

Enhancers and *trans*-Acting E2 Transcriptional Factors of Papillomaviruses

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The upstream regulatory regions of human papillomavirus (HPV) types 1, 6b, 7, 11, 16, and 18, bovine papillomavirus type 1, and cottontail rabbit papillomavirus were cloned into transcriptional enhancer assay plasmids which carry the simian virus 40 early promoter lacking its own enhancer and the bacterial gene encoding chloramphenicol acetyltransferase (EC 2.3.1.28) (CAT). Enhancer activity, reflected by CAT gene expression, was detected in all of the upstream regulatory regions tested only when the recombinants were cotransfected with plasmids which express an intact E2 open reading frame of HPV types 1 and 11 or bovine papillomavirus type 1. Each E2 protein stimulated the enhancer from the same virus and, to somewhat lesser degrees, also those from the heterologous viruses. Hence, the enhancer and the E2 protein are functionally conserved among papillomaviruses. There was some nonreciprocity in the extent of *trans*-activation in heterologous E2-enhancer interactions. Primer extension analyses demonstrated that the E2 proteins increased the abundance of CAT gene mRNA. Tandem multiplication of the HPV type 11 enhancer sequence dramatically increased its response to E2 stimulation; this is possibly relevant to the pathogenicity of papillomaviruses.

Papillomaviruses induce benign epithelial proliferations (warts) in many species of animals, including humans (for a review, see reference 35). Recently, the human papillomaviruses (HPVs) have received particular attention because some are associated with genital tract dysplasias and carcinomas (52). About 45 types of human HPVs have been identified, and each has a closed circular double-stranded DNA genome about 7,900 base pairs (bp) long. Nucleotide sequence analyses show that the genome organization is highly conserved among these viruses (4). Each type of papillomavirus has a tropism for a particular kind of epithelium. Moreover, elevated levels of viral DNA replication and RNA transcription are attained only in the epithelial strata consisting of more highly differentiated keratinocytes. As there is no permissive *in vitro* culture system for papillomaviruses, our understanding of the regulation of transcription and replication and the mechanism of pathogenesis is limited. The major exception is bovine papillomavirus type 1 (BPV-1), which can replicate in and transform certain mouse cell lines (14). In these cells, only the early genes are expressed and the functions of various open reading frames (ORFs) have been assigned based on elegant genetic analyses (for a review, see references 3a and 4).

Enhancer sequences stimulate transcription of linked genes in a fashion relatively independent of orientation and location and have been identified in papovaviruses, adenoviruses, hepatitis B virus, retroviruses, immunoglobulin genes, and interferon genes (for a review, see reference 41). Certain enhancers appear to be involved in host and tissue specificity. All papillomaviral DNAs have a long sequence which lacks apparent ORFs located downstream from the late region and preceding the early region. It is termed the upstream regulatory region (URR), synonymous with noncoding region or long control region. The replication origin (29, 48) and several promoters have been mapped in this region, based primarily on the 5' ends of viral transcripts (2a, 7, 12, 33, 43, 45, 47, 50; J. Choe and M. Botchan,

personal communication). Recently, an enhancer sequence was identified in the URR of BPV-1 (46). It is activated by a *trans*-acting factor encoded by the E2 ORF (46, 51). All of the sequenced papillomaviruses, including HPVs, cottontail rabbit papillomavirus (CRPV), and BPV-1, have an E2 ORF of analogous size and genomic location. Here, we describe the identification and characterization of the HPV-1 and HPV-11 E2 enhancer-stimulating proteins and the interactions of E2 proteins of HPV-1, HPV-11, and BPV-1 with enhancers of many different HPVs as well as those of BPV-1 and CRPV.

MATERIALS AND METHODS

Construction of CAT plasmids. Figure 1 shows the enhancer assay plasmids pCAT-A and pCAT-B. These vectors contain the bacterial chloramphenicol acetyltransferase (EC 2.3.1.28) (CAT) gene under control of the simian virus 40 (SV40) early promoter, which lacks the SV40 enhancer. The *SphI*-*BamHI* fragment isolated from pSV2CAT (20) was made blunt end with S1 nuclease and cloned into the *SmaI* site of pUC19. The URR sequences were then cloned into these plasmids. The pUR series plasmids except pUR14 and pUR15 were constructed by cloning DNA fragments (see Table 1 and Fig. 3) into the polylinker sites (*HindIII*, *PstI*, *SalI*, and *BamHI*) of pCAT-A. pUR14 and pUR15 were constructed by cloning the DNA fragment into the *BamHI* site of pCAT-B. The pUR plasmids carry the URR sequences in the same or opposite orientation relative to the CAT gene as to the papillomaviral ORFs. The DNA restriction fragments used for cloning were isolated by agarose gel electrophoresis and electroelution.

