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Differential promoter utilization by the bovine papillomavirus in transformed cells and productively infected wart tissues

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Expression of the 'late' genes of bovine papillomavirus type 1 (BPV-1) occurs only in the differentiated keratinocytes of the productively infected fibropapilloma. A detailed analysis of viral transcription in the fibropapilloma was performed and compared to BPV-1 specific transcription in transformed C127 cells. A cDNA library was constructed from bovine fibropapilloma mRNA using the method of Okayama and Berg. Analysis of full length cDNAs showed that the majority of viral transcripts in the fibropapilloma have 5′ termini near nt 7250 and utilize a common splice donor site at nt 7385. This mRNA start site was confirmed by the combination of primer extension and nuclease S1 analyses; it is not utilized in the BPV-1-transformed C127 cell, thus identifying it as a wart specific, 'late' promoter. Upstream of this mRNA start site is a tandemly repeated sequence element homologous to the SV40 late promoter sequence GGTACCTAACC, which has been shown to be important for the efficient utilization of the SV40 major late start site. Two additional mRNA start sites at nt 7185 and nt 7940 in the long control region (LCR) were identified and were found to be used in bovine warts as well as in BPV-1-transformed mouse cells. The promoter region upstream of the nt 7940 mRNA start site contains the E2 responsive enhancer mapping between nt 7611 and nt 7805 [Spalholz,B.A., Lambert,P.F., Yee,C. and Howley,P.M. (1987) *J. Virol.*, in press]. The previously identified mRNA start sites at nt 89, 2433 and 3080 were confirmed in this study and are utilized in both the fibropapilloma and the BPV-1-transformed mouse cell. These analyses show that viral transcription in a productive wart virus infection differs from that in transformed cells by the activation of a strong 'late' or wart specific promoter and by the use of a 'late' or wart specific polyadenylation site in addition to the early polyadenylation site.

Key words: papillomavirus/promoter/transcription/cDNA/fibropapilloma

Introduction

The papillomaviruses are small DNA viruses which induce squamous epithelial or fibroepithelial tumors in their natural hosts. Both animal and human papillomaviruses have been associated with squamous cell carcinomas. The bovine papillomavirus type 1 (BPV-1) has been the best studied of the papillomaviruses at a molecular level. BPV-1 induces cutaneous fibropapillomas in cattle and fibroblastic tumors in certain heterologous hosts including hamsters. It is capable of *in vitro* transformation of mouse cells, in which the viral DNA is maintained as a stable multicopy extrachromosomal plasmid (Law *et al.*, 1981). Late gene expression and virus production occur only in the terminally differentiated keratinocytes of the fibropapilloma (Amtmann and Sauer, 1982; Engel *et al.*, 1983). To date no one has obtained virus production with any papillomavirus in *in vitro* cell culture systems. The genetic analysis of BPV-1 has therefore been achieved through the combination of recombinant DNA technology and the analysis of *in vitro* transformation of rodent cells.

The entire 7945 bp circular double-stranded DNA genome of BPV-1 has been sequenced (Chen *et al.*, 1982). Analysis of this DNA sequence revealed that all of the open reading frames (ORFs) greater than 400 bp in size are located on one strand, suggesting that all transcription would be derived from one strand. Analyses of the RNAs from BPV-1 transformed cells and from fibropapillomas have confirmed this prediction (Engel *et al.*, 1983; Heilman *et al.*, 1982). Transcription of the BPV-1 genome in transformed cells has been analyzed in detail by cDNA cloning, by electron microscopy and by nuclease S1 protection (Stenlund *et al.*, 1985; Yang *et al.*, 1985a). Three potential viral promoters have been previously identified which are active in the transformed mouse cells; RNA start sites have been located at nt 89, nt 2443 and nt 3080. Multiple species of mRNA are then produced in BPV-1-transformed cells by differential splicing (Stenlund *et al.*, 1985; Yang *et al.*, 1985a). All species utilize a single polyadenylation site at nt 4203. There is no evidence of stable transcripts from the late region in BPV-1-transformed cells. Initial analysis of late viral transcription in a bovine fibropapilloma revealed a class of mRNAs which use a polyadenylation site at nt 7170 and a probable splice acceptor site at nt 5609. A detailed analysis of the splice patterns and promoter usage in the fibropapilloma has not been previously done, however.

The functions of the BPV-1 ORFs have been analyzed by recombinant DNA techniques and *in vitro* cell culture assays. The E5 and E6 ORFs have been shown to encode transforming proteins (DiMaio *et al.*, 1986; Groff and Lancaster, 1986; Rabson *et al.*, 1986; Schiller *et al.*, 1984, 1986; Yang *et al.*, 1985a,b). The E6 and E7 ORFs are involved in the maintenance of high plasmid copy number (Berg *et al.*, 1986; Lusky and Botchan, 1985). The 3′ E1 ORF encodes a factor required for plasmid replication and maintenance (Sarver *et al.*, 1984; Lusky and Botchan, 1985, 1986) and the 5′ E1 ORF apparently encodes a factor which acts as a modulator of viral DNA replication (Lusky and Botchan, 1986; Roberts and Weintraub, 1986). The E2 ORF encodes a factor which interacts with a conditional enhancer in the long control region (LCR) to activate transcription (Spalholz *et al.*, 1985; Yang *et al.*, 1985b). Recently it has been shown that the E2 protein is a DNA-binding protein with specificity for sequences with the core $ACCN_6GGT$ (Androphy *et al.*, 1987; Moskaluk and Bastia, 1987). The late ORF L1 encodes the major capsid protein (Engel *et al.*, 1983; Pilacinski *et al.*, 1984). No functions have been found yet for the BPV-1 E3, E4 or E8 ORFs. Although the product of the BPV-1 L2 ORF has not yet been identified, the L2 ORF of HPV-1a encodes a minor capsid protein (Komly *et al.*, 1986). The HPV-1a E4 ORF encodes an abundant cytoplasmic protein that appears to be ex-

pressed coordinately with the major capsid protein (Doorbar *et al.*, 1986).

In this study we present a detailed analysis of cDNA clones obtained from a BPV-1 fibropapilloma cDNA library. The promoter utilization in the fibropapilloma was determined by combined primer extension and nuclease S1 analyses. This usage is compared and contrasted with that in the BPV-1 transformed cell and that in the fibromatous portion of the fibropapilloma.

Results

Analysis of a bovine fibropapilloma cDNA library

A cDNA library was constructed from bovine fibropapilloma RNA using the eukaryotic expression vector developed by Okayama and Berg (1982, 1983). An initial screening of ~11 000 colonies with a BPV-1-specific probe showed that ~1.3% of the cDNA clones contained BPV-1 cDNA inserts. Of these, only 20% contained cDNAs mapping to the late region of BPV-1. The first 144 consecutively selected BPV-1 specific cDNA clones were analyzed in detail. Cleavage of the cDNA insert from the vector and agarose gel electrophoresis of the products was used to determine the approximate size of the cDNAs. All 144 clones were initially analyzed by hybridization to subgenomic fragments of the BPV-1 cloned into M13 to determine the presence of spliced exons. These M13 clones represented segments of nearly the entire genome and had each been previously sequenced (Chen *et al.*, 1982). The majority (>60%) of cDNAs from the transforming region were very short (<1100 bp) and appeared to be unspliced. These cDNAs were too short to have been transcribed from any known BPV-1 promoter and therefore were considered likely to be truncated cDNAs. The remaining 29 clones with cDNA inserts longer than 1100 bp were subjected to detailed restriction endonuclease cleavage analysis. Eight cDNAs with insert sizes of ~1100 bp hybridized to probes from the LCR and could be cleaved by *Mlu*I (nt 7352), indicating the presence of an exon not previously identified in the analysis of RNAs from BPV-1-transformed C127 cells. Sequence analysis revealed that the cDNA 5′ termini for these eight clones were heterogeneous and mapped to a short region between nt 7246 and nt 7255, suggesting that there is an RNA start site in the vicinity of nt 7250. Each of these eight

