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Promoters of Bovine Papillomavirus Type 1: In Vitro Activity and Utilization

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Five of six bovine papillomavirus type 1 (BPV-1) promoters which were previously mapped by determining the 5′ termini of viral mRNAs from bovine fibropapillomas and BPV-1-transformed cells were found to be active under in vitro transcription conditions. Transcription initiation at each of these promoters was accurate at the nucleotide level as determined by primer extension analysis. The most active promoter in vitro was P_{89}, a typical RNA polymerase II promoter with both TATA and CAAT boxes. The promoters P_{7185} and P_{2443} also have TATA-like boxes, but none of the other promoters contain these typical regulatory elements.

The papillomaviruses are small DNA viruses composed of a protein coat and a minichromosome of double-stranded closed circular DNA complexed with histones. These viruses induce squamous epithelial or fibroepithelial tumors in their natural hosts. In general, the papillomaviruses have a narrow host range for productive infection, and transmissibility to other species is often impossible. Bovine papillomavirus type 1 (BPV-1) belongs to a subgroup of papillomaviruses which can induce fibroblastic tumors in heterologous hosts such as mice (6), hamsters (10), and horses (31). In vitro they can also transform rodent cells. However, propagation of BPV-1, as of all other papillomaviruses, occurs only in vivo and is restricted to the terminally differentiated keratinocytes in the natural host.

BPV-1 has served as the prototype for the genetic and transcriptional analysis of the papillomaviruses. It contains 10 designated open reading frames (ORFs) (9), which all reside on one strand of the DNA, and only this strand is expressed as mRNA (2, 16). In the BPV-1-transformed cell, the early or transforming region of the virus is transcribed from at least five promoters (1, 4, 40) (Fig. 1). A sixth promoter (P_L) is active only in productively infected warts (4) and is responsible for the transcription of mRNAs in these tissues encoding the capsid proteins as well as some of the ORFs in the transforming region. Since the determination of these putative promoters was based on the mapping of the 5′ termini of different viral RNA species, it was possible that some of the positions mapped were not the result of transcription initiation but rather of some posttranscriptional processing mechanism. Therefore, we studied transcription from the BPV-1 genome in vitro with the Manley whole cell extract system (23), which has been shown in numerous cases to accurately initiate transcription in higher eucaryotes and their viruses (13, 15, 24, 30). By a combination of runoff and primer extension analyses, it was shown that five of the six promoters were active in vitro and that transcription initiation was accurate for each of these promoters.

MATERIALS AND METHODS

Cells. Nontransformed C127 cells, originally derived from a mammary tumor of an RIII mouse, and ID13 cells, which are BPV-1-transformed C127 cells, have been described previously (11, 22). CV-1 cells originate from African green monkey kidneys (18), and HeLa cells are from a human cervical carcinoma (35). All cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 4.5 g of glucose per liter, and 100 U of penicillin and 100 μg of streptomycin per ml.

Plasmids. Throughout this study, the following plasmids were used as templates for transcription in vitro. p142-6 is a plasmid containing the complete BPV-1 genome cloned at the *Bam*HI site of pML2d (34). pE$^-$XM contains the blunted *Xba*I-*Mlu*I fragment of BPV-1 (nucleotides [nt] 6134 to 7356) in the blunted *Xba*I site 5′ of the chloramphenicol acetyltransferase (*cat*) gene in p402, a pSV0CAT (14) expression vector lacking its own promoter.

In vitro transcription. Template DNA (0.5 to 1.0 μg) was incubated with 2 mM creatine phosphate, 1 mM each ATP, CTP, and GTP, 10 μCi (1 μM) of [α-^{32}P]UTP, 1,250 U of placental RNase inhibitor (Boehringer Mannheim) per ml, 4 mM dithithreitol, 0.3 mM EDTA, and 50% unfractionated HeLa whole-cell extract (23) in a total volume of 20 μl. The incubation was performed for 60 min at 30°C. The reaction was stopped by adding 230 μl of 25 mM Tris hydrochloride (pH 7.6)–100 mM KCl–0.2% sodium dodecyl sulfate, and the proteins were removed by two extractions with 1 volume of chloroform-isoamyl alcohol-phenol (24:1:25). The RNA was precipitated by the addition of 20 μl of 3 M sodium acetate and 600 μl of ethanol to the aqueous phase. The dried RNA pellet was taken up in 10 μl of loading buffer (80% formamide, 20% TBE electrophoresis buffer, 0.02% bromophenol blue, and 0.02% xylene cyanol) and separated on a 4% polyacrylamide–urea gel adjacent to ^{35}S-labeled fragments of pBR322 cleaved with *Hin*fI or *Msp*I as size markers.

