Differential Effects of the Splice Acceptor at Nucleotide 3295 of Human Papillomavirus Type 31 on Stable and Transient Viral Replication

DAVID J. KLUMPP, FRANK STUBENRAUCH, AND LAIMONIS A. LAIMINS*

Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, Illinois 60611

Received 23 May 1997/Accepted 5 August 1997

In human papillomavirus type 31 (HPV-31), the E1 Δ E4 and E5 open reading frames are expressed from polycistronic mRNAs. The major polycistronic mRNAs which encode E1 Δ E4 and E5 are spliced messages which utilize a splice acceptor at nucleotide (nt) 3295 (SPA³²⁹⁵). Our laboratory recently developed a recombinant system for the synthesis of HPVs following immortalization of primary keratinocytes with cloned HPV-31 genomes (M. G. Frattini et al., *Proc. Natl. Acad. Sci. USA* 93:3062–3067, 1996). These immortalized cell lines are capable of maintaining HPV-31 DNA as episomes and induce the synthesis of virions in organotypic raft culture. In this study, we used these methods to begin an analysis of the roles of E1 Δ E4 and E5 in HPV pathogenesis by mutating the major splice at nt 3295. Mutation of SPA³²⁹⁵ did not significantly alter the ability of HPV-31 genomes to replicate transiently in keratinocytes, nor did the mutation affect the immortalization potential of HPV-31. However, genomes carrying the SPA³²⁹⁵ mutation were not stably maintained as viral episomes, and the resulting immortalized keratinocyte cell line contained multiple, integrated copies of the mutated HPV-31 DNA. Northern analysis indicated that cell lines immortalized with the mutant HPV-31 expressed transcripts which were similar in size and abundance to wild-type messages, including those transcripts which rely on utilization of SPA³²⁹⁵. RNase protection and reverse transcription-PCR revealed that mutation of SPA³²⁹⁵ resulted in the utilization of a cryptic splice acceptor at nt 3298. These data suggest that the requirements for stable maintenance of HPV genomes are more stringent than those for transient replication and that factors which define these requirements rely on the major splice acceptor at nt 3295.

Human papillomaviruses (HPVs) induce a variety of hyperplasias in differentiating epithelia. Among the more than 70 HPV types which have been characterized, nearly one-third are specific for the anogenital track. The high-risk HPV types target the anogenital epithelium and are the etiologic agents of cervical cancer (13a). Two early gene products of high-risk HPVs, E6 and E7, encode oncoproteins which are capable of immortalizing keratinocytes by binding p53 and Rb, respectively (6, 25, 36, 37). The E1 and E2 proteins supply viral replication functions (4, 8, 35), where E1 functions as a sequence-specific initiator protein and E2 facilitates E1 binding onto the origin (7, 8, 24a). In contrast, little is known about the roles in viral pathogenesis of two other HPV gene products, E4 and E5. E4 is expressed as a fusion with the five N-terminal amino acids of E1 (26) and was initially thought to facilitate viral egress by associating with cytokeratins and resulting in the collapse of the cytokeratin network (5). This model of E4 function, however, remains controversial. E5 has weak transforming potential (2, 21, 27), probably mediated by its abilities to alter epidermal growth factor (EGF) receptor recycling (34) and to stimulate signal transduction pathways (10, 11). Still, the precise roles of HPV E4 and E5 in the viral life cycle remain unclear.

The HPV life cycle is strictly tied to epithelial differentiation (see reference 18 for a review). Following infection of basal keratinocytes, the HPV genome is maintained as episomes at

approximately 50 copies per cell. Transcription of mRNAs from the HPV early promoter, P97, directs the expression of immortalization functions encoded by E6 and E7 as well as the E1 and E2 factors required for viral replication. As keratinocytes differentiate, the HPV genome is amplified to several thousand copies per cell. Differentiation also activates the HPV late promoter, P742, in the upper strata of the epithelium (15). The first late gene transcript expressed from this promoter encodes the E1 Δ E4 fusion as well as E5. A subset of cells in the uppermost epithelial layers expressing E1 Δ E4 also express transcripts encoding the capsid protein L1.

HPV early proteins are translated from polycistronic messages originating from both P97 and P742 (Fig. 1). In CIN-612 cells, an immortalized keratinocyte line harboring episomal HPV type 31b (HPV-31b), P97 transcripts variously include the E6, E6*, E6 Δ E4, E7, E1, E2, E1 Δ E4, and E5 open reading frames (ORFs) (15), and this is similar to observations in other high-risk as well as low-risk HPVs (3, 29–33). Except for the unspliced E1 message, which contains all early ORFs, all P97 transcripts are spliced at least once. Messages encoding E2 use a splice acceptor at 2646, but all other spliced P97 transcripts employ an acceptor at position 3295 (SPA³²⁹⁵). Most P97 transcripts utilize a splice donor at 877 in conjunction with SPA³²⁹⁵ to generate an E6, E7, E1 Δ E4, E5 message. A less abundant mRNA species utilizes a donor at position 210 (within E6) in conjunction with SPA³²⁹⁵ to generate an E6 Δ E4, E5 message (15). In a similar manner, the P742 transcripts of HPV-31b have been characterized following differentiation of CIN-612 cells in raft cultures (15, 16). All P742 transcripts were found to utilize SPA³²⁹⁵. Each of the resulting polycistronic mRNAs contains the E1 Δ E4 ORF as well as E5, L2, or L1. Thus, all

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Northwestern University Medical School, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-0648. Fax: (312) 503-0649. E-mail: lal@merle.acns.nwu.edu.

FIG. 1. Map of HPV-31 ORFs and early and late transcripts. The uppermost portion indicates the ORFs relative to the 7.9-kb genome of HPV-31, where P97 and P742 are the constitutive and differentiation-dependent promoters, respectively. Below the map of HPV-31 are the structures of the early and late messages (14), and the encoded ORFs are listed at the right.

identified viral messages which encode E1ΔE4 and E5 require splicing to SPA³²⁹⁵.

Bovine papillomavirus type 1 (BPV-1) utilizes a splice acceptor at position 3225 (SPA³²²⁵) which is analogous to the HPV-31 SPA³²⁹⁵ (13a). Previous studies showed that when mutants of BPV-1 containing splice acceptors altered at nucleotide (nt) 3223 were examined in C127 cells, they failed to transform but could replicate as stable episomes (12). In BPV-1, transcripts from several promoters utilize SPA³²²⁵ for the expression of E5, the major BPV transforming function. BPV-1 utilizes at least five promoters, while only two have been identified in the high-risk HPV types. Also, BPV-1 primarily induces fibropapillomas whereas HPVs induce hyperplasias in stratifying epithelia, and so significant differences are likely to exist in their respective life cycles.

