JOURNAL OF VIROLOGY, June 1992, p. 3485–3493
0022-538X/92/063485-09$02.00/0

Vol. 66, No. 6

Late Promoter of Human Papillomavirus Type 8 and Its Regulation

FRANK STUBENRAUCH,[1] JACEK MALEJCZYK,[2] PAWEL G. FUCHS,[1] AND HERBERT PFISTER[1]*

Institut für Klinische und Molekulare Virologie der Friedrich-Alexander Universität, D-8520 Erlangen, Germany,[1] and Department of Embryology and Histology, Warsaw School of Medicine, 02004 Warsaw, Poland[2]

Received 26 December 1991/Accepted 16 March 1992

Human papillomavirus type 8 (HPV8) belongs to the HPV types associated with skin carcinomas of patients with epidermodysplasia verruciformis (EV). Its noncoding regulatory sequences (NCR) were shown to drive the expression of the reporter gene chloramphenicol acetyltransferase (*cat*) in transient assays with human epithelial cells (HT3 cells). This constitutive activity could be enhanced by coexpression of the HPV8 transactivator protein E2. The analysis of 5′ deletions of the NCR showed that the EV-specific sequence motif M33 and the neighboring AP1 site are essential for the promoter activity, whereas 44 nucleotides located immediately upstream of M33 are strongly inhibitory. The same effects were observed in simian virus 40-immortalized fetal keratinocytes (SV61 cells) and spontaneously immortalized skin keratinocytes (HaCaT cells). By using primer extension and RNase protection analyses two promoters could be identified within the HPV8 NCR. A nested set of weak signals, corresponding to start sites between positions 175 to 179, represented the previously described E6 promoter. The vast majority of transcripts was initiated at position 7535 and shown to undergo processing at an NCR-internal splice donor (positions 1 to 8). The promoter P_{7535} is similar to late promoters of other skin-associated papillomaviruses as far as localization, transcript structure, and sequence characteristics are concerned. To confirm that P_{7535}-initiated transcripts proceed indeed to the L1 gene for the major capsid protein, viral mRNAs from an HPV8-induced lesion of a patient with EV were characterized by RNase protection and sequence analysis of polymerase chain reaction-amplified cDNAs. The NCR leader (positions 7535 to 4) appeared in two messages with three exons each. The third exon started with the second ATG codon of L1 in both cases; the short central exons from the 3′ part of the early coding region were defined by a common splice acceptor site (position 3303) and different splice donor sites (positions 3443 and 3704).

Human papillomaviruses (HPV) induce epithelial proliferations of the skin and mucosa in a highly host- and tissue-specific manner. The viral life cycle strictly depends on the differentiation of the keratinocytes. The suprabasal cells of the epithelium permit only expression of the early viral functions and amplification of viral DNA, whereas synthesis of capsid proteins takes place in terminally differentiating cells within the apical part of the tumors (36). The HPV-induced lesions are at first benign, but it is well established that cutaneous macules of patients with epidermodysplasia verruciformis (EV) and cervical dysplasias progress to squamous cell carcinomas in a substantial proportion of cases (33, 50, 51). In skin carcinomas of patients with EV, no virus particles are detectable (39, 49). Malignant conversion is apparently linked to infections with specific HPV types, as reflected by the predominance of HPV type 5 (HPV5) and -8 in skin cancers and HPV16 and -18 in cervical carcinomas.

The cell differentiation-dependent gene expression of papillomaviruses is at least partially regulated at the level of promoter utilization. The majority of the relevant viral control signals resides within a noncoding genome region (NCR) between the L1 gene for the major capsid protein and the transformation-related E6 gene. A promoter in front of E6 is a consistent feature of all papillomaviruses studied so far. It drives the expression of several early genes and is regulated by *cis*-active elements of the NCR which bind cellular and viral transcription factors. Particularly, viral E2 proteins are involved as positive and negative *trans* regulators. The E2 proteins recognize and bind to a 12-bp palindromic sequence, $ACCN_6GGT$, appearing several times in the NCRs of papillomaviruses (22). Additional early promoters were identified in the NCR and within the early region of most papillomaviruses (2, 11, 47). HPV6 and -11, for example, revealed an E7-specific promoter which could not be detected in HPV16 and -18 (40). The late gene promoters could be characterized in bovine papillomavirus type 1 (BPV1), HPV1, and cottontail rabbit papillomavirus (3, 34, 48). They are located in the L1-proximal parts of the NCRs and produce messages consisting of short NCR leaders spliced to the late genomic sequences. The activity of the late promoters could so far be demonstrated only in productively infected skin tumors.

The NCRs of papillomaviruses belong to the less conserved genome segments. However, a more detailed analysis of papillomaviruses with similar tissue tropism and pathogenic properties revealed characteristic organization patterns among viruses associated with fibropapillomas, anogenital lesions, and EV tumors. This implies both a unique arrangement of common protein binding sites (e.g., for viral E2, AP1, and NF1) and subgroup-specific, highly conserved sequence elements such as M33 of EV-associated viruses (15, 22, 28). The functional relevance of individual elements was already intensively studied with BPV1 and genital HPVs. In the case of EV viruses, very little is known in this regard because biopsy material is rare and there are no human cell lines persistently infected with these viruses. This work identified HPV8 NCR promoters, which are active in human epithelial cells and defined *cis*-active regulatory elements.

MATERIALS AND METHODS

Cell culture. The cervical carcinoma cell line HT3 (16) and the skin keratinocyte cell line HaCaT (5) were maintained in Dulbecco's modified Eagle's medium (GIBCO-Bethesda Re-

* Corresponding author.

search Laboratories) supplemented with 10% fetal calf serum and 120 mg each of penicillin and streptomycin per liter. Simian virus 40 (SV40)-immortalized human fetal keratinocytes (SV61 cells [35]) were grown in Joklik's modified Eagle's medium supplemented as described above and additionally with 1 μM hydrocortisone.

Cell transfections and CAT assays. HT3, SV61, and HaCaT cells were transfected in 100-mm culture dishes at 70 to 80% confluency by standard calcium phosphate techniques (1). For transfections, 15 μg of chloramphenicol acetyltransferase (CAT) reporter plasmid and 15 μg of E2 expression vector or 15 μg of sonicated salmon sperm DNA as a carrier were used per plate. As a control for transfection efficiency, 1 μg of a Rous sarcoma virus long terminal repeat luciferase vector was included (14). The transfected cells were glycerol shocked after 6 h and refed with fresh medium.