Primer extension analysis. Primer extension reactions were carried out as described previously (4). Primers used in this study are listed in Table 1. Analyses of in vitro RNA used the RNA from a 120- to 150-μl reaction mix.

S1 analysis. Nuclease S1 analysis was performed by the aqueous hybridization method described by Lipshitz et al. (21). A single-stranded 5′-labeled S1 probe spanning nt 6960 to 7356 was generated by using the pr7356 oligonucleotide (Table 1) labeled as above and a single-stranded M13 DNA containing the BPV-1 long control region (LCR) following the prime-cut procedure of Biggins et al. (5). RNA (1 μg of polyadenylated wart RNA, 7.5 μg of total RNA from cycloheximide-treated ID13 cells, or RNA from a 65-μl in vitro

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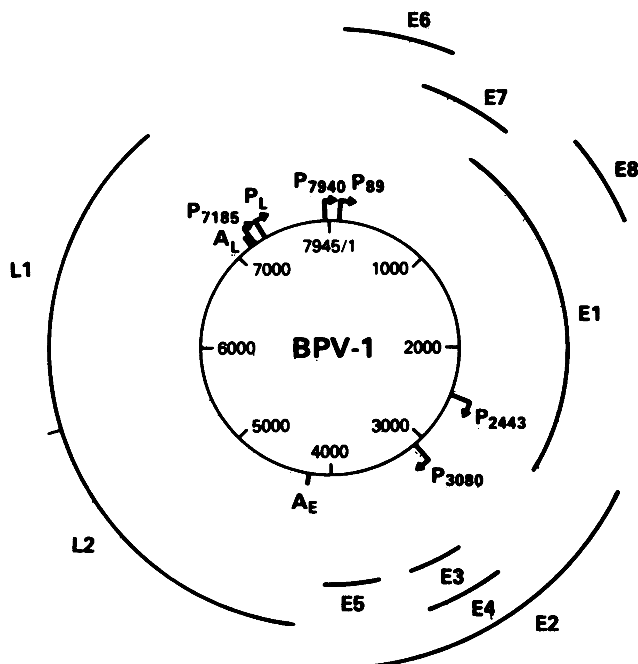

FIG. 1. Genomic organization of BPV-1. A circular representation of the BPV-1 genome is shown. Numbers within the circle are nucleotide positions. The arrows labeled with P and a number correspond to the known BPV-1 promoters and the approximate nucleotide positions of their respective RNA start sites. P_L is the wart-specific promoter whose RNA start site maps around nt 7250. A_E and A_L indicate the early and late polyadenylation sites, respectively. The locations of the major ORFs are indicated by the arcs outside the genomic circle.

transcription reaction mix) was coprecipitated with 2×10^5 cpm of single-stranded S1 probe and resuspended in 3.5 μl of H_2O. An equal volume of 1 M NaCl–80 mM PIPES (pH 6.4)–2 mM EDTA was added, and the mixture was heated to 85°C for 5 min, followed by hybridization at 50°C for 3 h. The hybridization solution was then added to 200 μl of S1 buffer (30 mM sodium acetate [pH 4.5], 0.25 M NaCl, 1 mM $ZnSO_4$, 5% glycerol) on ice. The sample was then split into two tubes and either 80 or 400 U of nuclease S1 (Boehringer Mannheim) was added and incubated at 15°C for 1 h. After extraction with phenol-chloroform, the nuclease S1-resistant products were precipitated in the presence of carrier tRNA. The samples were dissolved in loading buffer (see under in vitro transcription), heated to 90°C for 3 min, chilled on ice, and loaded onto 8% acrylamide–urea sequencing gels.