The plasmids carrying multiple copies of the HPV-11 enhancer sequence were constructed as follows. The *BamHI*-*BstEII* fragment of HPV-11 DNA (nucleotides [nt] 7072 to 7904) was first cloned into the *SalI* site of pCAT-A to make plasmid pUR21. The 5' sequences not necessary for the enhancer activity were deleted by BAL 31 nuclease resection. One to five copies of the resulting enhancer sequence (nt 7674 to 7904) were cloned into the *BamHI* site

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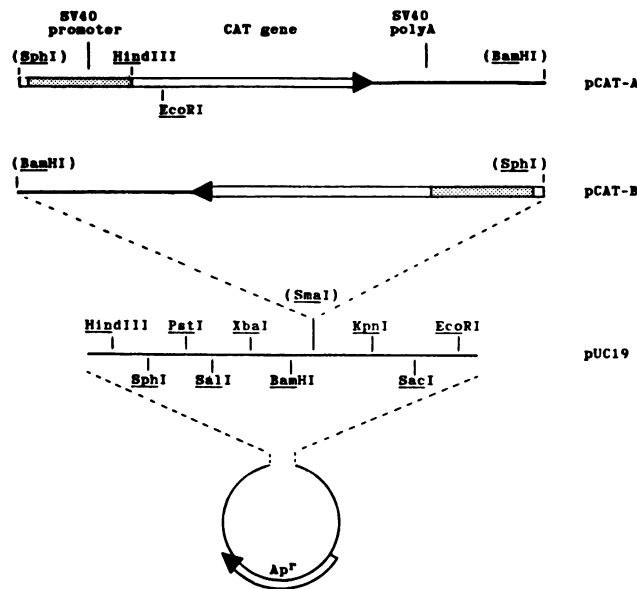


FIG. 1. Structures of enhancer assay plasmids. The *Sph*I-*Bam*HI fragment from pSV2CAT was cloned into the *Sma*I site of pUC19 in either orientation to generate pCAT-A and pCAT-B. These plasmids carry the CAT gene downstream from the SV40 early promoter but lack the SV40 enhancer sequence. The various restriction fragments of papillomaviral DNAs were recombined into the polylinker of these plasmids to test for enhancer sequences. Ap^r, Ampicillin resistance.

of pCAT-A in the sense orientation. The details of the construction of the enhancer deletion mutations and the analysis of enhancer activity will be described elsewhere (H. Hirochika et al., manuscript in preparation).

Construction of E2 ORF expression plasmids. Figure 2 shows the construction of eucaryotic expression plasmids of the HPV-1 E2 ORF, which extends from nt 2568 through nt 3794, with the first AUG at nt 2592. pRSE2(1-1), pRSE2(2-1), pRSE2(3-1), and pRSE2(4-1) were constructed as follows. The papillomaviral DNA fragments generated by restriction enzyme digestions of a whole genomic HPV-1 clone were purified and first inserted into polylinker sites of pUC19. Cloned fragments were isolated by digestion with *Sac*I and *Sal*I and ligated to the *Sac*I and *Xho*I sites in a Rous sarcoma virus (RSV) vector (kindly supplied by Joe Sorge) downstream of the long terminal repetition (LTR) but upstream of the AUG initiation codon of the *gag* gene. For construction of pRSE2(6-1) and pRSE2(7-1), an HPV-1 clone linearized with *Sac*I (nt 2359) was treated with BAL 31 nuclease, digested with *Bgl*II, and cloned into the *Sma*I and *Bam*HI sites of pUC19. The desired deletion mutations were then cloned into the RSV LTR vector as described above. pRSE2(6-1)BE was constructed as follows. pRSE2(6-1), which has three *Bst*EII sites, was partially digested with *Bst*EII to generate a linear molecule, and the overhanging ends were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I. Following blunt-end ligation, the clone with a 5-bp frameshift insertion in the E2 ORF at nt 2945 was selected.

The E2 ORF of HPV-11 extends from nt 2696 through nt 3823, with the first AUG at nt 2723. To construct pRSE2-11, the *Xmn*I fragment of HPV-11 DNA (nt 2661 to 4984) was first cloned into the *Sma*I site of pUC19 in the appropriate orientation and subsequently transferred into the RSV LTR

vector as described above. The deletion mutation pRSE2-11d was constructed as follows. pRSE2-11 was digested with *Bst*NI, and the ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I. After digestion with *Bam*HI located downstream of the HPV-11 sequence in the polylinker, the DNA fragment (nt 2756 to 4984) was isolated and cloned into the RSV LTR vector at the *Sac*I (blunt ended) and *Bam*HI sites.

