely impaired for the pD73I and pD73SM1.5 plasmids. Because the early region E6 and E7 RNAs of HPV-16 transcribed from the promoter p97 are polycistronic, a slight reduction in the promoter-proximal E6 EX1 exon and a substantial reduction in the E7 ORF sequences-containing second exon, suggests skipping of this E7 ORF sequences-containing second exon. In support of this, it has been shown that the mutation of the 5' splice site of an internal exon results in skipping of that internal exon (Robberson et al., 1990; Talerico and Berget, 1990; Niwa et al., 1992). The hypothesis that more of the E7 exon is skipped for the plasmids, pD73I, pD73SM1.5 and

pD73SM3.9, as compared with the plasmid pD73 by splicing of the nt 226 splice donor site to splice acceptor sites located downstream of E7 ORF sequences can be tested by using probes spanning the nt 2708 and 3357 splice acceptor sites. That the nt 880 splice site mutations for the pD73 series of plasmids results in E7 exon skipping is further supported by results of RNase protection experiments for the suboptimal nt 880 splice site containing pD73SM3.9 plasmid. For this plasmid, there was no detectable reduction for the promoter-proximal E6 EX1 exon RNA (Fig. 2.13A). However, a 2-3 fold reduction for the E7 RNA resulting from the E7 exon skipping, possibly because of impaired splicing of the promoter-proximal E6 EX1 exon to the E7 ORF sequences-containing second intron, was observed for this plasmid (Fig. 2.12A). The possibility that for most of the RNA for the nt 880 splice donor site mutated pD73 plasmid series, the nt 226 splice donor site was spliced to nt 526 splice acceptor site and that the protected RNA fragment corresponding to this more E7 proximal exon was not detected with the E6 probe used in the RNase protection assays, was also eliminated by the pD73SM3.9 plasmid. No reduction for the E7 RNA would be expected if the majority of the nt 226 splice donor sites are spliced to the nt 526 splice acceptor sites. The substantial reduction for the E7 RNA in the absence of any reduction for the E6 EX1 exon and impaired splicing of the nt 226 splice donor site to the nt 409 splice

acceptor site for the pD73SM3.9 plasmid indicated that the nt 880 splice donor site mutations resulted in E7 exon skipping. Thus, the low frequency of transformation for pD73 series of plasmids mutated for the nt 880 splice site appears to be a consequence of E7 exon skipping. This interpretation does not eliminate the importance of the E6 splice sites for stabilization of E7 RNA, because the mutation of the E6 splice donor sites in the pCMVE6ME7 plasmid resulted in loss of E6 and E7 RNA accumulation and transformation (Fig. 2.11, 2.12B). The importance of alternate splicing for the regulation of HPV-16 gene expression is further discussed in chapter 4.

The finding that the E6 intron of HPV-16 plays a role in stabilizing the early region polycistronic mRNAs was clearly demonstrated with plasmids pCMVE7, pCMVE6E7 and pCMVE6ME7 (Fig. 2.12B, 2.13B). The E6-E7 RNA accumulation and transformation was dependent on the presence of the functional E6 splice sites for the plasmid pCMVE6E7. The identity and source of the additional bands observed for the plasmids pCMVE7 and pCMVE6E7 are unclear (Fig. 2.12B, 2.13B). For the plasmid, pCMVE7, this band does not represent a specific signal, because this band was not seen in experiments in which SV40 large T antigen was used as an internal control instead of Cat (data not shown). Results obtained from these plasmids also indicate that the requirement for an intron in the E7 transcription unit can not be overcome by employing the

strong, heterologous promoter-enhancer and polyadenylation signal sequences. Previously, the efficient CMV promoter was shown to relieve the intron requirements of another gene (Neuberger and Williams, 1988).

However, heterologous sequences containing the SV40 small t intron functionally substituted for the nt 226 and 880 splice sites. Presence of this intron upstream of E7 in the sense orientation in the plasmid pY72t4 but not in the antisense orientation in the plasmid pY72t1, stabilized E7 RNA and conferred the transformation phenotype to a previously transformation incompetent plasmid, pY72 (Fig. 2.11, 2.12C). The transformation efficiency of the plasmid pY72t4 was lower than that of the plasmid pY71, although it produced consistently higher levels of E7 RNA (Fig. 2.11, 2.12C). The presence of the SV40-derived sequences upstream of the E7 ORF in this plasmid might have placed the E7 initiation codon in a relatively unfavourable configuration for translation, resulting in the synthesis of less E7 protein.

These studies indicate a role for the nt 880 and 226 splice sites in the stabilization of E6-E7 RNA. However, they do not exclude the possible role of the nt 1301 splice site. This splice site present in the nt 880 splice site mutated HPV-16 plasmids might have contributed to the transformation potential of these plasmids (Fig. 2.5). Contribution of the nt 1301 splice site to the stabilization of E6-E7 RNA may not

be significant. This interpretation is based on two important observations; (i) nt 1301 splice site has been reported to be rarely utilized, and (ii) no stabilizing effect of this splice site was detectable on E7 RNA, when it was tested in isolation in the plasmid pD7KB. Thus, it is more likely that the integrity of nt 226 and/or 880 splice sites determines, to a larger extent, the stability of early region polycistronic RNAs.

These studies demonstrate a role(s) for E6 splice sites, in posttranscriptional stabilization of HPV-16 early region transcripts. This is in addition to the previously assigned role for E6 splice sites in facilitating translation of E7 (Sedman et al., 1991).

Another observation of considerable interest was the presence of an additional protected band, of approximately 195 nt, in RNase protection assays for the plasmids pY72t4 and pD7 series (Fig. 2.7, 2.12A, 2.12C). Although the identity of this band could not be established with certainty, it is possible that it represents an RNA species initiated around nt 552, within the E6 or E7 ORFs. Several recent studies have suggested the presence of an internal initiation site within this early region (Doorbar et al., 1990; Rohlfs et al., 1991; Higgins et al., 1992).

Taken together, these results are in conformity with the observations that the presence of an intron in the

transcription unit facilitates higher expression of genes by promoting accumulation of RNA (Buchman and Berg, 1988; Huang and Gorman, 1990; Choi et al., 1991; Palmiter et al., 1991). However, for the pY71 and pY75 plasmids, functional splice sites, but not the process of splicing was required for accumulation of RNA. Although functional splice site and polyadenylation signal sequences were present for the RNA for the pY71 and pY75 plasmids, the majority of the RNA for these plasmids was not spliced and polyadenylated (Fig. 3.2, 3.7, 3.10, 3.11A). Further, the lack of splicing and polyadenylation did not destabilize these unprocessed RNAs or prevent their transportation to the cytoplasm, suggesting that the presence of functional splice sites and polyadenylation signal sequences were sufficient for the stabilization and possibly transportation of HPV-16 RNA. Similar observations have been made for the late mRNAs of polyoma virus. The stabilization of late mRNA for this virus was strictly dependent on the integrity of splice sites, but not on the actual process of splicing (Barrett et al., 1990; Lanoix et al., 1991). The likely explanation(s) for the requirement for the integral splice sites, but not splicing *per se*, is discussed further in chapter 4.

Although unlikely, a possible mechanism by which the nt 880 splice site mutations affected transformation could be through the activation of a cryptic splice site located within

the E7 coding region, resulting in the production of a truncated, nonfunctional E7 protein. Results of RNase protection assays with probes spanning the complete E7 ORF or different regions of the E7 ORF failed to detect products of such aberrant splicing (data not shown). Further, no potential cryptic splice donor site was present within the E7 ORF and the E7 upstream sequences of pY7 series of plasmids. It is also possible that the E7 RNA for the pY7 plasmid series with the mutated nt 880 splice donor site and the E6-E7 RNA for the pCMVE6ME7 plasmid with the mutated nt 226 splice donor site are retained within the nucleus and, consequently, rapidly degraded. The splicing apparatus assembles on nascent RNA transcripts, to form the splicesome, in which the actual process of splicing occurs (Green, 1991; Guthrie, 1991). Splicing has been shown to increase the efficiency of polyadenylation (Huang and Gorman, 1990; Niwa et al., 1990). The presence of poly(A) tails at the 3′ end of mRNAs is known to stabilize the mRNAs. The impaired splicing of E7 RNA for the nt 880 splice site mutated pY7 plasmid series and the E6-E7 RNA for the pCMVE6ME7 plasmid may interfere with efficient 3′ end processing of the E7 RNA contributing to destabilization of this RNA.

CHAPTER 3

NATURALLY OCCURRING AS HPV-16 RNA

3.1 INTRODUCTION

The genomes of viruses grouped under the family papovaviridae are small compared with the genomes of other double stranded DNA viruses such as adenovirus, herpes virus and pox virus. Members of the subfamily papovavirinae such as polyomavirus, SV40 and JC virus have a genome size of approximately 5 kb. The coding potential of the small genomes of these viruses is maximized by the presence of ORFs on both strands of the DNA and utilization of more than one promoter to produce a variety of mRNA species. In contrast to the papovaviruses, the members of the subfamily papillomavirinae have a genome size of approximately 8 kb and only one coding strand in their genome. Although more than one promoter has been mapped in the genomes of papillomaviruses such as BPV-1, CRPV and HPV-8, the majority of HPVs contain a single major promoter (Smotkin et al., 1989; Palermo-Dilts et al., 1990; Howley, 1990). Recently, an internal promoter has been mapped within the early region E6 of the low risk genital HPVs (Chow et al., 1987a; 1987b; Nasseri et al., 1987; Smotkin et al., 1989). Several studies have suggested the presence of a similar promoter within the genome of a high risk HPV, HPV-16 (Doorbar et al., 1990; Rohlfs et al., 1991; Higgins et al., 1992). The major promoter, termed p97, has been the only one

mapped to date in the genome of HPV-16 (Smotkin and Wettstein, 1986; Baker et al., 1987; Smotkin et al., 1989). Transcripts initiated at this promoter are differentially spliced to produce a variety of mRNAs with different coding potentials (Smotkin and Wettstein, 1986; Smotkin et al., 1989; Cornelissen et al., 1990; Doorbar et al., 1990; Johnson et al., 1990; Schneider-Maunoury et al., 1990; Nasseri et al., 1991; Rohlfs et al., 1991; Shirasawa et al., 1991; Sherman and Alloul, 1992; Sherman et al., 1992).

Recently a number of reports have indicated the presence of AS HPV-16 RNA in several cervical carcinomas and carcinoma-derived cell lines (Higgins et al., 1991; Vormwald-Dogan et al., 1992). The AS RNA corresponded to the NCR, E6-E7, L2 and L1 sequences or to the entire viral genome (Higgins et al., 1991; Vormwald-Dogan et al., 1992). The AS RNA may be a product of read-through transcription of the integrated HPV genome from flanking cellular promoter-enhancer sequences. Attempts to map the 5' ends of these AS transcripts by cDNA cloning were of limited success, because the 5' ends of these transcripts were heterogeneous and appeared to be non-authentic (Vormwald-Dogan et al., 1992).

The other possibility was that the AS RNA was transcribed from a novel viral promoter present on the negative strand of the viral genome. I have detected AS RNA corresponding to the E7 and E1 ORFs of HPV-16 in a transient expression assay

system which simulates the episomal state of the viral genome. The AS RNA was transcribed from an AS promoter located within the genome of HPV-16. The significance of the production of AS RNA for the life-cycle of the virus is discussed.

3.2 MATERIALS AND METHODS

3.2.1 Construction of recombinant plasmids

Standard molecular biological techniques were used in constructing plasmids (Sambrook et al., 1989). Plasmids pY71 and pY75 and pY713'Δ plasmid series have been described earlier in chapter 2. The pY713'Δ2 series of deletion mutants were constructed by Bal31 digestion of EcoRI-linearized pY71SE1176, that was derived from pY71 by inserting an EcoRI linker at the SspI site (nt 1179). The products of deletion were cleaved at the unique PvuI site within the pBR322 vector DNA. The smaller fragment containing the HPV-16 early region sequences for polyadenylation/cleavage was gel-purified and ligated to the flush-ended E7-containing larger EcoRI-PvuI fragment. All deletions were confirmed by sequencing. The plasmid pSV2HPA2 was constructed by ligating the blunt-ended small SspI-PvuI (nt 3978 of HPV-16-nt 3733 of pBR322) fragment of pY71, to the blunt-ended large HpaI-PvuI fragment of pSV2Cat. Plasmid pY7SA was constructed by ligating the blunt-ended HpaII-NcoI (nt 502-867) fragment of HPV-16 to the blunt-ended larger HindIII to BglII fragment of pSV2DHFR.

3.2.2 Cell culture and transfection

The techniques were as described in chapter 2.

3.2.3 RNA preparation and analysis

For total RNA preparation, Cos-1 cells transfected with 10 μg each of the indicated plasmids and pRSVCat, pRSVβGal internal control plasmids or pUC19 were used. The internal control Cat and βGal probes corresponded to nt 5018-4768 and nt 3063-3286 of pSV2Cat and pCH110 (Pharmacia), respectively.

Cytoplasmic RNA was prepared according to Sambrook et al., (1989).

Double RNase protection was performed according to Krystal et al., (1990), to analyze dsRNA. For this experiment, two samples of 10 μg each of total RNA was digested with 1000 U of RNase T1. After inactivating the RNase T1, RNase-resistant dsRNA was precipitated and hybridized to uniformly labelled strand-specific E7 or E7/E1 RNA probes. The nt 502-720 E7 probe has been described earlier in chapter 2. The E7/E1 probes used in this study corresponded to nt 720-955 and 792-1179 of HPV-16.

RNase protection experiments were performed with the indicated probes as described in chapter 2.

Primer extension analysis was performed as described by Ausubel et al., (1990). Ten μg of total RNA from Cos-1 cells transiently transfected with the plasmid, pY71, was used for analysis. The 5'-end-labelled primer sequences were nt 1292-

1312 continuing with nt 3763-3766, 25-mer oligonucleotide complementary to the negative strand of HPV-16 sequences present in the pY71 plasmid.

3.2.4 In situ hybridization

Forty eight hours after transfection, Cos-1 cells transfected with the indicated plasmids were trypsinized and plated in chamber slides. Cells were fixed with 4% paraformaldehyde (PFA) and 5 mM $MgCl_2$ at room temperature for 10 minutes. PFA-fixed cells were stored at 4°C, in 70% ethanol. In situ hybridization experiments were performed as described by Lawrence and Singer, (1986), with biotinylated sense and antisense E7 RNA probes. The hybridized probes were detected with the DNA detection system (Bethesda Research Labs), according to the manufacturer's recommendations.

