Transforming Properties of the Cottontail Rabbit Papillomavirus Oncoproteins LE6 and SE6 and of the E8 Protein

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Cottontail rabbit papillomavirus induces on cottontail and domestic rabbits papillomas which progress at a high frequency to carcinoma. The virus encodes three transforming proteins; one is translated from open reading frame (ORF) E7 and binds the retinoblastoma protein, and two, LE6 and SE6, are translated from the first and second ATGs of ORF E6, respectively. Here we show that neither of the E6 proteins coprecipitated with p53 *in vitro*, nor did they bind to a recently identified E6-binding protein (J. J. Chen, C. E. Reid, V. Band, and E. Androphy, *Science* 269:529–531, 1995). This protein was shown to bind to the E6 proteins of the high-risk human papillomavirus types 16 and 18 but not to the low-risk human papillomavirus types 6 and 11. In-frame deletions cloned into the pZipNeo vector were used to identify structural features of SE6 and LE6 important for transformation of NIH 3T3 cells. Three deletions covering the amino-terminal half of SE6 did not transform cells. In two of the three deletions, two Cys-X-X-Cys motifs were deleted, each deletion preventing the formation of one of the potential small Zn fingers of SE6. Among the LE6 deletions, only one had a reduced transformation efficiency, while seven transformed cells at least as efficiently as wild-type LE6. In each of three of these seven mutants, two Cys-X-X-Cys motifs were deleted. None of the three amino acid deletions which abolished transformation by SE6 reduced transformation by LE6. Furthermore, transformation did not correlate with the level of SE6 or LE6 proteins detectable. ORF E8 colinear with ORF E6, which could generate a 50-amino-acid protein with a hydrophobic segment, did not transform cells when cloned into the pZipNeo vector. However, mutation of the E8 ATG, which did not alter the amino acid sequence of LE6, increased transformation by LE6 without affecting the level of LE6 expression. The data suggest that transformation by the E6 proteins is not mediated by interfering with p53 function or through binding to the E6-binding protein. Furthermore, different structural features are important to maintain transformation functions and protein stability of LE6 and SE6. Finally, E8 seems not to be a transforming protein but rather appears to modulate transformation by LE6.

Papillomaviruses (PV) are small nonenveloped DNA viruses with a circular genome of approximately 8 kb. The viruses are strictly epitheliotropic, and virus replication is tightly linked to the differentiation of the epithelium. Infection with most PVs results in growth stimulation of the epithelium and formation of papillomas. Papillomas induced by some PVs, called high-risk PVs, often progress to carcinomas. The high-risk genital human PVs (HPVs) include HPV type 16 (HPV-16), HPV-18, HPV-31, HPV-33, and others (60); high-risk cutaneous HPVs include HPV-5 and -8 (43, 45). Cottontail rabbit PV (CRPV) induces papillomas on the hairy skin of cottontail and domestic rabbits, and these papillomas progress at a high frequency to carcinomas (39, 53). The high cancer risk appears to be associated with unique properties of E6 and E7 proteins (for the genital HPVs) (reviewed in reference 52) and the E6 protein (for HPV-8) (25, 31, 35). CRPV encodes three transforming proteins, LE6, SE6, and E7. Each of these proteins can induce anchorage-independent growth of NIH 3T3 cells (40, 50) and transform sflEp rabbit epithelial cells (40). Papilloma forma-

tion in rabbits, however, requires all three proteins (40, 59). The E7 protein has been shown to bind to the retinoblastoma protein (pRB) (16, 24, 50), but nothing is known about the transforming mechanisms of the E6 proteins. CRPV is unusual among PVs, as it encodes two proteins from open reading frame (ORF) E6 (5) and the ORF is considerably longer than those of other PVs (20). The two E6 proteins are translated from separate mRNAs initiated at two different promoters (15, 42, 56). The SE6 and E7 mRNAs are the major early transcripts in papillomas and carcinomas, while the LE6 mRNA represents a minor species. The two E6 proteins differ in several properties. LE6 is a nonphosphorylated, nuclear protein associated with the nuclear matrix (6) and consists of 273 amino acids; SE6 is initiated at the second ATG of ORF E6. It is a cytoplasmic phosphoprotein of 176 amino acids identical to the 176 carboxy-terminal amino acids of LE6 (5).

Both of the CRPV E6 proteins are larger than the genital HPV E6 proteins, which are approximately 150 amino acids in length. Furthermore, both CRPV E6 proteins contain more Cys-X-X-Cys motifs, eight and six for LE6 and SE6, respectively, compared with four in E6 of other PVs, and the spacing between some of the motifs is different (reviewed in reference 30). These motifs are important for Zn²⁺ binding (4, 23) and also affect the subcellular localization of the protein (33).