Recently, our laboratory developed a method for reconstituting the HPV-31 life cycle from cloned HPV DNA which had been transfected into primary human keratinocytes (9). This approach yields HPV-immortalized cell lines which stably maintain HPV-31 DNA as episomes. Following differentiation of these HPV-immortalized cells in the raft culture system, expression of late messages and production of HPV virions is induced. This recombinant HPV system can be used to characterize the role of HPV gene products in viral pathogenesis. Toward this end, we have initiated a mutational analysis of SPA³²⁹⁵ of HPV-31 to determine its role in the viral life cycle. Mutation of SPA³²⁹⁵ did not affect the ability of HPV-31 to replicate transiently, and mutated genomes efficiently immortalized primary human keratinocytes. However, analysis of viral DNA in these immortalized cell lines revealed that the mutant genomes were not maintained as episomes but were instead integrated into the host DNA. These results suggest differential requirements for transient replication versus stable maintenance of HPV-31 in keratinocytes, and factors influencing these requirements are likely to utilize SPA³²⁹⁵.

MATERIALS AND METHODS

Cell lines and cell culture. Primary human foreskin keratinocytes were purchased from Clonetics Inc. (San Diego, Calif.). SCC13 is an immortalized cell line derived from a human squamous cell carcinoma of the cheek. CIN-612 cells were isolated from a CIN I lesion (28). Primary human foreskin keratinocytes were maintained in KGM (Clonetics) for fewer than six passages. HPV-immortalized keratinocytes were plated onto mitomycin-treated NIH 3T3 feeders and were maintained in E medium supplemented with EGF (5 ng/ml) as previously described (28). SCC13 cells were maintained on 3T3 feeders in E medium (without supplemental EGF). Primary keratinocytes were transfected with 5 μg of ligated HPV DNA and 2 μg of pSV2neo, using LipofectAce (Gibco, Gaithersburg, Md.) in KGM, replated onto feeders the following day, and selected with G418, 200 μg/ml for 4 days followed by 150 μg/ml for 2 days and then 50 μg/ml for 2 days (9).

Raft culture of HPV-immortalized keratinocytes was performed as described previously (24). Briefly, 10⁶ keratinocytes were seeded atop a collagen plug containing live 3T3 feeders. Once the keratinocytes were confluent, the collagen plugs were lifted onto a wire mesh. The plugs were then fed with E medium from below while remaining at the liquid-air interface. Rafts were fed for 16 days and then harvested.

Hybridization analysis. For Southern analysis, total genomic keratinocyte DNA was isolated from a confluent 10-cm-diameter dish by proteinase K digestion followed by phenol extraction (1). Then 5 to 10 μg of keratinocyte DNA was digested with the appropriate restriction enzyme, electrophoresed through 0.7% agarose, denatured, and transferred to nitrocellulose. Probes were labeled with [³²P]dCTP by random-hexamer-primed synthesis using the complete HPV-31 genome as a template. Prehybridization and hybridization were performed at 42°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7])–50% formamide–5× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–0.1 mg of sonicated salmon sperm DNA per ml. Washes were performed at a final stringency of 0.1× SSPE–0.1% SDS at 60°C. For Northern analysis, total cellular RNA was purified from a confluent 10-cm-diameter dish of keratinocytes (or from tissue derived from raft cultures of keratinocytes), using Trizol reagent (Gibco-BRL, Gaithersburg, Md.) according to the manufacturer's protocol. Ten micrograms of total RNA was electrophoresed through 0.8% agarose containing 2.2 M formaldehyde in morpholinepropanesulfonic acid (MOPS) buffer and was transferred to GeneScreen Plus (DuPont-NEN, Boston, Mass.) in 10× SSC (1× is 0.15 M NaCl plus 0.015 M sodium citrate). Prehybridization and hybridization were performed at 42°C in 5× SSPE–50% formamide–5× Denhardt's solution–10% dextran sulfate–1% SDS–0.1 mg of sonicated salmon sperm DNA per ml. Final washes were performed with 0.1× SSPE–0.1% SDS at 60°C. Templates specific for E4/E5 and E6/E7 probes were generated by PCR and corresponded to nt 3278 to 5001 and 99 to 664, respectively.

Plasmids. Plasmid pUK-HPV31 contains wild-type HPV-31 in the *EcoRI* site of a modified pUC19 vector; this insertion site disrupts the E2 and E4 ORFs of HPV-31 at nt 3361. The SPAM mutant was created from pUK-HPV31 by using a Chameleon mutagenesis kit (Stratagene) and the mutation primer 5'-CTGAATCTGTATTTTCCAGTGACGAAATATCC-3' corresponding to nt 3279 to 3310 (mutation positions indicated in boldface); the resulting plasmid was named p31E4SPAM. Plasmid p31E4RESPAM was generated by amplifying two fragments followed by overlap extension PCR. The 5' fragment was generated with 5'-CACTAGATGGCAACCCTGTATC-3' (sense) and 5'-CCAGCA AAGGATATTTCTGCATGCTCTACAGATTCAGGAAAAACAATTAC C-3' (antisense; mutations indicated in boldface), yielding a product from nt 2417 to 3319. The 3' fragment was generated with 5'-GGTAAATGTTTTT CCTGAATCTGTAGAGGCATCTGACGAAATATCCTTTGCTGG-3' (sense; mutations indicated in boldface) and 5'-TTTTGGAATTCGATGTGGTG GTGTTGTGGC-3' (antisense), yielding a product from nt 3265 to 3371. After overlap extension, the mutant fragment was digested with *HindIII* and *EcoRI* and inserted into the corresponding sites of pUK-HPV31. pbs-P97 was constructed by inserting the *SpeI*-*HpaI* fragment (nt 7558 to 217) of pUK-HPV31-BS3 (a variant of HPV-31 where E2 binding site 3 is mutated [34a]) into the *SpeI* and *EcoRV* sites of Bluescript (Stratagene). pRP-P742 was constructed by amplification of nt 679 to 919 with primers containing *BamHI* sites and cloning the resulting PCR product into the *BamHI* site of pcDNA II (Invitrogen). p31SPA3295 was constructed by amplification of nt 3247 to 3578 of pUK-HPV31 (with an upstream or downstream primer containing a *BamHI* or *Sall* site, respectively) and cloning the product into the *BamHI* and *Sall* sites of pSP72 (Promega). pSPAM3295 was constructed by using the same strategy as used for p31SPA3295 except that the PCR product was generated by using p31E4RESPAM as the template. All plasmids were sequenced to confirm the presence of desired mutations.

RNAse protection assay. RNAse protections were performed on 6 to 8 μg of total RNA. Precipitated RNA pellets were hybridized to 250,000 cpm of anti-sense ³²P riboprobe in 10 μl of 40 mM piperazine-N,N-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–400 mM NaCl–1 mM EDTA–80% formamide overnight at 37°C. RNAse digestion was performed by the addition of 300 μl (ice-cold) of 10 mM Tris HCl (pH 7.5)–300 mM NaCl–5 mM EDTA containing RNAse A (10 μg/ml) and RNAse T₁ (40 U/ml). Incubation proceeded for 60 min at 37°C. The RNAse reaction was stopped by the addition of 3.5 μl of 20% SDS and 12.5 μl of

FIG. 2. The SPAM and RESPAM mutations of the splice acceptor at nt 3295 of HPV-31. The top portion shows the wild-type DNA sequence of HPV-31 in the region near nt 3295. In the SPAM mutation, nt 3293 to 3294 were converted from AG to TC by using a Chameleon mutagenesis kit (Stratagene). In the RESPAM mutation, overlap extension PCR mutagenesis was used to convert nt 3290 to 3297 from TTTAGCAG to GAGGCATC. The lower portion shows the wild-type HPV-31 E2 ORF, which overlaps SPA³²⁹⁵, and how the RESPAM mutation converts F₂₀₀ and S₂₀₁ to G₂₀₀ and A₂₀₁, respectively. The SPAM mutation is silent within the overlapping E2 ORF.

proteinase K (10 mg/ml) followed by digestion for 15 min at 37°C. Samples were then extracted with phenol-chloroform and precipitated with ethanol prior to resuspension in formamide loading buffer and resolution on a 6% urea-acrylamide gel. The riboprobe for examination of SPA³²⁹⁵ was generated by digesting p31SPA3295 (or pSPAM3295 for examining SPAM cell lines) with *Bam*HI and in vitro transcription with SP6 polymerase. The riboprobe for examination of the splice donor at nt 877 was generated by digesting pRP-P742 with *Spe*I and transcription with SP6 polymerase. The riboprobe for examination of splice donor was generated by digesting pbs-P97 with *Sca*I and transcribing with T3 polymerase.