The E2 ORF of BPV-1 extends from nt 2581 through nt 3834, with the first AUG at nt 2608. pRSE2-BP was constructed by cloning the *Bst*EII-*Bam*HI fragment (nt 2405 to 4451) into the *Bst*EII-*Bam*HI sites of the RSV LTR vector. *Bst*EII and *Bam*HI sites exist to the upstream side of the *Sac*I and *Xho*I sites, respectively, of the RSV LTR vector.

All manipulations were performed by procedures described by Maniatis et al. (30). Final constructions were confirmed by restriction enzyme analysis. Deletion endpoints were determined by M13 cloning and dideoxynucleotide sequencing (31, 39). All plasmid DNAs were amplified in DH1 cells (23) and purified by banding in CsCl-ethidium bromide equilibrium density gradients. Plasmid DNAs were examined by agarose gel electrophoresis before transfection into CV1 cells. Only preparations containing more than 80% of the plasmid DNA in monomeric covalently closed circular form were used.

Eucaryotic cell transfection and CAT assays. Monkey CV1 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The cells were split 1:7 24 h before transfection. One milliliter of the calcium phosphate-DNA coprecipitate containing a total of 15 μ g of

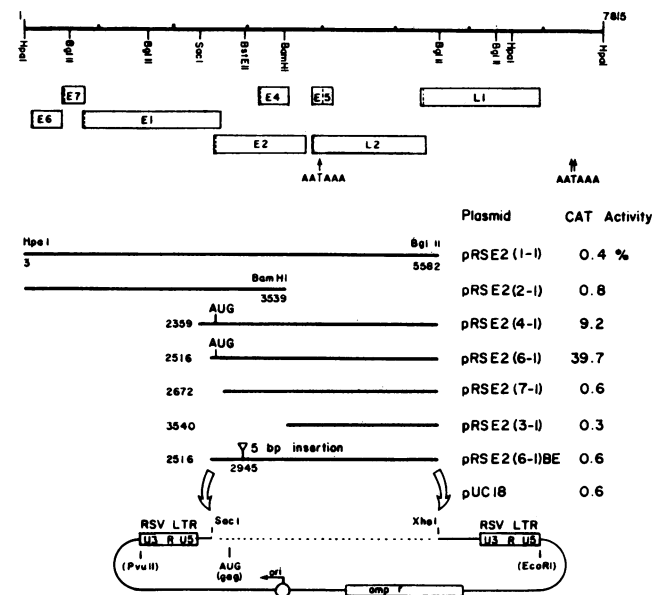


FIG. 2. Eucaryotic expression plasmids of the HPV-1 E2 ORF and their ability to *trans*-activate CAT activity. Various restriction fragments of HPV-1 were cloned into the RSV vector, as described in Materials and Methods. The nucleotide positions of the fragments are indicated and are aligned with the DNA restriction map and the ORFs. Ten micrograms of each of these plasmids or pUC18 control plasmid, together with 5 μ g of pUR1 (HPV-1 nt 6578 to 3 in pCAT-A), was transfected into a set of 100-mm (diameter) plates of CV1 cells. One-tenth of each cellular extract prepared 48 h posttransfection was assayed for CAT activity; the percentages of chloramphenicol acetylated are reported. ori, Origin of replication; amp^r, ampicillin resistance.

plasmid DNAs was applied to a 100-mm (diameter) plate of CV1 cells (22). At 4 h after transfection, the cells were treated with 15% glycerol for 3 min at room temperature. The cells were then washed and cultured in medium containing 1 mM sodium butyrate (18). Cell extracts were prepared 48 h after transfection as follows. The cells were harvested after trypsinization, and the cell pellets were suspended in 100 μ l of 0.25 M Tris hydrochloride (pH 7.8). Extracts were prepared by three freeze (-70°C)-thaw (37°C) cycles. Lysate (10 μ l) was added to 15 μ l of a reaction mixture which consisted of 0.25 M Tris hydrochloride (pH 7.8), 3.3 mM acetyl coenzyme A, and 0.2 μ Ci of [^{14}C]chloramphenicol (40 to 50 mCi/mmol; New England Nuclear Corp.). Incubation was usually at 37°C for 1 h unless otherwise stated. Samples were processed as described by Gorman et al. (20). The percent conversion of chloramphenicol to its acetylated forms was determined by liquid scintillation counting.

RNA preparation and primer extension analysis of CAT mRNA. Cytoplasmic RNA was prepared from CV1 cells transfected with various plasmids as described by Anderson et al. (1). An oligonucleotide 5' TCCATTTAGCTTCCT-TAGC 3' complementary to the 5' end of the CAT gene coding region was used as a primer for mapping the 5' ends of the CAT mRNA. Primer (0.0001 A_{260} unit) was labeled at the 5' end with [^{32}P]ATP by using T4 polynucleotide kinase, annealed with 20 μ g of cytoplasmic RNA, and extended by reverse transcriptase. The products were analyzed by electrophoresis on an 8% polyacrylamide sequencing gel.