An important aspect of cell transformation by several DNA tumor viruses is the ability of the viral transforming proteins to

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associate with two tumor suppressor proteins, pRB and p53. For the genital HPVs, the importance of this interaction in the development of malignancies is suggested by the observations that HPV-negative cervical carcinoma cell lines and primary cervical tumors contain p53 mutations and HPV-positive cervical carcinoma cell lines contain wild-type p53; similar observations were made with respect to pRB (13, 14, 48, 58). There are some exceptions to this general finding, as some HPV-negative cervical carcinomas or precancer lesions or vulvar carcinomas do not contain p53 mutations (7, 8, 37, 38, 44) and some HPV-positive cervical carcinomas contain p53 mutations (12).

In contrast to other DNA tumor viruses which only bind p53, HPV-16 and -18 E6 protein also mediate the degradation of p53 by the ubiquitin pathway (48, 55). This degradation requires the participation of a cellular protein, E6-AP (27, 28, 29). In addition to binding to p53 and E6-AP, E6 binds to several other cellular proteins (34). The inability of low-risk HPV E6 proteins to degrade p53 is a reflection of their inability to bind p53 or to bind p53 with lower affinity (11, 49). Finally, the E6 protein of a cutaneous high-risk PV, HPV-8, confers anchorage-independent growth to C127 and NIH 3T3 cells (50) but does not bind p53 (51). This finding suggests that p53 binding or degradation is not the transforming mechanism of all high-risk PV E6 proteins.

In this study, we showed that neither of the CRPV E6 proteins formed a stable complex with p53, nor did they bind to the E6-binding protein (E6BP) (10). A deletion analysis of the transforming properties of LE6 and SE6 suggests that the proteins transformed by different mechanisms. Finally, ORF E8 colinear with ORF E6, which could encode a protein with similarities to bovine PV type 1 (BPV-1) E5, is not a transforming protein.

MATERIALS AND METHODS

Plasmids. The CRPV LE6 and SE6 recombinant plasmids were cloned from pMSGneo-LE6 and pMSGneo-SE6, previously described (40). Briefly, pMSGneo-LE6 contains the CRPV *DdeI* fragment (nucleotides [nt] 144 to 1091) cloned into the *XhoI* site of pMSGneo (Pharmacia). In this LE6 insert, the ATG start codon for the SE6 was changed to ATA; thus, only LE6 can be made. pMSGneo-SE6 contains the *EaeI* (nt 316)-to-*EcoRI* (nt 1063) fragment inserted into the *XhoI* site of pMSGneo and can make only SE6. The LE6 and SE6 fragments were cleaved from pMSGneo-LE6 or -SE6 by *XhoI* digestion, the ends were filled in with Klenow DNA polymerase, *XbaI* linkers were added, and the LE6 and SE6 segments were subcloned into the *XbaI* site of the pBSKS phagemid (Stratagene) in which the *SacI* site had been changed to an *SphI* site. To generate M13mp19-LE6 and M13mp19-SE6, these segments were directionally subcloned into *SphI*- and *Asp* 718-digested M13 (Stratagene). To construct pBSKS-IgLE6 and pBSKS-IgSE6, pLAII-CRPVΔ*XbaI*SE6 (cannot express SE6) and pLAII-CRPVΔ*XbaI*LE6 (cannot express LE6), respectively, were used (40). pLAII-CRPVΔ*XbaI*SE6 and pLAII-CRPVΔ*XbaI*LE6 were digested with *HindIII*, filled in with Klenow DNA polymerase, and digested further with *BalI*. *XbaI* linkers were added, and fragments (nt 29 to 1093) were cloned into the *XbaI* site of the modified pBSKS described above.

(i) Site-specific mutants. Site-specific mutations were made for direct tests as well as to generate unique restriction sites to obtain in-frame deletions. For oligonucleotide-directed mutagenesis, the pBSKS-LE6 and -SE6 or pBSKS-IgLE6 and pBSKS-IgSE6 vectors were digested with *Asp* 718 and *SphI*, and the LE6 and SE6 fragments were isolated and directionally cloned into *Asp* 718- and *SphI*-digested M13mp19 (New England Biolabs). Site-directed mutagenesis of LE6 and SE6 was performed by using an oligonucleotide-directed in vitro mutagenesis system (Amersham) and oligodeoxynucleotides to produce the following mutations: 5'-CGTGGCAGGCGGCGCCCATCTGCTCTAAG-3' (LE6-2G3A/*NarI*), 5'-C GCAAAATATACAACCGGCGCCCAAGTCCTCAATG-3' (LE6-24G25A/*NarI*), 5'-GATCCAAAGCACAAGAGGCGCCAGTGCAGTCTCTG-3' (LE6-65G66A/*NarI*), 5'-CTGTACAACGTATGGCGCCGCTAGATA TTCTCTG-3' (LE6-96G97A/*NarI*), 5'-GACCTTTATCGATACAACAAAG-3' (LE6-125I126D/*ClaI*) and SE6-28I29D/*ClaI*), 5'-CTTCCGCAACTGTACAGGCGCCACGCGATGAACAGCTAG-3' (SE6-3G4/*AccI*), 5'-CCAAGTTCTC CAGGTCGACCGGAGTTAGATG-3' (LE6-176V177E/*AccI* and SE6-79V80E/*AccI*). The changed nucleotides are in boldface, and the restriction sites incorporated into the DNA backbone are underlined. The designation LE6-2G3A/*NarI* indicates that the LE6 DNA sequence was changed to incorporate a *NarI*