RESULTS

Transcription from P_{7940} and P_{89}. In vitro transcription of the promoters in the 3′ part of the LCR was studied with a BPV-1 fragment as template which extended from 7353 to 619 (*Mlu*I-*Xma*III). The RNA start sites for these in vitro transcription products were determined by primer extension analysis with a 5′-end-labeled oligonucleotide complementary to BPV-1 nt 189 to 208. The primer extension products were compared with those obtained with RNA from ID13 cells (Fig. 2A, lane 6). P_{89}, which is one of the most active promoters in BPV-1-transformed cells, was active under the in vitro transcription conditions used (Fig. 2A, lane 5). This is not unexpected, since P_{89} is a typical promoter with a

TABLE 1. List of primers used in this study

Name[a]	Sequence
pr208	5′-CAACATGAAAGTCTTTGACC
pr2500	5′-TCATCTGTGCATGGCTGCTC
pr3178	5′-GATCTCTTACAGAGTAATGC
pr7356	5′-CGCGTCCCATTGATGCTTAG
prCAT2[b]	5′-TCCATTTTAGCTTCCTTAGC

[a] The number indicates the BPV-1 nucleotide position corresponding to the 5′ terminus of the oligonucleotide.
[b] *cat* gene primer.

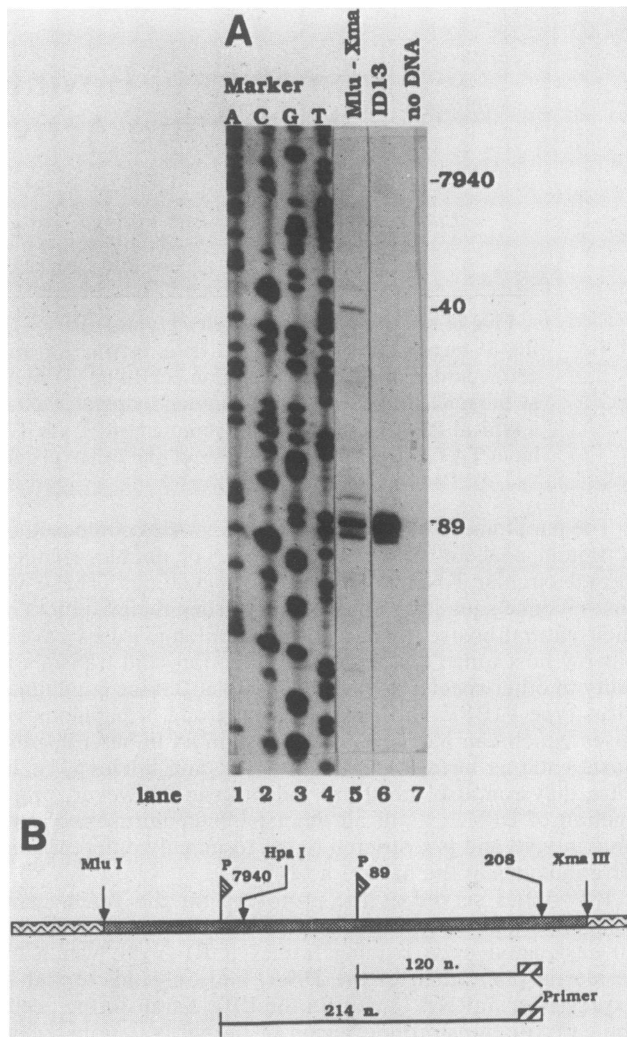

FIG. 2. Primer extension analysis of RNA transcribed in vitro from P_{7940} and P_{89}. (A) BPV-1 fragment extending from nt 7353 to 619 (*Mlu*I-*Xma*III) served as the DNA template for in vitro transcription. The resulting transcripts were analyzed by primer extension with a 5′-end-labeled synthetic oligonucleotide primer complementary to BPV-1 nt 189 to 208. Analysis of the primer extension products was performed on an 8% sequencing gel. Dideoxy sequencing products (lanes 1 to 4) obtained with the same oligonucleotide primer and BPV-1 DNA were used as markers. Primer extension products for in vitro and in vivo transcripts are shown in lanes 5 and 6, respectively. Lane 7 shows the analysis of a control in vitro transcription reaction containing no template DNA. Numbers to the right represent nucleotide positions in the BPV-1 genome. (B) Start sites of the promoters and the predicted sizes of the primer extension products. The position of the 5′ end of the primer (nt 208) is indicated.

