JOURNAL OF VIROLOGY, Nov. 1991, p. 5806–5812
0022-538X/91/115806-07$02.00/0

Vol. 65, No. 11

An Element in the Bovine Papillomavirus Late 3′ Untranslated Region Reduces Polyadenylated Cytoplasmic RNA Levels

PRISCILLA A. FURTH* AND CARL C. BAKER

Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, Maryland 20892

Received 30 May 1991/Accepted 6 August 1991

Expression of the two bovine papillomavirus type 1 (BPV-1) late genes, L1 and L2, coding for the two capsid proteins, is limited to terminally differentiated keratinocytes in bovine fibropapillomas. This pattern of expression is determined both by the activity of the late promoter and by the inhibition of late region expression in less well differentiated cells. Inhibition of L1 and L2 mRNA production in nonpermissive cells must occur since the late region potentially could be transcribed from early region promoters. Nuclear runoff analysis of the late region has demonstrated that up to 95% of transcripts which are initiated in the early region in nonpermissive cells terminate within the late region upstream of the late polyadenylation site (C. C. Baker and J. Noe, J. Virol. 63:3529–3534, 1989). However, very few of the primary transcripts which include the late polyadenylation site are processed into mRNA. In this study, we have used expression vectors to characterize an inhibitory element active in nonpermissive cells which is located in the late 3′ untranslated region (3′UTR). While the late polyadenylation site is functional in these cells, a 53-bp element in the late 3′UTR reduces levels of polyadenylated cytoplasmic RNA. This element inhibited chloramphenicol acetyltransferase (CAT) expression 6- to 10-fold when cloned in the sense orientation into the 3′UTR of a CAT expression vector. No block to expression was seen when the fragment was cloned immediately downstream of the poly(A) site, in an intron upstream of the CAT coding sequence, or in an antisense orientation in the 3′UTR. When the same fragment was deleted from a BPV-1 L1 expression vector, a sixfold increase in mRNA levels was seen. Actinomycin D chase experiments using BPV-1 L1 expression vectors indicated that the element does not destabilize cytoplasmic polyadenylated RNA. Therefore, the element must act before the mature mRNA reaches the cytoplasm. The data presented are consistent with effects on nuclear stability and/or inhibition of polyadenylation or nuclear transport.

Papillomaviruses are epitheliotropic circular double-stranded DNA viruses which are the etiological agents of squamous epithelial and fibroepithelial tumors in humans and a wide variety of animals. Some types of papillomaviruses are also associated with malignant transformation of infected tissues (32). In natural infection, papillomavirus gene expression can be divided into early and late phases according to the state of differentiation of the infected keratinocytes. Early genes are expressed throughout the infected epithelium, but the two late region genes encoding the capsid proteins (L1 and L2) are expressed only in terminally differentiated keratinocytes (16, 28, 29).

Infection of rodent cells in culture with bovine papillomavirus type 1 (BPV-1) or transfection of BPV-1 DNA into rodent cells results in expression of the early region, stable transformation of the cells in culture, and extrachromosomal maintenance of the papillomavirus genome (20). Late region mRNAs are present at levels approximately 4 orders of magnitude lower than those of early mRNAs (5). This expression pattern from the papillomavirus genome in the transformed cell resembles the pattern seen in the nonterminally differentiated keratinocytes in papillomavirus-infected epithelium (29).

Expression of the BPV-1 late region appears to be restricted at several different levels. In terminally differentiated keratinocytes, the late region genes are transcribed from a late promoter and polyadenylated at a single site (A_L) located at the end of the late region (6) (Fig. 1). Transcription from the late promoter cannot be demonstrated in the BPV-1-infected fibroblast of a bovine fibropapilloma, in BPV-1-transformed mouse C127 cells, or in vitro in HeLa cell extracts (6, 21). Keratinocyte differentiation-specific factors may be required for activation of the late promoter.

The late region can potentially be transcribed from early region promoters in nonproductively infected cells in culture, but nuclear runoff analysis demonstrates that up to 95% of these primary transcripts appear to terminate between the early and late polyadenylation sites (7). Few of the transcripts which do extend to the late polyadenylation site are processed into detectable mRNA. This observation led to investigation of additional mechanisms which could inhibit late gene expression.

This study demonstrates that the BPV-1 late polyadenylation site can be utilized in nonpermissive cells. However, a 53-bp region of the BPV-1 late 3′ untranslated region (3′UTR) inhibits expression of the BPV-1 L1 gene as well as expression from a reporter gene when cloned into its 3′UTR.

MATERIALS AND METHODS

Expression vectors. Nucleic acid manipulations, bacterial transformations, recombinant screening, and plasmid preparations were performed by standard methods (24). BPV-1 5′ deletion fragments of the late region were created as follows, using the exonuclease III deletion method of Henikoff (15). A 12-nucleotide (nt) *Hpa*I linker (TTAGTTAACTAA) was cloned into the *Hin*cII site of pUC18 to generate P18SCH. The BPV-1 *Xba*I-*Hpa*I fragment (nt 6134 to 0003) from p142-6 (25) was then cloned into the *Hpa*I site of P18SCH to generate CCB9. The BPV-1 *Xba*I site was recreated, and the fragment was oriented with the *Xba*I site adjacent to the

* Corresponding author.