Cellular extracts were prepared 48 h after transfection. Cells were rinsed thoroughly with phosphate-buffered saline, collected by scraping, and disrupted by four freeze-thaw cycles. Lysates were cleared by microcentrifugation, and the protein content was determined by the method of Bradford (6). CAT assays were performed for 4 h according to a standard protocol (1), with the amount of protein adjusted to keep the chloramphenicol conversion rate within the linear range (10 to 60 μg). The acetylation products were separated by ascending thin-layer chromatography. Percent acetylation was determined by quantitation of ^{14}C-activity in acetylated and unacetylated spots and was standardized against the luciferase activity of the corresponding extract which was determined by standard methods (14). Each transfection was repeated at least three times.

Recombinant DNAs. To generate pNCR8-CAT, the *Bam*HI-*Bgl*II fragment encompassing the *Eco*RI-*Eco*RV fragment of HPV8 (nucleotides [nt] 7077 to 558, according to Fuchs et al. [17]) was excised from plasmid pPF43 (37) and cloned in both orientations into the unique *Bgl*II site of the enhancer- and promoterless CAT vector pBLCAT3 (29). The 5′ deletions of pNCR8-CAT were constructed by means of the exonuclease III-nuclease S1 unidirectional digestion method of Henikoff (21) after restricting pNCR8-CAT with *Pst*I and *Bam*HI upstream of the viral insert. The extent of individual deletions was confirmed by sequencing.

pNCR8-CATΔM33/AP1 was constructed by deleting the sequences of HPV8 between nt 7429 and 7473 by the gapped duplex method of Stanssens et al. (42) and cloning the mutated fragment into the pBLCAT3 vector. The HPV8 E2 expression vector pPF82 contains the HPV8 open reading frame (ORF) E2 under the control of the Moloney murine leukemia virus long terminal repeat and has previously been described (23). pBS-NCR81 contains the 1,133-bp *Eco*RI-*Eco*RV fragment, pBS-NCR82, the *Nla*IV-*Alu*I fragment (nt 7359 to 243) of HPV8 cloned into the Bluescribe M13+ vector (Vector Cloning Systems). The vector pCR1000 (Invitrogen) was used for cloning of polymerase chain reaction (PCR)-amplified cDNAs.

RNA analysis. RNA was extracted from transfected cells after 48 h by the guanidinium lysis method of Chirgwin et al. (9). For primer extension analysis, 20 μg of total cellular RNA was coprecipitated with 10^5 cpm of purified ^{32}P-end-labeled primer and subjected to reverse transcription with MMLV reverse transcriptase (GIBCO-Bethesda Research Laboratories). Reaction conditions were as described by Baker and Howley (3). Extension products were separated on 5 to 8% denaturing polyacrylamide-urea gels. The 5′ end of primer 1 (28-mer) was located at HPV8 nt 312, primer 2 (30-mer) was located at pBLCAT3 nt 530 (29), and primer 3 (21-mer) was located at HPV8 nt 7590.

RNase protection experiments were performed essentially as described by Stamminger et al. (41). RNA probes were synthesized with T3 RNA polymerase from pBS-NCR81 after cutting the template plasmid with *Sty*I or *Eco*RI and with T7 RNA polymerase from *Bam*HI-linearized pBS-NCR82. Total RNA was hybridized with the probe (2.5×10^5 cpm) overnight at 37°C in 10 μl of hybridization buffer containing 80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4), 400 mM NaCl, and 1 mM EDTA. Digestion of single-stranded RNA was performed for 1 h at 37°C with 8 μg of RNase A per ml and 14 U of RNase T_1 (Boehringer Mannheim) per ml in RNase buffer containing 300 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl (pH 7.4). After recovery of RNA-RNA duplexes by ethanol precipitation, samples were analyzed on 5% denaturing polyacrylamide-urea gels.

First-strand cDNA was synthesized from 1.5 μg of total cellular RNA from an HPV8-induced lesion with HPV8-specific primer L1 (nt 5885 to 5909) or E4 (nt 3462 to 3486) and reverse transcriptase by using the GeneAmp RNA PCR Kit (Perkin-Elmer Cetus Corp.). The reaction mix containing RNA, oligonucleotide (0.75 μM), and 10× PCR buffer was heated to 65°C for 5 min; deoxynucleoside triphosphates (1 mM), RNasin (1 U/μl), and enzyme (50 U) were added; and the mixture was incubated at 42°C for 1 h. PCR amplification of cDNAs was initiated by the addition of 0.15 μM HPV8-specific primer N (nt 7562 to 7587) and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus Corp.) in a total reaction mixture volume of 100 μl. The amplification was performed in 35 cycles with 1 min of denaturation at 94°C, 40 s of primer annealing at 57°C, and 1 min of primer extension at 72°C. Reaction products were analyzed in 1.5% agarose gels, and the specificity of the amplimers was determined by Southern blot hybridization with a ^{32}P-nick-labeled HPV8 genome according to a standard protocol (1). Amplification products were directly cloned into the *Hph*I-linearized pCR1000 cloning vector (Invitrogen), and positive clones were identified by colony hybridization. The sequences of both strands of the viral inserts were determined.

Clinical material. The material was derived from a 34-year-old female suffering for 28 years from EV. Diagnosis has been confirmed by clinical and histopathological examinations. The course of the disease is stationary, and no malignancies have been observed. An HPV-induced premalignant lesion of the actinic keratosis type, with slight Bowen's atypia localized on the forehead of this patient, was surgically removed after the patient was given local anesthesia; the lesion was immediately frozen in liquid nitrogen and stored at −80°C until use. The lesion was then homogenized in a Dounce homogenizer, and nucleic acids were extracted by the guanidinium lysis method (9). The resuspended RNA was digested with RNase-free DNase I (Boehringer Mannheim), phenol extracted, and precipitated. DNA was dialyzed against Tris-EDTA buffer and cut with *Bam*HI and *Eco*RI. Southern blot hybridization (1) with an HPV8-specific probe revealed the characteristic fragments of extrachromosomally persisting HPV8 DNA (17).