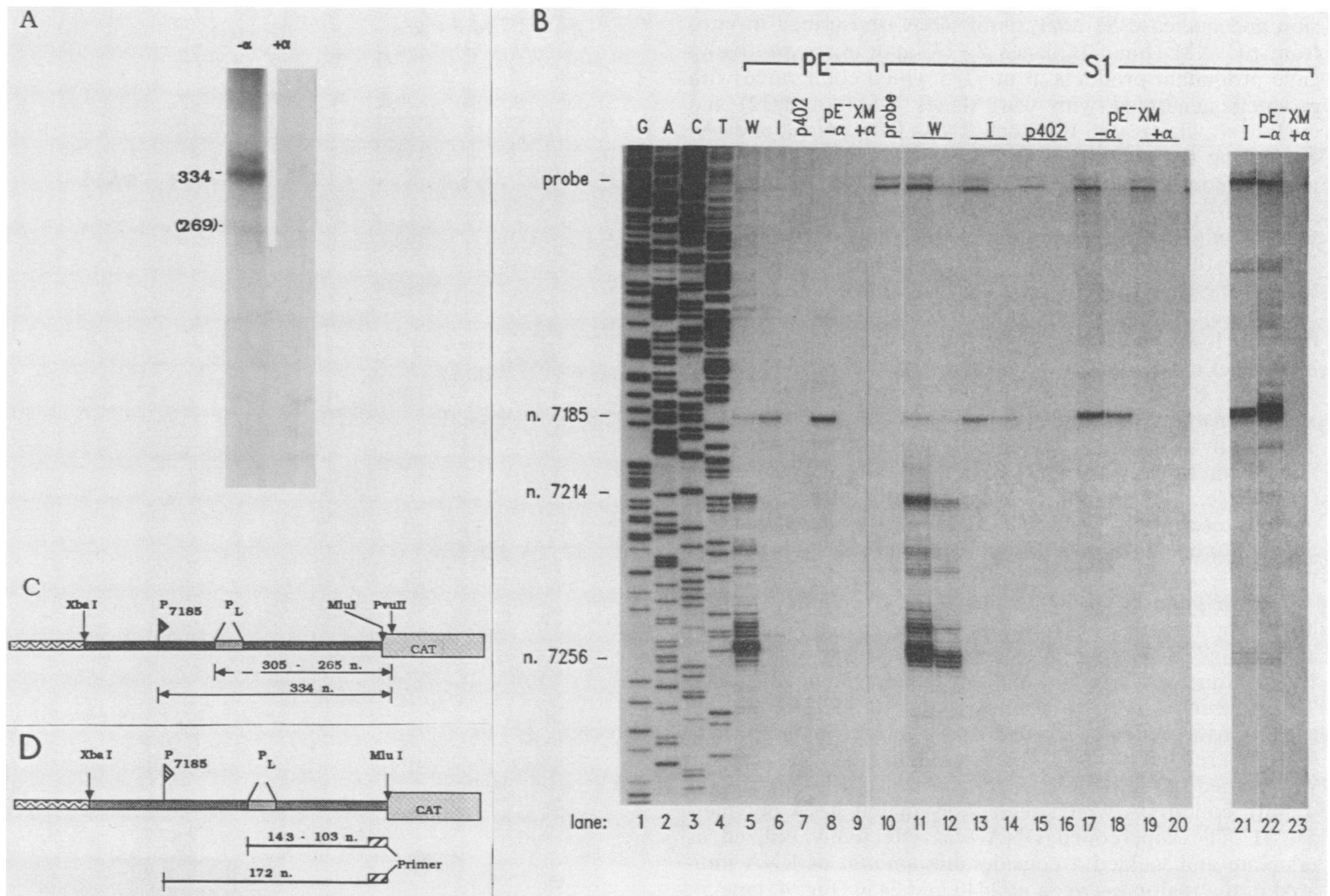

FIG. 3. Runoff, primer extension, and nuclease S1 analyses of RNA transcribed in vitro from P_{7185} and P_L. The template DNA (pE⁻XM) contains the BPV-1 *Xba*I-*Mlu*I fragment (nt 6134 to 7356) in a pSV0CAT background. (A) Radiolabeled RNA transcribed from *Pvu*II-cut pE⁻XM DNA in vitro was analyzed on a 4% polyacrylamide–urea gel. $+\alpha$ and $-\alpha$ refer to reactions in the presence and absence of α-amanitin, respectively. Numbers to the left indicate transcript length in nucleotides. (B) Primer extension: Nonradioactive RNA transcribed in vitro from pE⁻XM in the absence ($-\alpha$) or presence ($+\alpha$) of α-amanitin was analyzed by primer extension with a 5′-end-labeled synthetic oligonucleotide complementary to nt 7337 to 7356 (lanes 8 and 9). The same oligonucleotide was used for primer extension analysis of 0.75 μg of poly(A)-selected wart RNA (W, lane 5), 7.5 μg of total cellular RNA from cycloheximide-treated ID13 cells (I, lane 6), or RNA transcribed in vitro from p402 (lane 7). P402 is a pSV0CAT expression vector, the carrier plasmid of pE⁻XM. Analysis was performed on an 8% sequencing gel adjacent to markers (lanes 1 to 4) prepared by dideoxy sequencing of a single-stranded LCR DNA with the same synthetic oligonucleotide primer. Numbers to the left indicate BPV-1 nucleotide positions. S1 analysis: RNAs were the same as for primer extension. The amount of nuclease S1 used for each digest was 80 U (lanes 11, 13, 15, 17, and 19) and 400 U (lanes 12, 14, 16, 18, 20, and 21 to 23). Lane 10 shows the undigested probe extending from nt 6960 to 7356. Lanes 21 to 23 are overexposures (ca. 30-fold) of lanes 14, 18, and 20, respectively. (C and D) BPV-1 part of the pE⁻XM plasmid and the expected sizes of the runoff (C) and primer extension/S1 (D) products.

TATA box (nt 58 to 65) approximately 30 nt upstream of the initiation site and a sequence similar to a CAAT box at position −83. Cutting the template at the *Hpa*I site (nt 3) did not alter the signal intensity of the P_{89} transcription product (data not shown), indicating that transcriptional regulatory elements 5′ of the *Hpa*I site, such as the E2-responsive elements E2RE1 and E2RE2 (37), are not required for efficient transcription from P_{89} in vitro.

Runoff analyses of P_{7940} yielded a nonspecific ladder pattern, and the products were partially resistant to α-amanitin (data not shown). In addition, primer extension analysis did not show any products above background in the vicinity of nt 7940, although a few weak bands were seen in the region around nt 7900 and 50 (Fig. 2A, lane 5). Additional bands have also been observed with primer extension and S1 analysis of ID13 RNA, but these were very minor (4). These analyses indicate that P_{7940} is not utilized to a detectable degree in this in vitro transcription system.