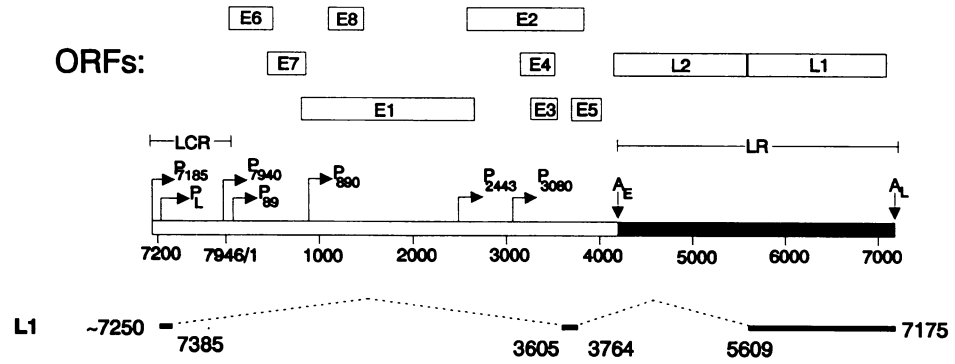

FIG. 1. Genomic organization of BPV-1 and structure of L1 mRNA. Numbers indicate nucleotide positions. The arrows labeled with P and a number correspond to the known BPV-1 promoters and the approximate nucleotide positions of their respective RNA start sites. P_L is the late promoter whose initiation sites map between nt 7214 and 7256. A_E and A_L indicate the early and late polyadenylation sites, respectively. The 5' and 3' ends and splice sites of the L1 mRNA are listed.

*Eco*RI side of the polylinker. Unidirectional exonuclease III deletions were made in CCB9 DNA linearized with *Xba*I and *Kpn*I, and the plasmids were religated. Deletion clones CCB9Del9, CCB9Del19, and CCB9Del25 have 5' deletion endpoints of nt 6547, 6977, and 7029, respectively.

A series of high-expressing simian virus 40 (SV40)-based chloramphenicol acetyltransferase (CAT) vectors which have polylinkers in the 3'UTR, downstream of the polyadenylation site, or in an intron upstream of CAT coding sequences were used to test for negative regulatory elements (4). CAT expression vectors to assay the BPV-1 late poly(A) site were created by cloning fragments of CCB9 or CCB9 deletion clones in the sense orientation into the *Sma*I site of pOBCAT28 (4), a CAT expression vector which lacks its own poly(A) site (Fig. 2). The specific fragments and resulting clones were as follows: CCB9 *Hin*cII-*Mlu*I, CCB216; CCB9Del19 *Eco*RI-*Mlu*I, CCB198; CCB9Del9 *Eco*RI-*Mlu*I, CCB199; and CCB9Del9 *Eco*RI-*Hin*cII, CCB200. The *Hin*cII and *Mlu*I cleavage sites are at BPV-1 nt 7145 and 7352, respectively. The *Eco*RI site is in the pUC polylinker adjacent to the deletion endpoint. All fragments were blunted with Klenow enzyme before ligation. CAT vectors for mapping the 3' negative element were created by cloning fragments of CCB9 deletion clones in the sense (S) or antisense (AS) orientation at the *Sma*I site in the 3'UTR of pOBCAT19 or pOBCAT20 (4), vectors which contain the SV40 early poly(A) site (Fig. 3). Fragments and resulting vectors were as follows: CCB9Del25 *Eco*RI-*Hin*cII, CCB242 (S), CCB229 (AS); CCB9Del9 *Eco*RI-*Hin*cII, CCB197 (S), CCB194 (AS); CCB9Del9 *Eco*RI-*Hin*dIII, CCB208 (S), CCB205 (AS); CCB9Del9 *Eco*RI-*Cla*I, CCB209 (S), CCB206 (AS); and CCB9Del9 *Eco*RI-*Nhe*I, CCB210 (S), CCB207 (AS). The *Hin*dIII, *Cla*I, and *Nhe*I sites are at BPV-1 nt 6959, 6836, and 6715, respectively. The BPV-1 *Hin*dIII-*Hin*cII fragment from p142-6 (26) was cloned in the sense orientation to give CCB251. Two complementary synthetic oligonucleotides (BPV-1 nt 7094 to 7146) (TCGACTAAA AGCTAAGTTTCTATAAATGTTCTGTAAATGTAAAAC AGAAGGTAAGTCA and GATCTGACTTACCTTCTGTT TTACATTTACAGAACATTTATAGAAACTTAGCTTT TAG) with *Sal*I and *Bgl*I compatible ends were cloned into pOBCAT19 and pOBCAT20 linearized at the *Sal*I and *Bgl*II polylinker cloning sites in the 3'UTR to give CCB250 (S) and CCB249 (AS). The 53-bp inserts in CCB249 and CCB250 were verified by DNA sequencing. The insert for CCB366 (S) and CCB365 (AS) was generated from CCB196 (similar to CCB197 except that the insert extends to the BPV-1 *Mlu*I site) by polymerase chain reaction (PCR), using a 5' primer in vector sequences and a 3' BPV-1 primer with the 5' end at nt 7096 (GACTCGAAGCTTATTTTTTTTTTTTTTTGCA GGCTTACTGG). The PCR fragment was cleaved with *Sst*I, which cuts in the polylinker, and the resulting BPV-1 fragment was cloned into the *Sma*I and *Sst*I sites of pOBCAT19 and pOBCAT20.