3.2.5 Immunofluorescence

Cos-1 cells transfected with the indicated plasmids were fixed with a 4% solution of PFA for 10 minutes followed by permeabilization with methanol for 2 minutes at room temperature. After fixation and permeabilization, cells were covered with an E7 specific monoclonal antibody, HBD4 (1:1000 dilution in PBS) and incubated at 4° C for 1 hour. Cells were washed thrice in PBS before applying the 1:5 diluted fluorescein isothiocyanate (FITC)-conjugated antimouse antibody. Cells were washed thrice in PBS and analyzed by fluorescent microscopy.

3.3 RESULTS

3.3.1 Detection of HPV-16 AS promoter

Several HPV-16 positive cervical squamous cell carcinomas and carcinoma derived cell lines have been shown to express AS HPV RNA (Higgins et al., 1991; Vormwald-Dogan et al., 1992). In all these tumors and cell lines, the viral genome was found to be integrated with the cellular genome. Expression of the AS HPV RNA in these cells may be a consequence of integration of the viral genome. Read-through transcription of the negative strand of the viral genome from the flanking cellular promoter-enhancer sequence could result in the appearance of AS HPV RNA. The AS HPV RNA may also result from the transcription of the viral negative strand from a hitherto unmapped viral promoter. However, no evidence for the presence of a promoter other than the previously mapped p97 promoter exists. The presence of AS RNA in only a few cell lines could be related to the pattern of viral integration. It has been shown that regions of 3' ORFs of the viral early region, especially the E1 and/or E2 ORFs, are generally deleted or disrupted as a consequence of integration of the viral genome (Matsukura et al., 1986; Durst et al., 1986; Baker et al., 1987; Wagatsuma et al., 1990). If the proposed AS promoter is located within the 3' early region ORFs, it is likely that this promoter is deleted in many cell lines and these cell lines fail to express AS HPV RNA. Cells in which the AS promoter is not deleted may continue to express AS HPV RNA.

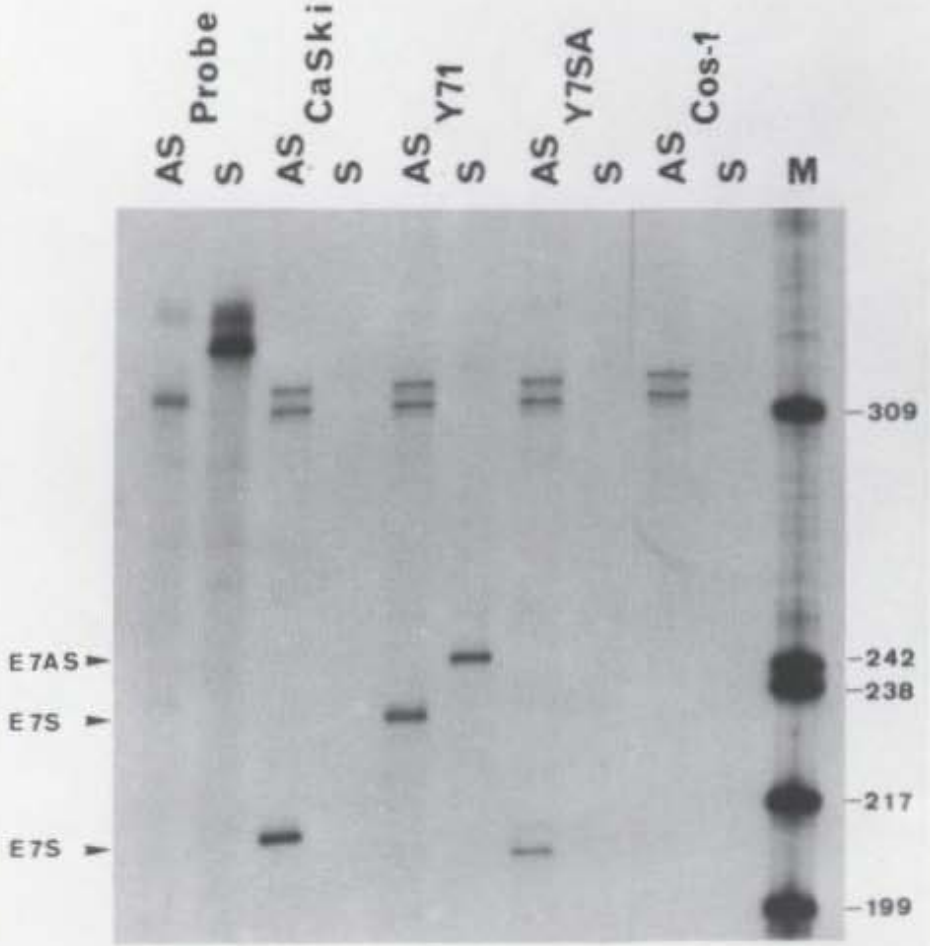
Based on the hypothesis that there is an AS promoter, the HPV-16 sequences that may contain this promoter were tested in the episomal form to avoid the effects of integration, because it has been shown that the cellular sequences flanking the viral sequences at the site of integration often influence the activity of the viral transcriptional regulatory elements (von knebel-Doeberitz et al., 1991). A plasmid, pY71, was constructed in which the 3' early region sequences downstream of E1 and/or E2 ORF were intact. This 3' early region contains the E5 ORF, a cryptic splice acceptor site and the viral early region polyadenylation signal (Seedorf et al., 1985). In addition to E5, sequences corresponding to the E7 ORF and a part of E1 ORF were also present in this plasmid (Fig. 3.1). To determine if this plasmid can result in the expression of AS HPV RNA, the plasmid was transfected into Cos-1 cells and the total RNA harvested from these cells was analyzed for the presence of S and AS HPV RNA by RNase protection assays, using S and AS E7 probes. The S and AS E7 RNAs are detected by the AS and S E7 probes, respectively. As shown in the fig. 3.1, both S and AS E7 RNAs were readily detected in cells transfected with the plasmid, pY71. The relative levels of AS RNA with respect to the S RNA was variable among different experiments. It was possible that this AS RNA was transcribed from a cryptic promoter located within the pBR322 vector sequences of pY71. To evaluate this

Figure 3.1 Diagrammatic representation of and RNase protection experiments for E7 expression plasmids.

The diagram is on top. Both plasmids are expressed from the SV40 early promoter-enhancer. The open boxes and the filled box represent, the HPV-16 E7 ORF and the early 3' region including the early polyadenylation signal sequences, and the SV40 small t intron and polyadenylation signal sequences, respectively. The deletion is indicated by the thin line. The numbers correspond to the HPV-16 nt sequence.

The results are shown on the bottom for 5 μg of total RNA from CaSki cells, untransfected Cos-1 cells and Cos-1 cells transfected with the indicated plasmids, analyzed by RNase protection assays with S and AS E7 probes. The E7 probes correspond to both strands of the StuI (nt 5190 of SV40) to SspI (nt 720 of HPV-16) fragment of pY71. The AS and S labels on the top of the lanes indicate the sense and antisense probes used in the assay. Control lanes and lanes for RNA from transfected cells are indicated on top as: probe, undigested E7 probes; CaSki, RNA from CaSki cells, Cos-1, RNA from untransfected Cos-1 cells; M, HpaII-digested pBR322 molecular size markers. The protected E7 S and E7 AS fragments are indicated by arrowheads. The protected E7 fragment for CaSki cells is small, because it lacks sequences present in the probe and E7 expression plasmids.


pY71
502
1312
3763
4338
pY7SA
502
867


AS
S
Probe
AS
S
CaSki
AS
S
Y71
AS
S
Y7SA
AS
S
Cos-1
M
309
242
238
217
199
E7AS
E7S
E7S

possibility, the pY7SA plasmid was constructed by replacing the HPV AS promoter region of pY71 with SV40 sequences (Fig. 3.1). Because the AS promoter region overlaps the splice and polyadenylation signal sequences a functionally analogous region consisting of the small t intron and the polyadenylation signal sequences was chosen from SV40. Only S, but not AS, E7 RNA was detected for this plasmid (Fig. 3.1). Similarly, no AS E7 RNA was detected for CaSki, a cervical squamous carcinoma cell line that constitutively expresses HPV-16 early region (Fig. 3.1). In this cell line, the 3' early region ORFs of HPV 3' to nt 3728 are deleted as a result of integration (Smits et al., 1991). Taken together, these results are suggestive of the presence of an AS promoter within the HPV-16 region of the plasmid pY71.

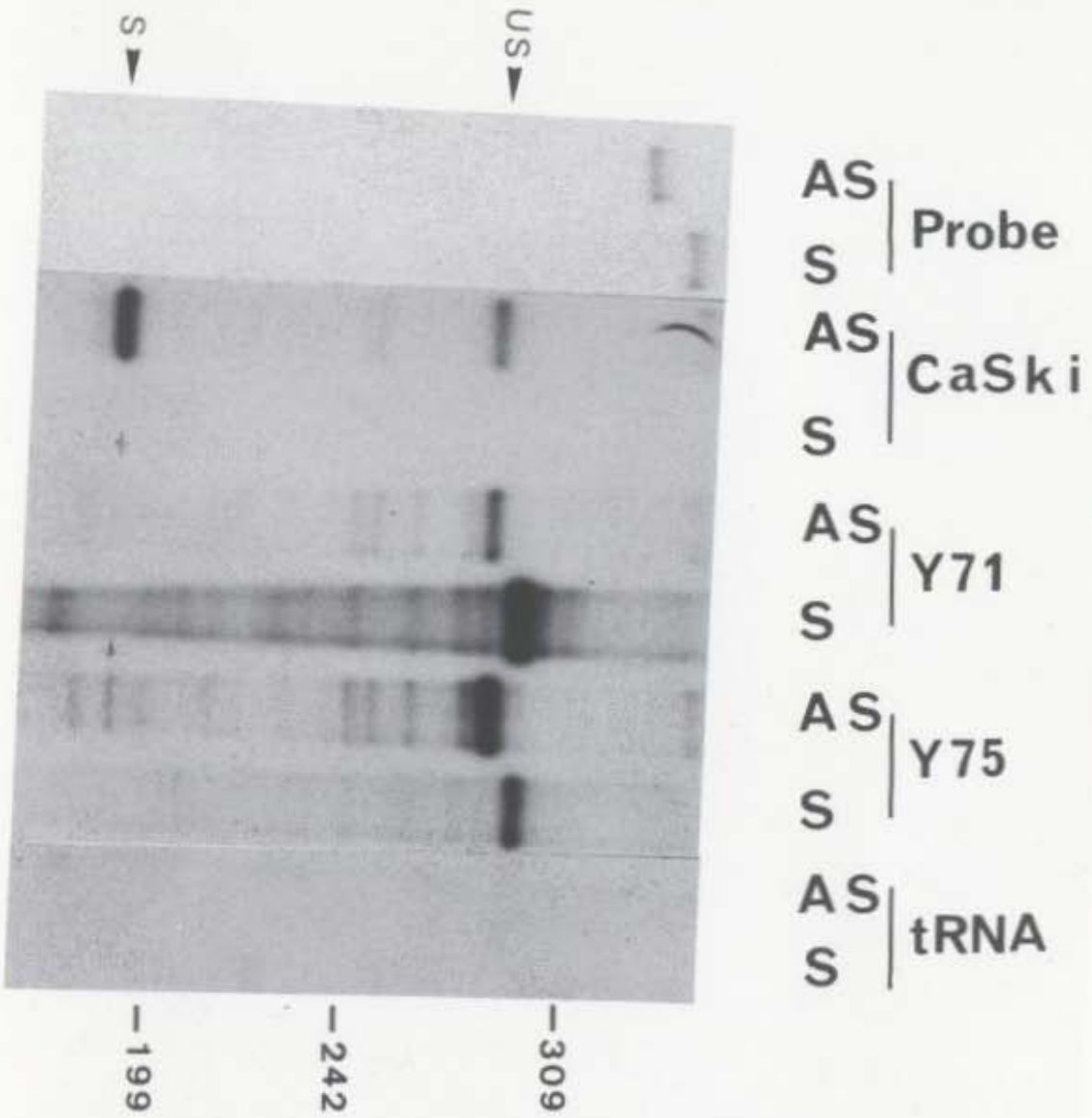
3.3.2 Localization of the AS promoter

The S probe used to detect the AS E7 RNA contained the nt 502-720 of HPV-16 and 25 nt of the SV40 early promoter region and additional nt derived from the pBSKS(+) vector. If the AS RNA was transcribed from a promoter located within the nt 502-720 sequences of HPV-16, not all of the HPV-16 sequences present in the probe would be protected from RNase digestion, resulting in a small size for the protected probe. In RNase protection assays, all of the HPV-16 and SV40-derived sequences present in this probe were protected indicating that the AS RNA was transcribed from the AS promoter located

further downstream of the probe sequences. Total RNA from Cos-1 cells transfected with the plasmids, pY71 and pY75, was analyzed by RNase protection assays with the probe spanning nt 720-955 at the 3′ end of the E7 and 5′ end of the E1 ORF sequences to address the possible location of the AS promoter within this region of the HPV-16 genome. The AS RNA was detected for both of the plasmids, pY71 and pY75 (Fig. 3.2). Higher levels of AS RNA relative to the S RNA was detected for the plasmid pY71 compared with the plasmid pY75. As observed earlier with the E7 probe, no AS RNA was detected for CaSki cervical carcinoma cells with this E7/E1 probe. All of the sequences between nt 720 and 955 were protected for the plasmids, pY71 and pY75, indicating that the AS promoter was located downstream of the nt 955. That the deletion between the nt 1176 and 1312 in the plasmid, pY75, did not eliminate AS RNA suggested that the AS promoter was not present within this region of HPV-16. However, this deletion lowered the level of AS RNA for the plasmid pY75. Further, the AS E7/E1 probe which detected S RNA indicated that the greater proportion of S RNA was spliced at the nt 880 splice site for CaSki cells. In contrast, less of S RNA was spliced for the plasmids, pY71 and pY75, at this splice site (Fig. 3.2). The effect of AS RNA on splicing of S RNA is discussed later in more detail.

Figure 3.2 RNase protection assays for plasmids pY71 and pY75 with the E7/E1 probes.