Transcription from the late region. The plasmid pE⁻XM, which contains the *Xba*I-*Mlu*I fragment of BPV-1 (nt 6134 to 7356) cloned into a pSV0CAT expression vector (p402), was used as template DNA for the study of P_{7185} and P_L. Runoff analysis of RNA transcribed from this template in vitro showed a definite α-amanitin-sensitive transcript of 334 nt, consistent with a transcript from P_{7185} (Fig. 3A). A very weak and indistinct transcript of about 270 nt was usually noted as well. This size would be consistent with an in vitro transcript initiated at P_L. The late promoter has been shown to have very heterogeneous start sites (nt 7214 to 7256) (4). Since the weak and indistinct nature of the 270-nt product could have been due to the heterogeneity in size of the transcripts, primer extension and nuclease S1 analyses were used to determine the in vitro polymerase II initiation sites. Primer pr7356 (Table 1) was 5′-end labeled with ^{32}P and used for both primer extension analysis and generation of the 5′-end-labeled single-stranded S1 probe. Both primer exten-

sion and nuclease S1 analysis of RNA transcribed in vitro from pE$^-$XM (Fig. 3B, lanes 8, 17, and 18, respectively) gave prominent products at nt 7185 which comigrated with products generated with wart (lanes 5, 11, and 12) and cycloheximide-treated ID13 cell RNAs (lanes 6, 13, and 14), confirming the activity of P_{7185} in vitro. In addition, very minor primer extension products were seen in the initiation region of P_L (nt 7214 to 7256) for both RNA transcribed in vitro from pE$^-$XM (lane 8) and cycloheximide-treated ID13 cell RNA (lane 6). Overexposure (approximately 30-fold) of the nuclease S1 analysis (lanes 21 to 23) revealed nuclease S1-resistant products with similar mobilities, indicating that there are low-abundance RNAs with 5′ termini in this region. No primer extension or nuclease S1-resistant products were seen with RNA transcribed in vitro from p402 (lanes 7, 15, and 16, respectively) or from pE$^-$XM in the presence of α-amanitin (lanes 9, 19, 20, and 23), indicating both template and polymerase II dependence of the products. We conclude from these analyses that P_{7185} is efficiently utilized in the in vitro transcription system used here. In addition, there appears to be a low level of transcription from P_L in vitro as well.