A cytomegalovirus (CMV) IE1 enhancer/promoter BPV-1 L1 expression vector was created by first substituting a PCR-amplified BPV-1 L1 cDNA fragment for the CAT coding sequence in a CMV IE1 CAT expression vector (14). The PCR-amplified fragment was made by using a BPV-1 L1 cDNA template from a BPV-1 fibropapilloma cDNA sublibrary (5), using a 5' primer containing CMV IE1 nt −19 to −1, which includes an *Sst*I site, fused to the BPV-1 late leader sequence (nt 7254 to 7273) (GCCGAGCTCGTTT AGTGAACCGATTGTGCTGGCTAGACTTCA) and a 3' primer composed of BPV-1 nt 6258 to 6239 with an *Eco*RI site added at its 3' end for use in cloning (CCGGAATTCCG GTAGATCTGATTTACTTGC). This intermediary construct was used to make both a wild-type L1 expression vector (BPVL1) and an L1 expression vector with a 53-bp deletion in the 3'UTR (BPVL1Δ3'UTR). BPVL1 was completed by cloning the 1,219-bp BPV-1 *Xba*I-to-blunted-*Mlu*I fragment from p142-6 into the *Xba*I and blunted *Eco*RI sites in the intermediary construct. BPVL1Δ3'UTR was completed by cloning the equivalent BPV-1 *Xba*I-to-blunted-*Mlu*I fragment from CCB311-1, a BPV-1 genomic construct in which the 53 bp 3'UTR element was deleted, into the intermediary construct. CCB311-1 was created as follows. The BPV-1 mutant p214-1 (26) contains a deletion from the *Hin*dIII site (nt 6959) to *Hin*cII (nt 7145) with insertion of a *Hin*dIII linker. The *Hin*dIII-*Hpa*I fragment (nt 6960 to 6963) of wild-type p142-6 was replaced by the p214-1 *Hin*dIII-*Hpa*I fragment to give plasmid CCB287. The L1 coding sequences deleted in CCB287 were replaced by insertion of a PCR-generated *Hin*dIII fragment in the sense orientation into the *Hin*dIII site of CCB287 to give plasmid CCB311-1. The PCR fragment was synthesized by using p142-6 as template, a 5' primer spanning the *Hin*dIII site (AAAGAAAAGCTTTCTT TGGAC), and a 3' *Hin*dIII-containing primer from BPV-1 nt 7096 to 7065 (GACTCGAAGCTTATTTTTTTTTTTTTT TGCAGGCTTACTGG). CCB311-1 contains a deletion of BPV-1 nt 7097 to 7145 with insertion of 5 bases of a *Hin*dIII

linker (CTTGG). The sequence of the PCR insert was verified by DNA sequencing.

A β-galactosidase expression vector (pCH110) was obtained from Pharmacia. A human α-globin expression vector (pSVα1) was provided by Michael Greenberg (27).

Cells. BPV-1-transformed C127 cells, CV-1 cells, COS-1 cells, and NIH 3T3 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, antibiotics, and glutamine.

Transfections. BPV-1-transformed C127 cells were transfected by the calcium phosphate method, glycerol shocked at 4 to 8 h after addition of the precipitate, and harvested after 48 h. Sodium butyrate (5 mM) was added to the BPV-1-transformed C127 cell transfections after the glycerol shock (4). NIH 3T3 cells were transfected either by using calcium phosphate followed by a glycerol shock and the addition of sodium butyrate (5 mM) or by electrotransformation (30) without the addition of sodium butyrate. The calcium phosphate transfections were harvested at 48 h; the electrotransformations were harvested at 24 h. Calcium phosphate transfections were performed on 10-cm plates, using 5 μg of the test construct, 2.5 μg of the expression vector used for transfection control, and 13.5 μg of salmon sperm DNA or pUC18 DNA as carrier. Electrotransformations were performed on 10^6 cells, using 40 μg of the L1 construct, 20 μg of the transfection control vector, and 10 μg of salmon sperm DNA as carrier. The cells from two such electrotransformations were pooled before being plated on four 10-cm plates. pCH110 was used as a transfection control for the CAT constructs, and pSVα1 was cotransfected with the L1 expression vectors.