restriction site, and this changed the second amino acid to glycine and the third amino acid to alanine. Analogous designations were used for other site-specific mutations.

The following site-specific mutants were made for direct tests. The antisense oligonucleotide 5'-CTAGGTCAACTAAATCC GGAGTTAGATGTGTC-3' was used to delete amino acids 176 to 192, giving LE6Δ176-192 and equivalently SEΔ79-95. The underlined nucleotides represent the triplets preceding and following the deletion. The antisense oligonucleotide 5'-GTCTCTGCAGGTCCC GTACGGCCACCCTTTATG-3' was used to change the ATG of ORF E8 which is colinear with E6 to ACG in the mutant LE6ΔE8atg. This mutation did not change any amino acid in LE6. The oligonucleotide 5'-GCAAACTGTACAAC GGACCTTGGCGCTTCTCTTGGGCATGAACAGCTAG-3' was used to insert the simian virus 40 large-T-antigen nuclear localizing signal (NLS), PKKKRKV (32), into SE6 following the ATG to generate SE6 (NLS).

(ii) LE6 deletion mutants. To obtain deletion mutants, the site-specific mutants were cloned into modified pBSKS vectors. In pBSKSΔB-E, the sequences from the *BamHI* site to the *EcoRV* site of the polycloning site were deleted; in pBSKSΔB-K, the sequences from the *BamHI* site to the *KpnI* site were deleted. Deletion mutants were generated by recombining two different site-specific mutants. A strategy was used to obtain stable proteins by leaving all Cys-X-X-Cys motifs intact. In pBSKS-IgLE6Δ3-24, LE6-2G3A/*NarI* was recombined with LE6-24G25A/*NarI* by cleaving of both plasmids with *NarI* and *SpeI*. A *SpeI* site is located at the end of the pBSKS polycloning site. The gel-purified fragments containing portions of E6 were then religated, deleting sequences coding for amino acids 3 to 24. In pBSKS-LE6Δ25-65, LE6-24G25A/*NarI* was recombined with LE6-65G66A/*NarI*, and this generated a deletion corresponding to amino acids 25 to 65. In pBSKS-LE6Δ66-96, LE6-65G66A/*NarI* was joined with LE6-96G97A/*NarI*, and this deleted amino acids 66 to 96. In pBSKS-IgLE6Δ97-125, LE6-96G97A/*NarI* was connected with LE6-125I126D/*ClaI* by combining *NarI* and *ClaI* restriction sites, which resulted in the deletion of amino acids 97 to 125. LE6-125I126D and LE6-176V177E were recombined by joining *ClaI* and *AccI* restriction sites, forming pBSKS-LE6Δ126-176, which removed amino acids 126 to 176. pBSKS-LE6Δ221-273 was constructed by digesting pBSKS-LE6 with *BamHI*, the ends were filled in with Klenow DNA polymerase, and *XbaI* linkers with universal termination signals were added. pBSKS-LE6Δ193-236 was constructed by cleaving pBSKS-LE6 with *AccIII*, filling in the ends with Klenow DNA polymerase, and ligation. This resulted in a deletion corresponding to amino acids 193 to 236. Maps of the LE6 deletions are presented in Fig. 4.

(iii) SE6 deletion mutants. The SE6 deletions were obtained similarly to the LE6 deletions. In SE6-3G4A, sequences which code for glycine at amino acid 3 and alanine at amino acid 4 were inserted into SE6. pBSKS-IgSE6-3G4A-28 was made by recombining IgSE6-3G4A/*NarI* and SE6-28I29D/*ClaI* by joining *NarI* and *ClaI* restriction sites, deleting amino acids 4 to 28. In pBSKS-SE6Δ29-79, *ClaI*-digested SE6-28I29D/*ClaI* was joined with *AccI*-digested SE6-79V80E/*AccI*. This deleted sequences coding for amino acids 29 to 79. pBSKS-SE6Δ79-95 was obtained by oligonucleotide-directed mutagenesis analogously to pBSKS-LE6Δ176-192. pBSKS-SE6Δ124-176 was obtained as pLE6Δ221-273. pBSKS-SE6Δ96-139 was made in the same way as pBSKS-LE6Δ193-236. The SE6 deletion mutants are schematically presented in Fig. 3.