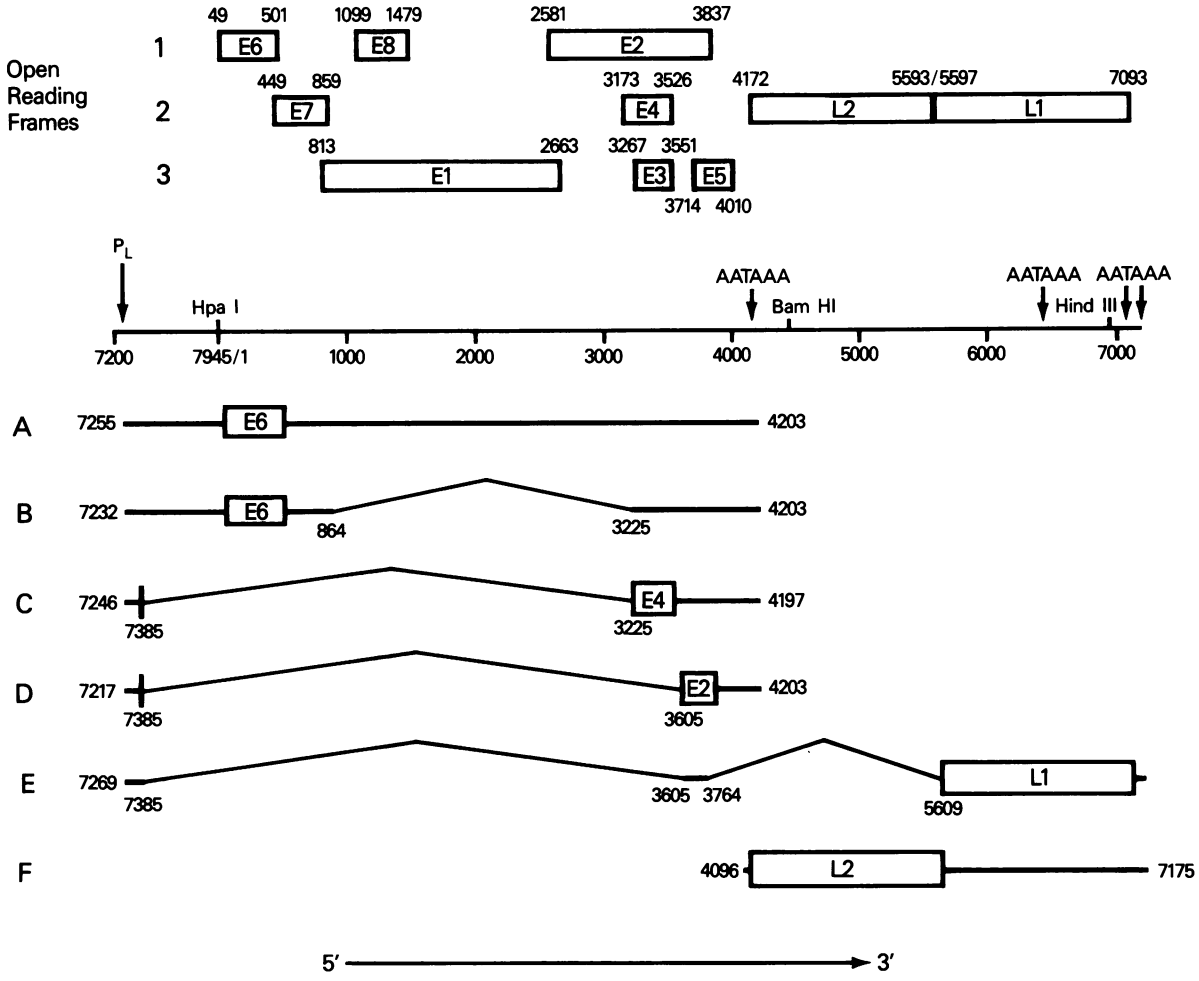

Fig. 1. Summary of bovine fibropapilloma specific cDNAs. The top half of the figure shows a linearized map of the genomic organization of bovine papillomavirus type 1. The major ORFs are depicted by open boxes and are labeled with numbers and the letters E or L. The E ORFs are located within the 69% transforming segment of the BPV-1 genome. The L ORFs map within a region only transcribed in the differentiated keratinocytes of a bovine fibropapilloma. The numbers at the 5′ and 3′ ends of the ORFs refer to the first nucleotide of the ORF and to the nucleotide preceding the stop codon, respectively. The arrow labelled P_L indicates the positon of the major late promoter. AATAAA indicates the positions of all potential polyadenylation signals determined from the coding strand (Chen *et al.*, 1982). The structure of six classes of fibropapilloma mRNAs (designated A–F) were determined from the sequence of cDNA clones and are shown schematically in the lower half of the figure. The nucleotide numbers indicate the first and last nucleotide of each exon. The open boxes indicate the first major ORF for each species and correspond to the ORFs shown at the top of the figure.

cDNAs used a splice donor at nt 7385; a complete analysis of these cDNAs (designated class C) is presented in Figure 1.

Several of the cDNAs had 5′ termini mapping around nt 3080 and likely represent unspliced mRNAs transcribed from the nt 3080 promoter (P_{3080}) previously identified by Stenlund *et al.* (1985) (data not shown). No cDNAs were found in the initial 144 clones which had 5′ termini mapping around nt 89 or near nt 2443, sites of RNA initiation previously mapped in BPV-1 transformed C127 cells (Stenlund *et al.*, 1985; Yang *et al.*, 1985a).

LCR-specific hybridization probes were used to screen ~100 000 additional clones from the cDNA library for cDNA clones containing the wart specific leader (nt 7250–7385). An additional four classes of cDNAs containing this leader were found and representative clones were sequenced. The results are summarized in Figure 1.

Structure and coding capacity of fibropapilloma cDNAs

Six classes of wart specific cDNAs, five of which combined the wart specific leader, were isolated from the fibropapilloma cDNA library and were analyzed. The structures of the cDNAs shown in Figure 1 represent only the longest cDNA in each class. For most classes, however, many shorter and apparently truncated cDNAs were also found. Each of the cDNAs which have been analyzed could be placed in one of these six classes. It could not be ruled out, however, that truncated cDNAs with 5′ termini downstream of the other early region promoters were not transcribed from these promoters rather than from the wart specific promoter near nt 7250.

The Class A cDNA was the longest cDNA (4894 bp) isolated from the library. It had its 5′ terminus at nt 7255 and its 3′ terminus at nt 4203. This polyadenylation site was the same for all the cDNAs from the early region and is the same polyadenylation site used exclusively in BPV-1-transformed C127 cells (Yang *et al.*, 1985a). This cDNA was unspliced and contained a very long 5′ noncoding region upstream of the first major ORF (E6). Since translation of this mRNA would be inefficient, we conclude that this species probably represents an unspliced nuclear precursor.

The class B cDNA had the same 5′ noncoding region upstream of the E6 ORF and may represent a splicing intermediate. The 5′ terminus was at nt 7232 and it was polyadenylated at nt 4203. The splice donor and acceptor of this cDNA (nt 864 and nt 3225) are identical to those used by the class I and IV BPV-1-transformed cell cDNAs described previously (Yang *et al.*, 1985a).