The results are shown for 5 μg of total RNA from CaSki cells and Cos-1 cells transfected with the indicated plasmids, analyzed by RNase protection assays with S and AS E7/E1 probes. The E7/E1 probes correspond to nt 720-955 of HPV-16. The AS and S labels on the top of the lanes indicate the sense and antisense probes used in the assay. Control lanes and lanes for RNA from transfected cells are indicated on top as: probe, undigested E7/E1 probes; CaSki, RNA from CaSki cells, HpaII-digested pBR322 molecular size markers are shown on the right. Labels are: US, unspliced E7 sense RNA; S, E7 sense RNA spliced at nt 880. The protected spliced and unspliced E7/E1 fragments are indicated by arrowheads.

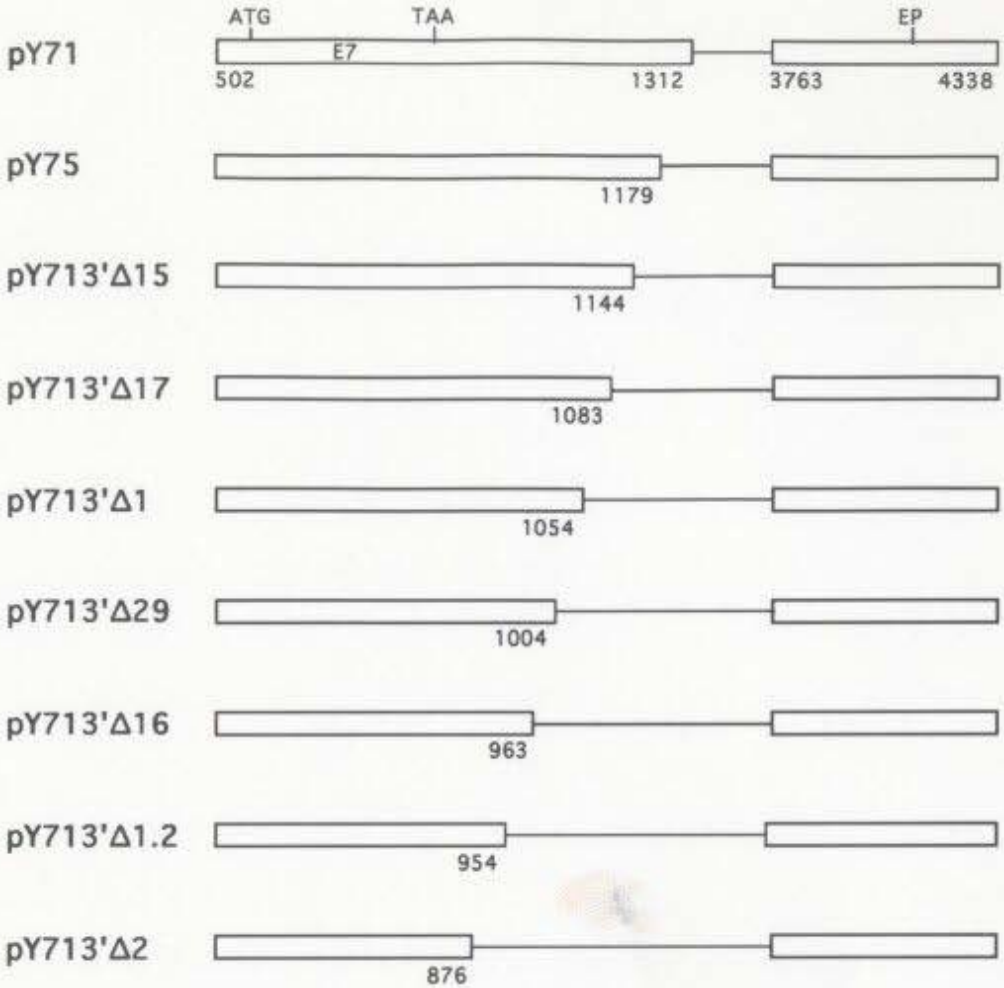

S
US
AS
S
Probe
AS
S
CaSki
AS
S
Y71
AS
S
Y75
AS
S
tRNA
—199
—242
—309

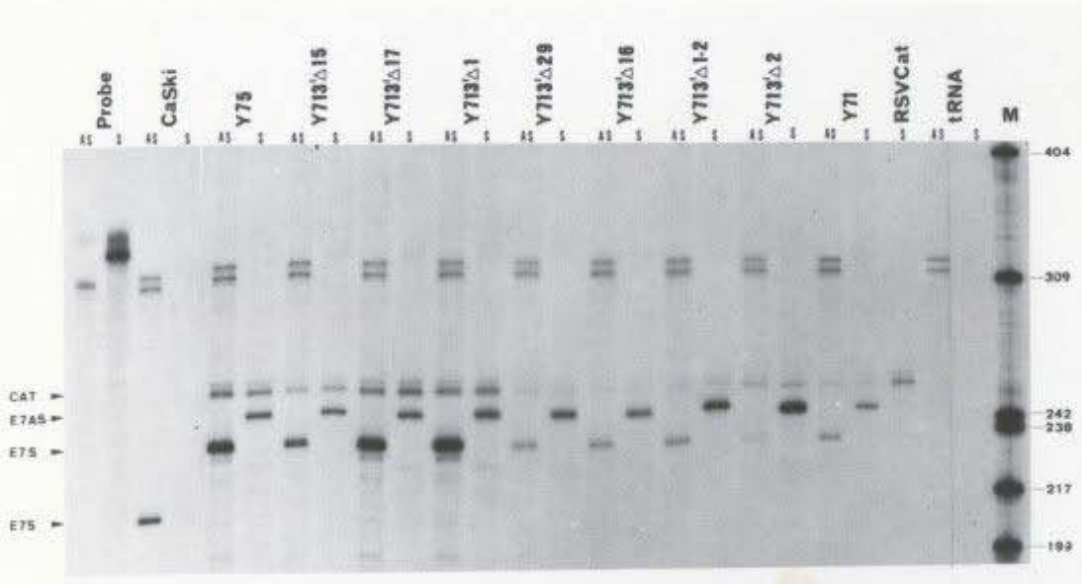
To map the location of the AS promoter, additional plasmids with deletions extending into the 5' end of the E1 ORF sequences were used (Fig. 3.3). Because the results of RNase protection assays for the pY71 plasmid had eliminated the presence of the AS promoter between the nt 1312 and 3763, deletions were made starting from the nt 1312 and extending towards the E7 ORF. Total RNA from Cos-1 cells transfected with these deletion plasmids and the internal control pRSVCat plasmid was analyzed by RNase protection assays with the previously described E7 probes and the AS Cat probe. As observed earlier, the plasmid, pY71, produced higher levels of AS RNA relative to S RNA compared with the plasmid pY75 (Fig. 3.3). Progressive deletions between the nt 1176 and 876 resulted in a gradual increase in the levels of AS RNA. For the plasmids, pY75, pY713'Δ15, pY713'Δ17 and pY713'Δ1, there was less of AS RNA relative to S RNA, whereas for the plasmids, pY713'Δ29, pY713'Δ16, pY713'Δ1.2 and pY713'Δ2, more of AS RNA relative to S RNA was observed (Fig. 3.3). As has been reported earlier (Belaguli et al., 1992), deletion of sequences between the nt 876 and 3763 greatly reduced the level of S E7 RNA (Fig. 3.3). Because the deletions between the nt 867-3763 did not eliminate AS RNA, results of these experiments indicated that the AS promoter is located downstream of the nt 3763.

Figure 3.3 Diagrammatic representation of and RNase protection experiments for pY713'Δ series of E7 expression plasmids.

The diagram is on top. All plasmids are expressed from the SV40 early promoter-enhancer. The boxes and thin lines represent the HPV-16 sequences and deletions, respectively. The numbers below the boxes correspond to HPV-16 nt. The abbreviations ATG, TAA and EP indicate the initiation and stop codons of E7 (E7) ORF and the HPV-16 early polyadenylation signal sequence, respectively.

The results are shown on the bottom for 5 μg of total RNA from CaSki cells and Cos-1 cells transfected with the indicated plasmids, analyzed by RNase protection assays with S and AS E7 probes. The E7 probes correspond to both strands of the StuI (nt 5190 of SV40) to SspI (nt 720 of HPV-16) fragment of pY71. The cotransfected pRSVCat plasmid was used as an internal control and the CAT probe corresponds to nt 5018-4768 of pSV2Cat. A mixture of undigested CAT probe (upper band) and AS and S E7 probes (lower bands) were run in the lanes marked Probe. Abbreviations used are: RSVCat, RNA from Cos-1 cells transfected with the pRSVCat plasmid; tRNA, E. coli tRNA. The protected E7 S and AS and CAT fragments are indicated by arrowheads. Conditions and other labels as in figure 3.1.


pY71
ATG
TAA
EP
E7
502
1312
3763
4338
pY75
1179
pY713'Δ15
1144
pY713'Δ17
1083
pY713'Δ1
1054
pY713'Δ29
1004
pY713'Δ16
963
pY713'Δ1.2
954
pY713'Δ2
876


Probe
CaSki
Y75
Y713'Δ15
Y713'Δ17
Y713'Δ1
Y713'Δ29
Y713'Δ16
Y713'Δ1-2
Y713'Δ2
Y71
RSVCat
tRNA
M
404
309
242
238
217
193
CAT
E7AS
E7S
E7S

It was likely that the AS promoter was present between the nt 3763 and 4338 of HPV-16, because all of the AS RNA expressing plasmids had these sequences in common and the results of RNase protection assays for the plasmids with progressive unilateral deletions in the E1 ORF sequences suggested that the AS promoter was located downstream of the nt 3763. Several plasmids with unilateral deletions extending from the nt 3763 towards the early polyadenylation signal sequences were constructed and tested by RNase protection assays for their ability to produce S and AS E7 RNA in Cos-1 cells, using E7 probes. All of the plamsids, pY713'Δ2.2, pY713'Δ2.5 and pY713'Δ2.14 produced low levels of AS RNA relative to S RNA (Fig. 3.4). The deletion of 268 bp in the plasmid pY713'Δ2.14 extending to nt 4031 did not eliminate AS RNA. This deletion plasmid localized the AS promoter activity to sequences between the nt 4031 and 4338 of HPV-16.

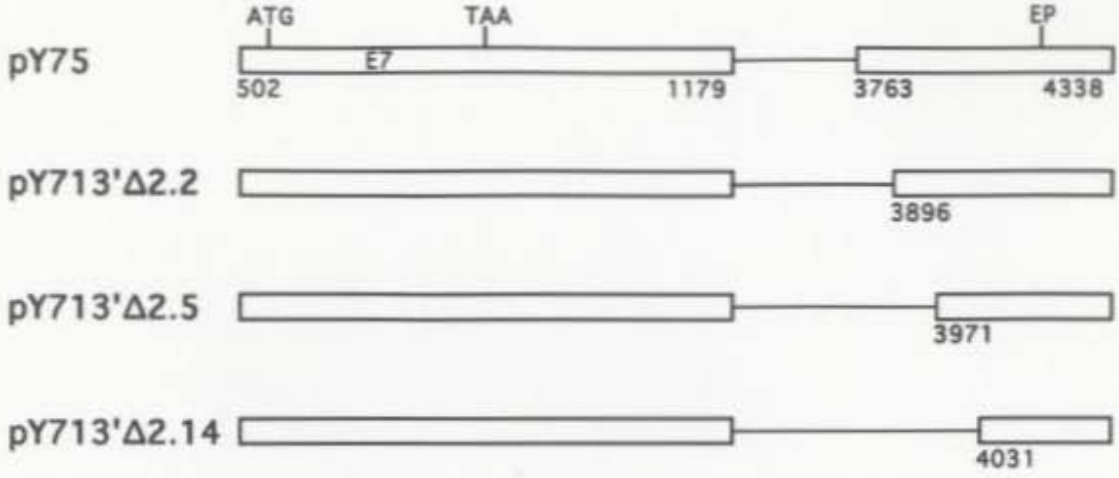
3.3.3 The activity of the AS promoter cloned downstream of Cat gene

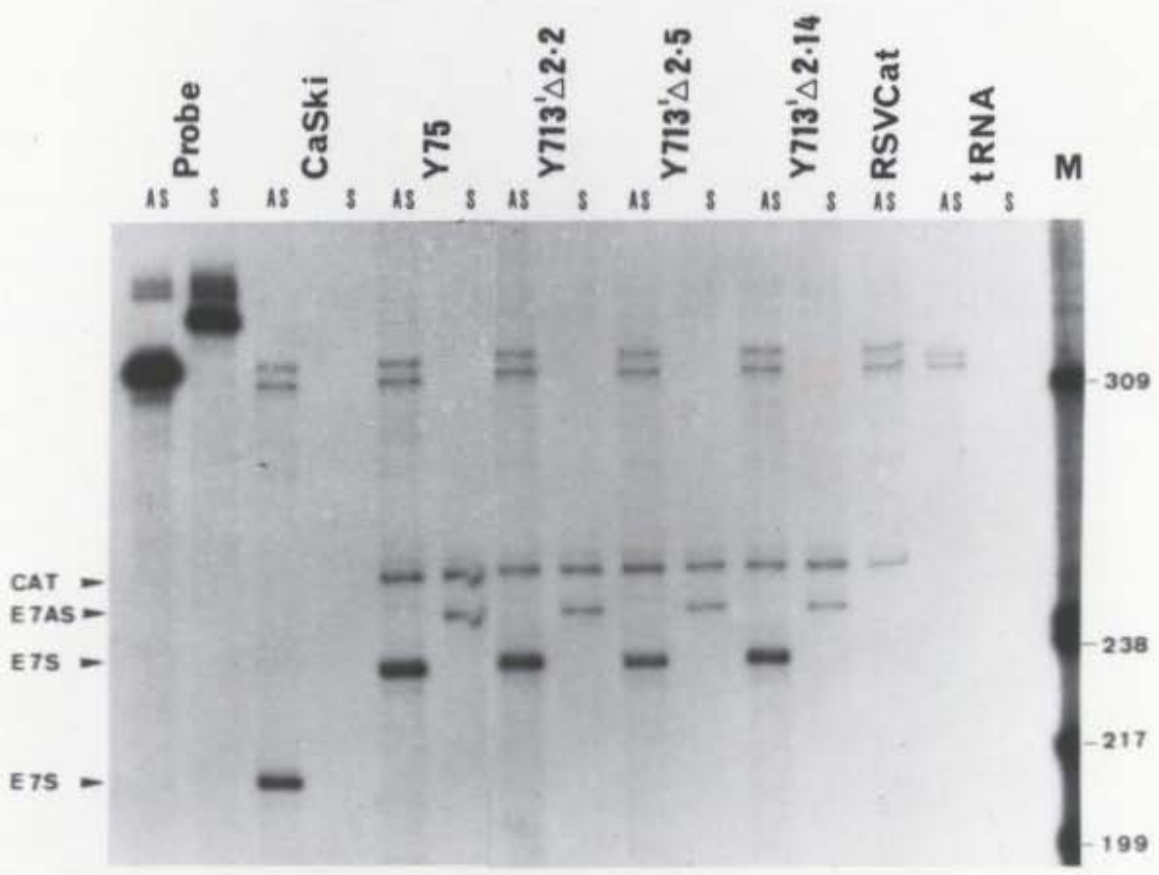
Cloning the AS promoter in the sense orientation upstream of a reporter gene would provide a convenient method of assay for the activity of this promoter. A 362 bp of HPV-16 nt 3976-4338 fragment containing the AS promoter was cloned upstream of the reporter Cat gene in the plasmid pAS(+)Cat and tested in the monkey kidney cell line, Cos-1, and the mouse fibroblast cell line, L cells. In both cell lines, the

Figure 3.4 Diagrammatic representation of and RNase protection experiments for pY713'Δ2 series of E7 expression plasmids.