RESULTS

Promoter activity within the NCR of HPV8. To detect and map the promoter activity of the HPV8 NCR in human cells, we cloned the *Bam*HI-*Bgl*II fragment from plasmid pPF43 into the *Bgl*II site of the promoter test plasmid pBLCAT3,

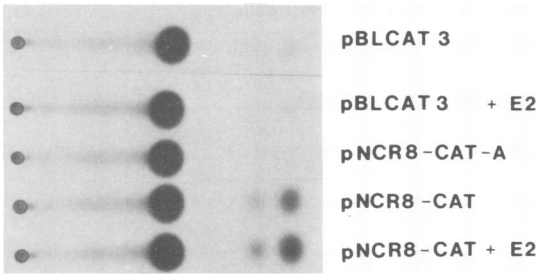

FIG. 1. CAT expression from pNCR8-CAT and pNCR8-CAT-A containing HPV8 control sequences in sense and antisense orientations relative to the *cat* gene. pBLCAT3 represents the promoter- and enhancerless parental plasmid. Fifteen micrograms of reporter plasmid was transfected in HT3 cells and assayed for *cat* gene expression as described in Materials and Methods. Cotransfection experiments were done with 15 μg of HPV8 E2-expression plasmid.

upstream of the *cat* reporter gene. The cloned fragment comprises the entire HPV8 NCR and parts of the flanking L1 and E6 genes (HPV8 nt 7078 to 558). The constructs, containing the viral insert in sense (pNCR8-CAT) and antisense (pNCR8-CAT-A) orientations relative to the *cat* gene, were tested for their promoter activities in transient CAT expression assays in the cervical carcinoma cell line HT3. As shown in Fig. 1, the viral sequences were able to induce the expression of the *cat* gene when cloned in sense relative to the reporter gene. Neither the vector alone nor the reversely cloned fragment was active. This orientation-dependent activity could be further stimulated (on average, fivefold) by cotransfection of an expression vector for the HPV8 transactivator protein E2. This effect also was strictly dependent on the sense orientation of the viral sequences (data not shown).

***cis*-active promoter control elements.** To characterize the sequences controlling the identified promoter activity, we constructed a nested set of 5′ deletion mutants of the promoter construct pNCR8-CAT. The structures and the *cat* expression levels of the individual deletion mutants are summarized in Fig. 2.

The removal of viral sequences up to the end of ORF L1 (pNCR8-CAT-7378) hardly influenced the NCR-driven *cat* expression. Further deletion of 44 nt (pNCR8-CAT-7422) down to the 5′ boundary of the EV-specific sequence motif M33 (HPV8 nt 7424 to 7456) resulted in a drastic enhancement of the promoter activity (30-fold), pointing to the presence of a *cis*-acting negative element. Deletion extending beyond the M33/AP1 region almost extinguished the ability of the HPV8 NCR to drive the expression of the reporter gene. In order to test for the role of the M33/AP1 region in the context of entire pNCR8-CAT, we removed this element by in vitro mutagenesis, creating plasmid pNCR8-CATΔM33/AP1. It showed only background activity, thus confirming the key role of the M33/AP1 element for the HPV8 NCR promoter activity. The residual activity observed with plasmid pNCR8-CAT-7474 in contrast to pNCR8-CATΔM33/AP1 is likely due to the absence of the previously identified negative sequences. The slight activity was abolished by all further deletions. We analyzed the activity of several representative constructs in two additional epithelial human cell lines. pNCR8-CAT was found to be active in SV40-immortalized human foreskin keratinocytes (SV61 cells) as well as in the keratinocyte cell line HaCaT (Table 1). Removal of upstream sequences down to

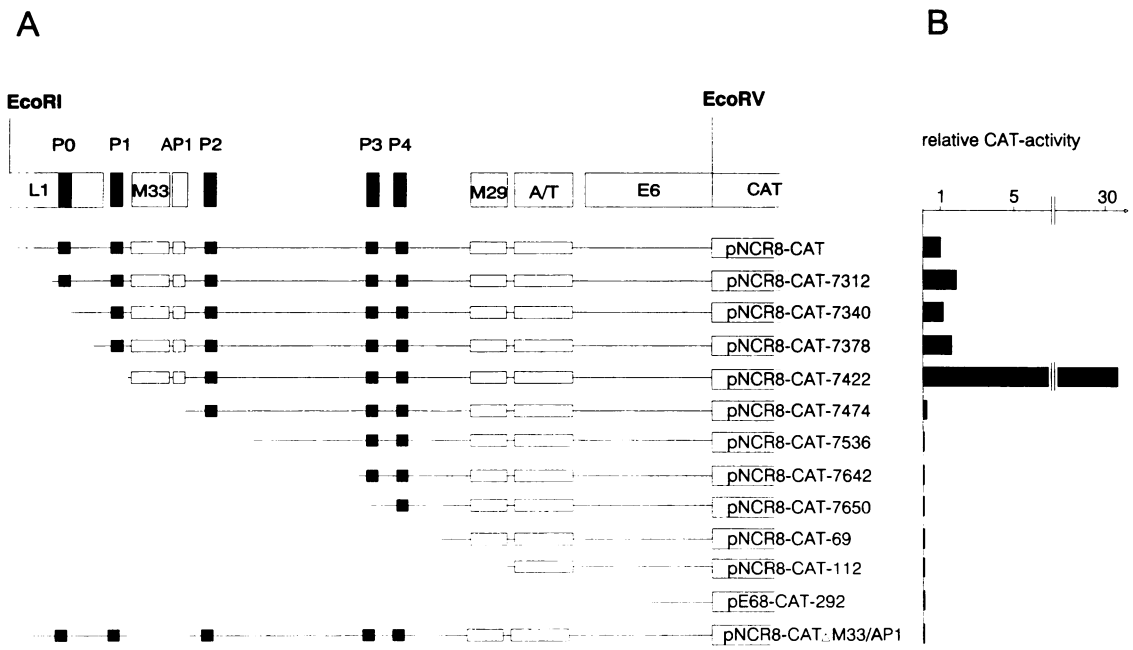

FIG. 2. (A) Deletion mutants of the HPV8 NCR in the promoter construct pNCR8-CAT. The HPV8 *Eco*RI-*Eco*RV fragment in front of the *cat* gene and typical sequence motifs characteristic of the NCRs of EV-associated viruses (M33, M29, A/T [28]) are shown on top. The boundaries of the E6 and L1 genes are marked. P0 to P4, E2-binding palindromes ($ACCN_6GGT$). The panel beneath depicts the structure of the individual deletion mutants, each designated with the position of the first nucleotide of the shortened HPV8 fragment. In plasmid pNCR8-CATΔM33/AP1, the M33 and AP1 elements were deleted by in vitro mutagenesis. (B) Relative CAT activity (based on the activity of the full-length construct) in extracts of HT3 cells transfected with the various NCR deletion mutants.