The most abundant RNA species present in the fibropapilloma was represented by cDNA class C. This cDNA species has 5′ termini between nt 7246 and nt 7255 and a 3′ terminus at nt 4197 (one clone sequenced). Although the exact site of polyadenylation differed slightly from the other classes in the one cDNA in which the 3′ terminus was sequenced, the same polyadenylation signal (AAUAAA) was used. The utilization of a splice donor at nt 7385 and an acceptor at nt 3225 spliced a short (135 nt) leader onto the 3′ early region exon. This late leader contains four AUG translation initiation codons. The first three initiation codons are reasonably strong by rules determined by Kozak (1986), but if used would generate only short polypeptides (14–26 amino acids); the first two of these short ORFs would be terminated before initiation at the fourth AUG. The third short ORF does not terminate within the leader. The fourth AUG (nt 7379) is in frame with the E4 ORF yielding a fusion ORF which encodes a 103 amino acid polypeptide. This AUG is a very poor translation initiation codon by Kozak rules, indicating that translation of E4 may be either inefficient or under additional translational control. This late leader contributes two amino acids to the E4 protein and thus is a coding exon.

The class D cDNA had a 5′ terminus at nt 7217 and polyadenylation site at nt 4203. Splicing linked the late leader to the 3′ early region at a splice acceptor at nt 3605. This splice acceptor has not been previously described. The fourth AUG in the leader described above is in frame with the 3′ portion of the E2 ORF. This species could encode an 80-amino-acid protein consisting of the C-terminal 78 amino acids of the E2 protein and two amino acids from the late leader. It is known that the E2 ORF encodes a *trans*-activator of transcription in BPV-1 (Spalholz *et al.*, 1985,1987) and it has recently been shown that E2 is a DNA-binding protein with specificity for sequences in the viral regulatory region (Androphy *et al.*, 1987; Moskaluk and Bastia, 1987). Although the precise DNA-binding domain of E2 has not yet been delineated, it is possible that the shortened E2 protein encoded during productive infection may have DNA-binding properties and may be involved in the regulated control of transcription during the life cycle of the virus.

Two different classes of cDNAs were isolated which could potentially encode the major capsid protein (L1). The class E cDNA spliced the late leader onto a second exon in the E2 region (nt 3605–3764) which was then spliced onto the L1 region at nt 5609. The 3′ terminus of this particular cDNA was at nt 7077, immediately upstream of a run of 16 As in the BPV-1 genomic sequence and not downstream of a polyadenylation signal. All other late region cDNAs which have been sequenced had the 3′ terminus of the cDNA insert at nt 7175. The major polyadenylation site for the late region mRNAs at nt 7175 was confirmed by nuclease S1 analysis (data not shown). Therefore it is most likely that synthesis of this particular cDNA was primed by oligo(dT) hybridization to this run of genomic As rather than to the As of the poly(A) tail. The first AUG in frame with the L1 ORF was at nt 5609 and is a strong translation initiation codon. The predicted size of the L1 protein is 515 amino acids, consistent with the observed size of 55 kd for the major capsid protein (Engel *et al.*, 1983; Pilacinski *et al.*, 1984). The fourth AUG in the late leader, however, was in frame with the 3′ part of the E2 ORF, which through a splice donor at nt 3764 and a splice acceptor at nt 5609 was in frame with a short ORF other than L1. This potential translation frame spanning all three exons would be predicted to encode a 72-amino-acid protein. However, the AUG in the late leader which would be predicted to initiate the synthesis of this protein is weak by the rules determined by Kozak (1986); thus it is not known whether or not this ORF is translated.

The only cDNA species in our analysis which could encode the L2 protein was class F and was rare. The 5′ terminus of this cDNA was at nt 4096, upstream of the first L2 AUG (nt 4187). The cDNA was unspliced and used the polyadenylation site at nt 7175. Both the L2 and L1 ORFs were present, so the L1 protein could potentially be encoded also by this species if it acted as a bicistronic mRNA. Very few molecules in this class were examined and it is not known if the 5′ terminus at nt 4096 maps to the vicinity of the mRNA 5′ terminus for a unique class of RNAs, or if this cDNA represents a truncated cDNA. Although the sequence TATATAC exists at nt 4073 and might serve as part of a second late transcriptional promoter, it is possible that this species of cDNA is truncated and is derived from RNAs

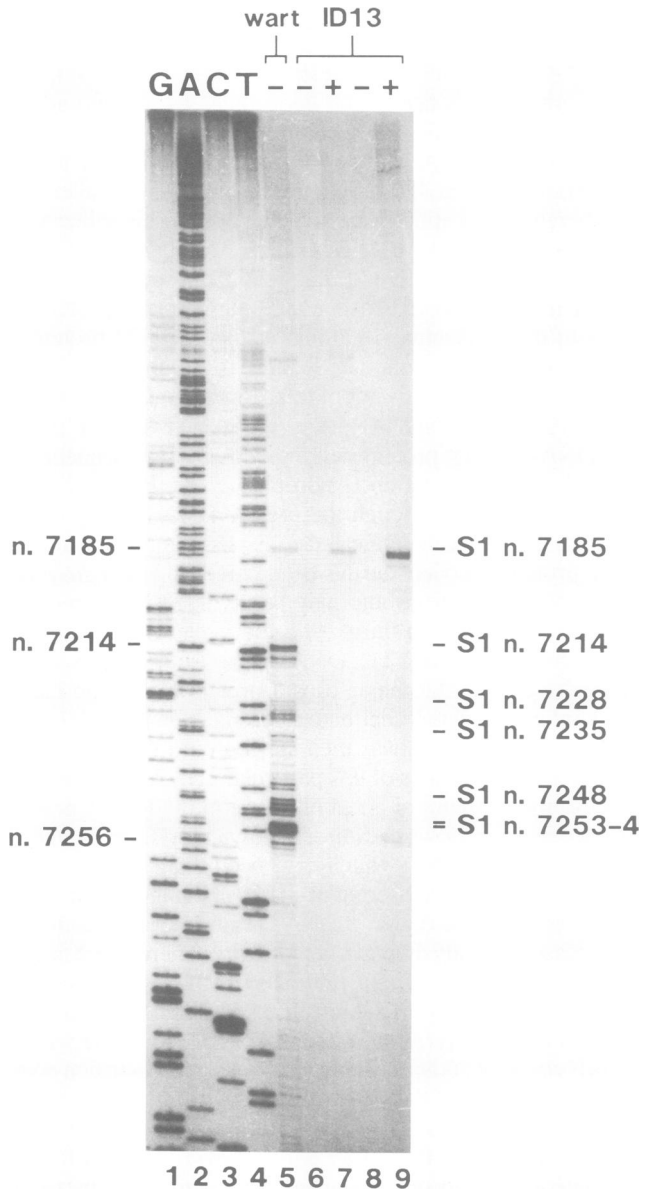

Fig. 2. Primer extension and nuclease S1 analysis of the major late promoter region. Primer extension analysis, using a 20-base oligonucleotide with 5′ end at nt 7356, was carried out with polyadenylated RNA (0.75 μg) from the fibropapilloma (**lane 5**) or ID13 cells (**lanes 6** and **7**) and total cellular RNA (7.5 μg) from ID13 cells (**lanes 8** and **9**). The +/− at the top of the lanes indicates whether or not the cells were treated with cycloheximide prior to harvesting of the RNA. The primer extension products were separated on an 8% sequencing gel adjacent to dideoxy sequencing markers (lanes labeled G, A, C and T) generated with the same synthetic oligonucleotide and single-stranded LCR DNA (**lanes 1–4**). The nucleotide numbers to the left of the autoradiograph indicate the nucleotide positions of the boundaries of the late promoter initiation region and the position of the P_{7185} RNA start site. The nucleotide numbers to the right of the autoradiograph indicate the positions of the major nuclease-S1-resistant fragments generated with the corresponding S1 probe DNA (data not shown).