Assays for CAT and β-galactosidase. Cellular extracts were prepared by freeze-thawing. CAT activity was assessed by standard methods; the acetylated and unacetylated forms of chloramphenicol were quantitated by either scintillation counting or radioanalytic imaging (AMBIS) and expressed as percent acetylation. β-Galactosidase activity was measured and used to normalize CAT activity as previously described (4).

Cytoplasmic RNA isolation, poly(A) selection, and quantitative Northern (RNA) blot analyses. Cytoplasmic RNA was isolated from transfected cells in Nonidet P-40 lysis buffer according to standard procedures (24). The RNA was quantitated on a Beckman spectrophotometer, and its integrity was analyzed on a formaldehyde agarose gel (11). Batch poly(A) selection was performed on 200 to 300 μg of total cytoplasmic RNA in a procedure utilizing microcrystalline oligo(dT)-cellulose (New England Biolabs) modified from the procedure of Aviv and Leder (3). Each poly(A)-selected sample was fractionated on a formaldehyde agarose gel, transferred to a nylon membrane (Dupont GeneScreen) by vacuum blotting (Pharmacia), and fixed on the membrane by UV irradiation (Stratagene). The membranes were prehybridized for 30 min and hybridized overnight at 65°C to random primer-labelled probes (13) in an aqueous buffer (10). L1 mRNA was detected by using a PCR-amplified fragment of the BPV-1 genome (nt 4983 to 7096). pSVα1 mRNA was detected with a 729-bp *Nco*I-to-*Bgl*I fragment isolated from the vector. BPV-1 early region mRNA isolated from BPV-1-transformed C127 cells was detected with a full-length BPV-1 genomic probe.

Measurement of steady-state levels and half-life of polyadenylated cytoplasmic RNA. Polyadenylated cytoplasmic RNA expressed from either BPVL1 or BPVL1Δ3′UTR was isolated from transfected cell cultures at 24 or 48 h after transfection for comparison of steady-state levels. mRNA half-lives were measured after inhibition of transcription with actinomycin D (Sigma). Actinomycin D (5 μg/ml) was added to the complete medium either 24 or 48 h after transfection, and the mRNA was isolated at various time points for up to 7.5 h. A 1-mg/ml solution of actinomycin D in 90% ethanol was prepared on the day of its use and diluted in complete medium before addition to the cell cultures. Expression levels were quantitated after Northern blotting by a radioanalytic imaging system (AMBIS) and/or computerized densitometry of radiographic films exposed at room temperature (Molecular Dynamics). To control for transfection efficiency and sample preparation, expression levels of BPV-1 L1 mRNA were normalized to pSVα1 expression levels.

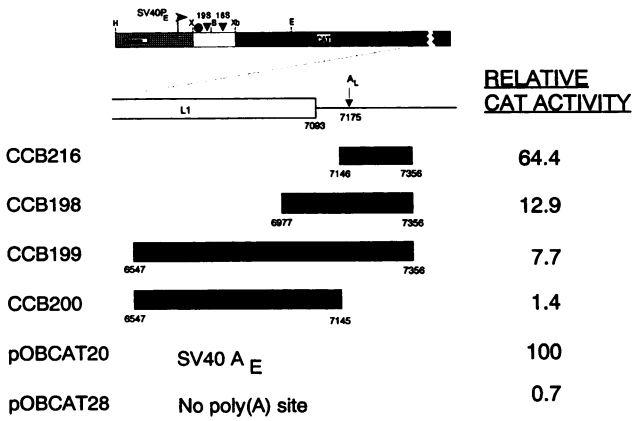

FIG. 2. Stimulation of CAT activity by fragments containing the BPV-1 late polyadenylation site. Fragments of the BPV-1 3′ late region (heavy bars) were cloned into pOBCAT28 (4), a CAT expression vector which lacks a polyadenylation site. Numbers underneath represent fragment endpoints. Numbers at the right represent CAT activity relative to the activity expressed from the vector utilizing the SV40 early polyadenylation site (A_E) as assayed by transient transfection into BPV-1-transformed C127 cells. Constructs are identified by name on the left. The L1 open reading frame is indicated by the open box. The SV40 early promoter is indicated by $SV40P_E$. Splice donor and acceptor sites are identified by ● and ▼, respectively. Abbreviations: H, *Hin*dIII; X, *Xho*I; B, *Bam*HI; Xb, *Xba*I; E, *Eco*RI.