The diagram is on top. All plasmids are expressed from the SV40 early promoter-enhancer. The boxes and thin lines represent the HPV-16 sequences and deletions, respectively. The numbers below the boxes correspond to HPV-16 nt. The abbreviations ATG, TAA and EP indicate the initiation and stop codons of E7 (E7) ORF and the HPV-16 early polyadenylation signal sequence, respectively.

The results are shown on the bottom for 5 μg of total RNA from CaSki cells and Cos-1 cells transfected with the indicated plasmids, analyzed by RNase protection assays with S and AS E7 probes. The E7 probes correspond to both strands of the StuI (nt 5190 of SV40) to SspI (nt 720 of HPV-16) fragment of pY71. The cotransfected pRSVCat plasmid was used as an internal control and the CAT probe corresponds to nt 5018-4768 of pSV2Cat. A mixture of undigested CAT probe (upper band) and AS and S E7 probes (lower bands) were run in the lanes marked probe. Abbreviations used are: RSVCat, RNA from Cos-1 cells cells transfected with the pRSVCat plasmid; tRNA, E. coli tRNA. The protected E7 S and E7 AS and CAT fragments are indicated by arrowheads. Conditions and other labels as in figure 3.1.


pY75
ATG
TAA
EP
E7
502
1179
3763
4338
pY713'Δ2.2
3896
pY713'Δ2.5
3971
pY713'Δ2.14
4031


Probe
CaSki
Y75
Y713'Δ2·2
Y713'Δ2·5
Y713'Δ2·14
RSVCat
tRNA
M
AS
S
CAT
E7AS
E7S
E7S
309
238
217
199

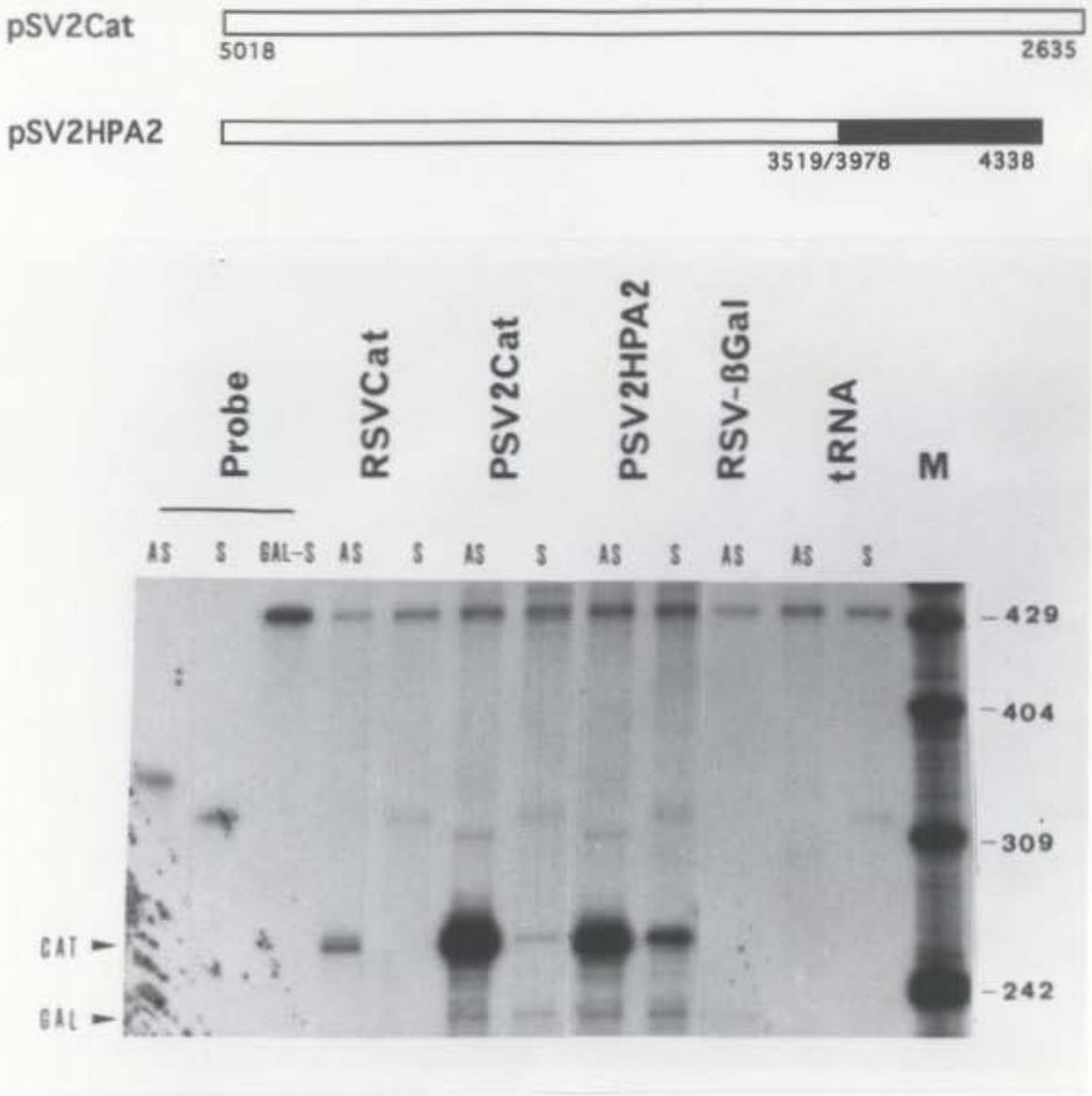
activity of this plasmid was several-fold lower than the promoterless parental plasmid, pBLCat3 (data not shown). The low level of Cat activity for the plasmid pAS(+)Cat compared with the plasmid, pBLCat3 could be related to the out-of-frame translation of the Cat mRNA, that would result in a nonfunctional Cat protein. Consistent with this possibility, several ATG triplets were present in the 5'region of AS RNA. Another possible explanation for the poor activity of this promoter could be related to the absence of enhancer sequences in the plasmid pAS(+)Cat.

To test the AS expression of heterologous sequences and because the activity for the AS promoter containing sequences cloned upstream of the reporter Cat gene in the enhancerless plasmid was low, the nt 3976-4338 AS promoter containing fragment was cloned downstream of the Cat gene in the SV40 early promoter-enhancer-containing plasmid, pSV2HPA2. The small t intron and the polyadenylation signal sequences of SV40 present in the plasmid, pSV2Cat, were replaced with HPV-16 nt 3976-4338 AS promoter containing sequences to construct the plasmid, pSV2HPA2 (Fig. 3.5). This plasmid was cotransfected with the internal control pRSVβgal plasmid into Cos-1 cells and the total RNA extracted from these cells was analyzed by the S and AS Cat probes and AS βgal probe. The HPV-16 AS promoter was functional for the AS transcription of the Cat gene. Compared with the control plasmid, pSV2Cat,

Figure 3.5 Diagrammatic representation of and RNase protection experiments for Cat plasmids.

The open boxes indicate the sequences of the pSV2Cat plasmid. The black box represent the HPV-16 sequences containing the AS promoter. The nt coordinates for the Cat and AS promoter region are from pSV2Cat and HPV-16, respectively.

Five μg of total RNA from Cos-1 cells transfected with the indicated plasmids were analyzed with S and AS CAT probes. Cotransfected pRSVβGal plasmid was used as the internal control. The CAT and βGal probes correspond to nt 5018-4768 and 3063-3286 of the pSV2Cat and pRSVβGal plasmids, respectively. Symbols CAT and GAL indicate the protected Cat and βGal fragments. Conditions and other abbreviations as in the figure 3.3.


pSV2Cat
5018
2635
pSV2HPA2
3519/3978
4338
Probe
RSVCat
PSV2Cat
PSV2HPA2
RSV-ßGal
tRNA
M
AS
S
GAL-S
CAT
GAL
429
404
309
242

significantly higher levels of AS Cat RNA were detected for the pSV2HPA2 plasmid (Fig. 3.5). These results confirm the presence of an AS promoter between nt 3978 and 4338 of HPV-16 and show that this promoter can be used to transcribe heterologous sequences.

3.3.4 Mapping the 5′ ends of AS RNA

Primer extension analysis was used to map the 5′ ends of the AS RNA. Total RNA from Cos-1 cells transfected with the plasmid, pY71, was used for this analysis. For CaSki cells, which were consistently negative for AS RNA expression, no primer extension product was detected (data not shown). The results of the experiments done in duplicate for the plasmid, pY71, indicated that the AS RNA has heterogeneous 5′ ends initiated in an AT-rich region around nt 4100 (Fig. 3.6).

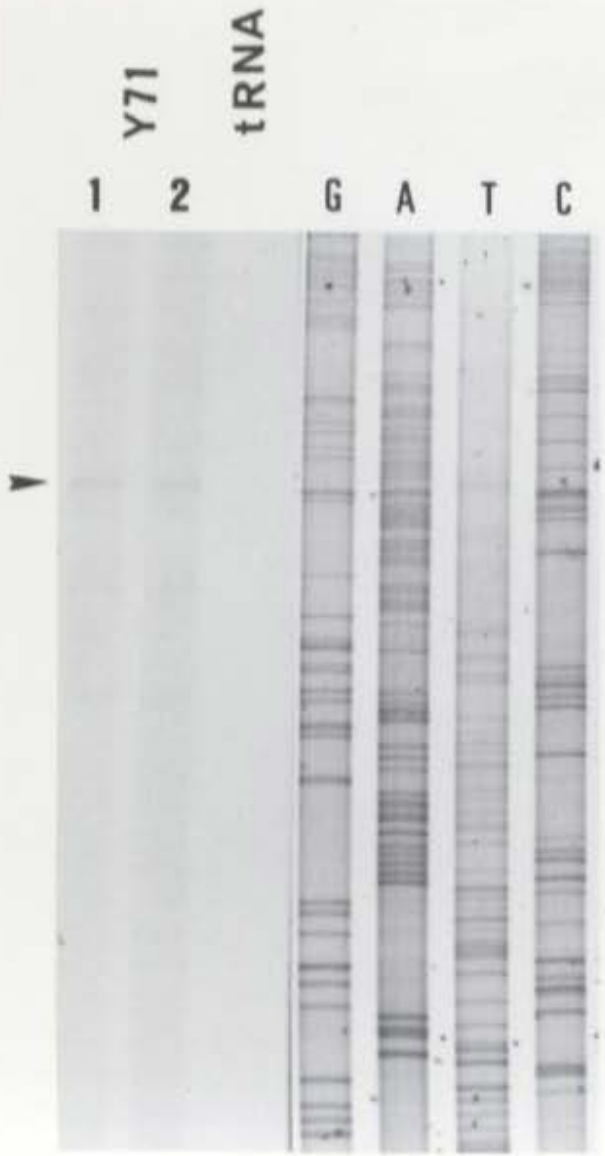
To confirm the results of primer extension analysis regarding the 5′ ends of AS RNA, RNase protection experiments were performed on total RNA from pY71- and pY75-transfected Cos-1 cells with S and AS RNA probes corresponding to nt 4107-4311. If the AS transcripts are initiated around the nt 4100, no signal corresponding to the AS RNA would be expected for this probe. Consistent with the previous observations with the E7 and E7/E1 probes, no AS RNA was detected for CaSki cells (Fig. 3.7). Because the 3′ early region ORF sequences of HPV-16 3′ to the nt 3728 are deleted as a consequence of integration of the viral genome, no S RNA was detected for

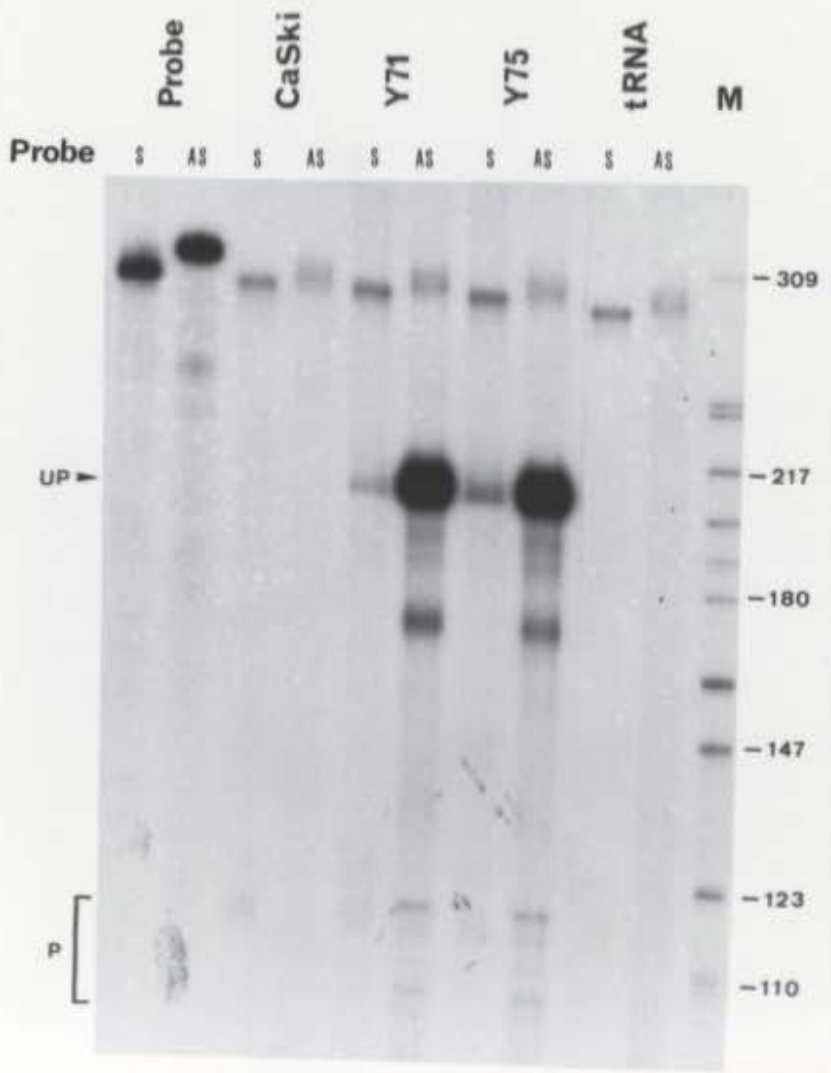
Figure 3.6 Primer extension analysis.