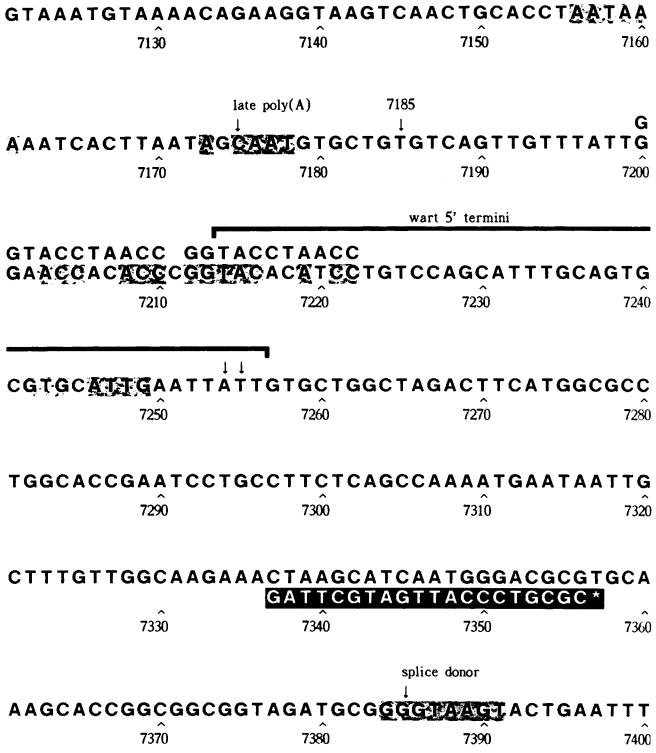

Fig. 3. Sequence of the late promoter region. The sequence of the late promoter region of BPV-1 is shown. The positions of the P_{7185} and P_L transcription start sites as well as the late polyadenylation site and late leader splice donor site are indicated above the sequence. The late promoter initiation region extends from nt 7214–7256 and is indicated by a heavy bar over the sequence. The two arrows at nt 7254–5 indicate the two most frequently utilized RNA start sites. Sequences matching the AATAAA polyadenylation signal (nt 7156–7161), and sequences highly homologous to the CCAAT box consensus (nt 7173–7178), SV40 late promoter sequence (nt 7200–7210 and 7212–7222) (Brady *et al.*, 1982,1984), enhancer core sequence (nt 7242–7249) (Weiher *et al.*, 1983), and the mapped splice donor (nt 7384–7391) are shown shaded in gray. The eleven base sequence element of the SV40 late promoter is indicated above each of the homologous BPV-1 sequences (nt 7200–7210 and nt 7212–7222). The oligonucleotide used for primer extension analysis is shown highlighted in black and * refers to the 5′ [^{32}P]phosphate.

transcribed from either the LCR late promoter or one of the other promoters defined in the transforming region.

Analysis of the major late promoter region

The 5′ termini of the cDNA inserts for classes A–E cDNAs mapped between nt 7217 and nt 7269, suggesting that there might be a fibropapilloma specific promoter in this region. The actual 5′ termini of the mRNAs from a fibropapilloma were determined by primer extension and nuclease S1 analysis. A 20-nt-long synthetic oligonucleotide (5′ nt 7356) complementary to RNA sequences upstream of the splice donor at nt 7385 was labeled with ^{32}P at its 5′ end, annealed to polyadenylated RNA, and extended with reverse transcriptase. The products were separated on a sequencing gel alongside a dideoxy sequencing ladder generated with the same 5′ labeled oligonucleotide and single-stranded BPV-1 LCR DNA cloned into M13 (Figure 2). This type of analysis permitted the direct determination of the nucleotide positions of the 5′ ends of the RNAs. The lane labeled 'wart' shows a prominent set of primer extension products mapping RNA 5′ termini between nt 7214 and nt 7256. Nuclease S1 analysis produced a similar set of products, confirming that these positions represent the actual 5′ termini of RNAs in the fibropapilloma. This indicates the presence in this region of a transcriptional promoter with multiple initiation sites. An additional RNA 5′ terminus mapped to nt 7185; however, species of RNA with this 5′ terminus were much less abundant in the wart tissue than those RNAs with 5′ termini between nt 7214 and nt 7256. Similar analyses were performed on RNA from ID13 cells with or without treatment with cycloheximide

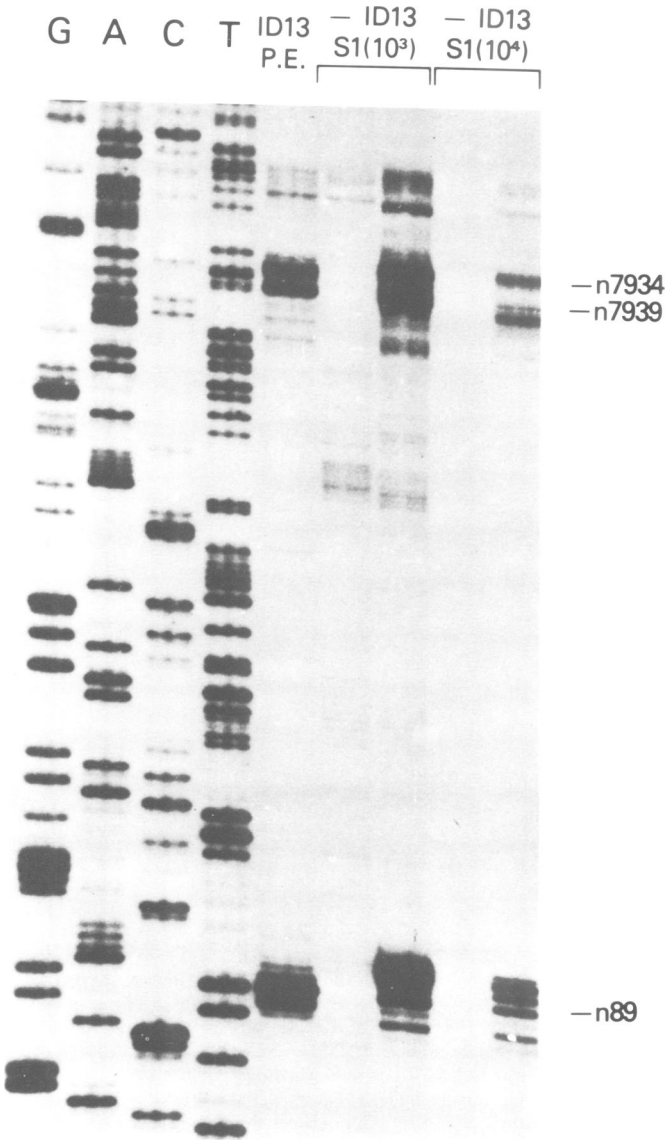

Fig. 4. Primer extension and nuclease S1 analysis of the P_{7940} and P_{89} promoter region. Total cellular RNA (7.5 μg) from ID13 cells treated with cycloheximide prior to extraction was analyzed by primer extension and nuclease S1 using a 5′ labeled oligonucleotide with 5′ end at nt 208 and S1 probe DNA generated with this oligonucleotide. The reaction products were run on an 8% polyacrylamide–urea gel along side a dideoxy sequencing ladder (lanes labeled G, A, C and T) generated with the same oligonucleotide primer. Primer extension and S1 reactions are indicated by P.E. and S1, respectively. The (–) above S1 lanes indicates the control reaction done with carrier RNA only. The nuclease S1 analyses were done using 10^3 and 10^4 units S1 per reaction (lanes labeled 10^3 and 10^4, respectively). Nucleotide positions of the ends of the most prominent primer extension and nuclease S1 products are indicated at the right of the figure.