Transcription from P_{2443} and P_{3080}. Applying electron microscopy, nuclease S1, and cDNA analyses, Stenlund et al. (40) and Yang et al. (43) described classes of RNAs in BPV-1-transformed cells with 5′ ends mapping near nt 2443. To determine whether a promoter could be detected in this region of the genome, in vitro transcription was performed with supercoiled p142-6 DNA as the template. The reaction was carried out in the presence or absence of α-amanitin (1 μg/ml), conditions which selectively inhibit RNA polymerase II. The supercoiled DNA was effectively utilized as template and yielded a considerable amount of RNA initiated in the region between nt 2440 and 2450 (Fig. 4, lane 5). In vitro-transcribed RNA as well as RNA from transformed cells also showed transcripts from additional start sites near nt 2410 (1). The in vitro-transcribed RNAs were α-amanitin sensitive (Fig. 4, lane 6), indicating that they were transcribed by RNA polymerase II. Thus, it is most likely that P_{2443} is an authentic promoter with heterogeneous start sites and that the in vivo RNAs with 5′ termini mapping in the vicinity of nt 2443 originate from transcription initiation at this promoter and not from a posttranscriptional processing event.

The last promoter region we studied is P_{3080}. Primer extension and nuclease S1 analysis of in vivo RNA from BPV-1-transformed cells revealed major RNA start sites at nt 3070 to 3080 and minor start sites around nt 3010 (1, 4, 33). Supercoiled p142-6 DNA was used as the template for in vitro transcription, and the in vitro transcripts were subjected to primer extension analysis with a 5′-end-labeled primer complementary to nt 3159 to 3178. The transcription initiation sites between nt 3070 and 3080 were utilized in vitro (Fig. 5, lane 5), and transcription in the presence of α-amanitin abolished the RNAs starting at these positions (Fig. 5, lane 6), indicating that they were synthesized by RNA polymerase II. Overexposure of the autoradiograph showed additional larger primer extension products in vitro as well, confirming the heterogeneity of P_{3080}.

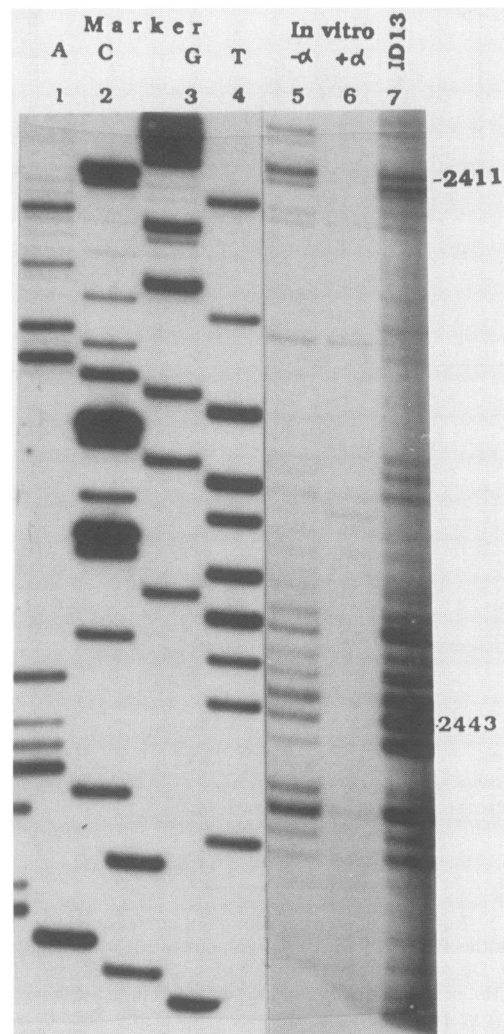

FIG. 4. Primer extension analysis of RNA transcribed in vitro from P_{2443}. Supercoiled plasmid DNA containing the entire BPV-1 genome was used as the template in this experiment. The transcription was performed in either the absence (−α) or presence (+α) of α-amanitin. The resulting transcripts were analyzed by primer extension (lanes 5 and 6) with a synthetic oligonucleotide primer complementary to nt 2481 to 2500. Total cellular RNA from ID13 cells (lane 7) was analyzed in a similar manner for comparison. Markers (lanes 1 to 4) were prepared by dideoxy sequencing of a single-stranded BPV-1 DNA cloned into the *Sal*I site of M13mp7 with the same primer mentioned above. Numbers to the right indicate BPV-1 nucleotide positions. Starting at nt 2420, the sequence pattern is that of M13mp7 (nt 6248).