RESULTS

Identification of the HPV-1 gene encoding the enhancer-stimulating factor. To screen for potential papillomavirus enhancer sequences, assay vectors were constructed (Fig. 1). These plasmids contained the CAT gene linked to an SV40 early promoter which lacked its own enhancer sequences. The enhancer activities of papillomaviral DNA fragments incorporated into the vector were monitored by assaying expression of the CAT gene after transfection into CV1 cells. This CAT assay system has been used in many similar studies (19, 20, 21, 25, 27, 34, 38, 46).

We first searched for the enhancer sequences and enhancer-stimulating factor in HPV-1 which causes plantar warts and the transcription of which has been studied in our laboratory (6, 7). It is also of interest to compare HPV-1 and BPV-1 because they differ in many of their biological properties (11). A DNA fragment containing the URR of HPV-1 (nt 6578 to 3) was cloned into the *Bam*HI site of pCAT-A (Fig. 1). The resulting plasmid, pUR1, carried the URR in the same orientation relative to the CAT gene as to HPV ORFs (the sense orientation). This plasmid showed little CAT activity when transfected by itself into CV1 cells (Fig. 3). To identify the gene coding for an enhancer-stimulating factor, different regions of HPV-1 DNA were cloned into an RSV LTR expression vector in the sense orientation. These plasmids were cotransfected into CV1 cells together with pUR1. The structures of the plasmids and the resulting CAT activities are shown in Fig. 2. pRSE2(4-1) and pRSE2(6-1), which contained the intact E2 ORF immediately adjacent to the LTR promoter, elevated CAT activity 15- and 66-fold, respectively. The weaker activity by pRSE2(4-1) than by pRSE2(6-1) can be rationalized by a diminution of translation of the E2 ORF caused by additional out-of-frame AUG codons preceding the putative E2 initiation codon. When the 5' portion of the E2 ORF was deleted as in pRSE2(7-1), the activity was lost. pRSE2(6-1)BE, which has a frameshift

TABLE 1. E2-dependent enhancer activity of HPV-1 DNA fragments^a

Plasmid	Nucleotide position of fragment cloned (length [bp])	Orientation ^b	Site ^b	CAT activity
pUR8	3-4527 (4,524)	Sense	5'	0.65
pUR9	3-4527 (4,524)	Antisense	5'	0.83
pUR6	4527-744 (4,032)	Sense	5'	25.9
pUR7	4527-744 (4,032)	Antisense	5'	6.6
pUR1	6578-3 (1,240)	Sense	5'	62.1
pUR2	6578-3 (1,240)	Antisense	5'	11.0
pUR14	6578-3 (1,240)	Sense	3'	8.1
pUR15	6578-3 (1,240)	Antisense	3'	3.5
pUR12	7235-3 (583)	Sense	5'	32.4
pUR13	7235-3 (583)	Antisense	5'	5.5
pUR10	7235-97 (677)	Sense	5'	15.5
pUR11	7235-97 (677)	Antisense	5'	4.6
pCAT-A				2.3
pCAT-B				1.7
pSV2CAT				94.7

^a Five micrograms of each of the plasmids, together with 10 μ g of pRSE2(6-1), was transfected onto a set of 100-mm (diameter) plates of CV1 cells. After 48 h, protein extracts were made and 1/10 of each extract was assayed. CAT activity is expressed as percent conversion of [^{14}C]chloramphenicol to monoacetylated forms during incubation.

^b These are relative to the CAT gene of the cloning vector.

insertion of 5 bp in the 5' portion of the E2 ORF, had no activity either. This 5-bp insertion precedes the E4 ORF and affects only the E2 ORF. A 4-bp insertion at the *Bam*HI site (nt 3539) near the 3' terminus of the E2 ORF also abolished enhancer-stimulating activity (data not shown). Together, these results clearly demonstrate that the E2 ORF encodes a *trans*-acting enhancer-stimulating factor.

We also examined the possibility of additional enhancer *trans*-activating genes by expressing other regions of HPV-1 DNA in the RSV LTR vector (Fig. 2). For instance, pRSE2(2-1) carries the E6, E7, and E1 ORFs; it showed no stimulatory activity. pRSE2(1-1) contains E6, E7, E1, and the intact E2 ORF but had no activity either. This might be explained by the extensive upstream coding sequences blocking initiation of translation at E2. However, a similar recombinant plasmid of BPV-1 showed significant activity (46), perhaps due to the presence of a promoter near the beginning of the E2 ORF (50). No corresponding promoter has been detected in HPV-1, HPV-6, or HPV-11 (5a, 7).