(cycloheximide treatment indicated by + in Figure 2). Cycloheximide treatment of ID13 cells has been shown to produce a 10- to 100-fold enrichment in BPV-1-specific polyadenylated RNA (C.C.Baker and Y.-C.Yang, unpublished results; Kleiner *et al.*, 1986). The RNA species with a 5′ terminus at nt 7185 could also be detected in ID13 cells treated with cycloheximide, but was 10- to 100-fold less abundant without cycloheximide treatment. Little if any RNA with 5′ termini between nt 7214 and nt 7256 could be detected in ID13 cells, even after cycloheximide treatment. These data indicate that there are two promoters in this region of the LCR which are controlled independently.

Table I. Analysis of relative BPV-1 promoter utilization in BPV-1 infected tissues and transformed cells

RNA source	P_L	P_{7185}	P_{7940}	P_{89}	P_{2443}	P_{3080}
Wart poly(A) plus	5+	2+	1+	2+	3+ (2+)	3+
Wart epidermis total	4+	1+	1+	2+	3+ (2+)	3+
Fibroma total	–	–	2+	3+	4+ (2+)	4+
ID13 – CH total	+/–	1+	3+	4+	4+ (2+)	3+
ID13 + CH total	1+	4+	4+	5+	4+ (2+)	4+

The values 1+ to 5+ indicate the relative steady-state levels of RNA transcripts from each of the promoters. The numbers are relative to the level of RNA from the late promoter. The relative scale (1+ to 5+) is non-linear and represents a range of ~1000-fold as estimated by the intensity of primer extension products from gels similar to those shown in Figures 2, 6 and 7. –, No transcripts were detected from the promoter. Values in parentheses for P_{2443} refer to the unspliced mRNA only. CH indicates cycloheximide treatment.

The promoter with RNA start sites between nt 7214 and nt 7265 produces significant levels of RNA only in the epithelial part of a fibropapilloma. This promoter is not used in either ID13 cells (Figure 2) or in the fibroma portion of the fibropapilloma (Table I). Since the activity of this promoter is limited to those tissues which also express late viral products (capsid proteins and virus) and since the mRNA encoding the capsid protein is transcribed from this promoter, we have named this promoter the major late promoter (P_L). Its control would appear to be linked to the state of differentiation of the keratinocyte. In contrast, the promoter with RNA start site at nt 7185 (P_{7185}) is active at low levels in both the fibropapilloma and ID13 cells, although the level of activity is quite variable. Whether or not P_{7185} directs the synthesis of RNAs with small coding exons in the LCR is currently under investigation.

The DNA sequence of the late promoter region is presented in Figure 3. It is of interest that two copies of an 11-nt sequence element which each match eight of 11 nucleotides of the SV40 late promoter element GGTACCTAACC map to the region upstream of the late 5′ start sites. This element has been shown by Brady *et al.* (1982, 1984) to be important for the specification and efficient utilization of the SV40 major late RNA start site. The presence of two tandemly arrayed copies of this element in BPV-1 may be responsible for the heterogeneity of the RNA start site of the BPV-1 late promoter. A CCAAT box is present at nt 7173–7178 and overlaps the polyadenylation site for late region RNAs (nt 7175). A core enhancer sequence (Weiher *et al.*, 1983) is indicated from nt 7242 to nt 7249, although its physiologic significance is untested at this point. Also of interest is the proximity of plasmid maintenance sequence I (PMS I) to the late promoter; the region of homology between PMS I and PMS II is nt 7103–7171 (Lusky and Botchan, 1984).

Analysis of the P_{7185} region reveals a potential TATA box at nt 7155–7161, 30 nucleotides upstream from the RNA start site at nt 7185. It is of interest that overlapping this TATA box is the AATAAA polyadenylation signal for the late region RNAs.

Mapping of a new promoter upstream of nt 89

Analysis of BPV-1 transcription in transformed C127 cells by Yang *et al.* (1985a) and Stenlund *et al.* (1985) suggested that at least three viral promoters were active in the BPV-1-transformed cell. RNA start sites for these promoters were mapped to nt 89, nt 2443 and nt 3080. We refer to these promoters as P_{89}, P_{2443} and P_{3080}, respectively. Primer extension analyses using oligonucleotides with 5′ termini at nt 208, nt 2505, nt 2551 and nt 3178 were carried out to assess the steady-state level of

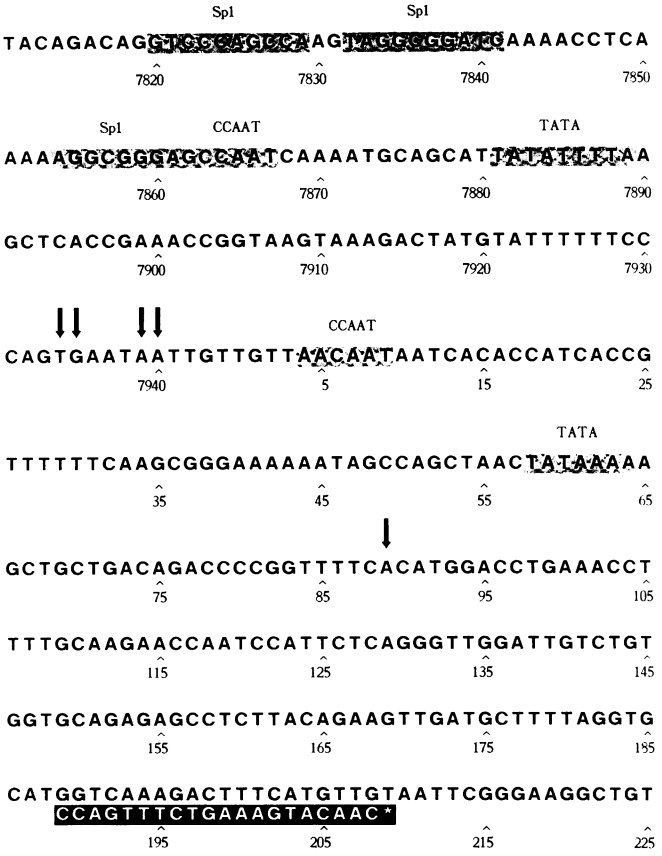

Fig. 5. Sequence of the P_{7940} and P_{89} promoter regions of BPV-1. The sequence of the region in BPV-1 containing the P_{7940} and P_{89} promoters is shown. The location of the major transcriptional start sites as defined by primer extension and nuclease S1 analyses are indicated by the heavy down arrows. The sequences homologous to the SP1 binding site consensus sequence, sequences similar to the CCAAT box consensus sequence, and the TATA box consensus are shown shaded in gray. The synthetic oligonucleotide primer is shown highlighted in black and the * refers to the 5′ [^{32}P]phosphate.