RESULTS

The BPV-1 late polyadenylation site is functional in nonpermissive cells. To determine whether the BPV-1 late polyadenylation site could function in BPV-1-transformed cells, fragments of the BPV-1 late region which include the BPV-1 late polyadenylation site were cloned into the 3′UTR of an SV40-based CAT expression vector lacking a polyadenylation site. CAT activity was measured 48 h after calcium phosphate transfection of BPV-1-transformed C127 cells, normalized to β-galactosidase levels from cotransfected pCH110, and expressed as relative CAT activity (Fig. 2). BPV-1-transformed C127 cells were used for the experiment presented here because the BPV-1 early region is expressed in these cells but only very low levels of mRNA polyadenylated at the endogenous late poly(A) site can be detected. Insertion of a BPV-1 fragment (nt 7146 to 7356) containing the late polyadenylation signal and only 30 nt of 3′UTR increased CAT expression 92-fold above that of the parent vector without a poly(A) site. This level of CAT expression was nearly as high as that obtained with the SV40 early

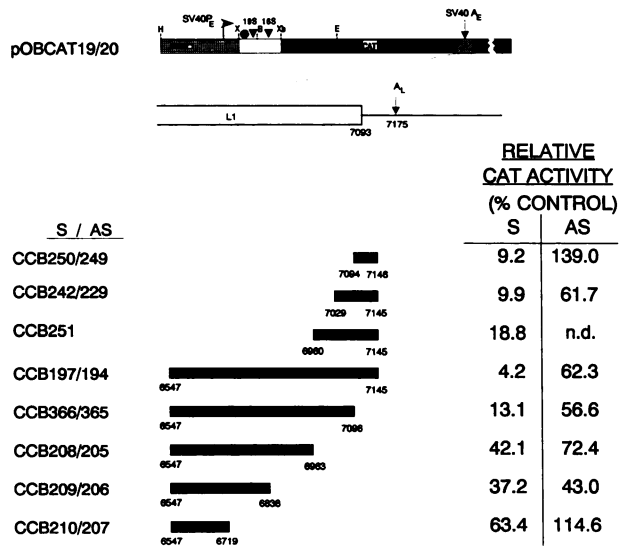

FIG. 3. Comparison of relative CAT activity expressed from vectors which contain fragments of the BPV-1 L1 open reading frame and 3'UTR cloned into the 3'UTR of a CAT expression vector which utilizes the SV40 early polyadenylation site. The fragments were inserted in both sense (S) and antisense (AS) orientations. Expression vector at top is either pOBCAT19 or pOBCAT20 (4) and is similar to the vector in Fig. 2 except that it contains the SV40 early polyadenylation site (A_E). Symbols and abbreviations are described in the legend to Fig. 2. Numbers at the right represent CAT activity relative to that of the appropriate parent vector as assayed by transient transfection into BPV-1-transformed C127 cells. n.d., not done.

poly(A) site. Similar results were obtained in uninfected NIH 3T3 cells. These data demonstrate that the late polyadenylation site can be efficiently utilized in nonproductively infected cells when fused to the CAT coding sequences. However, inclusion of additional BPV-1 sequences upstream of the late poly(A) site resulted in lower levels of CAT expression and suggested that a negative regulatory element is present in the BPV-1 L1 open reading frame and/or the late 3'UTR (Fig. 2).

A 53-bp fragment from the BPV-1 late 3'UTR inhibits CAT activity in an orientation- and position-dependent manner. To localize the inhibitory sequence, fragments of the BPV-1 late region which include the 3'UTR but not the BPV-1 late polyadenylation site were cloned in both sense and antisense orientations into the 3'UTR of a CAT expression vector which contains the SV40 early polyadenylation site. CAT expression levels were measured 48 h after calcium phosphate transfection into BPV-1-transformed C127 cells. All fragments containing 3'UTR sequences upstream of nt 7145 inhibited CAT expression in an orientation-dependent manner (Fig. 3). The most marked difference in CAT activity between sense and antisense orientations was observed with a 53-bp fragment composed of nt 7094 to 7146. The removal of BPV-1 sequences upstream of nt 7094 did not reduce the inhibitory activity of the fragments containing the 3'UTR when cloned in the sense orientation, indicating that sequences upstream of nt 7094 are not required for the full function of the 3'UTR element. However, a fragment from nt 6547 to 7096 inhibits CAT expression with partial orientation dependence, suggesting that additional inhibitory elements may lie upstream of nt 7096. Further mapping demonstrates that BPV-1 sequences between nt 6547 and 6963 do not inhibit CAT activity in an orientation-dependent manner.

The inhibitory 53-bp fragment composed of nt 7094 to 7146 encompasses almost the entire 3'UTR, as the translation termination codon lies at nt 7094 to 7096 and the late polyadenylation site is at nt 7175. This 53-bp fragment reduced CAT expression ninefold in BPV-1-transformed C127 cells. This inhibition was also found in NIH 3T3 and CV-1 cells, indicating that BPV-1-encoded factors are not required for its function. Steady-state levels of CAT mRNA and CAT activity expressed from the vector containing the 53-bp fragment were equivalently reduced, indicating that this fragment affects mRNA levels rather than translation (data not shown). This fragment had no effect on CAT expression levels in BPV-1-transformed C127 cells when cloned in either orientation in an intron upstream of CAT coding sequences or immediately downstream of the SV40 early polyadenylation site. This orientation and position dependence suggested that the element functions at the RNA level. The observation that a fragment from nt 6547 to 7096 inhibits CAT expression with some orientation dependence suggests that additional inhibitory sequences may lie upstream of nt 7096.