TABLE 1. Promoter activities of HPV8 deletion mutants in HaCaT and SV61 cells[a]

Transfected plasmid	Activity in HaCaT cells per expt			Mean value	Activity in SV61 cells per expt			Mean value
	1	2	3		1	2	3	
pBLCAT3	0.06	0.07	0.09	0.07	0.05	0.1	0.1	0.08
pNCR8-CAT	1	1	1	1	1	1	1	1
pNCR8-CAT-7312	0.9	1.2	2.0	1.4	2.7	1.8	3.2	2.6
pNCR8-CAT-7340	3.5	1.5	0.9	2.0	3.4	2.5	3.1	3.0
pNCR8-CAT-7378	3.0	1.2	4.0	2.7	5.3	3.1	3.4	3.9
pNCR8-CAT-7422	9.7	6.2	9.2	8.4	39.0	16.5	8.1	21.2
pNCR8-CAT-7642	0.2	0.5	0.2	0.3	0.1	0.1	0.1	0.1

[a] Chloramphenicol conversion rates were determined with cell extracts from three independent experiments and were normalized against luciferase activity; rates are expressed as activities relative to that in extracts from pNCR8-CAT-transfected cells.

nt 7422 led to an 8-fold (HaCaT cells) or 20-fold (SV61 cells) increase of promoter activity.

Cap sites within the NCR and characterization of transcripts. To determine the transcription initiation site(s) within the NCR, we set up primer extension and RNase protection analyses with total cellular RNA isolated from HT3 cells transiently transfected with promoter constructs. In a first approach we performed primer extension assays with two primers, one complementary to sequences within E6 (primer 1) and one at the 5′ region of the *cat* gene (primer 2). In order to increase the level of specific transcripts, pNCR8-CAT was cotransfected with the E2 expression vector pPF82. As shown in Fig. 3A, a group of weak signals which corresponded to cDNAs of 132 to 136 nt could be seen with primer 1. The extension of primer 2 resulted in a prominent cDNA of 218 nt. An identical major extension product could be observed with RNA isolated from cells transfected with both pNCR8-CAT alone and the most active deletion mutant pNCR8-CAT-7422, whereas the weak cluster of cDNAs could be detected only with pNCR8-CAT-7422 (data not shown). These signals were specific since they were absent from the control reactions performed with RNA from pBLCAT3-transfected HT3 cells (data not shown).

Projected onto the map of pNCR8-CAT (Fig. 3D2), the results suggest the existence of two transcription start points. The first one can be localized between the A/T box and the first ATG of E6 at nt 175 to 179 and corresponds most probably to the E6 promoter. The second 5′ end could map within the coding region of E6, but a sequence analysis of the corresponding E6 region revealed no element reminiscent of a typical RNA polymerase II promoter.

To gain more information about the structure of the major transcript, we challenged the above observation with RNase protection analyses. Two labeled antisense RNA probes were synthesized in vitro after linearization of pBS-NCR81 with *Sty*I or *Eco*RI. Probe 1 covered the *Sty*I-*Eco*RV fragment (nt 7596 to 558) and probe 2 encompassed the entire *Eco*RI-*Eco*RV fragment (nt 7082 to 558) of HPV8. We transfected in parallel both pNCR8-CAT with and without the E2 expression vector and the highly active deletion mutant pNCR8-CAT-7422. As expected (Fig. 3B), a specific 124-nt RNA fragment appeared to be protected when probe 2 and RNA from pNCR8-CAT-7422-transfected HT3 cells were used. After prolonged exposure, the same bands could also be observed with RNA from cells transfected with pNCR8-CAT alone or cotransfected with the HPV8 E2 expression vector (Fig. 3B). The relative intensities of these signals confirmed the E2 inducibility of the corresponding promoter and the extraordinarily high activity of pNCR8-CAT-7422 relative to pNCR8-CAT observed in CAT assays. In the case of an active promoter within the E6 gene, identical protected fragments should be seen with both probes. Surprisingly, however, a different protected fragment of 67 nt showed up when probe 1 was used. The only possibility for interpreting these results is to postulate a promoter localized in the 5′ part of the NCR, giving rise to a spliced transcript with a leader exon overlapping the 5′ end of probe 1 (Fig. 3D1). This can also explain the large protected fragments visible in Fig. 3B as due to an unspliced precursor of the major transcript. Degradation products in the range of 400 nt prevent the detection of the possible weak signals originating from the cap sites in front of E6. To confirm the localization of the 5′ end of the major NCR initiation site, we performed another primer extension experiment (Fig. 3C) with an oligonucleotide (primer 3) binding within the sequences immediately upstream of the *Sty*I site (Fig. 3D2). As shown in Fig. 3C, the reverse transcription of RNA from HT3 cells transfected with pNCR8-CAT-7422 produced a single specific cDNA of 56 nt. The same extension product could be observed when analyzing RNA from HT3 cells transfected with pNCR8-CAT with or without the E2 expression vector after prolonged exposure (data not shown). These results map the initiation site of the major transcript to HPV8 sequence position 7535. According to the RNase protection data, the leader exon of 124 nt ends at a splice donor site at position 4 of the HPV8 sequence. From the lengths from the extension product of primer 2 (218 nt) and the leader exon (124 nt), the position of the splice acceptor can be calculated to coincide with the boundary of the viral insert and the multiple cloning site of the vector (Fig. 3D3).

Mapping of a natural splice acceptor of transcripts starting at position 7535. The location of the cap site proximal to L1 and the use of an NCR-internal splice donor site are strongly reminiscent of late transcripts of cutaneous papillomaviruses. We therefore wanted to see whether the identified, HPV8-specific NCR exon is part of naturally occurring mRNAs with the potential to encode capsid proteins. Total RNA was extracted from an HPV8-induced lesion from a patient with EV and used for cDNA synthesis. Reverse transcription was initiated with an antisense oligonucleotide homologous to sequences downstream from a potential splice acceptor site at the beginning of L1 (Fig. 4). After PCR amplification of the cDNA copies with a second primer specific for the NCR exon, distinct bands which were absent from the negative control assay with HaCaT cell RNA could be visualized after electrophoretic separation (data not shown). Having confirmed the HPV8 specificity by Southern blot hybridization (data not shown), the amplimers were cloned and sequenced (Fig. 4). Two different cDNA frac-