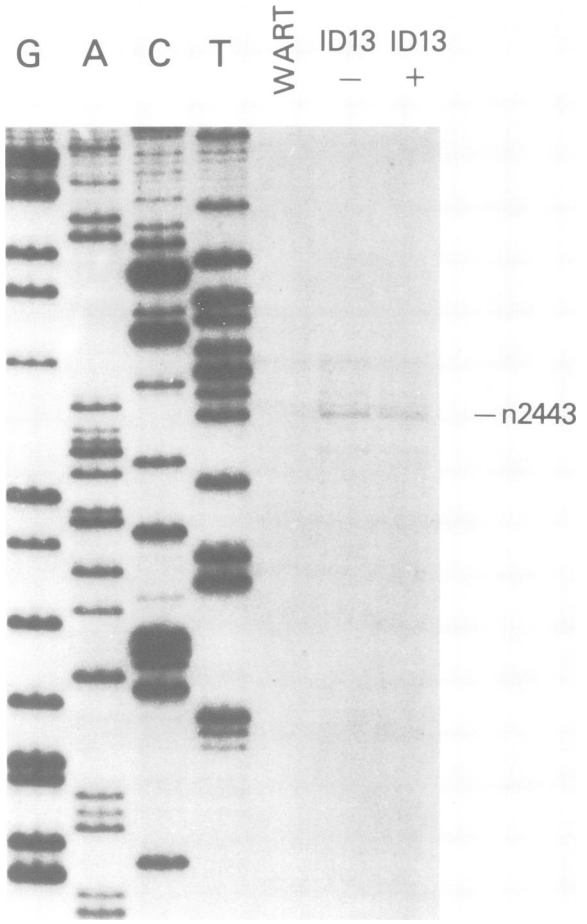

Fig. 6. Primer extension analysis of the P_{2443} promoter region. Primer extension analysis of polyadenylated RNA (0.75 μg) from a fibropapilloma (labeled wart) or total RNA (7.5 μg) from ID13 cells was carried out using a 20-base oligonucleotide primer (nt 2551−2532) ^{32}P-labeled at the 5′ end. The ID13 RNA was isolated from cells which were either treated (+) or untreated (−) with cycloheximide. Products were separated on 8% polyacrylamide−urea gels along with dideoxy sequencing reactions (labeled G, A, C and T) as markers. The number to the right of the autoradiograph indicates the nucleotide position of the 3′ end of the most prominent primer extension product.

RNA transcribed from each of these promoters in the fibropapilloma relative to the level of RNA transcribed from the major late promoter (P_L). In addition the abundance of RNA from each of these promoters in the fibropapilloma was compared to the abundance in BPV-1-transformed cells.

Primer extension analysis of RNA from ID13 cells treated with cycloheximide was carried out using the nt 208 oligonucleotide (Figure 4). In addition to the expected set of primer extension products mapping around nt 89, there were two prominent doublets at nt 7934−5 and nt 7939−7940, with approximately one-third the intensity of the nt 89 products. Nuclease S1 analysis using a single-stranded DNA probe 5′ labeled at nt 208 and spanning the entire LCR confirmed that the positions mapped by primer extension at nt 7934−5 and nt 7939−40 corresponded to authentic RNA 5′ termini (Figure 4). We refer to the promoter directing the synthesis of these RNAs as P_{7940}. The same primer extension products were detected in the analysis of RNA from ID13 cells not treated with cycloheximide and were approximately one-tenth the intensity of the products obtained with RNA from cells treated with cycloheximide (Table I). Therefore cycloheximide affected the relative amounts of the RNAs from these promoters but did not result in the activation of additional promoters. RNA from fibropapillomas was also examined using the nt 208 oligonucleotide (Table I). P_{7940} and P_{89} produced approximately the same steady-state levels of RNA in ID13 cells as they did in the fibromatous part of a fibropapilloma, but produced only ~10% of these levels in the epithelial portion of the fibropapilloma. In the epithelial part of the fibropapilloma, P_L-derived transcripts were ~100-fold more abundant than those from either P_{7940} or P_{89} (Table I).

The DNA sequence of the P_{7940} and P_{89} promoters is presented in Figure 5. The positions of the major RNA start sites are indicated by large arrows. Upstream of nt 89 is a TATA box (nt 58−65) and a CCAAT box (nt 4−9). Further upstream of the CCAAT box are the start sites for P_{7940}. A TATA box (nt 7881−7890) and a CCAAT box (nt 7861−7867) are also found upstream of these start sites. Three potential SP1 binding sites (Dynan and Tjian, 1983; Gidoni *et al.*, 1984) are present at nt 7820−7829, nt 7832−7841 and nt 7853−7862. Immediately upstream of the SP1-binding sites is the LCR conditional enhancer (nt 7611−7805) (B.A.Spalholz, P.F.Lambert, C.Yee and P.M.Howley, in preparation). It has been shown that both P_{7940} and P_{89} are *trans*-activated by the E2 gene product through this conditional enhancer (Spalholz *et al.*, 1987).

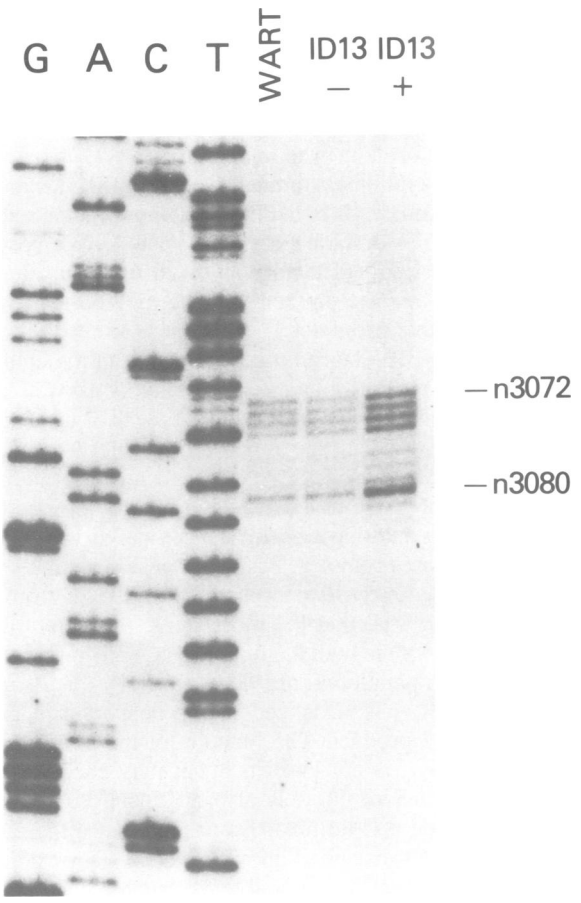

Fig. 7. Primer extension analysis of the P_{3080} promoter region. Primer extension analysis of polyadenylated RNA (0.75 μg) from a fibropapilloma (labeled wart) or total RNA (7.5 μg) from ID13 cells was carried out using a 20-base oligonucleotide primer (nt 3178–3159) ^{32}P-labeled at the 5′ end. The ID13 RNA was isolated from cells which were either treated (+) or untreated (−) with cycloheximide. Products were separated on 8% polyacrylamide–urea gels along with dideoxy sequencing reactions (labeled G, A, C and T) as markers. The numbers to the right of the autoradiograph indicate the nucleotide positions of the 3′ end of the upper and lower most primer extension products.

Relative activities of the P_{2443} and P_{3080} promoters

The transcription mapping of BPV-1-transformed C127 cells by Yang *et al.* (1985a) and Stenlund *et al.* (1985) revealed two species of RNAs produced from P_{2443}; one species is unspliced and likely encodes the full E2 product, and the other species is spliced (nt 2505–3225) and could potentially encode the E5 product. Two oligonucleotides with 5′ termini at nt 2505 and nt 2551 were used to confirm the RNA start site at nt 2443 and to quantitate the relative abundance of these two RNAs. RNAs with the nt 2443 start site were detected in both the fibropapilloma and in the ID13 cells (Figure 6). The quantity of RNA from P_{2443} was approximately the same in ID13 cells and in the fibromatous portion of the fibropapilloma, but was reduced somewhat (~30%) in the epithelial portion of the fibropapilloma (Table I). The unspliced RNAs which could encode the full E2 gene product represented only about 10% of the RNAs generated by this promoter; the remaining 90% of the RNAs were spliced (Table I). There is no significant effect of cycloheximide on levels of RNA from P_{2443}. Therefore P_{2443} may not be under the transcriptional control of a short-lived *trans*-repressor or *trans*-activating factor.