RT-PCR analysis. HPV sequences were amplified from 1 µg of total keratinocyte RNA by using rTth DNA polymerase and EZ buffers (Perkin-Elmer, Foster City, Calif.) according to manufacturer's recommendations. The reverse transcription (RT) reaction was performed for 30 min at 60°C followed by amplification through 40 cycles. The primers corresponded to nt 741 to 767 and 3804 to 3830 for the upstream and downstream primers, respectively. Following amplification, PCR products were purified on QIAquick columns (Qiagen, Hilden, Germany), and the DNA sequence was determined by using an fmol DNA sequencing kit (Promega, Madison, Wis.) and 10% of the product.

Transient replication assay. A 2.5-µg aliquot of plasmid DNA containing an HPV genome was digested with a restriction enzyme to excise the genome from the vector. The cut DNA was then ligated at 10 µg/ml to promote intramolecular ligation. Circular HPV genomes were then transfected into 60-mm-diameter dishes of SCC13 cells or primary human foreskin keratinocytes (approximately 30% confluent), using 15 µl of Lipofectamine in OptiMEM (Gibco) or in KGM (for primary cells; Clonetics) according to the manufacturer's recommendations. The following day, the transfected cells were split onto 100-mm-diameter dishes containing mitomycin-treated feeders, allowed to grow 4 more days in E medium, and harvested by scraping into phosphate-buffered saline. Low-molecular-weight DNA was then purified by the Hirt procedure (13), with following modifications: cell pellets were digested in 400 mM NaCl–10 mM EDTA–10 mM Tris HCl (pH 7.5)–50 µg of proteinase K per ml–0.2% SDS at 55°C for 3 h; NaCl was added to 1 M, and DNAs were precipitated at 4°C overnight followed by centrifugation for 60 min in 4°C at 14,000 rpm; supernatants were then extracted once with phenol-chloroform-isoamyl alcohol and once with chloroform before precipitation with isopropanol. Each sample was then digested with 5 U of *Dpn*I, 15 U of *Ban*II, and 50 µg of RNase A per ml for 5 h prior to Southern analysis.

RESULTS

Mutation of the splice acceptor at nt 3295. The major splice acceptor of HPV-31 early and late transcripts is located at nt 3295 (15). This splice acceptor, SPA³²⁹⁵, contains the consensus AG at the 3' end of the intron (nt 3293 to 3294; Fig. 2). To examine the role of this acceptor in viral pathogenesis, we constructed mutant viral genomes in which the consensus AG was converted to TC. We chose this alteration of AG to TC, which results in a silent mutation within the overlapping E2 ORF, so that E2 function would not be altered. We designated this mutation SPAM, for splice acceptor mutant, and genomes

FIG. 3. The HPV-31 genomes carrying the SPAM or the RESPAM mutations replicate transiently in keratinocytes. SCC13 cells (A) or primary human foreskin keratinocytes (B) were transfected with 2.5 µg of ligated HPV genomes and maintained for 5 days. Low-molecular-weight DNA purified from the transfected cells was then analyzed by digestion with *Dpn*I (to degrade unreplicated input DNA) and *Ban*II (to linearize HPV-31) followed by Southern analysis. A probe was generated from the entire HPV-31 genome. Arrows indicate replicated HPV-31. (A) M (marker; lane 1) is linearized HPV-31 DNA. Lane 2 is an HPV-31 mutant which fails to replicate due to a stop codon which interrupts the E1 ORF. Lanes 3 to 5 indicate replication of wild-type HPV-31 DNA, 31-E4SPAM DNA, and 31-E4RESPAM DNA, respectively, in SCC13 cells. (B) Lanes 1 and 2 indicate replication of wild-type HPV-31 DNA and 31-E4SPAM DNA, respectively, in primary keratinocytes. Lane 3 is an HPV-31 mutant which fails to replicate due to a stop codon which interrupts the E1 ORF.

containing this mutation were called 31-E4SPAM. We also constructed a second mutation of SPA³²⁹⁵, RESPAM, which is described below.

31-E4SPAM replicates transiently. To determine which viral functions were affected by this mutation, we first performed transient replication assays with mutant HPV-31 genomes. 31-E4SPAM and wild-type HPV-31 genomes were isolated and ligated under dilute conditions to favor intramolecular ligation. The ligated genomes were then transfected into the SCC13 keratinocyte line. At 120 h posttransfection, total cellular DNA was isolated, digested with *Dpn*I and *Xba*I, and analyzed by Southern blotting (Fig. 3A). *Dpn*I-resistant, replicated HPV DNA was detectable in SCC13 cells transfected with either wild-type or 31-E4SPAM DNA, although the 31-E4SPAM genomes replicated slightly less efficiently than wild-type HPV-31 in some experiments (62% of the wild-type level). A mutant HPV-31 containing a translational stop codon near the amino terminus of E1 did not replicate transiently (lane 2). We then repeated the transient replication analysis using human primary keratinocytes (Fig. 3B) and found that wild-type HPV-31 DNA and 31-E4SPAM genomes replicated to similar levels (lanes 1 and 2), whereas the E1 mutant virus did not replicate (lane 3). We conclude that although there may be a slight reduction in the efficiency of transient replication of 31-E4SPAM genomes, the SPAM mutation does not significantly reduce the capability of HPV-31 to replicate transiently in primary keratinocytes or an immortalized keratinocyte cell line.