The left panel shows the results. The major primer extension product is indicated by the arrowhead. Lanes 1 and 2, are for total RNA from Cos-1 cells transfected with the pY71 plasmid. The tRNA lane is for E. Coli tRNA. Lanes G, A, T and C indicates the dideoxy sequencing reactions performed with the primer that was used for primer extension.

Figure 3.7 RNase protection experiments with the nt 4107-4311 probe.

The probes correspond to nt 4107-4311 of HPV-16. Abbreviations used are: UP, unprocessed RNA; P, RNA that has undergone 3' end cleavage downstream of the early polyadenylation signal. All other conditions and abbreviations are as in figure 3.3.


Y71
tRNA
1
2
G
A
T
C


Probe
CaSki
Y71
Y75
tRNA
M
Probe
S
AS
UP
P
309
217
180
147
123
110

CaSki cells with the nt 4107-4311 probe. For the plasmids, pY71 and pY75, very low levels of AS RNA relative to the S RNA were detected (Fig. 3.7). The representative figure shown was made from an overexposed autoradiogram to reveal the 3' end processing for S RNA. In lighter exposures, the AS RNA was not detected for the plasmids, pY71 and pY75, with the nt 4107-4311 probe. These results confirm that the AS RNA transcripts are initiated within a narrow region around the nt 4100.

3.3.5 Detection of AS RNA in the cytoplasm

Analysis of the HPV-16 AS RNA in cervical cancers and cervical cancer-derived cell lines has indicated that the HPV-16 AS RNA is predominantly found in the nucleus (Higgins et al., 1991; Vormwald-Dogan et al., 1992). The AS RNA detected in cervical cancers and cancer-derived cell lines may be the product of read-through transcription of the integrated HPV-16 genome from flanking cellular promoter-enhancer sequences. In contrast, the AS RNA detected for the HPV-16 expression plasmids used in my study were initiated from the viral AS promoter. It is possible that the intracellular location of the AS RNA produced from the HPV-16 expression plasmids such as, pY71 and pY75, might be different from the AS RNA detected in cervical cancers and cervical cancer-derived cell lines. To determine whether the AS RNA for the HPV-16 expression plasmids is present in the cytoplasm, the cytoplasmic RNA was

isolated from Cos-1 cells transfected with the pY71 and pY75 plasmids and analyzed by RNase protection assays, with E7 probes. For CaSki cells, only the S, but not the AS RNA was detected. For the plasmids, pY71 and pY75, both the S and AS RNAs were detected (Fig. 3.8A). The levels of the AS RNA relative to S RNA for the plasmids, pY71 and pY75, were comparable for the experiment shown. However, the relative levels of AS RNA were variable among different experiments for these plasmids.

Contamination of the cytoplasmic RNA with the nuclear RNA during the process of isolation of the cytoplasmic RNA might have resulted in AS cytoplasmic RNA for the pY71 and pY75 plasmids. To eliminate this possibility, in situ hybridization experiments were performed on Cos-1 cells transfected with the pY71 plasmid, using strand-specific biotinylated E7 RNA probes. The pY7SA plasmid which does not express AS RNA was used as the negative control. Under low magnification, the S and AS RNAs were detected for the pY71 plasmid transfected-Cos-1 cells (data not shown). Because only a small population of transfected cells take up the transfected plasmid DNA, heterogeneity for the expression of S and AS E7 RNA was observed. Under higher magnification, the AS RNA for the plasmid pY71-transfected Cos-1 cells was predominantly cytoplasmic (Fig. 3.8B). Low levels of both S and AS E7 RNAs were also present in the nucleus (Fig. 3.8B).

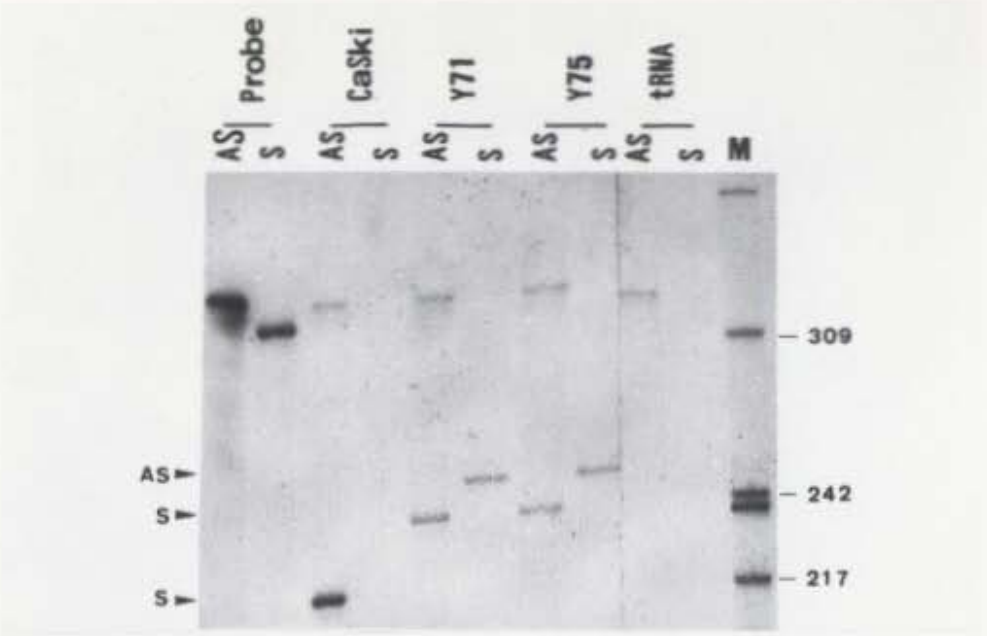
Figure 3.8 RNase protection and in situ hybridization experiments for cytoplasmic RNA.

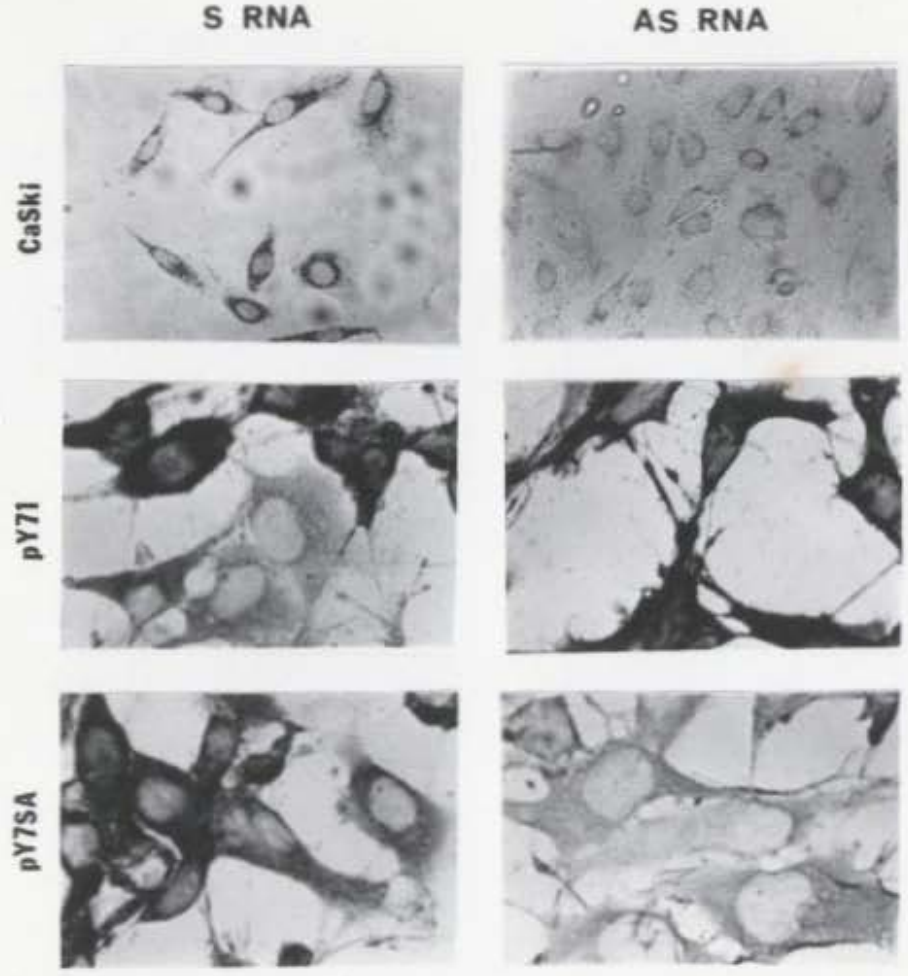
A. RNase protection experiments.

The results are shown on the top for 5 μg of total RNA from CaSki cells and Cos-1 cells transfected with the indicated plasmids, analyzed by RNase protection assays with S and AS E7 probes. The E7 probes correspond to both strands of the StuI (nt 5190 of SV40) to SspI (nt 720 of HPV-16) fragment of pY71. The AS and S labels on the top of the lanes indicate the sense and antisense probes used in the assay. Control lanes and lanes for RNA from transfected cells are indicated on top as: Probe, undigested E7 probes; CaSki, RNA from CaSki cells, tRNA, E. coli tRNA; M, HpaII-digested pBR322 molecular size markers. The protected S and AS E7 fragments are indicated by arrowheads.

B. In situ hybridization experiments.

CaSki cells and Cos-1 cells transfected with the pY71 and pY7SA plasmids were hybridized with S and AS biotinylated E7 RNA probes. Labels are: S RNA, assays for E7 sense RNA with AS probe; AS RNA, assays for E7 AS RNA with S probe; CaSki, CaSki cervical carcinoma cells; pY71, Cos-1 cells transfected with the plasmid pY71; pY7SA, Cos-1 cells transfected with the plasmid pY7SA. Magnification: 25x for CaSki cells and 40x for Cos-1 cells.


AS
S
Probe
AS
S
CaSki
AS
S
Y71
AS
S
Y75
AS
S
tRNA
M
309
242
217
AS
S
S


S RNA
AS RNA
CaSki
pY71
pY7SA

In agreement with the results of RNase protection, only S, but not AS E7 RNA was detected in the cytoplasm for CaSki cells and Cos-1 cells transfected with the pY7SA plasmid (Fig. 3.8B). That the AS RNA detected for the pY71 plasmid was not because of hybridization of the S RNA probe to denatured template DNA was suggested by the absence of AS RNA for the pY7SA plasmid. Compared to CaSki cells, Cos-1 cells transfected with the plasmids, pY71 and pY7SA, expressed very high levels of S RNA and this could be related to the amplification of these SV40 origin of replication-containing plasmids, in Cos-1 cells. The results of in situ hybridization experiments confirmed the cytoplasmic location of the AS RNA for the pY71 plasmid.

Only a few of the reported naturally occurring AS RNAs have the coding potential and these AS RNAs have been detected in the cytoplasm (Williams and Fried, 1986; Adelman et al., 1987; Kimelman and Kirschner, 1989; Lazar et al., 1989; Miyajima et al., 1989; van Duin et al., 1989; Dolnick, 1993). The nt sequence of the naturally occurring AS RNA to the bFGF mRNA of Xenopus is highly conserved among different species of animals and encodes a protein of 25 kDa (Kimelman and Kirschner, 1989). The AS RNA to the rat c-erbAα-2 mRNA encoded a 56 kDa protein (Lazar et al., 1989). Because the HPV-16 AS RNA was also detected in the cytoplasm, it was possible that the AS RNA could code for a protein. The

possible coding potential of the HPV-16 AS RNA was examined by prediction of ORFs in these AS RNAs by Ficket's method. No significant ORFs were detected in HPV-16 AS RNAs.

3.3.6 Presence of AS RNA in the RNA-RNA duplex

The concurrent presence of the S and AS RNAs in the same cell could allow hybridization of these two species of RNAs resulting in dsRNA. To examine this possibility, the double RNase protection assays were used. The double RNase protection assay depends on the sensitivity of single stranded RNA, but not the dsRNA, to degradation by single strand-specific RNase, RNase T1. The double RNase protection assay includes two steps. In the first step, the RNA samples are treated with the single strand specific-RNase, RNase T1. Only dsRNA, but not the single stranded RNA remains after the RNase treatment. At the end of the first step, the RNase is inactivated. In the second step, hybridization is set up with uniformly labelled strand-specific RNA probes and the standard RNase protection assay is performed. The probes used for the double RNase protection assays were from the E7 ORF 5' region sequences. The pD71 plasmid which expressed low level of AS RNA was used as the negative control. For CaSki cells, which does not express AS RNA, total RNA treated with RNase T1 prior to hybridization to the probe resulted in total degradation of E7 RNA (data not shown). In contrast, a significant proportion of both the S and AS RNAs for the pY71 and pY75

plasmids remained RNase resistant when treated with RNase prior to hybridization (Fig. 3.9). Approximately comparable levels of RNase resistant S and AS E7 RNAs were observed for the pY71 and pY75 plasmids. The RNase treatment prior to hybridization of total RNA from cells transfected with the plasmid, pD71, resulted in the drastic reduction of the S RNA signal (Fig. 3.9). These results clearly indicate that significant proportions of the AS E7 RNA for the pY71 plasmid exist in the form of RNA-RNA duplex with the S E7 RNA.