BPV-1-transcription mapping in ID13 cells by Stenlund *et al.* (1985) revealed an unspliced RNA species with a 5′ terminus near nt 3080. The existence of several potential RNA start sites between nt 3020 and nt 3080 was demonstrated by nuclease S1 analysis (Stenlund *et al.*, 1985). We used an oligonucleotide with 5′ terminus at nt 3178 to confirm the mapping of the P_{3080} RNA start site and to quantitate the RNA from this promoter (Figure 7). Primer extension analysis revealed a set of primer extension products with ends mapping between nt 3072 and nt 3080 (Figure 7). Some additional products were noted as far upstream as nt 3012 (not shown). These positions correspond with those mapped by nuclease S1 by Stenlund *et al.* (1985), indicating that the P_{3080} promoter has a very heterogeneous RNA start site. Comparison of different primer extension analyses revealed that transcripts from P_{2443} and P_{3080} are the most abundant BPV-1 transcripts in the fibromatous portion of the fibropapilloma (Table I). In ID13 cells, transcripts from P_{3080} are less abundant than those from either P_{89} or P_{2443} and show only a 2- to 3-fold increase in abundance after cycloheximide treatment (Figure 7). This increase is consistent with the 2-fold RNA stabilization effect of cycloheximide seen by Kleiner *et al.* (1986) and suggests that P_{3080} is not likely to be under the transcriptional control of a short-lived *trans*-acting factor.

Discussion

The papillomaviruses produce epithelial and fibroepithelial tumors in animals and man. BPV-1 produces fibropapillomas in cattle and can transform rodent cells *in vitro*. Vegetative viral DNA synthesis, late gene expression, and virus production are limited to the differentiating keratinocytes of a fibropapilloma and have not been detected in cell culture systems. Detailed genetic and transcriptional studies of BPV-1 have thus far been limited to BPV-1-transformed mouse cells. This study presents a detailed comparison of transcription both in transformed cells and in a productively infected bovine fibropapilloma.

There are two major qualitative differences between BPV-1 transcription in the fibropapilloma and in the transformed cell. First, a wart specific promoter (P_L) in the LCR is activated in epithelial cells and produces steady-state levels of RNA which are 10- to 100-fold more abundant than levels of RNA derived from any other BPV-1 promoter. This promoter is responsible for transcription of the mRNA for the major capsid protein encoded by the L1 ORF. Second, the late region is expressed in the fibropapilloma through the use of a fibropapilloma-specific polyadenylation site at nt 7175. Analysis of transcription in nuclei isolated from ID13 cells has shown that most of the late region is transcribed, but that transcription appears to terminate just upstream of the late polyadenylation site (C.C.Baker, unpublished results). It is possible that transcription from the late promoter (P_L) is necessary to read through the termination region and allow utilization of the late polyadenylation site. Use of the P_L does not mandate the use of the late polyadenylation site, however, since the majority of late promoter transcripts in the fibropapilloma utilize the early polyadenylation site at nt 4203. Alternatively, attenuation of termination upstream of the late polyadenylation site may involve factors specific for differentiated keratinocytes. It is also possible that BPV-1 DNA amplification during vegetative viral DNA synthesis could result in the effective titration of a termination factor(s).

Although late region RNAs are transcribed from the late promoter, the late promoter produces predominantly RNAs from the transforming region. These RNAs are unique to the

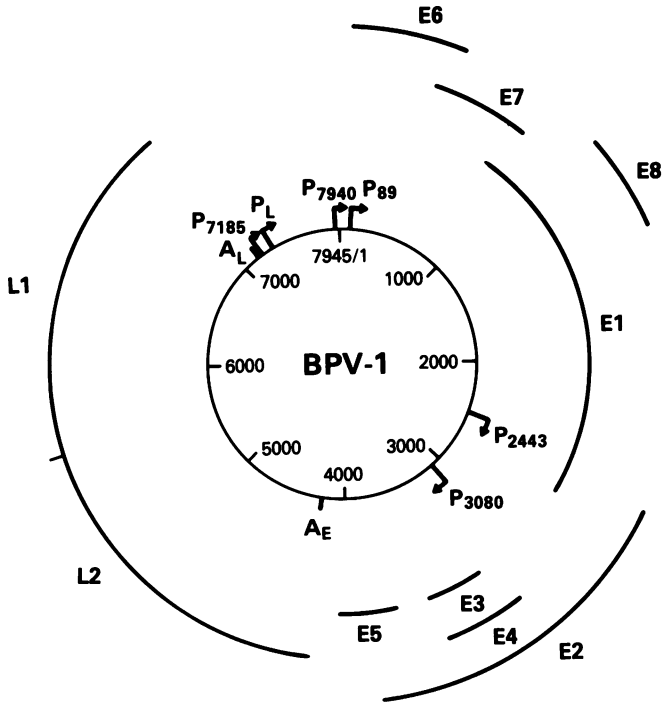

Fig. 8. The promoters of BPV-1. A circular representation of the BPV-1 genome is shown. Numbers within the circle are nucleotide positions. The known promoters of BPV-1 are indicated by P followed by the approximate nucleotide position of the RNA start site except for the late promoter which is designated by P_L. Polyadenylation sites for the early and late regions are indicated by A_E and A_L, respectively. The locations of the major ORFs are indicated by the heavy arcs outside the genomic circle.

fibropapilloma. The most abundant of these is the E4 mRNA (class C; Figure 1), suggesting that the E4 protein is actually a late viral protein. This is consistent with the data of Doorbar *et al.* (1986) indicating that the E4 protein of HPV-1a is most abundant in those cells of an HPV-1a-induced wart which are also producing L1 protein. An E4 cDNA has also been shown to be the most abundant species isolated from a cDNA library made from an HPV-11 condyloma (L.Chow and T.Broker, personal communication). We should point out, however, that E4 may also serve as a domain of a protein in transformed cells; a cDNA species with the E6 ORF fused in frame to the E4 ORF was identified in a cDNA library made from BPV-1-transformed mouse cell RNA (Yang *et al.*, 1985a).

A wart-specific early region mRNA with a potential regulatory product is the C-terminal E2 encoding species (class D; Figure 1). The full E2 ORF is known to encode a transcriptional regulatory factor which activates the LCR conditional enhancer (Spalholz *et al.*, 1985). The E2 protein has recently been demonstrated to be a specific DNA-binding protein (Androphy *et al.*, 1987; Moskaluk and Bastia, 1987). It is possible that proteins which contain only a portion of the E2 ORF, either alone or fused to other ORFs, may also have a transcriptional regulatory function and that this function may differ from that of the full-length E2 protein. Such regulatory functions could be mediated through the same element in the LCR responsive to the full E2 gene product, or alternatively through interactions with other regulatory elements or factors.

Three new promoters in the LCR of BPV-1 have been identified (P_{7185}, P_L and P_{7940}) (Figure 8). In addition, the RNA start sites at nt 89, nt 2443 and nt 3080 have been confirmed. The major late promoter (P_L) is the only promoter which is specific to the fibropapilloma. This promoter is not active in the fibromatous portion of the fibropapilloma and is therefore epithelial-cell specific. Its activity is likely to be specific for the differentiated epithelial cells. The promoter is not solely under the negative regulation of short-lived repressor in BPV-1-transformed cells since it shows minimal stimulation by the protein synthesis inhibitor cycloheximide. It is likely that this promoter requires activation by cellular or viral regulatory factors. We postulate that cellular transcriptional factors involved in the program of terminal differentiation in the keratinocyte may be involved in the activation of this promoter.