31-E4SPAM efficiently immortalizes primary keratinocytes. To examine if the SPAM mutation affected the immortalization of primary keratinocytes, we next transfected ligated 31-E4SPAM and wild-type genomes into human foreskin keratinocytes. A drug-selectable marker (pSV2neo) was cotrans-

TABLE 1. HPV-immortalized cell lines used in this study

Cell line	DNA	Source ^a
CIN-612	HPV-31b	Isolated from CIN I lesion
31wt-1	HPV-31	Transfected primary HFK; cloned
31wt-3	HPV-31	Transfected primary HFK; cloned
SPAM-2	31-E4SPAM	Transfected primary HFK; cloned
SPAM-3	31-E4SPAM	Transfected primary HFK; cloned
SPAM-4	31-E4SPAM	Transfected primary HFK; cloned
SPAM-P	31-E4SPAM	Transfected primary HFK; pooled
SPAM-P2	31-E4SPAM	Transfected primary HFK; pooled
31wt-P5	HPV-31	Transfected primary HFK; pooled
RESPAM-P	31-E4RESPAM	Transfected primary HFK; pooled

^a HFK, human foreskin keratinocytes.

fectured with the HPV genomes, and G418-resistant colonies were isolated. This procedure has been used previously to isolate cell lines which stably maintain episomal copies of HPV-31 and which, following growth in raft cultures, induce differentiation-dependent HPV late functions (9). Transfection with either wild-type HPV-31 or 31-E4SPAM yielded similar numbers of G418-resistant colonies (a total 30 colonies for the mutant and a total of 48 colonies for wild-type genomes over three experiments). Thus, we found no noticeable difference in immortalization potential between wild-type and 31-E4SPAM DNA. In some experiments, one or two G418-resistant colonies grew on plates transfected only with pSV2neo but did not survive splitting. Therefore, we deemed any culture in which keratinocytes survived three passages as immortalized. Individual colonies were then isolated, or pooled cultures were established in which all colonies from a single transfected dish were combined and expanded. Table 1 summarizes the HPV-31-immortalized keratinocyte lines isolated by this approach and examined in detail in this study. The SPAM cell lines did not differ noticeably from wild-type lines in appearance or growth rate (not shown). We next characterized these cell lines for the impact of the SPAM mutation on the maintenance of HPV-31 genomes, viral gene expression, and mRNA splicing.

31-E4SPAM integrates into host genome. Since the SPAM mutation did not affect the capability for transient replication, we used Southern analysis to examine the ability of 31-E4SPAM to be stably maintained as viral episomes (Fig. 4). Total genomic DNA was purified from SPAM cell lines as well as from wild-type HPV-31-immortalized lines and from a biopsy-derived cell line harboring episomal HPV-31b, CIN-612 (28). The state of HPV DNA was then analyzed by Southern blotting followed by hybridization to an HPV-31 probe. DNA from the CIN-612 cell line contained bands consistent with supercoiled, episomal HPV DNA as well as a family of bands representing nicked circular and concatemeric HPV. These bands were detectable after digestion with *Bam*HI, an enzyme which cuts neither HPV-31 nor HPV-31b (lane 1). Digestion of CIN-612 DNA with *Xba*I (lane 2; *Xba*I cuts once in HPV-31 and -31b) generated a single band of 7.9 kb corresponding to full-length linear HPV DNA. Both of the cell lines which had been transfected with HPV-31 DNA, 31-1 and 31-3, exhibited the same hybridization pattern seen with the CIN-612 DNA (lanes 3 through 6), indicating the presence of episomal HPV. In contrast, all cell lines containing 31-E4SPAM DNAs yielded a different pattern of hybridizing bands. No band corresponding to supercoiled SPAM DNA was detectable in the *Bam*HI-digested samples even after prolonged exposure (not shown), nor was nicked circular DNA detectable (lanes 7, 9, 11, 13, and 15). Instead, the *Bam*HI digests of SPAM DNAs contained only high-molecular-weight HPV DNA. The absence of circu-

FIG. 4. SPAM and RESPAM mutations disrupt stable episomal maintenance of HPV-31. Southern analysis was performed on 5 μ g (lanes 1 to 16) or 10 μ g (lanes 17 to 20) of total keratinocyte cellular DNA purified from the cell lines described in Table 1. DNAs were digested with *Bam*HI (B) or *Xba*I (X). Migration of DNA marker is shown at the left in kilobases, and the positions of supercoiled (S); indicates episomal HPV), full-length linear (L), and concatemeric (C) DNAs are shown at the right. The *Xba*I digest shown in lane 20 was incomplete. WT, wild type.

lar forms of HPV is most consistent with integrated copies of 31-E4SPAM. Digestion of the SPAM cell line DNAs with *Xba*I indicated that HPV sequences were full length (lanes 8, 10, 12, 14, and 16) and were integrated as head-to-tail concatemers. Junction fragments were detectable in the SPAM-2 DNA (lane 8) in Fig. 4 and were evident for other cell lines upon prolonged exposure (not shown). Taken together, these results suggest that HPV-31 genomes containing the SPAM mutation were unable to establish and maintain viral episomes despite their ability to replicate efficiently in transient assays.

The possibility existed that the 31-E4SPAM DNA became integrated due to imprecise ligation prior to transfection. If such imprecise ligation occurred, E2 function could be disrupted because our HPV-31 plasmid is cloned into the *Eco*RI site in E2. Since cells immortalized by integrated HPV DNA are thought to hold a growth advantage over cells harboring episomal HPV (17), imprecisely ligated SPAM DNAs which might become integrated might also outgrow episomal cells, thereby masking the initial presence of stably maintained episomes. To exclude this possibility, we performed PCR sequence analysis on SPAM DNA (data not shown). Sequencing of the region surrounding the *Eco*RI site revealed an intact E2 ORF, which demonstrated that correct ligation of the 31-E4SPAM DNA had occurred. Thus, integration of the genomes containing the SPAM mutation was an inherent property of the mutant HPV genomes.

SPAM transcripts are similar to 31wt. The simplest explanation for the integration of 31-E4SPAM genomes was that failure to splice efficiently at the 3295 acceptor grossly altered the stability and/or forms of HPV mRNAs. To investigate this possibility, we used Northern analysis to examine the HPV transcripts expressed in both SPAM and wild-type cells (Fig. 5). Ten micrograms of total cellular RNA was purified from monolayer cultures and examined with an E4/E5-specific probe. RNA isolated from CIN-612 cells was included as a control, and three major species of HPV transcripts were detected (Fig. 5A, lane 2). The most abundant message (II), about 1.7 kb in size, represents transcripts originating at P97 which utilize the major splice donor at nt 877 in E1 and the major splice acceptor at nt 3295 (15). The resulting mRNAs potentially encode the E6, E7, E1 Δ E4, and E5 ORFs. Another

FIG. 5. The HPV transcripts expressed in SPAM cell lines are similar to wild-type HPV-31 messages. Northern analysis of 10 μ g of total cellular RNA purified from wild-type (WT) HPV-31 cell lines (lanes 2 to 4) or from SPAM cell lines (lanes 5 to 9) indicates three major transcripts (I, II, and III) expressed in HPV-immortalized cell lines. All RNAs were isolated from monolayer cultures. The blot was probed with an E4/E5 probe (A) corresponding to nt 3278 to 5001, and the blot was stripped and reprobed with an E6/E7 probe (B) corresponding to nt 99 to 664. Class I transcripts are unspliced transcripts originating at P97 and terminating at the early polyadenylation site. Class II transcripts originate from P97, encode E6, E7, E1 Δ E4, and E5, and terminate at the early poly(A) site. Class III transcripts originate from P742, encode E1 Δ E4 and E5, and terminate at the early poly(A) site. SCC13 cells (lane 1) contain no HPV sequences. Position of migration of RNA markers are indicated in nucleotides at the left of each panel.

transcript (III) of about 1.1 kb likely represents low-level induction of expression from P742, the late promoter at nt 742. The 1.1-kb band likely represents messages encoding E1 Δ E4 and E5 (15). To confirm that the 1.1-kb message originated from P742, the blot was stripped and reprobed with an E6/E7-specific probe. The E6/E7 probe (nt 99 to 664), which detects only early transcripts originating from P97, did not hybridize to the 1.1-kb transcript (Fig. 5B, lane 2), indicating that these transcripts arise from P742. Finally, CIN-612 cells express a transcript of about 4 kb which is an unspliced early message that encodes E1 and is believed to be the primary message for expression of this replication protein (15, 15a, 18a).