3.3.7 Interference with splicing of E7 S RNA by AS RNA

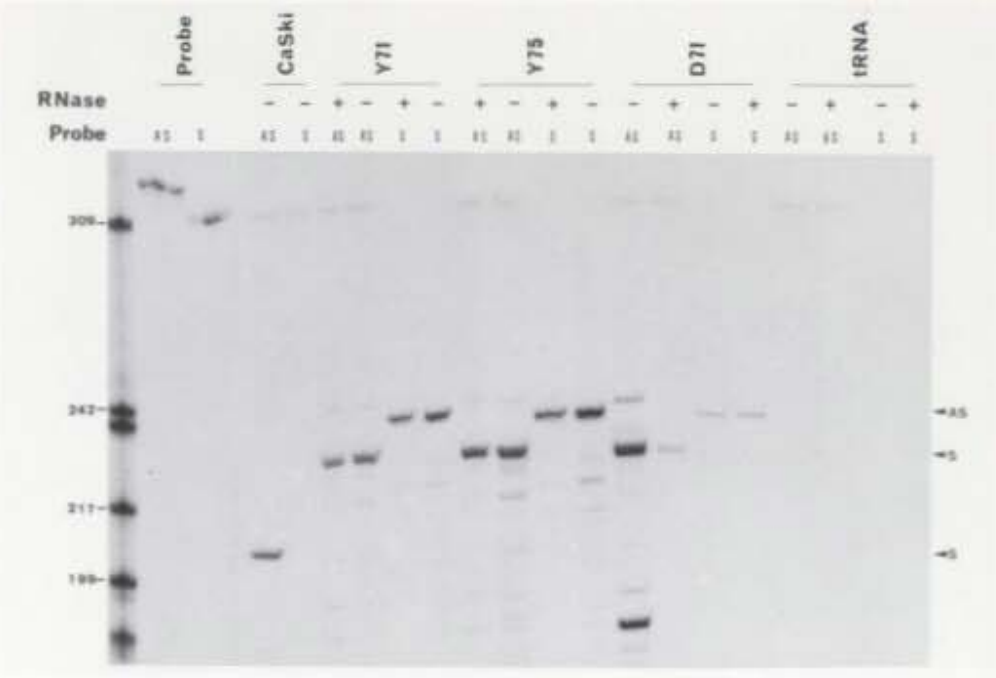
Modulation of expression of specific genes by AS RNA is dependent upon hybridization of the AS RNA to its target sequences present in specific pre-mRNA and mRNA. The formation of such hybrids with pre-mRNA and mRNA has been shown to interfere with the processes required for gene expression (Helene and Toulme, 1990; Takayama and Inouye, 1990). This interference may occur at any or all of the levels of RNA processing and mRNA transportation and translation and mRNA metabolism. The AS strategy has also been used to modulate the expression of high risk HPV E6 and E7 genes (von Knebel-Doeberitz et al., 1988; 1992; Helene and Toulme, 1990, Takayama and Inoue, 1990; Storey et al., 1991). Because the results presented in chapter 2 have indicated an important role for the nt 880 splice donor site for the expression of E7 RNA, the possible interference with splicing

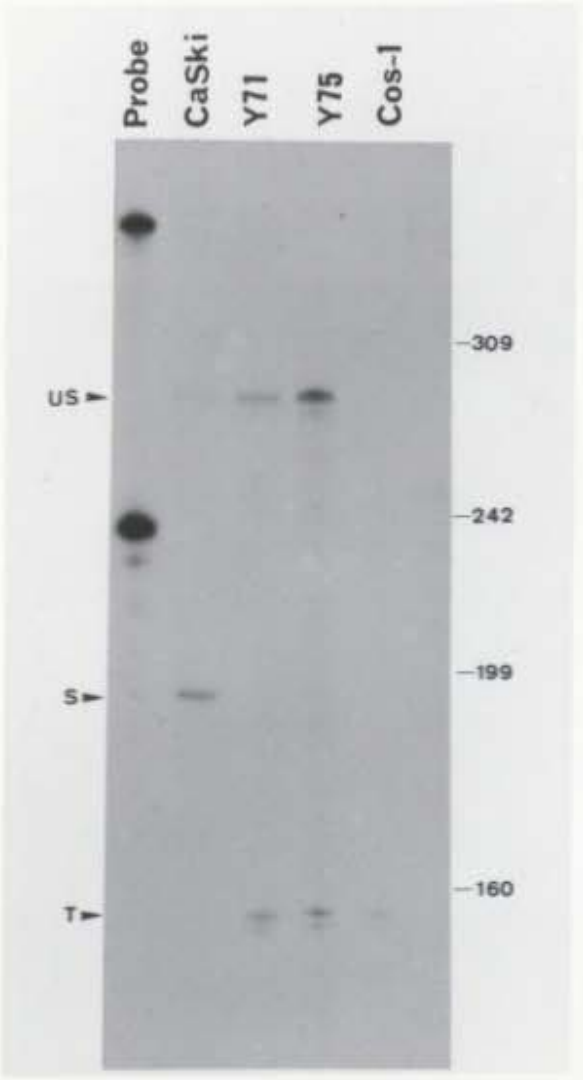
Figure 3.9 Double RNase protection experiments with the E7 probe.

The experiments were performed as described in materials and methods. The E7 probes correspond to both strands of the StuI (nt 5190 of SV40) to SspI (nt 720 of HPV-16) fragment of pY71. The lane labels are: +, RNA treated with RNase T1 before hybridization; -, RNA not pretreated with RNase T1. Protected S and AS E7 fragments are indicated by arrowheads. Conditions and other abbreviations are as in figure 3.3.

Figure 3.10 RNase protection experiments with the E7/E1 probe.

The results are shown for 5 μg of total RNA from CaSki cells and Cos-1 cells transfected with the indicated plasmids and analyzed by AS E7/E1 probe. The T antigen which is constitutively expressed in Cos-1 cells was used as an internal control. The E7/E1 and the T antigen probes correspond to nt 720-955 of HPV-16 and 3072-3225 of SV40, respectively. Control lanes and lanes for RNA from CaSki cells and transfected Cos-1 cells are shown on top for: Probe, a mixture of undigested E7/E1 (upper band) and T antigen probes (lower band); CaSki, RNA from CaSki cells; Cos-1, RNA from Cos-1 cells. Molecular size markers are shown on the right side. Protected E7 and T antigen fragments are indicated by arrowheads. Labels are: US, unspliced E7 RNA; S, spliced E7 RNA; T, internal control T antigen protected fragment.


Probe
CaSki
Y71
Y75
D71
tRNA
RNase
Probe
309
242
217
199
US
S


Probe
CaSki
Y71
Y75
Cos-1
US
S
T
309
242
199
160

of S RNA by AS RNA was evaluated by RNase protection assays, with the E7/E1 nt 720-955 region probe. For CaSki cells, which does not produce AS RNA, a significant proportion of S RNA was spliced. For the plasmids, pY71 and pY75, that produce high levels of AS RNA, all of the S RNA and the major proportion of S RNA remained unspliced, respectively (Fig. 3.2; 3.10). Further, an inverse correlation between the levels of spliced RNA and the AS RNA was observed for the plasmids pY71 and pY75. Less of the RNA was spliced at the nt 880 splice site for the plasmid, pY71, which expressed high level of AS RNA relative to the S RNA. For the pY75 plasmid, which expressed lower level of AS RNA compared with the S RNA, a slightly higher proportion of RNA was spliced (Fig. 3.2).

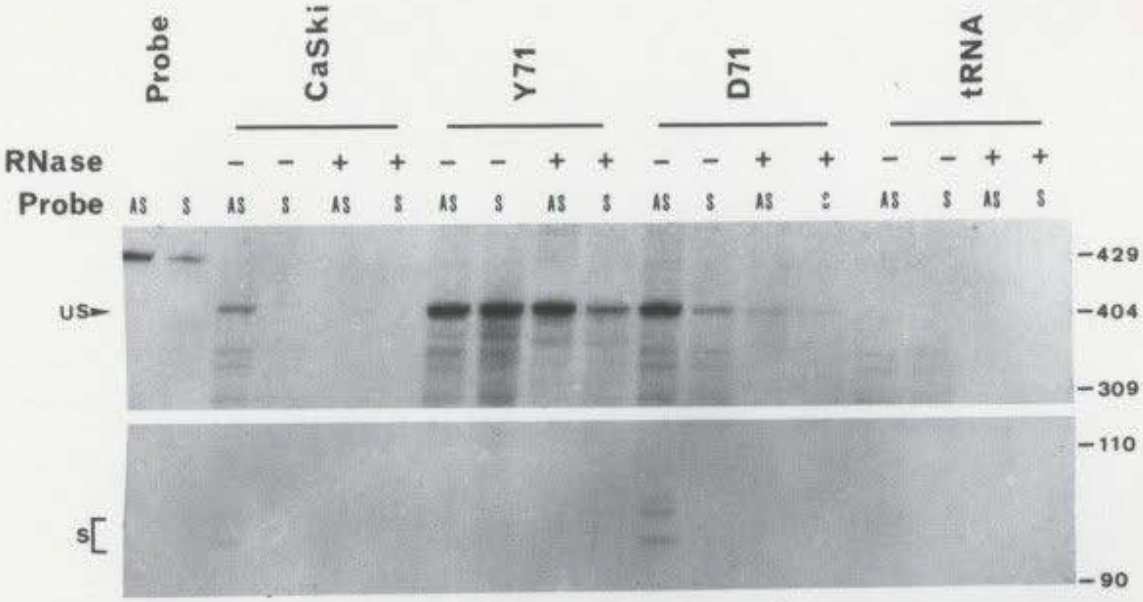
The possibility that the nt 880 splice site and the flanking sequences present in the S RNA hybridized with the AS RNA and formed dsRNA was addressed by double RNase protection assays, with the nt 792-1179 E7/E1 region probe. For this probe, the relative intensities of signals for the protected unspliced and spliced RNA fragments do not clearly reflect the proportions of spliced and unspliced RNAs, because fewer labelled U residues are present in the protected spliced RNA compared with the unspliced RNA. For CaSki cell and the pD71 plasmid transfected Cos-1 cell RNAs not treated with RNase T1, splicing at the nt 880 splice site was observed. Consistent with the results of the double RNase protection experiments

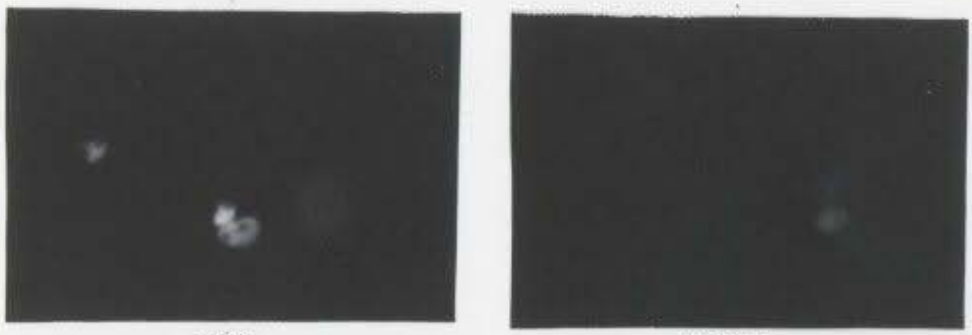
Figure 3.11A Double RNase protection experiments with the E7/E1 probe.

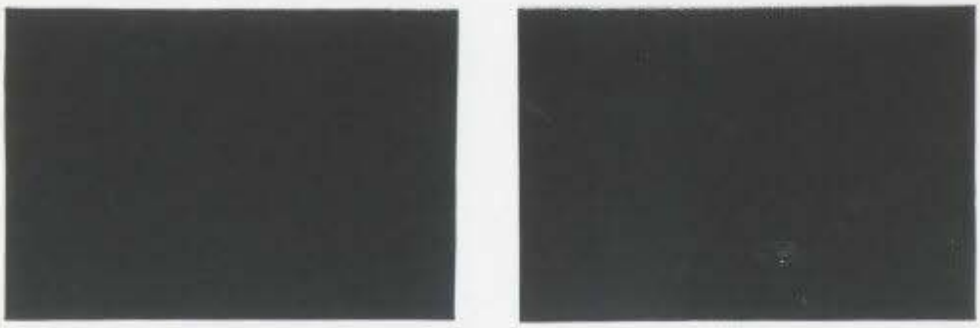
The experiments were performed as described in materials and methods. The probe corresponds to nt 792-1179 of HPV-16. The lane labels are: +, RNA treated with RNase T1 before hybridization; -, RNA not pretreated with RNase T1. S and US are abbreviations for the protected spliced and unspliced RNAs. Conditions and other abbreviations are as in figure 3.3.

Figure 3.11B Immunofluorescence experiments.

CaSki cells and Cos-1 cells transfected with the indicated plasmids were analyzed for the E7 protein specific immunofluorescence with an anti E7 monoclonal antibody. Labels are: D71, Cos-1 cells transfected with the plasmid pD71; Y71, Cos-1 cells transfected with the plasmid pY71; Y7SA, Cos-1 cells transfected with the plasmid pY7SA; CAT, Cos-1 cells transfected with the plasmid pSV2Cat. What might appear to be the fluorescence for the pSV2Cat plasmid (CAT) is a photographic artefact. No signal was detected in the original prints.


Probe
CaSki
Y71
D71
tRNA
RNase
Probe
AS
S
US
S
-429
-404
-309
-110
-90


D71
Y7SA


Y71
CAT

with E7 probes, treatment of RNA from CaSki cells with RNase T1 prior to hybridization to the probe resulted in total degradation of E7/E1 RNA (Fig. 3.11A). For the pD71 plasmid-transfected Cos-1 cell RNA, similar treatment with RNase T1 prior to hybridization to the probe resulted in degradation of most of the S E7/E1 RNA. For the pY71 plasmid, which produced high level of AS RNA, no spliced RNA was detected. Further, approximately comparable levels of unspliced E7/E1 S and AS RNAs remained RNase resistant. The S RNA detected in the RNase resistant dsRNA was not spliced (Fig. 3.11A). Considered together, these results suggest that the AS RNA interferes with splicing of the S RNA at the nt 880 splice site by forming an RNA duplex with the S RNA.