Deletion of the 3'UTR element from a BPV-1 L1 expression vector increases mRNA levels. The inhibitory effect of the late 3'UTR element in its normal context in the BPV-1 genome was tested by comparing the steady-state level of mRNA expressed from a wild-type BPV-1 L1 CMV IE1 expression vector (BPVL1) with that expressed from a vector with the 53-bp fragment deleted from the 3'UTR (BPVL1Δ3'UTR) (Fig. 4). These vectors were constructed by using an L1 cDNA with genomic sequences substituted at and 181 bp 3' to the late polyadenylation site. These vectors should initiate transcription at one of the predominant 5' start sites used by the late promoter in the fibropapilloma. Expression levels from these vectors were measured after transient transfection into NIH 3T3 cells. Cytoplasmic polyadenylated RNA

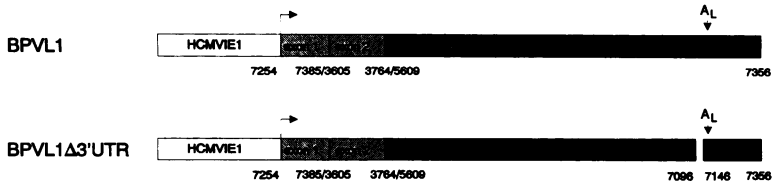

FIG. 4. Structures of BPV-1 L1 expression vectors. BPVL1 expresses an L1 mRNA identical in structure to the L1 mRNA expressed in the fibropapilloma. BPVL1Δ3'UTR is the same vector except for a 53-bp deletion in the 3'UTR. The numbers underneath indicate the BPV-1 nucleotide sequences included in the vectors. HCMVIE1 refers to the human CMV immediate-early gene 1 promoter. The three L1 exons are labeled. The third exon is fused with genomic sequences to give an intact late poly(A) site. The horizontal arrows on the top indicate the transcriptional start site. A_L refers to the late polyadenylation site at nt 7175.

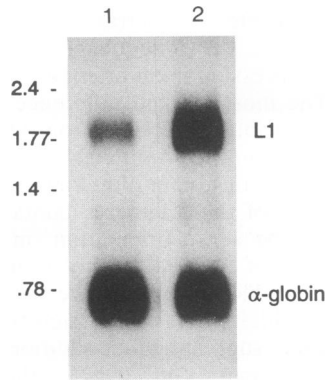

FIG. 5. Northern blot analysis of steady-state mRNA expressed from BPVL1 (lane 1) and BPVL1Δ3'UTR (lane 2). Each construct was cotransfected with an SV40 α-globin expression vector into NIH 3T3 cells. Total cytoplasmic RNA was isolated 24 h after transfection. Each lane represents poly(A) RNA selected from 200 μg of total cytoplasmic RNA. L1 refers to the BPV-1 L1 RNA with and without the 53-bp deletion, and α-globin refers to the RNA used as a transfection control. The blot was sequentially hybridized to the probes for L1 and α-globin. The radiograph was obtained after a 4-day exposure at room temperature. Positions of size markers are indicated at the left in kilobases.

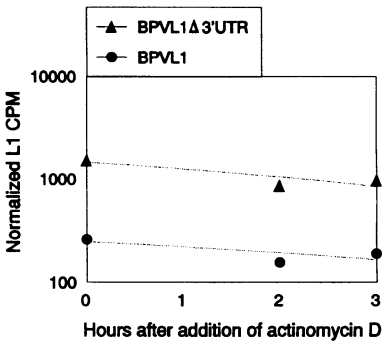

FIG. 6. Measurement of the stability of polyadenylated cytoplasmic RNA expressed from BPVL1 and BPVL1Δ3'UTR after transcription was inhibited by actinomycin D (5 μg/ml). Northern blots were prepared as described for Fig. 5. RNA levels were measured by radioanalytic imaging of Northern blots and are normalized for the level of α-globin expressed from the cotransfected expression vector. Data are plotted on a semilogarithmic graph.

was analyzed on a Northern blot and quantitated by radioanalytic imaging (AMBIS). Steady-state levels of L1 mRNA expressed from BPVL1Δ3'UTR were at least six times higher than levels expressed from BPVL1 after normalization to a coexpressed α-globin mRNA (Fig. 5). The same results could be demonstrated in CV-1 cells with the same vectors and in COS-1 cells with similar vectors utilizing the SV40 early promoter (data not shown).

The location of the negative element in the 3'UTR of the BPV-1 late region and its orientation dependence suggested that it might act as a cytoplasmic mRNA destabilization element. Therefore, the half-life of BPVL1 mRNA was compared with the half-life of BPVL1Δ3'UTR mRNA after inhibiting transcription with actinomycin D. Actinomycin D was added 24 h after electrotransformation of NIH 3T3 cells, and poly(A) mRNA was isolated 0, 2, and 3 h later. The half-life of the wild-type mRNA was 5.5 h, and the half-life of the mutant mRNA with the 53-bp deletion was 4.1 h (Fig. 6). This finding indicates that the element has no destabilizing effect on L1 mRNA. Similar results were obtained when NIH 3T3 cells were transfected by using calcium phosphate and treated with sodium butyrate as well as in experiments in which the transfection efficiency was low. In comparison, BPV-1 early region mRNAs in BPV-1-transformed cells treated with actinomycin D have a relatively short half-life (Fig. 7) (19). These results indicate that the 3'UTR element has minimal effect on cytoplasmic L1 mRNA stability as measured by actinomycin D chase experiments.