DISCUSSION

In this study we have shown that five BPV-1 promoters previously mapped in vivo (1, 4, 40) are active in HeLa whole-cell extracts. RNA processing is usually negligible in these extracts (24 and references therein) and has been observed only in a few cases and with low efficiency (<2%) when high concentrations of template DNA (200 μg/ml) were used (19), incubation periods were several hours long, or very active promoters like the adenovirus type 2 major late promoter served as templates (32). In these respects the transcription conditions applied in this study were suboptimal. Therefore, the results presented here provide good evidence that each of these sites is a transcriptional promoter. This is particularly important for P_{2443} and P_{3080}, which lie downstream of the LCR of BPV-1. It was a formal possibility that the RNA 5′ termini mapping in the vicinity of nt 2443 and 3080 were not derived from transcription initiation but rather from some posttranscriptional processing

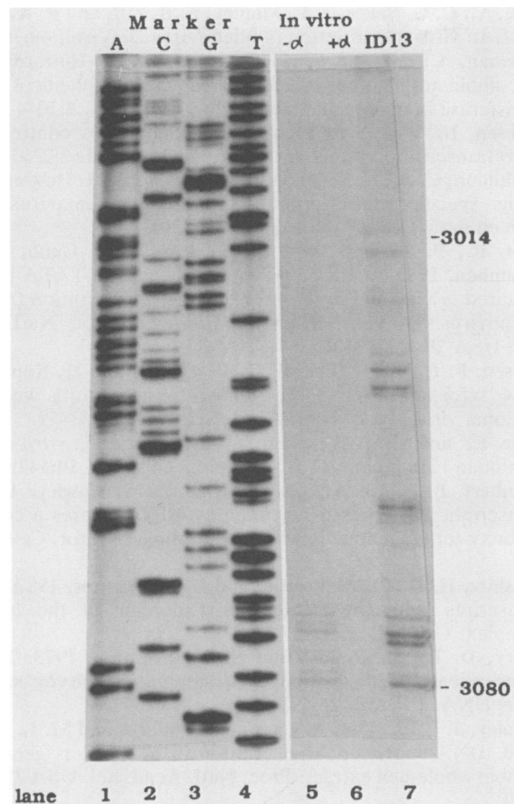

FIG. 5. Primer extension analysis of RNA transcribed in vitro from P_{3080}. A supercoiled plasmid containing the entire BPV-1 genome was transcribed in vitro in the presence (+α) or absence (−α) of α-amanitin. The transcripts were annealed to a 5′-end-labeled primer (nt 3178 to 3159), and the primer was extended with reverse transcriptase (lanes 5 and 6). Total cellular RNA from ID13 cells (lane 7) was also analyzed for comparison. The primer extension products were separated on an 8% sequencing gel next to size markers (lanes 1 to 4), which are the dideoxy sequencing products obtained with a single-stranded BPV-1 DNA fragment cloned into M13mp7 and the same synthetic primer. Numbers to the right indicate BPV-1 nucleotide positions. The sequence pattern represents vector sequences (nt 3080 [BPV] = nt 6218 M13).

event. The establishment of promoters at these positions is important because these promoters are most likely responsible for the transcription of mRNAs encoding the full-length E2 transactivator (38) and the truncated E2 repressor (20). Thus, modulation of the activity of the E2-responsive promoters in the 3′ LCR (P_{7940} and P_{89}) could be mediated through regulation of P_{2443} and P_{3080}.

The most active BPV-1 promoters in the in vitro transcription system were P_{89} and P_{7185}. The activity of P_{89} in vitro was not unexpected, since it is one of the most active promoters in vivo and has both TATA and CAAT box signals. These elements, which are present in many eucaryotic promoters, have been shown to be sufficient for accurate and efficient transcription initiation in vitro (15, 17). The LCR E2-responsive element (E2RE1) required for efficient in vivo utilization of P_{89} (37) was not required for the in vitro activity of P_{89}, since this promoter was equally active with and without these sequences. However, transcription from P_{7940}, which requires E2 transactivation of the upstream enhancer element as well, was not detected under the in vitro conditions. E2RE1 cannot be expected to have an effect in the HeLa cell extracts because it requires the product of the papillomavirus E2 ORF for its function. Although HeLa cells contain and express the human papillomavirus type 18 genome, the E2 ORF has been disrupted by integration, and thus HeLa cells are incapable of making the E2 protein (36). In the case of P_{89}, the presence of the TATA and CAAT box signals appears to be sufficient for a basal level of transcription in vitro. P_{7940}, which lacks a TATA box at the appropriate distance upstream, however, might only be recognized after specific transcriptional activation.