3.3.8 Interference with synthesis of E7 protein by AS RNA

Several experimental studies have indicated that the AS RNA targeted to the cap site and the 5' untranslated region of mRNA efficiently interferes with translational initiation (Helene and Toulme, 1990; Takayama and Inouye, 1990). The HPV-16 AS RNA detected for the HPV-16 expression plasmids was complementary to the 5' and 3' regions and the coding region sequences of the E7 RNA and formed RNA duplex with the E7 S RNA (Fig. 3.9). To examine the possible interference with the synthesis of E7 protein by AS RNA, immunofluorescence experiments were performed on Cos-1 cells transfected with the plasmid, pY71, which produces high levels of the S and AS RNA

and the plasmids, pD71 and pY7SA, which produces high levels of S RNA, but low levels of AS RNA and the control plasmid, pSV2Cat. The Cos-1 cells transfected with the plasmids, pD71 and pY7SA, showed distinct nuclear staining for the E7 protein. The nucleoli were clearly excluded (Fig. 3.11B). Similar nuclear staining for the E7 protein has been reported earlier (Sato et al., 1989a). That the nuclear staining for the plasmids, pD71 and pY7SA, was specific was demonstrated by the absence of staining for the negative control plasmid, pSV2Cat. Under similar experimental conditions, no E7 protein was detected for the pY71 plasmid (Fig. 3.11B). The inverse correlation between the AS RNA expression and the E7 protein synthesized for the plasmids, pY71, pD71 and pY7SA, suggests that the AS RNA may interfere with the synthesis of E7 protein.

3.4 DISCUSSION

In genital HPVs, the majority of transcripts are initiated from a promoter located in the LCR, immediately upstream of the E6 ORF. Recently an internal promoter was detected in the E6 early region of the low risk HPVs (Chow et al., 1987a; 1987b; Nasseri et al., 1987; Smotkin et al., 1989). Presence of an internal promoter within the E6-E7 region of HPV-16 has been suggested by several recent studies (Rohlfs et al., 1991; Higgins et al., 1992). My studies

provide evidence for the presence of a novel promoter within the genome of HPV-16. Because of its orientation with respect to the major promoter p97, I have termed this novel promoter the AS promoter.

The evidence for the presence of the AS promoter within the genome of HPV-16 comes from the plasmids, pY71, pY7SA and pSV2HPA2. The AS RNA was detected for the plasmid pY71. No AS E7 transcripts were detected for the plasmid pY7SA, in which the AS promoter was not present, confirming the presence of the AS promoter within the HPV-16 sequences (Fig. 3.1). The AS promoter containing sequences cloned downstream of the Cat gene in the plasmid, pSV2HPA2, was transcriptionally active and gave rise to AS Cat transcripts (Fig. 3.5). In contrast to the AS E7 RNA, the levels of AS Cat RNA was always lower compared with the S Cat RNA (Fig. 3.5). This could be related to the differences in the stability of AS Cat and AS E7 RNAs.

By using various deletion mutants, the AS promoter was localized to a region between nt 4031 and 4338 (Fig. 3.3; 3.4). Progressive deletions extending towards the E7 ORF from the nt 1176 resulted in a gradual increase in the levels of AS E7 RNA (Fig. 3.3). This could be related to the distance of the probe from the AS promoter. The AS RNAs may have heterogeneous ends resulting from random termination of AS transcripts a short distance from the AS promoter and lack of

3' end processing for these AS transcripts. The deletions in the pY713'Δ series of plasmids which brings the E7 probe sequences close to the AS promoter might have facilitated inclusion of E7 ORF sequences in the AS RNA by preventing random termination of AS RNA. In support of this, increased distance between the E7 probe sequences and the AS promoter for the pD71 plasmid resulted in very low levels of AS RNA. According to this model the deletions extending towards the AS promoter region for the pY713'Δ2 plasmid series were expected to increase the levels of AS RNA. However, no increase in the levels of AS RNA attributable to the progressive deletions in these plasmids were observed (Fig. 3.4). Although the deletions in these plasmids bring the AS promoter close to the probe sequences, the deletions might have resulted in the loss of transcription initiation sites, resulting in no net effect on the levels of AS RNA. In support of this hypothesis, several minor initiation sites were detected for the deleted regions of the pY713'Δ2 series of plasmids by primer extension analysis (Fig 3.6).

It was reported that the 5' ends of AS RNA detected in cervical squamous carcinoma cells were heterogeneous and mapped to the NCR and the 5' region of L1 ORF sequences (Vormwald-Dogan et al., 1992). These authors suggested that the production of AS RNA was a consequence of integration of the viral genome, since no AS RNA corresponding to the NCR,

E6-E7 and the L1 ORFs was detected in premalignant lesions harboring the HPV-16 genome in the episomal form. Inability to detect the AS RNA in premalignant lesions could be related to the lower levels of AS RNA and the low sensitivity of the in situ hybridization technique and the long distances between the AS promoter and the E6-E7, LCR and L1 region probes used in that study. Contrary to their findings and suggestion, AS E7 and E7/E1 RNAs were detected in the transient expression assay system in which the transfected plasmids remain predominantly episomal, indicating that the integration of the viral genome is not essential for the production of AS RNA. The 5′ ends of the AS RNAs were heterogeneous and found to be initiated over an AT-rich region around nt 4100. Several minor transcription initiation sites were detected (Fig. 3.6). This observation was further confirmed by RNase protection experiments with the nt 4107-4311 probe corresponding to the upstream sequences of this putative initiation sites. Very low levels of AS RNA, as compared with the S RNA, were detected for this probe (Fig. 3.7). The low levels of AS RNA seen with longer autoradiography may correspond to transcripts originating from the pBR322 vector sequences of the plasmid.

Approximately 30 nt upstream of the initiation site for the major AS RNA species was a TATA element which might represent the potential TATA box element for these AS transcripts (Seedorf et al., 1985). The TATA element binding

protein, TBP, has been shown to be required for the activity of RNA polymerase I, II and III promoters (Greenblatt, 1991; Rigby, 1993). Thus, the HPV-16 AS RNA could be transcribed by RNA polymerase I or II or polymerase III. RNA polymerase II generally requires the TATA box element. Presence of the TATA box element at an appropriate distance upstream of the major AS RNA transcription initiation site suggests that this polymerase is involved in the transcription of AS RNA.

The pD71 plasmid consistently expressed lower levels of AS E7 and E7/E1 RNA (Fig. 3.9, 3.11A). There are several possible explanations for this observation: (i) The AS RNA produced from this plasmid has termination sequences downstream of E7 and is not detectable by the E7 probe. (ii) The plasmid, pD71, possesses splice signals for the AS RNA, that are deleted from the plasmids of the pY7 series, and the AS RNA undergoes splicing. If the probe used to detect the AS RNA corresponded to the intronic region, it would only detect unspliced AS RNA. (iii) The downregulation of the AS promoter by the viral gene products E1 and/or E2 which are encoded in the pD71 plasmid could result in lower levels of AS RNA. (iv) Certain destabilizing sequences are present in the AS RNA produced from the plasmid pD71, but not for the plasmids of pY7-series. (v) As suggested earlier, the lower AS RNA levels for the pD71 plasmid could be related to the distance of the probe from the AS promoter.

Previous reports have indicated that the AS RNA is predominantly nuclear in cervical carcinoma cells (Higgins et al., 1991; Vormwald-Dogan et al., 1992). In contrast, the AS E7 RNA was detected in both the nucleus and cytoplasm of Cos-1 cells (Fig. 3.8, 3.9). Several possible reasons could underlie this discrepancy regarding the localization of the AS RNA. In cervical carcinoma cells, in which the viral genome is integrated with the host cell genome, the AS RNA may arise as a result of read-through transcription from flanking cellular promoter-enhancer sequences and are of high molecular weight. Such high molecular weight AS RNAs may not be transported efficiently to the cytoplasm. Some of the naturally occurring AS RNAs transcribed from the cellular promoters are high molecular weight RNAs of heterogeneous size distribution and localized to the nucleus (Khochbin and Lawrence, 1989; Khochbin et al., 1992; Dolnick, 1993). Because the HPV-16 AS RNA detected in Cos-1 cells are transcribed from the viral AS promoter, it is less likely that this AS RNA accumulates in the nucleus as high molecular weight RNA. The cytoplasmic location of the HPV-16 AS RNA detected in Cos-1 cells could be related to the high copy number of the template DNA from which the AS RNA is transcribed in Cos-1 cells. The SV40 based E7 expression plasmids undergo amplification in Cos-1 cells.

My studies indicate that the AS RNA interferes with the expression of S RNA by forming an RNA duplex with the S RNA (Fig. 3.9, 3.11A, 3.11B). This interference due to AS RNA can occur at different levels such as, splicing, mRNA 3′ end formation, mRNA transportation, translation and RNA metabolism. Splicing and polyadenylation of pre-mRNA are important posttranscriptional steps essential for the production of mature mRNA. The efficiency of splicing of the nt 880 splice site was lower for the plasmids, pY71 and pY75, compared with the plasmid, pD71 (Fig. 3.2, 3.10, 3.11B). The formation of duplex RNA for the plasmids, pY71 and pY75, might exclude the assembly of spliceosomes over the splice site sequences. The duplex formation could prevent the base-pair interactions between the components of splicing machinery such as, U1 snRNP, U2 snRNP and U5 snRNP, and the cis-acting splice site sequences and other elements essential for the function of splice sites. It has been established that the binding of U1 snRNP to the 5′ splice site is essential for the assembly of spliceosome (Green, 1991). Binding of the U1 snRNP involves base-pair interactions between the first 9 nt of U1 snRNA and the 5′ splice site sequence. The duplex formation between the nt 880 5′ splice site and the flanking region sequences of S RNA and the AS RNA might prevent the binding of U1 snRNP to the nt 880 splice site. This interference with the binding of U1 snRNP and other essential splicing factors

might have resulted in reduced splicing for the plasmids, pY71 and pY75. Further support for this hypothesis comes from studies which have shown that the AS RNA interferes with efficient splicing of S RNA by forming an RNA-RNA duplex with the S RNA (Munroe, 1988; Munroe and Lazar, 1991). Very low levels of spliced product detected for the pY71 and pY75 plasmids could have resulted from splicing of unhybridized E7/E1 S RNA (Fig. 3.2). The other possibility for reduced splicing for the pY71 and pY75 plasmids is the suboptimal cryptic splice acceptor site sequences for these plasmids compared with the authentic splice acceptor sites for the plasmid, pD71. This is an unlikely possibility because, the deletion of the authentic splice acceptor sites did not affect transformation and RNA levels for the pY71 and pY75 plasmids compared with the pD71 plasmid.

The possible interference with the 3′ end formation for S RNA by AS RNA is considered now. In addition to reduced splicing, the efficiency of 3′ end formation was lower for the pY71 and pY75 plasmids (Fig. 3.7). This could be related to the formation of duplex RNA for the polyadenylation signal containing region of the S RNA. It is less likely that the AS RNA directly interfered with the process of 3′ end formation because, no AS RNA was detected for the nt 4107-4311 probe, which spans the early polyadenylation signal sequence containing region of the HPV-16 (Fig. 3.7). The other

possibility for the low efficiency of 3′ end formation for the pY71 and pY75 plasmids could be related to the reduced splicing for these plasmids. In support of this argument, it has been reported that for terminal exons containing the polyadenylation signal sequences, the splicing of the upstream splice sites increases the efficiency of 3′ end formation (Niwa et al., 1990). It is more likely that the reduced 3′ end formation for the pY71 and pY75 plasmids is a consequence of reduced splicing of the upstream splice sites for these plasmids. Thus, the AS RNA for the pY71 and pY75 plasmids might indirectly interfere with the process of 3′ end formation by interfering directly with splicing of the nt 880 splice site.

The transportation of mRNA from the nucleus to cytoplasm is an essential prerequisite for translation of mRNA. Some of the naturally occurring and experimentally produced AS RNAs have been shown to interfere with this step, resulting in accumulation of S RNA in the nucleus (Helene and Toulme, 1990; Takayama and Inouye, 1990). Irrespective of their ability to produce AS RNA, the S RNA for the pY71 and pY75 plasmids was detected in the cytoplasm (Fig. 3.8B). Only the single stranded AS RNA which did not hybridize with the S RNA was detected in in situ hybridization experiments, because the cells were not denatured prior to the addition of the probe. In in situ hybridization experiments for the plasmid pY71,

high level of S RNA was detected in the cytoplasm and there was no evidence for the accumulation of S RNA in the nucleus. Thus, it is unlikely that the HPV-16 AS RNA interfered with the transportation of S RNA.

For the pY71 plasmid-transfected Cos-1 cells, no E7 protein was detected by immunofluorescence experiments. Distinct nuclear staining was detected for the plasmid pD71 and pY7SA-transfected Cos-1 cells. A similar pattern of nuclear staining has been reported for Cos-1 cells transfected with SV40 early promoter-enhancer-based E7 expression plasmids (Sato et al., 1989a). For CaSki cells, a similar nuclear staining pattern was observed. However, the staining was not reproducible in all the experiments. The difficulty in demonstrating the E7 protein for CaSki cells by immunofluorescence has been shown to be due to the low level of E7 expression compared with the SV40 promoter containing E7 expression plasmid-transfected Cos-1 cells and the masking of the E7 epitopes in the nucleus of CaSki cells (Kanda et al., 1991). The cytoplasmic location of the AS RNA, the hybridization of the AS RNA with the E7 S RNA to form RNA duplex and the inverse correlation between the level of AS RNA and the E7 protein synthesis suggested that the AS RNA interfered with synthesis of E7 protein.

The AS RNA has been shown to destabilize S RNA (Hildebrandt and Nellen, 1992; Nellen et al., 1992). The effect of AS RNA on the stability of S RNA may be due to

degradation of dsRNA by dsRNA-specific RNases. The dsRNA-specific activity called unwindase, has been shown to induce A to I conversion in dsRNA (Bass and Weintraub, 1987; Rebagliati and Melton, 1987). Such modifications alter the stability of dsRNA (Bass and Weintraub, 1988; Kimelman and Kirschner, 1989). The unwindase activity is present in a variety of cell types including epithelial cells, which are the natural target cells for HPV-16 (Wagner et al., 1990). Although the HPV-16 AS RNA formed the RNA-RNA duplex with the S RNA, the AS RNA-induced destabilization of S RNA was not apparent. This could be due to the lower stability of AS RNAs. Heterogeneous 5′ and 3′ ends for AS RNAs might be an indication of their lower stability.