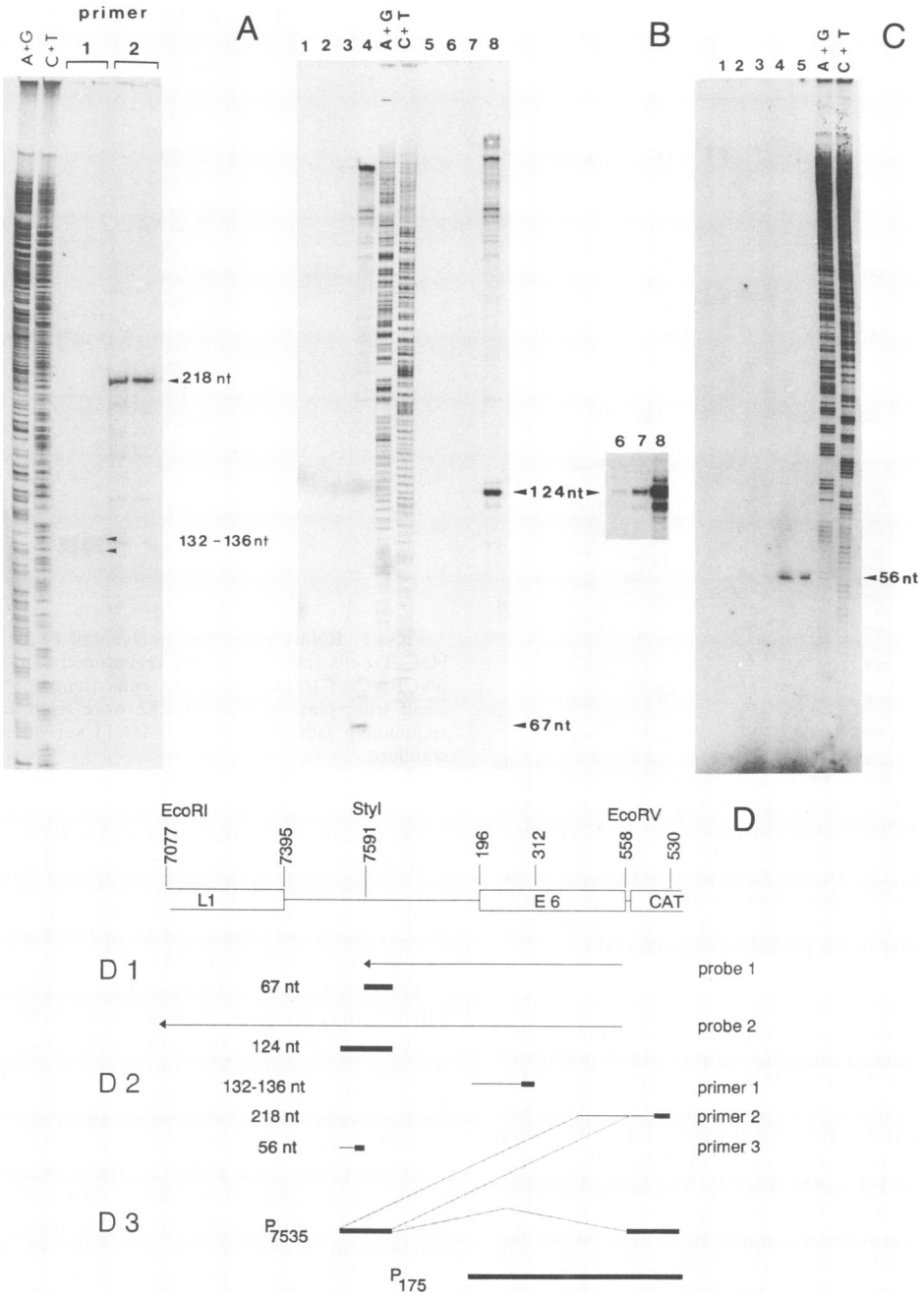

FIG. 3. Analysis of transcripts initiated within the HPV8 NCR. HT3 cells were transfected with HPV8-NCR-CAT constructs, and total cellular RNA was isolated. Twenty micrograms of RNA from each was analyzed for HPV8-specific transcripts. A sequencing ladder of plasmid pNCR8-CAT generated by extension of primer 1 or 2 was included as a molecular size standard (A+G and C+T). Specific extension products or RNase-resistant fragments on the autoradiograms are marked by arrowheads. (A) Primer extension analysis of RNA expressed from plasmid pNCR8-CAT cotransfected with the HPV8 E2 expression vector. The localizations of primers 1 and 2, which were used for reverse transcription, are shown in panel D2. (B) RNase protection analysis of RNAs expressed from pNCR8-CAT (lanes 2, 3, 6, and 7) and pNCR8-CAT-7422 (lanes 4 and 8). RNAs shown in lanes 3 and 7 were cotransfected with the HPV8 E2 expression vector. Yeast (*Saccharomyces cerevisiae*) tRNA was used as a negative control (lanes 1 and 5). Probe 1 was used in lanes 1 to 4 and probe 2 was used in lanes 5 to 8 (see panel D1). A longer exposure of lanes 6 to 8 is shown for greater detail. (C) Primer extension analysis of RNA expressed from plasmids pBLCAT3 (lane 1), pNCR8-CAT (lanes 2 and 3), and pNCR8-CAT-7422 (lanes 4 and 5). RNA shown in lane 3 was extracted from cells cotransfected with the HPV8 E2 expression vector. The map position of primer 3 is shown in panel D2. (D) Schematic representation of RNase protection (D1), primer extension results (D2), and deduced transcript structure (D3) in relation to the genetic map of pNCR8-CAT, which is shown above D1.

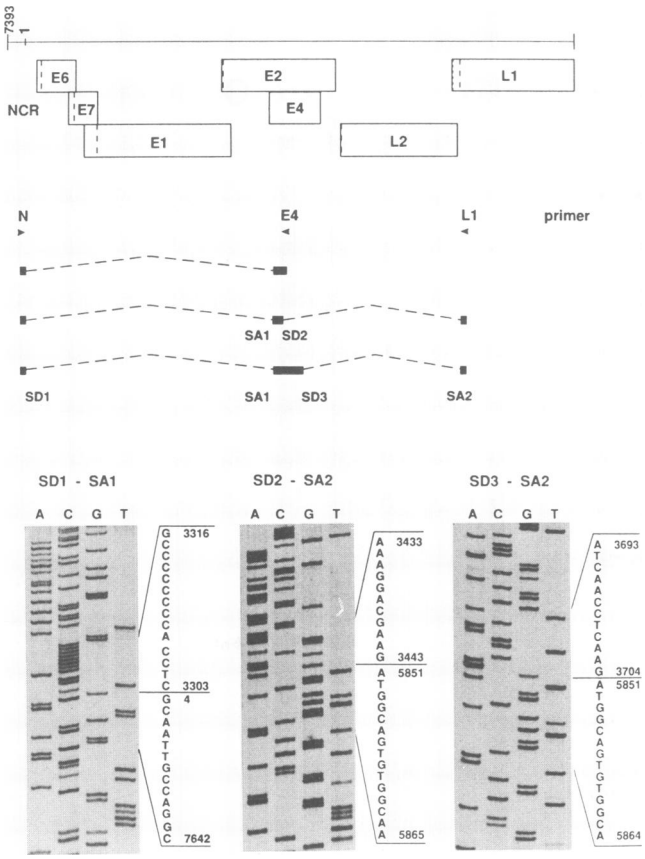

FIG. 4. Structure of PCR-amplified HPV8-specific cDNAs. The genome organization of HPV8 and the map positions of PCR primers are shown at the top. The dashed lines within the ORFs represent the translation codons (17). Details from sequencing gels at the bottom represent splice junctions between three different splice donor sites (SD1 to 3) and two splice acceptor sites (SA1 to 2). Nucleotide positions refer to the published sequence (17).