DISCUSSION

These experiments demonstrate that a 53-bp fragment from the 3'UTR of the BPV-1 late region inhibits both homologous and heterologous gene expression in transfection studies in cell culture. In contrast, the BPV-1 late polyadenylation site appears to function efficiently in the absence of the 3'UTR element in nonpermissive cells.

The location of the negative element in the late region 3'UTR, its orientation and position dependence, and its AT-rich base composition are all consistent with characteristics of previously described cytoplasmic mRNA instability elements (8). Under our experimental conditions, this element had no effect on the half-life of cytoplasmic BPV-1 L1 mRNA. Although the use of actinomycin D may have interfered with destabilization of the message by incorporation into double-stranded RNA or inhibited the transcription of mRNAs encoding short-lived proteins needed for mRNA destabilization (2), our data suggest that this element works through a different mechanism.

The presence of an inhibitory element in the late region 3'UTR may be a common feature of papillomaviruses. Sequences upstream of the late region polyadenylation site in human papillomavirus type 16 also inhibit expression of a reporter gene (17). In contrast to the BPV-1 3'UTR element, these human papillomavirus type 16 sequences affected RNA stability in an in vitro assay, but these results were not verified by an in vivo assay (18).

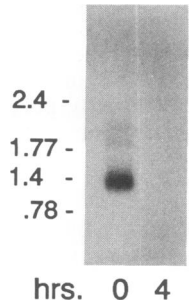

FIG. 7. Northern blot analysis of BPV-1 early region mRNAs expressed from the extrachromosomal BPV-1 in transformed C127 cells before and after the addition of actinomycin D. Cytoplasmic RNA was isolated at the times indicated after transcription was inhibited with actinomycin D (5 μg/ml). Each lane represents poly(A) RNA isolated from 24 μg of total cytoplasmic RNA. The blot was hybridized with a full-length BPV-1 genomic probe. The radiograph was obtained after a 72-h exposure with a screen. Positions of size markers are indicated at the left in kilobases.

Comparison of computer-predicted RNA secondary structures reveals a similar structural motif in papillomavirus late region 3'UTRs. An analysis of 10 papillomavirus genomic sequences demonstrates a 6- to 7-bp stem with a 3- to 7-bp loop downstream of the L1 termination codon. The BPV-1 stem loop (nt 7116 to 7137) is contained within the 53-bp 3'UTR element but by itself did not inhibit heterologous gene expression when cloned into the 3'UTR of a CAT expression vector (data not shown). If the stem-loop structure is required for activity of the inhibitory element, then the surrounding RNA sequences must be required for the proper RNA secondary structure and/or protein RNA interaction.

The experimental data indicate that the element inhibits gene expression before the polyadenylated RNA reaches the cytoplasm. Effects on efficiency of polyadenylation, nuclear RNA stability, and/or transport out of the nucleus are consistent with the data, and examples of RNA sequences which affect some of these processes have been identified. Upstream sequences which influence polyadenylation in SV40 (9), adenovirus (12), ground squirrel hepatitis virus (23), and human immunodeficiency virus (31) have all been characterized. However, in contrast to the BPV-1 element described here, all act to increase polyadenylation efficiency. In germ cells from *Xenopus laevis* and *Caenorhabditis elegans* (1), 3'UTR elements which affect poly(A) tail length have been identified, but these elements do not appear to affect RNA levels. Human immunodeficiency virus contains an orientation-dependent RNA element which is required for efficient nuclear export of incompletely spliced RNA species (22).

In summary, the experiments reported here support the concept that expression of the BPV-1 late region is regulated at several different levels. We have shown that a 53-bp fragment from the BPV-1 late region 3'UTR is capable of inhibiting gene expression in nonpermissive cells. Identification of elements which block expression of the BPV-1 late region in nonpermissive cells is critical to understanding the mechanisms which limit the expression of papillomavirus late genes to terminally differentiated keratinocytes.

ACKNOWLEDGMENTS

We thank Fred Delvecchio for helpful advice and discussion.

This work was partially supported by a research associateship to P.A.F. from the National Research Council. P.A.F. is on a leave of absence from the University of Maryland Medical School.