Localization and characterization of the E2-dependent enhancer sequence of HPV-1. We examined the effect of the orientation and location of the URR enhancer sequences relative to the SV40 promoter in the CAT gene expression vector. The URR sequence (nt 6578 to 3) was cloned in both the sense (pUR1) and antisense (pUR2) orientations. pUR2 produced significant but reduced CAT activity relative to pUR1 (Table 1). When the same fragment was cloned in either orientation to the 3' side of the CAT gene (pUR14 and pUR15), activity was reduced further but was still obvious and some orientation dependence was also observed. When a much longer genomic fragment (nt 4527 to 744) including the URR was tested (pUR6 and pUR7), the activity was also reduced. In combination, these results indicate that the distance between the enhancer and the promoter can affect transcriptional activity. When a smaller URR fragment (nt 7235 to 3) was used (pUR12 and pUR13), the activity was lower than that generated by pUR1, suggesting that the sequences between nt 6578 and 7235 can supplement the enhancer located between nt 7235 and 3. The addition of the short adjacent sequence (nt 3 to 97) caused a reduction in

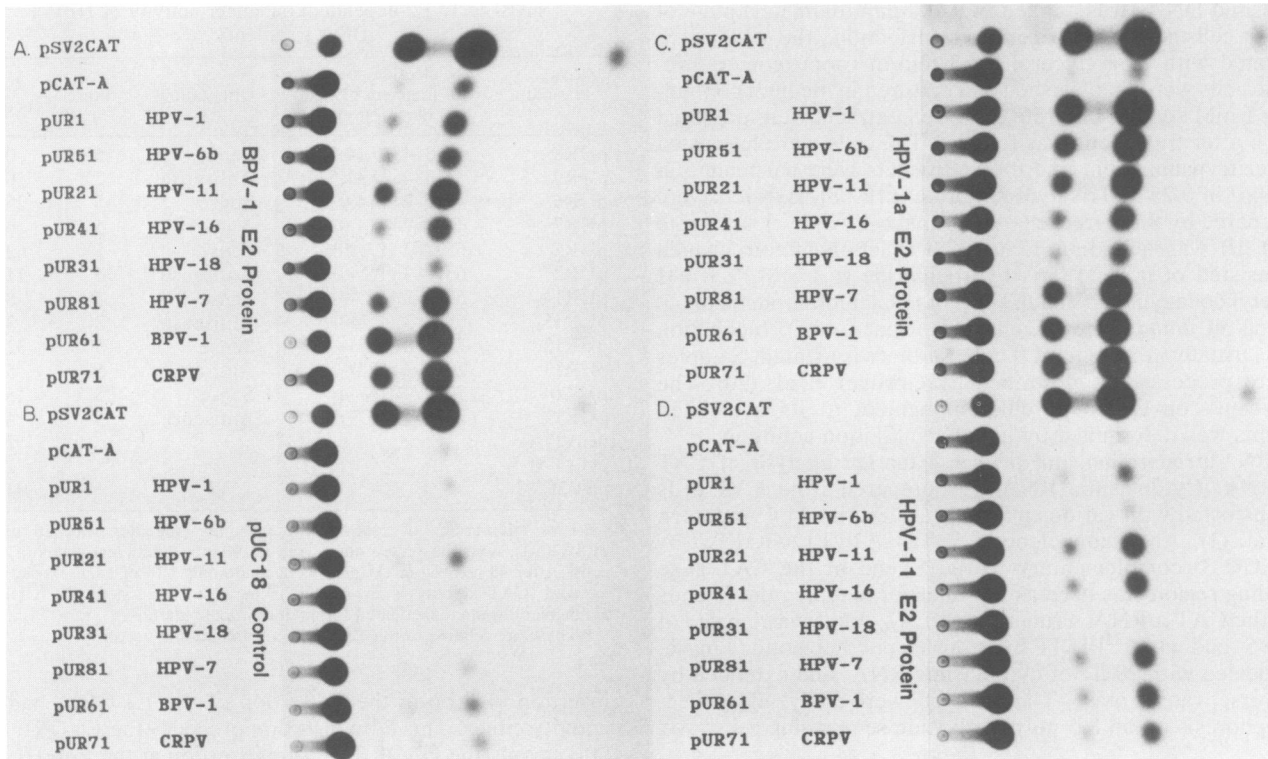


FIG. 3. Identification of papillomavirus enhancers and enhancer-stimulating proteins and demonstration of cross-activation. Sections A, B, C, and D show the CAT activities resulting from cotransfections of monkey CV1 cells with pRSE2-BP, pUC18 control, pRSE2(6-1), and pRSE2-11, respectively, together with the pUR series of plasmids. The pUR plasmids contain, in the natural sense orientation, the upstream regulatory regions of each of the papillomavirus types immediately preceding the SV40 promoter in the pCAT-A expression vector. The URR segments present in the plasmids are HPV-1 (nt 6578 to 3), HPV-6b (nt 6499 to 236), HPV-11 (nt 7072 to 7904), HPV-16 (nt 6359 to 280), HPV-18 (nt 6809 to 119), HPV-7 (nt 6900 to 48), BPV-1 (nt 6958 to 3), and CRPV (nt 7106 to 187). Transfections and CAT assays were performed as described in the legend to Fig. 2.