All of the other BPV-1 promoters are active in the epithelial and fibromatous portions of a fibropapilloma as well as in BPV-1-transformed cells. Two short cDNAs with 5′ termini near nt 3080 were identified in the fibropapilloma library and may represent full length transcripts from P_{3080}. cDNAs with 5′ termini at nt 7185, nt 7940, nt 89 or nt 2443 were not identified, but these classes would have been rare compared with species derived from the late promoter. Primer extension analyses were used to measure the steady-state levels of RNA derived from each promoter and suggested that the most active promoters (other than the late promoter) in both the fibromatous and epithelial portions of the fibropapilloma are P_{2443} and P_{3080}. A minor transcript from P_{2443} is unspliced and probably encodes the *trans*-activating E2 product. The function of the major spliced transcript from P_{2443} is not known, but it may encode the E5 protein. The product of P_{3080} is also an unspliced transcript which could encode a C-terminal E2 product since there is an ATG at nt 3091. As discussed above, since it shares domains with the full E2 product, it is possible that such a gene product could also have a transcriptional regulatory function. Recent experiments by P.F.Lambert, B.A.Spalholz and P.M.Howley (submitted for publication) have demonstrated that cDNAs directing the synthesis of the E2 C-terminal domain may have such a function.

Materials and methods

Cells and tissue

BPV-1-transformed C127 cells (ID13 cells) (Law *et al.*, 1981) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cycloheximide treatment was performed by addition of cycloheximide to the medium at a final concentration of 25 μg/ml, incubation at 37°C for 3 h, and the harvesting of cells for RNA preparation. Bovine fibropapilloma tissue was obtained from fibropapillomas induced by inoculation with the same strain of BPV-1 (isolate #307) sequenced by Chen *et al.* (1982). The tissue was separated into epithelial and fibromatous components by blunt dissection.

Preparation of RNA

Total cellular RNA was isolated from ID13 cells (BPV-1-transformed C127 cells) and from previously quick-frozen bovine fibropapilloma tissue by the method of Chirgwin *et al.* (1979). Polyadenylated RNA was prepared by selection on oligo(dT)-cellulose for the fibropapilloma RNA by the method of Aviv and Leder (1972) or by selection on mAP paper (Amersham) for ID13 RNA using procedures supplied by the manufacturer.

Construction of cDNA library

A eukaryotic expression cDNA library was constructed from polyadenylated RNA from bovine fibropapilloma and the vectors pcDV1 and pL1 using the plasmid primer method described by Okayama and Berg (1982, 1983) with the following modifications. First strand cDNA synthesis conditions were as follows: 50 mM Tris–HCl pH 8.0, 8 mM $MgCl_2$, 30 mM KCl, 5 mM DTT, 0.5 units/ml RNasin, 100 μg/ml actinomycin D, 2 mM dNTPs, 600 c.p.m./pmol [α-^{32}P]-dCTP, 133 μg/ml polyadenylated RNA, 75 μg/ml vector-primer DNA, and 12.5 units avian reverse transcriptase/μg RNA. The reaction was allowed to proceed at 37°C for 30 min. The remainder of the procedure was according to the protocols in the above references. Enzymes were from the following suppliers: avian reverse transcriptase (Seikagaku America), RNasin (BRL), terminal

transferase (Pharmacia), *Escherichia coli* DNA ligase (Pharmacia), *E. coli* DNA polymerase I (Boehringer Mannheim), and *E. coli* RNase H (Pharmacia). Bacterial transformation was carried out using *E. coli* strain DH1 following the procedure of Hanahan (1983) or using competent *E. coli* strain HB101 from BRL.

Isolation and characterization of BPV-1 cDNA clones

BPV-1-specific cDNA clones were identified by colony hybridization (Maniatis *et al.*, 1982) using nick-translated agarose gel purified BPV-1 DNA probes (Rigby *et al.*, 1977). All BPV-1-specific cDNA clones were initially characterized by restriction endonuclease digestion and slot blot hybridization as follows. Miniprep plasmid DNA (Maniatis *et al.*, 1982) was digested with *Xho*I (a no-cut enzyme for BPV-1) and analyzed by agarose gel electrophoresis to determine the length of the cDNA insert. Slot blot analysis was carried out by treatment of miniprep DNA with 0.25 N HCl at room temperature for 5 min followed by treatment with 0.2 N NaOH at 65°C for 5 min and subsequent application to nitrocellulose filters using a Schleicher and Schuell slot blot manifold. The nitrocellulose bound DNA was hybridized to unlabeled single-stranded subgenomic fragments of BPV-1 cloned into M13 mp7 (Chen *et al.*, 1982) and nick-translated (Rigby *et al.*, 1977) M13 mp7 DNA. All clones with inserts >2 kb in length and clones showing evidence of splicing by the slot blot procedure were analyzed in detail by restriction endonuclease cleavage analysis and direct DNA sequencing (Maxam and Gilbert, 1977).

Direct analysis of RNA

Primer extension analyses were carried out using a modification of the procedure of Danos *et al.* (1985). Synthetic oligonucleotides (20-mers) with 5′ nucleotides at BPV-1 nt 208, 2502, 2551, 3178 and 7356 were used as primers. The oligonucleotides were labeled at their 5′ termini with ^{32}P as follows. For a 12.5 μl kinasing reaction: 5.5 μl oligonucleotide (0.3 A_{260} units/ml) is incubated at 37°C for 1 h with 5.75 μl [γ-^{32}P]ATP (500 Ci/mmol; 10 mCi/ml aqueous solution) and 5 units polynucleotide kinase (Pharmacia PL; 10 units/ml) in 50 mM Tris−HCl pH 9, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA. The oligonucleotide was then incubated at 70°C for 10 min to inactivate polynucleotide kinase. The 5′ labeled oligonucleotide was used to prime cDNA synthesis as follows. RNA (0.75 μg polyadenylated RNA at 7.5 μg total RNA) was incubated with 1.3×10^{-4} A_{260} units 5′ labeled oligonucleotide in 10 μl 100 mM NaCl, 20 mM Tris−HCl pH 8.3, 0.1 mM EDTA at 90°C for 3 min and 55°C for 10 min, followed by slow cooling to room temperature over ~30 min. cDNA synthesis was initiated by the addition of 10 μl 2 × AMV reverse transcriptase buffer (80 mM Tris−HCl pH 8.3, 80 mM KCl, 12 mM $MgCl_2$, 10 mM DTT, 200 μg/ml actinomycin D, 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP) and 10 units AMV reverse transcriptase (Seikagaku America) or the addition of 10 μl 2 × M-MLV reverse transcriptase buffer (80 mM Tris HCl pH 8,3, 150 mM KCl, 6 mM $MgCl_2$, 20 mM DTT, 100 μg/ml actinomycin D, 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP) and 200 units cloned M-MLV reverse transcriptase (BRL). Reactions were incubated for 1 h at 37°C and stopped by the addition of 80 μl 12.5 mM EDTA and extraction with phenol/chloroform (1:1). The cDNAs were precipitated by the addition of 10 μl 3 M NaAc, 10 μg tRNA and 3 vol EtOH. Nuclease S1 analyses were performed essentially as described by Baker and Ziff (1981). Single-stranded 5′ labeled S1 probes were generated using the oligonucleotides labeled as above and subgenomic fragments of BPV-1 cloned into M13 (Chen *et al.*, 1982) following the prime-cut procedure of Biggins *et al.* (1984). All primer extension and nuclease S1 samples were dissolved in formamide-dye-buffer described by Maxam and Gilbert (1977). Dideoxy sequencing ladders were generated using the 5′ labeled oligonucleotides and the M13 subgenomic BPV-1 clones. All samples were heated to 90°C for 3 min, chilled on ice and loaded onto 8% acrylamide−urea sequencing gels.

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