The strong activity of P_{7185} in vitro was surprising, since it is the weakest BPV-1 promoter in vivo. This promoter does not have a classical TATA box, although the sequence TAATAAAAAT is present approximately 30 nt upstream of the RNA start site. It is likely that this AT-rich element, which also contains the polyadenylation signal (A_2UA_3) used by late-region mRNAs (4, 12), serves as a functional TATA box. Stenlund et al. (39) have shown that a sequence element which lies immediately downstream of the P_{7185} cap site is required for in vitro transcription from P_{7185}. This downstream region contains both an $ACCN_6GGT$ motif, which has been shown to bind the viral E2 protein (3, 27, 28), and the sequence motif CCACACCC, which is an upstream domain of the β-globin promoter and is homologous to the BPV-1 distal enhancer and a simian virus 40 (SV40) enhancer domain (39, 41).

Despite the rather strong activity of P_{7185} in the in vitro transcription system with the HeLa whole-cell extract, we were unable to detect transcripts coming from this promoter in transiently transfected HeLa cells (data not shown). One reason for this discrepancy between the in vitro and in vivo activity of P_{7185} could be the lack of competition between many different promoters under in vitro transcription conditions, which might enable us to observe transcripts coming from this start site, whereas under in vivo conditions, the necessary transcription factors would have been bound by stronger promoters. The results of two recently reported expression studies could indicate another possibility. Stenlund et al. (39) reported the in vivo expression of P_{7185} in ID13 and C127 cells. They observed very low levels of CAT activity (2 and 8%, respectively, after overnight incubation) with a BPV-1 DNA fragment comprising nt 6960 to 7276 (*Hin*dIII-*Nar*I). Spalholz et al. (37) did not observe expression of CAT in CV-1 cells from BPV-1 DNA extending from the *Hin*dIII site through approximately nt 7400. It is conceivable that the additional BPV-1 sequences in our vector (nt 6134 to 7352) and those of Spalholz et al. (37) contain *cis* elements inhibitory to transcription in vivo. The dramatic stimulation of P_{7185} by cycloheximide (4) suggests that this promoter is under the control of a short-lived repressor in vivo. It has been shown for a number of promoters that transcription in vitro does not necessarily respond to all the sequences controlling expression in vivo. For example, utilization of the SV40 early promoter in vitro seems largely independent of the presence of the SV40 enhancer (15; unpublished data) or at most confers some transcription stimulation when it is in its original 5′ position (8, 42). The late SV40 promoter is as well efficiently transcribed in vitro and does not require T-antigen binding for in vitro activity (7, 15).

Both P_{2443} and P_{3080} were active in HeLa cell extracts as well, although to a lesser degree than P_{89} and P_{7185}. These promoters showed the same heterogeneity in initiation in vitro as in vivo. Like P_{7185}, P_{2443} does not have a classical TATA box but does have the sequence TAATATT (BPV-1 nt 2414 to 2420) which may function as a TATA box. P_{3080},

however, does not have any recognizable TATA box. Faithful in vitro transcription has been shown previously for the adenovirus IVa2 (26, 29) and EIIaE (13, 25) promoters and the SV40 late promoter (7), all of which lack TATA boxes.

The BPV-1 late promoter (P_L) was previously shown to be responsible for transcription of most of the BPV-1-specific mRNA in the fibropapilloma (4). This promoter produces no detectable RNA in the fibroblasts of the fibropapilloma and has only very low basal activity in ID13 cells. It is likely that this promoter requires factors specific for the differentiating keratinocytes for efficient utilization in vivo. The fact that P_L is active in HeLa cell extracts, although at a very low level compared with P_{7185}, raises the possibility that these cells contain regulatory transcription factors that can simulate the situation in the wart. To study this possibility further, future experiments will include binding studies to determine possible factors in the HeLa extract which might be involved in the utilization of this BPV-1 promoter and to compare the pattern with similar extracts from warts. The extremely low activity of the late promoter in the in vitro transcription system should also allow the use of this system to assay for possible *trans*-acting factors present in productively infected tissues.

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