CAT activity (pUR10 and pUR11). This region has a putative HPV promoter (see below) which might have interfered with the distal SV40 promoter, as has been demonstrated in other systems (26, 40, 49).

We have recently performed electron microscopic R-loop analysis of mRNAs recovered from HPV-1-induced plantar warts (7). The 5' end of most RNAs mapped to the 3' end of the E7 ORF, with much less frequent 5' ends located at the 5' end of the E6 ORF. This suggests the possibility of another enhancer sequence near the E7 ORF. Lusky et al. (28) found a constitutive enhancer sequence 5' to the L2 ORF in BPV-1 DNA. pUR8 and pUR9 carry both of these regions from HPV-1 (nt 3 to 4527). Neither had any enhancer activity in the CAT assay. We conclude that only the region located between nt 7235 and 3 of HPV-1 has an E2-dependent enhancer sequence active in CV1 cells.

Specificity of enhancers and enhancer-stimulating factors. To express the potential enhancer-stimulating factor of HPV-11, as well as that of BPV-1 (46) as a positive control for comparison, DNA fragments of HPV-11 (nt 2661 to 4986) and BPV-1 (nt 2405 to 4451) carrying intact E2 ORFs were cloned into the RSV LTR vector. The URRs from HPV types 6b, 7, 11, 16, and 18; CRPV; and BPV-1 were cloned into pCAT-A. The CAT activities obtained by transfection with these plasmids are shown in Fig. 3. Both the HPV-1 and BPV-1 E2 proteins activated enhancers in the URR of each virus type except for HPV-18, which responded relatively weakly to HPV-1 E2 and marginally, if at all, to BPV-1 E2. Enhancer stimulation by the HPV-11 E2 plasmid (pRSE2-11)

was relatively weak but nonetheless reproducible. It did not activate the enhancers of HPV-6b or HPV-18. Another HPV-11 URR enhancer assay plasmid, pUR27, was constructed and tested. It contained nt 7072 to 64 and thus was more comparable than pUR21 to enhancer plasmids from the other papillomaviruses. pUR27 had a lower basal activity than pUR21 (Fig. 3 and 4), but the response to HPV-11 E2 was still weak (Fig. 4). However, the activity of HPV-11 E2 was much more obvious when CAT plasmids carrying multiple copies of enhancer sequences were used (see below).



FIG. 4. Mutational analysis of the HPV-11 E2 ORF. Transfection and the CAT assay were performed as described in the legend to Fig. 2.

To demonstrate unequivocally that the E2 ORF of HPV-11 indeed encodes a *trans*-acting factor, a deletion mutation of the E2 expression plasmid pRSE2-11d (nt 2756 to 4984) was constructed. It lacked the 5' end of the E2 ORF including the first AUG codon (nt 2723 to 2725); the deletion did not affect any other ORF. There are two out-of-frame AUG codons before the next in-frame AUG (nt 2813 to 2815) located 30 codons downstream of the first. pRSE2-11d did not *trans*-activate either pUR21 or pUR27 carrying the HPV-11 URR (Fig. 4). Based on these results, we conclude that the HPV-11 E2 ORF also encodes an enhancer-stimulating factor.

trans-Activation of enhancers by the three E2 proteins has some specificity. Each stimulated the enhancer from the same virus type more than those from other viruses. However, the heterologous interactions were not strictly reciprocal. For instance, the HPV-1 E2 protein strongly activated the enhancers of BPV-1 and most other papillomaviruses, but the HPV-1 enhancer was only weakly activated by BPV-1 E2 protein. HPV-6b and HPV-11 have an overall sequence homology of about 85% (13). Interestingly, these two viruses had different patterns of activation by two of the three E2 proteins. The HPV-18 URR showed only weak enhancer activity but nevertheless exhibited five- to eight-fold stimulation by BPV-1 and HPV-1 E2 proteins, respectively, relative to its intrinsic activity in the absence of any E2 protein (Fig. 3). The low activity of the HPV-18 enhancer might be explained by the presence of the putative E6 promoter (43) between the enhancer and the SV40 promoter, as discussed above for the HPV-1 enhancer. The effect of E2 proteins on the SV40 enhancer was also examined (Fig. 3). Activation of pSV2CAT was not obvious. This result is in contrast to a previous report (46).