The two HPV-31 wild-type-transfected lines (lanes 3 and 4) expressed the same three major transcripts as were detected in CIN-612 cells. Surprisingly, the SPAM lines also expressed three major transcripts of similar size (lanes 5 to 9) and exhibited similar patterns of hybridization to either an E4/E5- or an E6/E7-specific probe (Fig. 5A or B, respectively). Since the 1.1- and 1.7-kb wild-type mRNAs utilize SPA³²⁹⁵, SPAM messages of similar size were presumably generated via splicing to a cryptic acceptor site near nt 3295. To confirm that the SPAM cell lines indeed contained mutant HPV genomes and not simply integrated copies of wild-type HPV-31 (which would presumably express transcripts of wild-type size), we performed cycle sequencing. The HPV sequences from SPAM cell DNA confirmed that the splice acceptor mutation (AG to TC [Fig. 2]) was present in each SPAM cell line (data not shown).

SPAM utilizes a cryptic splice acceptor. To examine the nature of SPAM transcripts within the region near the mutated 3295 splice acceptor, we next performed RNase protection assays (Fig. 6A). Antisense probes which originated in the C terminus of E4 and terminated at nt 3247 upstream of 3295 were prepared (Fig. 6B). These probes allowed us to discriminate between spliced and unspliced transcripts in the region around 3295. Two separate probes were used to examine wild-type and SPAM RNAs. The SPAM probe was generated from the mutated DNA so that the AG-to-TC splice acceptor mutation, potentially present in SPAM messages, would not generate shorter protected species when hybridized to a wild-type

FIG. 6. HPV-31 transcripts expressed in SPAM cell lines utilize a cryptic splice acceptor near SPA³²⁹⁵. (A) RNase protection assays performed on 8 μ g of total cellular RNA purified from wild-type (WT) HPV-31 cell lines (lanes 5 to 7) or SPAM cell lines (lanes 8 to 11). RNAs were purified from monolayer cultures. Protected fragments corresponding to unspliced E1 or E2 messages and spliced E1 Δ E4 (and E6 Δ E4) mRNAs are indicated at the right. Lane 1 (marker [M]) is an end-labeled DNA size standard with the corresponding sizes in base pairs indicated to the left; lanes 2 and 3 are untreated probes derived from wild-type and SPAM sequences, respectively. (B) Structure of the riboprobe and the expected protected fragments. Shaded regions correspond to HPV-31 sequences, and vector sequences present in the riboprobe are represented as thick lines; the sizes of predicted protection products are shown at the right.

probe. Two major protected species were detected in wild-type RNAs (lanes 5 to 7): (i) a protected product of 331 nt representing unspliced E1 or E2 message and (ii) a protected product of 283 nt representing message spliced at the 3295 acceptor (E1 Δ E4 or E6 Δ E4). It was also possible that any HPV 31 DNA which contaminated the RNA preparation also contributed to the levels of the 331-nt product. The majority of products were found to be spliced at the SPA³²⁹⁵, but unspliced products were also evident. Consistent with the results of Northern analysis (see above), the sizes of fragments protected by SPAM RNAs (lanes 8 to 11) were similar to wild-type protection products. However, the major product protected by SPAM RNA—which represents utilization of a splice acceptor near 3295—appeared slightly smaller than the corresponding product protected by wild-type RNAs. This finding indicated that the spliced SPAM transcripts utilized a cryptic acceptor downstream of nt 3295 within the E4 ORF.

To specifically identify the splice acceptor used to generate the spliced SPAM mRNAs, we used RT-PCR (Table 2). Using a sense primer within E7 and an antisense primer at the C terminus of E4, we generated cDNAs from both wild-type (CIN-612) and SPAM RNAs. Each RT-PCR generated a single major product (data not shown), and the nucleotide sequences of these products were then determined. The cDNA amplified from CIN-612 RNA consisted of E7 and E1 sequences spliced to E4 sequences at nt 3295. Such a cDNA was expected from the amplification of E7 and E1 Δ E4 sequences

TABLE 2. Identification of splice acceptors by RT-PCR

mRNA	Identified splice acceptor(s) ^a
612	3295
SPAM-2.....	3298, 3508 ^b
SPAM-3.....	3298
SPAM-4.....	3298
RESPAM-1.....	3332 ^c

^a Splice acceptors identified by RT-PCR followed by sequence analysis of PCR products. The upstream primer was located in E7, and the downstream primer was located in E5.

^b Additional cryptic acceptor identified in a separate experiment.

^c Sequence of RT-PCR product became heterogeneous upstream of this position, indicating utilization of nt 3332 as well as upstream sites as splice acceptors.

present in either the 1.1- or 1.7-kb transcripts observed in the Northern analysis (Fig. 5A). In contrast, cDNAs amplified from SPAM-2, SPAM-3, and SPAM-4 RNAs were found to utilize a cryptic splice acceptor at position 3298, only 3 nt downstream of the wild-type acceptor. A second RT-PCR experiment using a different primer pair confirmed the utilization of the 3298 acceptor and identified another cryptic acceptor at position 3508. These data indicate that SPAM mRNAs are spliced within E4 via the joining of a 5' exon to cryptic splice acceptors.

The fact that SPAM transcripts could be spliced to a cryptic acceptor 3 nt downstream of the wild-type acceptor suggested that SPAM cells might be capable of synthesizing proteins from the E4 region. To test this possibility, we grew SPAM-2 cells in raft culture and examined the resulting tissue for E4-like immunoreactivity with anti-E1 Δ E4 serum. SPAM-2 raft tissue contained cells expressing E4-like immunoreactivity, whereas raft tissue derived from primary keratinocytes did not contain any cells which were reactive with the anti-E1 Δ E4 serum. The number of E4-positive cells observed in SPAM-2 raft tissue, however, was significantly reduced from the number of E4-positive cells detected in wild-type HPV-31 rafts (data not shown). Although it was not possible to determine if putative E4-positive cells represented bona fide synthesis of E1 Δ E4 or E6 Δ E4 protein, this observation suggests that the spliced SPAM transcripts generated by utilization of a cryptic splice acceptor can support translation of a portion of the E4 ORF.

Other splice sites are unaffected by SPAM. A change in one splice site is able to influence splicing at other splice sites (14). It was possible, then, that the SPAM mutation also altered splicing at sites distant from the 3295 acceptor. To investigate this possibility, we used RNase protection assays to determine if the SPAM mutation had altered splicing at either of the two major splice donors located at nt 210 or 877 (Fig. 7). Using a probe spanning the donor at nt 210, we found no significant difference in the relative levels of spliced and unspliced transcripts which use the 210 donor (Fig. 7A; compare lanes 6 and 7). Likewise, a probe spanning the donor at nt 877 did not reveal any significant differences in splicing activity at the 877 donor between wild-type and SPAM transcripts (Fig. 7B; compare lanes 6 and 7). Given the apparent absence of effects of the SPAM mutation on distant splicing events, this finding suggests that the mutation exerts its influence primarily by altering splicing at SPA³²⁹⁵.