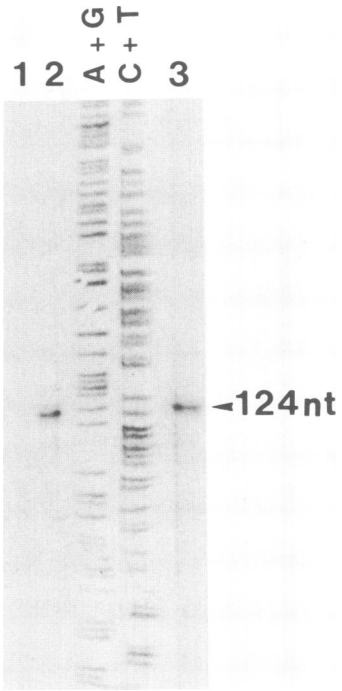

FIG. 5. RNase protection analysis of 10 μg each of RNAs from HaCaT cells (lane 1), an HPV8-induced skin lesion (lane 2), and pNCR8-CAT-transfected HT3 cells (lane 3). Transcripts synthesized from plasmid pBS-NCR82 were used as a probe. An M13 sequencing ladder (A+G and C+T) served as a molecular size standard.

tions were analyzed and shown to contain two splice junctions each. The first linked the splice donor site at position 4 to nucleotide 3303 in both cDNAs; the second junction connected different splice donor sites at positions 3443 and 3704 with the predicted splice acceptor in L1. The splice junction 4^3303 could be confirmed in cDNAs which were amplified with primers E4 and N (Fig. 4). The cloned cDNAs thus consist of three exons. The first represents 98 of 124 nt of the previously characterized exon from the NCR; the second ones are derived from the overlapping ORFs E2 and E4 and comprise 141 and 402 bp, respectively, without any ATG codon; and the third starts with the translation initiation codon of ORF L1 at position 5851. To confirm the existence of the full-length NCR exon, we performed an RNase protection experiment and detected a fragment of 124 nt, which corresponds exactly to the expected size (Fig. 5). These data strongly suggest that the cap site at position 7535 is used in vivo for the generation of mRNAs encoding the L1 protein of HPV8.

DISCUSSION

Two promoters could be mapped within the NCR of the EV-associated HPV8. Both were active in human epithelial cells. One led to transcripts with slightly heterogeneous 5′ ends between nt 175 and 179 (P_{175}) and contributed less than 5% to the overall promoter activity of the HPV8 NCR. Transcripts from the second promoter started at nt 7535 (P_{7535}) and were processed at an NCR-internal splice donor site at positions 3 to 10 (CGGTAAGT).

The constitutive activity of P_{7535} in pNCR8-CAT could be further increased by coexpression of the HPV8 E2 protein. Analysis of deletion mutants of the NCR sequences identified the EV-specific M33 motif and the adjacent AP1 site as an essential promoter element. The M33/AP1 region is located within the E2-dependent enhancer core of HPV8 (37) and acted as an enhancer element on its own when tested as a trimer (21a). This DNA segment is involved in multiple sequence-specific interactions with cellular proteins, and one of the footprints within M33 coincides with a potential recognition sequence for members of the *ets* oncogene family (30). The importance of AP1-binding sites in the regulation of HPV transcription could be clearly shown for genital HPV16 and -18 (10, 12, 32). Moreover, it should be noted that upstream regulatory sequences of several eucaryotic promoters (e.g., collagenase, urokinase plasminogen activator, stromelysin, and polyomavirus) contain an arrangement of close *ets*- and AP1-binding sites which were shown to cooperate in transcriptional regulation (20, 38, 45, 46).

It was most remarkable that the activity of P_{7535} was severely reduced by 44 bp (nt 7378 to 7421) immediately preceding the positive control element. A similar constellation of positive and negative regulatory elements has been reported at a comparable position in BPV4 (25). Deletion of the HPV8-specific negative element increased *cat* gene

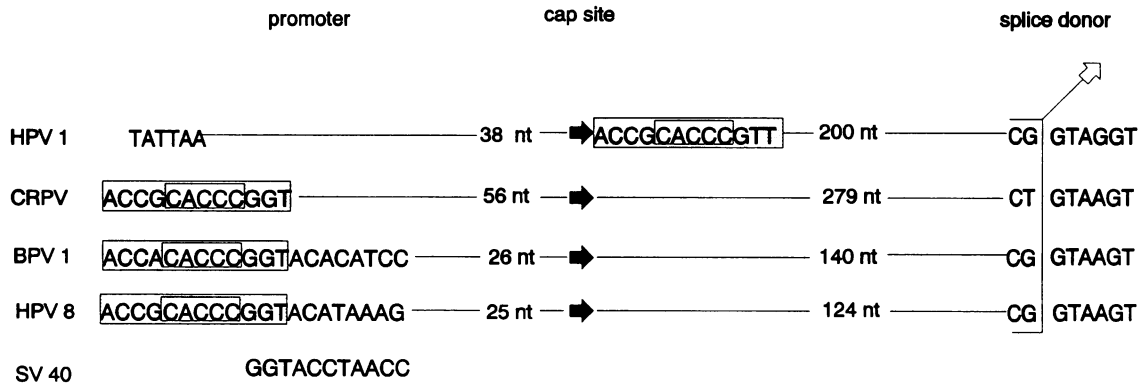

FIG. 6. Similarities between P_{7535} of HPV8 and late gene promoters of skin-specific papillomaviruses. The main initiation sites are indicated by solid arrows. As BPV1 and HPV1 use several cap sites, the most prominent ones have been chosen for calculation of the distances shown. The potential E2-binding sites with homologies to the β-globin CACCC element (boxed), homologies to the SV40 major late promoter, and sequences surrounding the splice donor sites are presented. Positions of the E2-binding palindromes are as follows: for HPV1, nt 7508 to 7519 (13); for cottontail rabbit papillomavirus, nt 7455 to 7466 (19); for BPV1, nt 7203 to 7214 (8); and for HPV8, nt 7491 to 7502 (17).

expression approximately 20-fold (pNCR8-CAT-7378 versus pNCR8-CAT-7422) when tested in the HT3 cell line. Although less pronounced, the effect occurred in two more epithelial cell lines examined in this study. The sequence in question encompasses the E2-binding palindrome P1, and one might speculate that the viral E2 protein interferes with that negative function.