Analysis of CAT gene mRNA by primer extension. To ascertain that the enhancement of CAT gene activity by E2 protein was due to an elevated level of CAT gene mRNA, we examined the CAT gene transcripts by quantitative primer extension analysis. A synthetic oligonucleotide 5' TCCATT-TTAGCTTCCTTAGC 3' complementary to the 5' end of the CAT gene coding region was used as a primer. Reverse transcription with cytoplasmic RNA prepared from pSV2CAT-transfected cells generated four major products (Fig. 5, lane 3). Their lengths indicated the CAT messages were initiated from the SV40 early cap sites (16). The same primer extension products were formed with RNAs prepared from CV1 cells cotransfected with pUR1 carrying the HPV-1 URR and the HPV-1 E2 expression plasmid pRSE2(6-1) (lane 5). No CAT mRNA was detected when pRSE2(6-1) was omitted from the transfection (lane 4). These results indicate that the CAT activity reflects the level of CAT mRNA and that the HPV-1 URR activates the SV40 promoter only in the presence of the HPV-1 E2 protein. The same conclusion can be drawn from the cross-activation of the HPV-11 URR by the BPV-1 E2 protein (lanes 6 and 7).

Augmentation of enhancer strength by increasing the copy number. Recently, duplications of DNA sequences were found in the URRs of several oncogenic HPVs (3, 10, 37). These duplications might augment the enhancer effect, as has been shown for the SV40 enhancer element (51). To examine this possibility, we introduced one to five copies of the HPV-11 enhancer in tandem array into pCAT-A. To minimize the size of the final plasmid, the URR sequences not necessary for enhancer activity, as defined in our fine-structure mapping (Hirochika et al., in preparation), were deleted before ligation of the multiple copies of the enhancer. The plasmid carrying one copy of the DNA segment

between nt 7674 and 7904 had the same activity as pUR21. Multiplication of the enhancer copy number up to four dramatically increased CAT activity in the presence of the E2 expression plasmid (Fig. 6). The basal level of these plasmids also increased with enhancer copy number. The reason for the slight decline in the stimulation of CAT activity in the presence of five copies of the enhancer sequence is not clear.

DISCUSSION

The enhancer and the *trans*-acting factor originally demonstrated for BPV-1 (46) are generalized to many papillomaviruses by our results. We have shown that intact E2 ORFs of HPV-1 and HPV-11, in addition to that of BPV-1, encode a *trans*-acting protein which functions by increasing the level of mRNA transcription (Fig. 5). The E2 proteins of these three viruses activate the enhancers from a number of HPV types, CRPV, and BPV-1 (Fig. 3). However, each protein has some specificity and is most effective with the homologous enhancer. In addition, the heterologous E2-enhancer interactions are not strictly reciprocal. For example, BPV-1 E2 protein activated the BPV-1 enhancer strongly but stimulated the HPV-1 enhancer only mildly, whereas HPV-1 E2 protein activated both enhancers very well.

HPV-11 E2 protein consistently exhibited relatively low activities (Fig. 3). Nonetheless, deletion analysis of the HPV-11 E2 ORF clearly demonstrated that the observed

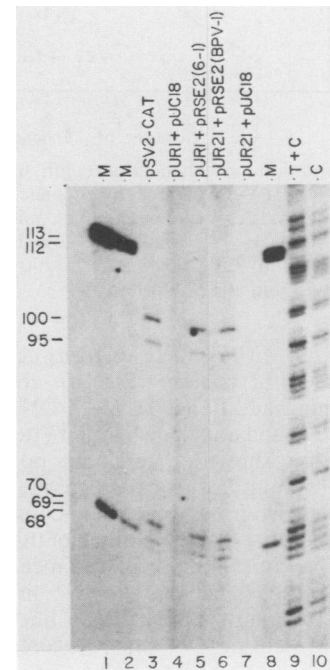


FIG. 5. Primer extension analysis of CAT mRNA. CV1 cells were transfected with various plasmids, and cytoplasmic RNA was extracted and assayed as described in Materials and Methods. Lanes: 1, 2, and 8, length markers (M) generated from pUC19 cleaved with *Hpa*II and 3' end labeled by filling the recessed ends with *E. coli* DNA polymerase I; 3, pSV2CAT plus pUC18; 4, pUR1 plus pUC18; 5, pUR1 plus pRSE2(6-1); 6, pUR21 plus pRSE2-BP; 7, pUR21 plus pUC18; 9 and 10, T + C and C sequence ladders from Maxam-Gilbert (30a) reactions of an unrelated DNA segment serving as length markers. The primary extension products of 100, 95, 70, and 68 nt are indicated on the left.