Evaluating the different mechanisms by which the AS RNA could interfere with gene expression, it is more likely that the HPV-16 AS RNA interfered with the expression of the E7 gene by interfering with splicing and/or polyadenylation and/or translation of E7 S RNA. Polyadenylation has been shown to enhance the stability and translatability of mRNA (Huang and Gorman, 1990; Jackson and Standardt, 1990). My results indicated that the AS RNA interfered with polyadenylation of S RNA (Fig. 3.7). Further, the complementarity between the 5′ and 3′ flanking regions and the coding region sequences of the E7 S RNA and the AS RNA suggested the possible interference with translation of E7 S

RNA by AS RNA. Thus, the AS RNA could lower the expression of E7 directly by interfering with the translation of S RNA and indirectly by interfering with the process of polyadenylation.

Only the pD71 plasmid, but not the pY71 plasmid, expressed detectable levels of E7 protein in transiently transfected Cos-1 cells. Because the expression of E7 is essential for transformation of BRK cells, the pY71 plasmid which did not express detectable levels of E7 protein may be expected to be nontransforming. However, there were no significant differences in the transformation frequency for the pD71 and pY71 plasmids, although there were clear differences in the levels of E7 protein expressed for these plasmids (Fig. 2.1, 3.11B). The discrepancy between the level of E7 protein expressed and the transformation competency for the pY71 plasmid could be due to the presence of a low level of E7 protein expression for this plasmid that could not be detected by immunofluorescence. Because the epitopes of E7 protein are masked, it may not be possible to detect the E7 protein unless it is expressed at high levels (Greenfield et al., 1991; Kanda et al., 1991). A more sensitive technique such as immunoprecipitation might have detected the E7 protein for the pY71 plasmid. The other possibility for the observed discrepancy could be the differences in cell types and the assays used. Transient transfections were used for Cos-1 cells, whereas stable expression was used for transformation

of BRK cells. The transient transfections in Cos-1 cells did not depend on the integration of the transfected plasmid DNA into the cellular DNA and the pY71 plasmid expressed high levels of AS RNA, which interfered with the processing of E7 S RNA, resulting in low levels of E7 protein (Fig. 3.10, 3.11A, 3.11B). The stable expression in BRK cells, which resulted in transformation was dependent on integration of the transfected plasmid DNA with the cellular DNA. It is possible that during the process of integration, deletion of the AS promoter region sequences from the plasmid pY71 might have prevented the interference with the processing and translation of the E7 S RNA by AS RNA. This was shown to occur in CaSki cells in which the AS promoter had been deleted (Smits et al., 1991).

There is considerable evidence that HPV gene expression is closely coupled to the differentiation status of the host epithelial cells (Crum et al., 1988; Sterling et al., 1990; Dollard et al., 1992; Durst et al., 1992; Higgins et al., 1992; Meyers et al., 1992; Stoler et al., 1992). The activity of the AS promoter may also be coupled to the differentiation status of cells. Differential regulation of the AS promoter could have an influence on the growth properties of cells expressing high levels of AS E7 RNA. Consistent with this hypothesis, AS nucleic acids targeted to the E6-E7 genes suppressed the growth rate of HPV-16 and HPV-18 positive

transformed cells (von Knebel-Doeberitz et al., 1988; Crook et al., 1989; Storey et al., 1991; von Knebel-Doeberitz et al., 1992). Further, the AS promoter may be regulated by the viral early proteins. Thus, the AS RNA may either directly or indirectly regulate HPV-16 gene expression posttranscriptionally.

The significance of expression of the AS RNA to the life-cycle of the virus is not clear. Interfering with the expression of E7 does not appear to be the primary effect of AS RNA, because for the pD71 plasmid, which expresses the majority of the early region, very low level of AS E7 RNA was detected and this AS RNA did not inhibit the expression of E7 (Fig. 3.9, 3.11A, 3.11B). If all the early region ORFs such as E1, E2 and E4 are intact, the AS RNA may terminate randomly a short distance from the AS promoter within these sequences. Deletion of these sequences from the pY7 series plasmids could have resulted in the inclusion of E7 ORF sequences in the AS RNA. If this is the case, under the conditions of natural infection in which the complete viral genome is maintained in an episomal form, S transcripts corresponding to ORFs immediately downstream of the AS promoter are likely to be found in an RNA duplex. In support of this, AS RNA corresponding to the E4, E2 and E1 ORF were detected in several naturally occurring tumors (Higgins et al., 1991).

The dsRNA is a potent inducer of interferons (IFNs), which interfere with the virus life-cycle. The effect of IFNs

is mediated by IFN inducible genes. The DNA tumor viruses have evolved a mechanism to prevent the activation of IFN inducible genes. The adenoviral oncogene, E1A, has been shown to impair the formation of IFN inducible transcription initiation complex (Lengyel, 1993). It is possible that similar to E1A, the HPV-16 E7 may provide anti-IFN activity to the virus.

Accumulation of dsRNA might be important for the life-cycle of the virus. One of the mechanisms by which viruses such as, Epstein-Barr virus, adenoviruses and HIV, overcome the cellular defence mechanism, the global inhibition of protein synthesis, is by producing high levels of dsRNA or single stranded RNA with a potential to form double stranded secondary structure (Mathews and Shenk, 1991). High concentrations of dsRNA inhibit the activity of dsRNA dependent protein kinase (PKR), an important enzyme involved in the shut-off of the host's translational machinery (Hunter et al., 1975; Farrell et al., 1977; Hovanessian, 1989; Kostura and Mathews, 1989). Production of AS RNA by HPV-16 might be a mechanism to inhibit the activity of PKR. Recent studies have implicated PKR as a tumor suppressor gene (Clemens, 1992; Koromilas et al., 1992; Meurs et al., 1993). The outcome of inhibition of PKR provides distinct advantages to the virus: (i) the virus can overcome the translational inhibitory effects of PKR and (ii) inhibition of this candidate tumor suppressor gene can enhance the ability of the virus to induce deregulated proliferation of host cells.

CHAPTER 4

FUTURE DIRECTIONS

Posttranscriptional events play an important role for the expression of HPV-16 genes. This is reflected by the ability of the viral genome to produce a number of alternately spliced mRNAs from a limited number of primary transcript(s). It is possible that certain genes are selectively expressed or repressed by preferential utilization of specific splice sites. This can be best exemplified by the complex splicing pattern of transcripts corresponding to the early region ORFs, E6, E7, E1 and E2 (Fig. 1.1).

It has been reported that the splicing of the E6 intron, nt 226 splice donor site spliced to either nt 409 or 526 splice acceptor sites, upregulates the expression of E7 by stabilizing the E6-E7 mRNA and facilitating the translation of E7 (Smotkin et al., 1989; Sedman et al., 1991; results presented in chapter 2). The same splice donor site can also be used to downregulate the expression of E7. This can be achieved by skipping the E7 exon from the primary transcript by splicing the nt 226 splice donor site to nt 2708 or 3357 splice acceptor sites located downstream of E7 (Doorbar et al., 1990; Sherman et al., 1992).

The production of the E2 protein can be regulated by alternate splicing. The mRNA encoding the full length E2 protein is produced by splicing the nt 226 or 880 splice donor

sites to the nt 2581 or 2708, but not the 3357 splice acceptor sites (Nasseri et al., 1991; Rohlfs et al., 1991; Sherman and Alloul, 1992; Sherman et al., 1992). Another mechanism by which alternate splicing downregulates the activity of E2 is by producing transcripts encoding the E2 repressor. Splicing out the 5' region of E2 from the primary transcript of BPV-1 resulted in the production of mRNA encoding the E2 repressor. This truncated form of E2 protein interferes with the activity of full length E2 by competing for the E2 binding sites (Ham et al., 1991; McBride et al., 1991). Similar HPV-16 transcripts capable of encoding the E2 repressor can be generated by splicing the nt 1301 splice donor site to the nt 3357 splice acceptor site (Doorbar et al., 1990; Sherman et al., 1992). The full length E2 protein modulates the activity of the viral promoter, p97, and is required for the replication of the viral genome. Thus the regulated production of the full length E2 transactivator protein or the truncated E2 repressor protein by alternate splicing facilitates indirectly, regulation of the viral gene expression and replication. DNA replication is generally closely tied to transcription, but more so for the PV system, because the E2 transactivator protein has an important and direct role in replication of BPV and HPV genomes.

Another early region splice site, the activity of which may be important for the HPV-16 life-cycle is the nt 1301

splice donor site. Splicing of this site to the nt 5637 generates mRNA encoding the major capsid protein L1. The L1 protein can also be expressed from a double-spliced mRNA, the production of which depends on the utilization of the nt 1301 splice donor site (Doorbar et al., 1990; Sherman et al., 1992). The restricted expression of late genes to differentiating and differentiated keratinocytes could be related to the preferential utilization of the nt 1301 splice site in these cells.

The results presented in chapter 2 clearly demonstrated the role of the early region splice donor sites located at nt 226 and 880, for the expression of E6-E7 RNA. Both of the nt 226 and 880 splice sites have been shown to be utilized predominantly (Smotkin et al., 1989; Dilts et al., 1990; Doorbar et al., 1990; Sherman et al., 1992). For the whole HPV-16 plasmids mutated for the nt 880 splice donor site, the transforming activity was dependent on the presence of functional nt 1301 splice donor site. Deletion of both the nt 880 and 1301 splice donor sites resulted in loss of transformation, whereas deletion of only the nt 880 splice donor site severely reduced, but did not abolish transformation (Pater et al., 1992b). Although the role for the nt 1301 splice donor site for the expression of the early region of HPV-16 genes was evident, my studies did not investigate the role of this splice donor site in sufficient

detail. A mutational analysis of this splice site in the context of the wild type and mutant nt 226 and 880 splice sites would contribute to a greater understanding of the function of this splice site for posttranscriptional regulation of HPV-16 gene expression.

The complex pattern of the alternate splicing of HPV-16 transcripts may be regulated by the interaction of tissue enriched and tissue restricted *trans*-acting splicing factors with certain *cis*-acting elements present in the flanking exon-intron sequences of these alternately spliced splice sites. Presence of a specific splicing factor or differences in the abundance of ubiquitous splicing factors such as ASF/SF2, DSF and SR proteins could affect the choice of a splice site (Harper and Manley, 1991; Lamond, 1991; Zahler et al., 1993). The possible relation between the mechanism(s) and the process of splicing for the production of specific alternately spliced RNAs and the differentiation program of the target epithelial cells deserves further investigation. The possible correlation between the restricted expression of late genes to differentiating and differentiated keratinocytes and the utilization of the nt 1301 splice donor site for the production of spliced late region RNAs would be interesting to pursue further. The use of an *in vitro* splicing system should facilitate a better understanding of the mechanisms of complex splicing pattern of HPV-16 transcripts.

Several studies have indicated that the presence of a spliceable intron in the transcription unit promotes stabilization and accumulation of mature RNA (Buchman and Berg, 1988; Huang and Gorman, 1990; Choi et al., 1991; Palmiter et al., 1991). The results for the plasmids of pD73 and pCMV series confirmed this observation. For the pY7 plasmids series, the integrity of the nt 880 splice site, but not the actual process of splicing, was essential for the accumulation of E7 RNA. Similarly, for the accumulation of polyomavirus late region mRNAs, the splice sites, but not splicing, was essential (Barrett et al., 1990; Lanoix et al., 1990). The requirement for the functional splice sites but not the splicing process can be explained if the splice donor site sequences are also essential for function/s other than splicing. Recently, the compartmentalization of splicing machinery to discrete subnuclear foci was elegantly demonstrated (Fu and Maniatis, 1990; Spector, 1990; Carmo-Fonseca et al., 1991a; 1991b; Huang and Spector, 1991b; Spector et al., 1991). It was also suggested that the splicing of pre-mRNA occurs in these foci (Lawrence et al., 1989; Huang and Spector, 1991a; Wang et al., 1991; Carter et al., 1993; Xing et al., 1993). It is possible that the splice signals target the nascent transcripts to these splicing compartments. Transcripts with mutated splice sites may undergo degradation due to failure to reach these splicing

compartments. Appropriate experiments can be designed to test such hypotheses.

In chapter 3, I have provided evidence for the presence of an AS promoter within the genome of HPV-16. Similar to the major promoter, p97, the activity of the AS promoter may also be closely coupled to the differentiation program of the host epithelial cells. Further studies can be done to test whether the activity of the AS promoter is dependent on the cell type or the differentiation status of cells.

The promoter may be regulated by viral transcriptional transactivator proteins, such as E7 and E2. For the pD71 plasmid, the level of AS RNA was low. The low level of AS RNA expression for this plasmid could be related to the downregulation of the AS promoter activity by the viral E2 protein, which was encoded in the same plasmid.

The AS RNA appeared to interfere with the expression of E7 gene, possibly by blocking the splicing of E7 RNA at the nt 880 splice donor site and by interfering with the synthesis of E7 protein. The AS RNA may affect the production of other viral proteins such as, E5 and E2, by interfering with the production of mRNAs encoding these proteins. The E2 protein encoding mRNAs are produced by splicing of the nt 226 or 880 splice donor sites to the nt 2581 or 2708 splice acceptor sites. By forming an RNA duplex with these splice acceptor containing regions of the S RNA, the AS RNA may block the

splicing to these splice acceptor sites. Thus, the AS RNA may interfere with the production of the E2 protein. In addition, by forming an RNA duplex with the mRNA containing the E5 ORF sequences, the AS RNA may directly interfere with the translation of the E5 RNA. By regulating the production of E2, the AS RNA may indirectly affect the activity of the viral promoter p97. More studies are needed to determine whether the AS RNA undergoes posttranscriptional processing such as splicing and polyadenylation. It is also important to map the termination sites for these AS RNAs. Depending on the extent of overlap, the AS RNA may affect the splicing pattern of the early region transcripts.

The possible role of the AS RNA in overcoming the global translational inhibition, a protective cellular mechanism against the viral infection, can also be investigated. The cellular dsRNA dependent protein kinase, PKR, when activated by dsRNA, inhibits the initiation of translation by phosphorylating the α-subunit of the eukaryotic initiation factor, eIF-2. However, the activation of PKR is prevented by high concentrations of dsRNA (Hovanessian, 1989). Because it is likely that high levels of dsRNA accumulate during the life-cycle of HPV-16, the dsRNA may inhibit the activity of PKR and facilitate the complete life-cycle of the virus (Mathews and Shenk, 1991). In addition, inhibition of the activity of this tumor suppressor protein may enhance the oncogenic potential of HPVs (Clemens, 1992).

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