P_{175}, localized in front of the first ATG of the E6 gene, is most likely responsible for the expression of early genes. All papillomaviruses characterized in this respect present a promoter at this position. A corresponding early mRNA cap site of HPV8 could be mapped in mouse fibroblasts (24), and the cap sites of the HPV47 E6 promoter exactly occur at homologous positions (27). The surprisingly low activity of P_{175} relative to P_{7535} in the context of pNCR8-CAT may result from the additional splice in P_{7535}-initiated mRNAs, because splicing has been shown to affect the CAT expression level of reporter plasmids. Alternatively, it could be due to suppression by the dominant promoter P_{7535}. A deletion of the upstream promoter did not lead to detectable P_{175}-derived messages, but this could be a consequence of the simultaneous removal of the M33/AP1 region, which might also be essential for P_{175}. A more refined dissection of control elements will therefore be required to exclude promoter interference.

The analysis of transcripts from an HPV8-induced skin lesion suggested that P_{7535} gives rise to mRNAs consisting of three exons: an NCR leader, a short segment from the early region, and the L1 gene. P_{7535} thus appears to be the late promoter of HPV8. It bears a striking similarity to the late gene promoters of BPV1, cottontail rabbit papillomavirus, and HPV1 (3, 34, 47). All these promoters are localized in the L1-proximal part of the NCR, and their messages include a short NCR exon. The transcripts are spliced first to short exons from the early region, whose map positions differ among the four viruses studied so far, and then directly to a splice acceptor at the beginning of the L1 gene. There is no classical TATA box recognizable in most of the late promoters, but, as shown in Fig. 6, both BPV1 P_L and HPV8 P_{7535} share some homology with the SV40 major late promoter (3, 7, 28). Furthermore, all mentioned papillomaviruses display an E2-binding site with an identical CACCC core in close proximity to the cap sites. This element is well known to occur in the upstream region of the β-globin gene (31) and represents an essential part of the constitutive enhancer of BPV1. This E2-binding site of BPV1 is involved in specific interactions with cellular factors (43, 44). Also, in HPV8 a sequence-specific protein binding takes place at palindrome P2 (30).

The activity of the late promoter P_{7535} in a variety of poorly differentiated epithelial cells is surprising (11), but this unusual property might be due to the lack of control elements within the used NCR fragment, which normally down-regulate late transcription in a cell differentiation-dependent manner. In BPV1 and HPV16, both transcription termination and the stability of the late messages appear to be influenced by *cis*-active sequences (4, 18, 26).

Supplementing the hypothesis of release from a negative control, our study showed that P_{7535} is further down-regulated by NCR sequences which had to be removed in order to allow high promoter activity. The presence of this silencer in conjunction with the M33/AP1 enhancer could in fact imply a tight regulation, which would meet the requirements of a late promoter.

ACKNOWLEDGMENTS

We thank R. Zimmermann and T. Stamminger for providing plasmid pNCR8-CATΔM33/AP1 and pRSV-luciferase, respectively. We are indebted to Stephania Jablonska for stimulating discussions and support.