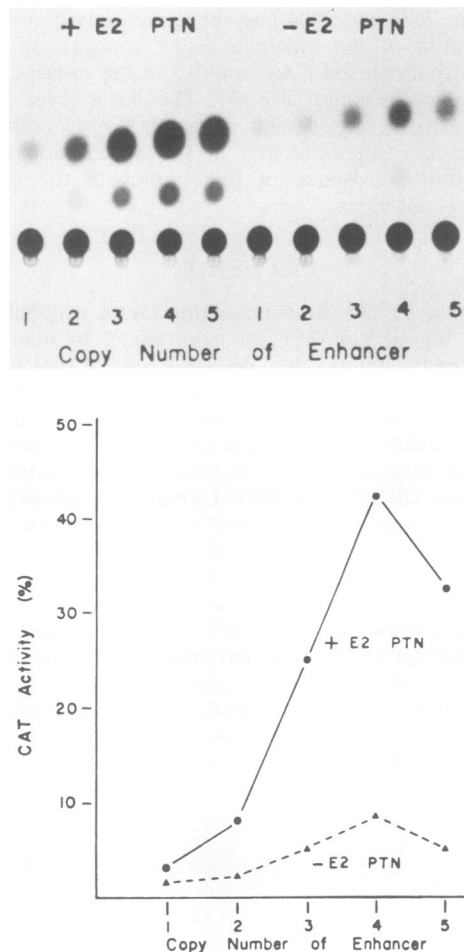


FIG. 6. Augmentation of enhancer strength by increasing the copy number of the HPV-11 enhancer sequence. Portions (5 μ g each) of the CAT plasmids carrying one to five copies of the HPV-11 enhancer (nt 7674 to 7904) were transfected together with 20 μ g of pRSE2-11 or pUC18 into CV1 cells. The CAT assays were performed at 37°C for 20 min. PTN, Protein.

CAT activities were in response to *trans*-activation of the enhancer by the viral protein (Fig. 4). RSV expression plasmids containing additional HPV-11 DNA upstream of the E2 ORF were tested and had slightly better, yet still low, activities (data not shown). We could not rule out the possibility that instability or translational inefficiency of the E2 mRNA derived from our particular constructs contributed to the low activity. However, the fact that CAT activity increased with the copy number of enhancers in the assay plasmid (Fig. 6) suggested that the E2 protein was not limiting and that the low activity was likely an inherent property of the protein in CV1 cells. Recently, HPV-16 E2 protein has also been shown to activate the enhancer sequence of the homologous virus, and its effect was also relatively weak, yielding 2- to 10-fold stimulation over background (36).

The URR is one of the most diverged regions of many papillomaviruses (5, 9). However, almost all of the enhancer sequences were activated by each E2 protein. These results suggest two explanations for the mechanism of E2 protein action. One possibility is that the E2 protein induces or interacts with cellular factors which, in turn, interact with different motifs in the enhancer sequences. The other inter-

pretation is that a short conserved sequence is recognized directly by E2 proteins. A search for such a sequence showed that the tract ACCGNNNCGGT is found in one or multiple copies in all of the sequenced papillomaviruses in the URRs (4, 9, 13, 17). Recently, BPV-1 E2 fusion proteins expressed in *E. coli* were shown to interact with DNA sequences containing this 12-bp consensus sequence (2, 32). This interaction may be important for transcriptional activation of the enhancer. However, this 12-bp consensus sequence alone is not sufficient for specificity because the URRs of HPV-6b and HPV-11, which have the identical 12-bp sequences, show different activation patterns in response to the different E2 proteins (Fig. 3). Our recent results with BAL 31 nuclease deletion mutants of HPV-11 indicate that the 12-bp conserved sequence is the E2 responsive element, and about 150 bp of URR sequence is necessary for full enhancer activity (Hirochika et al., in preparation). Studies of the SV40 enhancer show that it is composed of multiple components which function more or less additively (24, 51). Each component might be recognized by different cellular factors. This interaction may play an important role in the host and tissue specificity of the enhancer (8, 15, 42, 44). Papillomaviruses are highly host and tissue specific. The sequence divergence of enhancers in the URR may be involved in these specificities. We have studied enhancer activity only in monkey CV1 cells. To examine the possibility that it confers host or tissue specificity, experiments in other cells, particularly keratinocyte cultures, in conjunction with the HPV transcriptional promoters are necessary.

We have shown here that tandem copies of the HPV-11 sequence (nt 7674 to 7904) spanning the enhancer increase its strength. A duplication found in HPV-6 subtype d (3) corresponds to the enhancer region identified in the HPV-11 enhancer. This isolate was recovered from a highly differentiated squamous tumor, whereas HPV-6 is usually associated with benign tumors (condylomata). Another variant, HPV-6VC, isolated from an invasive verrucous carcinoma, also carries a short insertion duplication in the same region (37). HPV-33 is associated with cervical cancer. It contains a 78-bp tandem duplication in the URR (10). Our results with artificially assembled tandem multiplications of the enhancer suggest that naturally selected duplications of the enhancer sequence are involved in the higher tumorigenic potential of these viruses by augmenting transcriptional activity.

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