Instability of SPAM is not due to enhanced splicing to nt 3298. It was possible that, due to our efforts to design a mutation which was silent in E2, the SPAM mutation altered the levels of unspliced messages which contain this sequence. The conversion of AG to TC in the SPAM mutation extends a

series of pyrimidine bases upstream of the cryptic acceptor at 3298. Such polypyrimidine tracts are conserved features of splice acceptor sites. Therefore, the SPAM mutation could actually enhance splicing within the area near SPA³²⁹⁵, which would result in depletion of the pool of unspliced messages. A reexamination of the level of unspliced messages (Fig. 5 and 6) indicated that the SPAM samples contained slightly reduced levels of unspliced messages relative to wild-type cell lines.

To determine whether enhanced splicing near nt 3295 contributed to the phenotype of the 31-E4SPAM, we constructed another mutation of SPA³²⁹⁵, RESPAM. RESPAM abolished both AGs as well as the polypyrimidine tract constituting the 3295 and 3298 splice acceptors (Fig. 2). This mutation is not silent within the overlapping E2 ORF; however, other deletion or substitution mutations in this region of the E2 ORF do not affect episomal maintenance (17a) (this region of E2 corresponds to the unstructured hinge region between the transcriptional activation domain and the DNA binding domain). HPV-31 genomes containing the RESPAM mutation were first tested for the ability to replicate transiently (Fig. 3A). Although transient replication appeared less efficient than for the wild type, 31E4-RESPAM genomes retained the ability to replicate transiently in SCC13 cells (55% of the wild-type level [lane 5]).

We next tested the RESPAM mutant for its ability to immortalize primary keratinocytes. 31-E4RESPAM efficiently immortalized primary human foreskin keratinocytes, and DNA from the resulting RESPAM cells was analyzed by Southern blotting (Fig. 4). Wild-type HPV 31 replicated as stable episomes, as indicated by the presence of supercoiled genomes (lane 17). Similar to the results obtained with 31E4-SPAM, 31E4-RESPAM DNA was found to be integrated into host sequences (lane 19), since no band corresponding to supercoiled HPV-31 could be detected even upon extended autoradiography (not shown). As seen for 31E4-SPAM, 31E4-RESPAM integrated as head-to-tail concatemers (lane 20; the *Xba*I digest was incomplete in this experiment).

Northern analysis indicated that RESPAM cells also expressed transcripts similar in size to those expressed by wild-type cells (not shown). Since it appeared likely that 31-E4RESPAM transcripts were also utilizing a cryptic splice acceptor near position 3295, we performed RT-PCR analysis to identify the splice junction utilized by the RESPAM mutant (Table 2). Cycle sequencing of the PCR products revealed that E1 sequences were joined to E4 sequences via a cryptic acceptor at position 3332. However, the DNA sequence became heterogeneous upstream of nt 3332, despite the fact that the RT-PCR product ran as a single band on an agarose gel. These results suggest that the RESPAM mutation forces utilization of multiple splice acceptors in lieu of a single preferred acceptor.

DISCUSSION

To initiate an examination of the roles of E1 Δ E4 and E5 in HPV pathogenesis, we have used an assay which can result in the synthesis of HPVs from transfected viral DNA. In this study, we mutated the splice acceptor at nt 3295 of HPV-31 which is utilized by all spliced viral transcripts potentially encoding E1 Δ E4 or E5. We found that genomes containing this mutation, 31E4SPAM, retained the ability to replicate transiently (albeit at a reduced rate) and to efficiently immortalize primary human keratinocytes. The cell lines immortalized by SPAM DNA, however, were found not to maintain HPV sequences as stable episomes. Instead, the SPAM DNA had become integrated as tandem repeats. One possible explana-

FIG. 7. The SPAM mutation does not affect utilization of upstream splice donors at nt 877 (A) or 210 (B). The upper portion shows RNase protection assays performed on 8 μ g of total cellular RNA purified from wild-type (WT) HPV-31 cell lines (lanes 4 to 6) or SPAM cell lines (lanes 7 to 10). RNAs were purified from monolayer cultures. In panel A, protected fragments corresponding to unspliced E1 messages and spliced E1 Δ E4 mRNAs originating at P97 are indicated at the right. In panel B, protected fragments corresponding to unspliced E6 messages and spliced E6* mRNAs originating at P97 are indicated at the right. Lane 1 (M [marker]) is DNA size standard with the corresponding sizes in base pairs indicated to the left; lane 2 is untreated probe derived from wild-type HPV-31 sequences. The lower portion indicates the structure of the riboprobe and the expected protected fragments. Shaded regions correspond to HPV-31 sequences where the promoters are indicated by arrows, and vector sequences present in the riboprobe are represented as thick lines; the sizes of predicted protection products are shown at the right.

tion for these effects was that the SPAM mutation disrupted splicing patterns so severely that early HPV transcripts could not supply sufficient levels of immortalizing functions and replication factors necessary for stable episomal maintenance. Surprisingly, the size and abundance of viral transcripts in the SPAM cells were similar to those detected in lines immortalized by wild-type HPV-31. We also observed comparable levels of utilization of the splice donors (relative to wild type) at nt 210 and 877 and conclude that SPAM did not influence the processing at other splicing elements. SPAM mutants were found to utilize a cryptic splice acceptor at nt 3298, only 3 nt downstream of the wild-type acceptor at nt 3295. Utilization of this cryptic acceptor resulted in a shorter E1 Δ E4 ORF, but these transcripts appeared to encode translatable protein. Finally, we found that a second mutant which was designed not to utilize either the wild-type acceptor or the cryptic acceptor at 3298 behaved like the SPAM mutant in both transient and stable assays. The behavior of this second mutant indicated that the phenotype of SPAM was not simply the result of increased splicing near SPA³²⁹⁵. Taken together, these observations demonstrate the importance of the acceptor at 3295 and suggest that a factor(s) encoded by transcripts spliced at

this sequence may be necessary for stable episomal maintenance of HPV 31.

The altered splicing pattern observed in the SPAM mutant lines is consistent with recent studies in yeast. In these studies, the utilization of otherwise equivalent splice acceptors was found to be dependent on their proximity to the branchpoint adenosine within the intron (22). The acceptor which is proximal to the branch point is utilized in favor of a distal acceptor, but a distal acceptor can be used preferentially when its sequence more closely resembles a consensus acceptor. For HPV-31, an adenosine is located 13 nt upstream of the SPA³²⁹⁵ at position 3282. The optimal distance from the branchpoint adenosine to the acceptor is 13 to 22 nt in yeast. Therefore, nt 3282 in HPV-31 may serve as the branchpoint adenosine for the utilization of SPA³²⁹⁵. In SPA³²⁹⁵ mutants, a cryptic acceptor is utilized at 3298 which lies 16 nt from the putative branchpoint adenosine. This is consistent with our observations that transcripts of SPAM cell lines are similar in abundance and size to those expressed in wild-type HPV-31 cell lines. However, factors other than a convenient branchpoint site may also direct splicing to the region near SPA³²⁹⁵. Recent studies have shown that use of the analogous BPV-1

acceptor, SPA³²²⁵, is regulated by a splicing enhancer comprised of both positive and negative regulatory elements (38). If similar *cis* elements regulate SPA³²⁹⁵ utilization in HPV-31, splicing may be strongly directed to the region near SPA³²⁹⁵ instead of skipping further downstream, and this possibility is also consistent with our results.