This work was supported by a grant (Hi-291/5-1) from the Deutsche Forschungsgemeinschaft to P.G.F.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Wiley Interscience, New York.
2. **Baker, C. C.** 1990. Bovine papillomavirus type 1 transcription, p. 91–112. *In* H. Pfister (ed.), Papillomaviruses and human cancer. CRC Press, Inc., Boca Raton, Fla.
3. **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.
4. **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.
5. **Boukamp, P., R. T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, and N. E. Fusenig.** 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. **106:**761–771.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Brady, J., M. Radonovich, M. Vodkin, V. Natarajan, M. Thoren, G. Das, J. Janik, and N. P. Salzman.** 1982. Site-specific base substitution and deletion mutations that enhance or suppress transcription of the SV40 major late RNA. Cell **31:**625–633.
8. **Chen, E. Y., P. M. Howley, A. D. Levinson, and P. H. Seeburg.** 1982. The primary structure and genetic organization of bovine papillomavirus type 1 genome. Nature (London) **299:**529–534.
9. **Chirgwin, J. M., A. E. Przybla, R. J. McDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
10. **Chong, T., W.-K. Chan, and H.-U. Bernard.** 1990. Transcriptional activation of human papillomavirus 16 by nuclear factor 1, AP 1, steroid receptors and a possibly novel transcription factor, PVF: a model for the composition of genital papillomavirus enhancers. Nucleic Acids Res. **18:**465–470.
11. **Chow, L. T., S. S. Reilly, T. R. Broker, and L. B. Taichman.** 1987. Identification and mapping of human papillomavirus type 1 RNA transcripts recovered from plantar warts and infected epithelial cell cultures. J. Virol. **61:**1913–1918.
12. **Cripe, T. P., A. Alderborn, R. D. Anderson, S. Parkkinen, P. Bergman, T. H. Haugen, U. Pettersson, and L. P. Turek.** 1990. Transcriptional activation of the human papillomavirus-16 P97 promoter by an 88-nucleotide enhancer containing distinct cell-dependent and AP-1-responsive modules. New Biol. **2:**450–463.
13. **Danos, O., M. Katinka, and M. Yaniv.** 1982. Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among papovaviridae. EMBO J. **1:**231–236.
14. **DeWet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
15. **Ensser, A., and H. Pfister.** 1990. Epidermodysplasia verruciformis associated human papillomaviruses present a subgenus-specific organization of the regulatory genome region. Nucleic Acids Res. **11:**3919–3922.
16. **Fogh, J. (ed.).** 1975. Human tumor cells in vitro, p. 115–159. Plenum Publishing Corp., New York.
17. **Fuchs, P. G., T. Iftner, J. Weninger, and H. Pfister.** 1986. Epidermodysplasia verruciformis-associated human papillomavirus 8: genomic sequence and comparative analysis. J. Virol. **58:**626–634.
18. **Furth, P., and C. C. Baker.** 1991. An element in the bovine papillomavirus late 3′ untranslated region reduces polyadenylated cytoplasmic RNA levels. J. Virol. **65:**5806–5812.
19. **Giri, I., O. Danos, and M. Yaniv.** 1985. Genomic structure of the cottontail rabbit (Shope) papillomavirus. Proc. Natl. Acad. Sci. USA **82:**1580–1584.
20. **Gutman, A., and B. Wasylyk.** 1990. The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. EMBO J. **9:**2241–2246.
21. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
21a. **Horn, S., and P. G. Fuchs.** Unpublished data.
22. **Iftner, T.** 1990. Papillomavirus genomes: sequence analysis related to functional aspects, p. 181–202. *In* H. Pfister (ed.), Papillomaviruses and human cancer. CRC Press, Inc., Boca Raton, Fla.
23. **Iftner, T., P. G. Fuchs, and H. Pfister.** 1989. Two independently transforming functions of human papillomavirus 8. Curr. Top. Microbiol. Immunol. **144:**167–173.
24. **Iftner, T., G. Sagner, H. Pfister, and F. O. Wettstein.** 1990. The E7 protein of human papillomavirus 8 is a nonphosphorylated protein of 17 kDa and can be generated by two different mechanisms. Virology **179:**428–436.
25. **Jackson, M. E., and M. S. Campo.** 1991. Positive and negative E2-independent regulatory elements in the long control region of bovine papillomavirus type 4. J. Gen. Virol. **72:**877–883.
26. **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.
27. **Kiyono, T., K. Nagashima, and M. Ishibashi.** 1989. The primary structure of major viral RNA in a rat cell line transformed with type 47 human papillomavirus DNA and the transforming activity of its cDNA and E6 gene. Virology **173:**551–565.
28. **Krubke, J., J. Kraus, H. Delius, L. T. Chow, T. R. Broker, T. Iftner, and H. Pfister.** 1987. Genetic relationship among human papillomaviruses associated with benign and malignant tumours of patients with epidermodysplasia verruciformis. J. Gen. Virol. **68:**3091–3103.
29. **Luckow, B., and G. Schütz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res. **15:**5490.
30. **May, M., V. Helbl, H. Pfister, and P. G. Fuchs.** 1991. Unique topography of DNA-protein interactions in the non-coding region of epidermodysplasia verruciformis-associated human papillomaviruses. J. Gen. Virol. **72:**2989–2997.
31. **Myers, R. M., K. Tilly, and T. Maniatis.** 1986. Fine structure of a β-globin promoter. Science **232:**613–618.
32. **Offord, E. A., and P. Beard.** 1990. A member of the activator protein 1 family found in keratinocytes but not in fibroblasts required for transcription from a human papillomavirus type 18 promoter. J. Virol. **64:**4792–4798.
33. **Orth, G.** 1987. Epidermodysplasia verruciformis, p. 199–243. *In* N. P. Salzman and P. M. Howley (ed.), The papovaviridae, vol. 2. Plenum Publishing Corp., New York.
34. **Palermo-Dilts, D. A., T. R. Broker, and L. T. Chow.** 1990. Human papillomavirus type 1 produces redundant as well as polycistronic mRNAs in plantar warts. J. Virol. **64:**3144–3149.
35. **Parkinson, E. K., P. Grabham, and A. Emmerson.** 1985. A subpopulation of cultured human keratinocytes which is resistant to the induction of terminal differentiation-related changes by phorbol, 12-myristate, 13-acetate: evidence for an increase in the resistant population during transformation. Carcinogenesis (London) **4:**857–861.
36. **Pfister, H.** 1984. Biology and biochemistry of papillomaviruses. Rev. Physiol. Biochem. Pharmacol. **99:**111–181.
37. **Reh, H., and H. Pfister.** 1990. Human papillomavirus type 8 contains cis-active positive and negative transcriptional control sequences. J. Gen. Virol. **71:**2457–2462.
38. **Rørth, P., C. Nerlov, F. Blasi, and M. Johnsen.** 1990. Transcription factor PEA3 participates in the induction of urokinase plasminogen activator transcription in murine keratinocytes stimulated with epidermal growth factor or phorbol ester. Nucleic Acids Res. **18:**5009–5017.
39. **Ruiter, M., and P. J. van Mullem.** 1970. Behavior of virus in malignant degeneration of skin lesions in epidermodysplasia verruciformis. J. Invest. Dermatol. **54:**324–331.
40. **Smotkin, D., H. Prokoph, and F. O. Wettstein.** 1989. Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. **63:**1441–1447.
41. **Stamminger, T., H. Fickenscher, and B. Fleckenstein.** 1990. Cell type-specific induction of the major immediate early enhancer of human cytomegalovirus by cyclic AMP. J. Gen. Virol. **71:**105–113.
42. **Stassens, P., C. Opsomer, Y. K. McKeown, W. Kramer, M. Zabeau, and H.-J. Fritz.** 1989. Efficient oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternating selectable markers. Nucleic Acids Res. **17:**4441–4454.
43. **Stenlund, A., and M. Botchan.** 1990. The E2 trans-activator can act as a repressor by interfering with a cellular transcription factor. Genes Dev. **4:**123–136.

44. **Vande Pol, S. B., and P. M. Howley.** 1990. A bovine papillomavirus constitutive enhancer is negatively regulated by the E2 repressor through competitive binding for a cellular factor. J. Virol. **64:**5420–5429.
45. **Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince, and D. Stehelin.** 1990. The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. Nature (London) **346:**191–193.
46. **Wasylyk, C., A. Gutman, R. Nicholson, and B. Wasylyk.** 1991. The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. EMBO J. **10:**1127–1134.
47. **Wettstein, F. O.** 1990. State of viral DNA and gene expression in benign vs. malignant tumours, p. 155–179. *In* H. Pfister (ed.), Papillomaviruses and human cancer. CRC Press, Inc., Boca Raton, Fla.
48. **Wettstein, F. O., M. S. Barbosa, and M. Nasseri.** 1987. Identification of the major cottontail rabbit papillomavirus late mRNA cap site and mapping and quantitation of an E2 and minor E6 coding mRNA in papillomas and carcinomas. Virology **159:**321–328.
49. **Yabe, Y., M. Yasui, N. Yoshino, T. Fujiwara, N. Ohkuma, and N. Nokara.** 1978. Epidermodysplasia verruciformis: viral particles in early malignant lesions. J. Invest. Dermatol. **71:**225–228.
50. **zur Hausen, H.** 1977. Human papillomaviruses and their possible role in squamous cell carcinomas. Curr. Top. Microbiol. Immunol. **78:**1–30.
51. **zur Hausen, H.** 1989. Papillomaviruses as carcinomaviruses, p. 1–26. *In* G. Klein (ed.), Advances in viral oncology, vol. 8. Raven Press, New York.