REFERENCES

1. **Ahringer, J., and J. Kimble.** 1991. Control of the sperm-oocyte switch in *Caenorhabditis elegans* hermaphrodites by the *fem*-3 3' untranslated region. Nature (London) **349**:346–348.
2. **Atwater, J. A., R. Wisdom, and I. M. Verma.** 1990. Regulated mRNA stability. Annu. Rev. Genet. **24**:519–541.
3. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
4. **Baker, C. C.** 1990. An improved chloramphenicol acetyltransferase expression vector system for mapping transcriptional and post-transcriptional regulatory elements in animal cells, p. 75–86. *In* M.-L. Huhtala and A. Vaheri (ed.), Recombinant systems in protein expression. Elsevier Science Publishers B.V., Amsterdam.
5. **Baker, C. C.** Unpublished data.
6. **Baker, C. C., and P. M. Howley.** 1987. Differential promoter utilization by the bovine papillomavirus in transformed cells and productively infected wart tissues. EMBO J. **6**:1027–1035.
7. **Baker, C. C., and J. S. Noe.** 1989. Transcriptional termination between bovine papillomavirus type 1 (BPV-1) early and late polyadenylation sites blocks late transcription in BPV-1-transformed cells. J. Virol. **63**:3529–3534.
8. **Brawerman, G.** 1989. mRNA decay: finding the right targets. Cell **57**:9–10.
9. **Carswell, S., and J. C. Alwine.** 1989. Efficiency of utilization of the simian virus 40 late polyadenylation site: effects of upstream sequences. Mol. Cell. Biol. **9**:4248–4258.
10. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81**:1991–1995.
11. **Davis, L. G., M. D. Dibner, and J. F. Battey.** 1986. Basic methods in molecular biology. Elsevier, New York.
12. **DeZazzo, J. D., and M. J. Imperiale.** 1989. Sequences upstream of AAUAAA influence poly(A) site selection in a complex transcription unit. Mol. Cell. Biol. **9**:4951–4961.
13. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–267.
14. **Ghazal, P., H. Lubon, B. Fleckenstein, and L. Hennighausen.** 1987. Binding of nucleoprotein complex on the human cytomegalovirus enhancer. Proc. Natl. Acad. Sci. USA **84**:3658–3662.
15. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–359.
16. **Jenson, A. B., J. D. Rosenthal, C. Olson, F. Pass, W. D. Lancaster, and K. Shah.** 1980. Immunologic relatedness of papillomaviruses from different species. J. Natl. Cancer Inst. **64**:495–500.
17. **Kennedy, I. M., J. K. Haddow, and J. B. Clements.** 1990. Analysis of human papillomavirus type 16 late mRNA 3' processing signals in vitro and in vivo. J. Virol. **64**:1825–1829.
18. **Kennedy, I. M., J. K. Haddow, and J. B. Clements.** 1991. A negative regulatory element in the human papillomavirus type 16 genome acts at the level of late mRNA stability. J. Virol. **65**:2093–2097.
19. **Kleiner, E., W. Dietrich, and H. Pfister.** 1986. Differential regulation of papilloma virus early gene expression in transformed fibroblasts and carcinoma cell lines. EMBO J. **5**:1945–1950.
20. **Law, M. F., D. R. Lowy, I. Dvoretzky, and P. M. Howley.** 1981. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. Proc. Natl. Acad. Sci. USA **78**:2727–2731.
21. **Linz, U., and C. C. Baker.** 1988. Promoters of bovine papillomavirus type 1: in vitro activity and utilization. J. Virol. **62**:2537–2543.
22. **Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 *rev trans*-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature (London) **338**:254–256.
23. **Russnak, R., and D. Ganem.** 1990. Sequences 5' to the polyadenylation signal mediate differential poly(A) site use in hepatitis B viruses. Genes Dev. **4**:764–776.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Sarver, N., J. C. Byrne, and P. M. Howley.** 1982. Transformation and replication in mouse cells of a bovine papillomavirus–pML2 plasmid vector that can be rescued in bacteria. Proc. Natl. Acad. Sci. USA **79**:7147–7151.
26. **Sarver, N., M. S. Rabson, Y. C. Yang, J. C. Byrne, and P. M. Howley.** 1984. Localization and analysis of bovine papillomavirus type 1 transforming functions. J. Virol. **52**:377–388.
27. **Shyu, A.-B., M. E. Greenberg, and J. G. Belasco.** 1989. The c-*fos* transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes Dev. **3**:60–72.
28. **Spalholz, B. A., and P. M. Howley.** 1989. Papillomavirus-host cell interactions, p. 27–53. *In* G. Klein (ed.), Advances in viral oncology, vol. 8. Raven Press, New York.
29. **Stoler, M. H., and T. R. Broker.** 1986. In situ hybridization

detection of human papillomavirus DNAs and messenger RNAs in genital condylomas and a cervical carcinoma. Hum. Pathol. **17**:1250–1258.

30. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. **10**:449–457.
31. **Valsamakis, A., S. Zeichner, S. Carswell, and J. C. Alwine.** 1991. The human immunodeficiency virus type 1 polyadenylation signal: a 3′ long terminal repeat element upstream of the AAUAAA necessary for efficient polyadenylation. Proc. Natl. Acad. Sci. USA **88**:2108–2112.
32. **zur Hausen, H.** 1989. Papillomaviruses as carcinomaviruses, p. 1–26. *In* G. Klein (ed.), Advances in viral oncology, vol. 8. Raven Press, New York.