The phenotype of HPV-31 genomes carrying the SPAM mutation is different from the phenotype of a previously characterized BPV-1 mutant (12). The BPV-1 mutant p1039-1 failed to transform mouse C127 cells which were efficiently transformed by wild-type BPV-1. In contrast, the SPAM mutation does not appear to alter the immortalizing potential of HPV-31. The BPV-1 mutant likely exhibits reduced transforming activity because the E5 ORF, which encodes the major transforming protein of BPV, lies downstream of the mutated splice acceptor and transcripts encoding E5 utilize this acceptor. In HPV-31, the immortalizing functions are encoded by E6 and E7, and transcripts encoding these genes also utilize the 3295 acceptor. It is possible that, in contrast to the cryptic acceptor used in the HPV-31 SPAM mutants, no suitable cryptic acceptor exists in BPV-1 to permit expression of E5. Finally, another significant difference lies in our observation that SPAM mutant genomes failed to be stably maintained as episomes, while the corresponding BPV-1 mutant could stably replicate.

If the diversity and abundance of HPV 31 early transcripts expressed from genomes carrying the SPAM mutation are similar to those expressed from wild-type genomes, why then is SPAM unable to maintain viral episomes? It is possible that E1ΔE4 plays a previously uncharacterized role in stable replication of HPVs. This possibility is unlikely, since HPV-31 genomes containing a stop codon near the amino terminus of E4 are able to immortalize primary keratinocytes and are stably maintained as episomes (17a). Similarly, reduced expression of E5 is an unlikely explanation because the use of a cryptic splice site within E4 probably would not affect the E5 ORF. In addition, we find that an E5 knockout mutation does not prevent stable episomal maintenance of HPV episomes (17a). Since alterations of E4 and E5 expression are not likely to destabilize HPV-31 maintenance in genomes carrying the SPAM mutation, SPAM probably exerts its influence by altering the replication functions of HPV.

When HPV DNA is introduced into keratinocytes by transfection of ligated genomes, several modes of replication probably occur. First, the HPV DNA is quickly replicated to about 50 copies per cell, and this process is most likely mirrored in transient replication assays. Following this initial phase, replication must then be down-regulated such that episomes are stably maintained and viral replication occurs in concert with cellular replication to maintain a roughly constant copy number of HPV genomes. Finally, in our assays, transfected keratinocytes must also express levels of E6 and E7 high enough to immortalize cells. The SPAM mutation likely exerts its effect(s) at one or more of these steps, and this effect may be either qualitative and/or quantitative.

Our studies showed that both SPAM and RESPAM DNAs replicated in transient assays, though at rates slightly lower than for the wild type (ranging from 40 to 100% of the wild-type rate). In HPV-31, the transcripts encoding full-length E1 and E2 ORFs do not rely on splicing at 3295 to generate their messages (15, 15a, 18a). However, BPV-1 encodes a transcriptional repressor (20), the E8-E2 protein, which is generated via SPA³²²⁵, the acceptor analogous to SPA³²⁹⁵ in HPV-31. HPV-11 also expresses a partial E2 protein which utilizes a splice acceptor that is analogous to the SPA³²⁹⁵ of HPV-31 (23). If such a partial E2 protein is also expressed in oncogenic

HPVs, the SPAM mutation could interfere with the function of these truncated E2 proteins because utilization of the cryptic splice acceptor at nt 3298 would eliminate an amino acid (corresponding to Ser 201 in the full-length E2). Alternatively, it is possible that the SPAM mutation disrupts the proper temporal accumulation of E8ΔE2 messages by forcing the utilization of less efficient splice acceptors.

If HPVs have a stringent requirement for a minimal level of initial replication in order to establish genomes as stable episomes, then even a slight reduction in replication efficiency could affect maintenance. In our Northern analysis of SPAM early RNAs, a reduction in the levels of transcripts of approximately 4 kb in size was observed. Previous studies suggested that this transcript encoded all early ORFs and was the only transcript in which an intact E1 ORF could be detected (15, 15a, 18a). It is possible that the reduction in levels of 4-kb transcripts observed in SPAM cells results in reduced amounts of E1 protein which are insufficient to support stable HPV replication. In the BPV-1 p1039-1 mutant, reduction in E2 levels to 50% of the wild-type level was detected (19), and a corresponding reduction in E2 repressor levels was also observed. It is unclear whether these reductions were due to slower splicing kinetics, altered message stability, or decreased mRNA transport, as these potential mechanisms could contribute to a decrease in unspliced SPAM messages. Despite these changes in the levels of full-length and repressor forms of E2 and the mechanism underlying these changes, the p1039-1 mutant was able to stably replicate at levels similar to the wild-type level. The differences in replication ability of the HPV-31 and BPV-1 mutants suggest that the requirements for stable maintenance of bovine genomes in C127 cells and human viruses in keratinocytes may be distinct.

In summary, our data suggest that a factor(s) involved in the stable episomal maintenance of HPV-31 relies on the utilization of the major splice acceptor at nt 3295. Since HPV DNA carrying the SPAM mutation replicated transiently, the SPAM mutation also revealed differential requirements for transient versus stable replication of HPV in keratinocytes. It thus appears that the requirements for stable maintenance of HPV genomes in keratinocytes are more stringent than those for transient replication, and characterizing the basis of these differences will further illuminate the role of HPV factors in pathogenesis.

ACKNOWLEDGMENTS

We thank Kathleen Rundell for careful reading of the manuscript and members of the Laimins laboratory for suggestions. We also thank Mary Hummel and Hock Ben Lim for many helpful discussions.

This work was supported by grants RO1 CA-59655 from the National Cancer Institute (L.A.L.), F32 AI09494-01 from the National Institute of Allergy and Infectious Diseases (D.J.K.), and Stu218/(1-1) from the Deutsche Forschungsgemeinschaft (F.S.).

REFERENCES

1. Bell, G. I., J. H. Karam, and W. J. Rutter. 1981. Polymorphic DNA region adjacent to the 5' end of the insulin gene. *Proc. Natl. Acad. Sci. USA* **78**:5759-5763.
2. Bouvard, V., G. Matlashewski, Z.-M. Gu, A. Storey, and L. Banks. 1994. The human papillomavirus type 16 E5 gene cooperates with the E7 gene to stimulate proliferation of primary cells and increases viral gene expression. *Virology* **203**:73-80.
3. Chow, L. T., M. Nasseri, S. M. Wolinsky, and T. R. Broker. 1987. Human papillomavirus types 6 and 11 mRNAs from genital condylomata acuminata. *J. Virol.* **61**:2581-2588.
4. DelVecchio, A. M., H. Romanczuk, P. M. Howley, and C. C. Baker. 1992. Transient replication of human papillomavirus DNAs. *J. Virol.* **66**:5949-5958.
5. Doorbar, J., S. Ely, J. Sterling, C. McLean, and L. Crawford. 1991. Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of

- the epithelial cell intermediate filament network. *Nature* **352**:824–827.
6. **Dyson, N., P. M. Howley, K. Münger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**:934–936.
 7. **Frattini, M. G., and L. A. Laimins.** 1994. Binding of the human papillomavirus E1 origin-recognition protein is regulated through complex formation with the E2 enhancer-binding protein. *Proc. Natl. Acad. Sci. USA* **91**:12398–12402.
 8. **Frattini, M. G., and L. A. Laimins.** 1994. The role of the E1 and E2 proteins in the replication of human papillomavirus type 31b. *Virology* **204**:799–804.
 9. **Frattini, M. G., H. B. Lim, and L. A. Laimins.** 1996. In vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late expression. *Proc. Natl. Acad. Sci. USA* **93**:3062–3067.
 10. **Ghai, J., R. S. Ostrow, J. Tolar, R. C. McGlennen, T. D. Lemke, D. Tobolt, Z. Liu, and A. J. Faras.** 1996. The E5 gene product of rhesus papillomavirus is an activator of endogenous Ras and phosphatidylinositol-3'-kinase in NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA* **93**:12879–12884.
 11. **Gu, Z.-M., and G. Mathlshewski.** 1995. Effect of human papillomavirus type 16 oncogenes on MAP kinase activity. *J. Virol.* **69**:8051–8056.
 12. **Hermonat, P. L., and P. M. Howley.** 1987. Mutational analysis of the 3' open reading frames and the splice junction at nucleotide 3225 of bovine papillomavirus type 1. *J. Virol.* **61**:3889–3895.
 13. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365–369.
 - 13a. **Howley, P. M.** 1996. Papillomavirinae: the viruses and their replication, p. 947–978. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fundamental virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
 14. **Huang, C. H., M. Reid, G. Daniels, and O. O. Blumenfeld.** 1993. Alteration of splice site selection by an exon mutation in the human glycophorin A gene. *J. Biol. Chem.* **268**:25902–25908.
 15. **Hummel, M., J. B. Hudson, and L. A. Laimins.** 1992. Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. *J. Virol.* **66**:6070–6080.
 - 15a. **Hummel, M.** Unpublished observations.
 16. **Hummel, M., H. B. Lim, and L. A. Laimins.** 1995. HPV 31b late gene expression is regulated through protein kinase C-mediated changes in RNA processing. *J. Virol.* **69**:3381–3388.
 17. **Jeon, S., and P. F. Lambert.** 1995. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. *Proc. Natl. Acad. Sci. USA* **92**:1654–1658.
 - 17a. **Klumpp, D. J.** Unpublished observations.
 18. **Laimins, L. A.** 1993. The biology of human papillomaviruses: from warts to cancer. *Infect. Agents Dis.* **2**:74–86.
 - 18a. **Laimins, L. A.** 1996. Human papillomaviruses target differentiating epithelia for virion production and malignant conversion. *Semin. Virol.* **7**:305–313.
 19. **Lambert, P. F., N. L. Hubbert, P. M. Howley, and J. T. Schiller.** 1989. Genetic assignment of multiple E2 gene products in bovine papillomavirus-transformed cells. *J. Virol.* **63**:3151–3154.
 20. **Lambert, P. F., B. A. Spalholz, and P. M. Howley.** 1987. A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. *Cell* **50**:69–78.
 21. **Leptak, C., S. Ramon y Cajal, R. Kulke, B. H. Horwitz, D. J. Riese II, G. P. Dotto, and D. DiMaio.** 1991. Tumorigenic transformation of murine keratinocytes by the E5 genes of bovine papillomavirus type 1 and human papillomavirus type 16. *J. Virol.* **65**:7078–7083.
 22. **Luukkonen, B. G. M., and B. Seraphin.** 1997. The role of branchpoint-3' splice site spacing and interaction between intron terminal nucleotides in 3' splice site selection in *Saccharomyces cerevisiae*. *EMBO J.* **16**:779–792.
 23. **Maran, A., C. A. Amell, T. P. DiLorenzo, K. J. Auburn, L. B. Taichman, and B. M. Steinberg.** 1995. Human papillomavirus type 11 transcripts are present at low abundance in latently infected respiratory tissues. *Virology* **211**:285–294.
 24. **Meyers, C., M. G. Frattini, J. B. Hudson, and L. A. Laimins.** 1992. Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science* **257**:971–973.
 - 24a. **Mohr, I. J., R. Clark, S. Sun, E. Androphy, P. MacPherson, and M. Botchan.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science* **250**:1694–1699.
 25. **Münger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley.** 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J.* **18**:4099–4105.
 26. **Nasseri, M., R. Hirochika, T. R. Broker, and L. T. Chow.** 1987. A human papillomavirus type 11 transcript encoding an E1 Δ E4 protein. *Virology* **159**:433–439.
 27. **Pim, D., M. Collins, and L. Banks.** 1992. Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. *Oncogene* **7**:27–32.
 28. **Rader, J. S., T. R. Golub, J. B. Judson, D. Patel, M. A. Bedell, and L. A. Laimins.** 1990. In vitro differentiation of epithelial cell from cervical neoplasias resembles in vivo lesions. *Oncogene* **5**:571–576.
 29. **Rotenberg, M. O., L. T. Chow, and T. R. Broker.** 1989. Characterization of rare human papillomavirus type 11 mRNAs coding for regulatory and structural proteins, using the polymerase chain reaction. *Virology* **172**:489–497.
 30. **Schneider-Gadicke, A., and E. Schwartz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. *EMBO J.* **5**:2285–2292.
 31. **Schwarz, E., U. K. Freese, L. Gissman, W. Mayer, B. Roggenbuck, A. Stremmel, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **314**:111–114.
 32. **Schwarz, E., A. Schneider-Gadicke, and H. zur Hausen.** 1987. Human papillomavirus type-18 transcription in cervical carcinoma cell lines and in human cell hybrids. *Cancer Cells* **5**:47–53.
 33. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. USA* **83**:4680–4684.
 34. **Straight, S. W., P. M. Hinckle, R. J. Jewers, and D. J. McCance.** 1993. The E5 oncoprotein of HPV 16 transforms fibroblasts and effects the down-regulation of the epidermal growth factor receptor in keratinocytes. *J. Virol.* **67**:4521–4532.
 - 34a. **Stubenrauch, F., et al.** Unpublished data.
 35. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. *EMBO J.* **10**:449–457.
 36. **Vousden, K. H.** 1994. Interactions between papillomavirus proteins and tumor suppressor gene products. *Adv. Cancer Res.* **64**:1–24.
 37. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**:76–79.
 38. **Zheng, Z.-M., P. He, and C. C. Baker.** 1996. Selection of the bovine papillomavirus type 1 nucleotide 3225 3' splice site is regulated through an exonic splicing suppressor. *J. Virol.* **70**:4691–4699.