(iv) LE6 and SE6 expression vectors. The expression vector pZipNeo (9), kindly provided by T. Iftner, was modified to incorporate *NotI* and *ApaI* restriction sites at the *BamHI* site. The LE6 and SE6 segments containing the site-specific and deletion mutants were released from the pBSKS vectors by digestion with *NotI* and *ApaI* and directionally cloned into modified pZipNeo.

(v) E8 expression vectors. Two expression vectors were constructed to evaluate the role of E8 in transformation. An expression vector which can express only E8, pZipNeo-E8, was constructed from a modified pBSKS-LE6 plasmid in which an *ApaI* site was introduced at the *SylI* site of CRPV (nt 528). The modified pBSKS-LE6 was cleaved with *EaeI* (CRPV nt 316) and *ApaI*. The 269-bp segment was isolated and cloned into the modified pZipNeo expression vector digested with *NotI* and *ApaI*. pZipNeo-LE6ΔE8atg was obtained by inserting the *NotI*-to-*ApaI* fragment containing LE6ΔE8atg from pBSKS-LE6ΔE8atg into the modified pZipNeo digested with *NotI* and *ApaI*. This vector expresses only LE6.

DNA preparations. For cloning purposes, DNA was prepared with a Qiagen plasmid or M13 kit (Qiagen, Chatsworth, Calif.). For cell culture transfections, plasmid DNA was prepared by the alkaline lysis procedure of Sambrook et al. (46) and further purified on CsCl gradients.

Sequencing. Site-specific mutants were verified by restriction enzyme digestion and DNA sequencing. Furthermore, the entire ORF E6 was sequenced in both directions. DNA sequencing was performed by the dideoxy-chain termination method (47) with 7-deaza-dGTP, using M13, T7, or T3 primers (Stratagene), [α -³⁵S]dATP, and Sequenase (United States Biochemical Corp.) on double-stranded templates. Reaction products were examined on denaturing 6% polyacrylamide sequencing gels and exposed to Kodak XAR film.

Cell culture and transfections. NIH 3T3 were obtained from the American Type Culture Collection (Rockville, Md.) and grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and with penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (25 μg/ml).

All transfections were performed by calcium phosphate precipitation (22). Transfections for G418 (Geneticin; Gibco BRL) selection were carried out as follows. NIH 3T3 cells were seeded at 4×10^5 cells per 60-mm-diameter plate. Twenty four hours later, the cells were transfected with 5 μg of pZipNeo re-

combinant or parental DNA and 5 μ g of sonicated salmon sperm DNA per plate. After 4 to 6 h, the DNA precipitate was removed, and the cells were washed once with 1 $\times$ phosphate-buffered saline (PBS), shocked with 2 ml of 15% (vol/vol) glycerol in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered solution (pH 7.05) (17) for 2 min, washed twice, and incubated at 37°C for 12 to 18 h in complete medium containing 5 mM sodium butyrate (21). Sodium butyrate was removed, complete medium was added, and the cultures were incubated for an additional 48 h. For G418 selection, cells from one 60-mm-diameter plate were passaged into three 100-mm-diameter plates and incubated with complete medium containing 500 μ g of Geneticin per ml, and the medium was changed twice weekly. Resistant colonies were pooled after 16 days, and the pools were expanded for anchorage independence assays. All cell cultures were maintained at 37° in 5% CO₂.

Anchorage independence growth assays. Soft agar assays have been described previously (41). Briefly, pooled and expanded G418-resistant cells were plated at a density of 10⁵ cells per 60-mm-diameter plate in medium containing 0.3% Noble agar (Difco). The plates were prepared with a bottom layer of 0.5% Noble agar in medium. Cells were fed twice weekly with 2 ml of 0.3% Noble agar in medium. Four weeks after plating cells, colonies with at least 20 cells were counted. Four to six plates were counted for each experiment.

Metabolic labeling and immunoprecipitations. The procedures used were as described previously (40). Briefly, 60-mm-diameter plates of G418-resistant NIH 3T3 cells, 80 to 100% confluent, were labeled for 2 h with approximately 0.5 μ Ci of L-[³⁵S]cysteine (Amersham Corp.) in Dulbecco's modified Eagle medium without cysteine and containing 1/10 the normal concentration of methionine and 2% dialyzed fetal bovine serum. In some experiments, the 2-h labeling period was followed by a chase with complete medium for 1.5 or 3 h. Cells were harvested in 0.5 ml of radioimmunoprecipitation assay buffer (0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 mM Tris-HCl [pH 7.4]) containing 0.2 mM phenylmethylsulfonyl fluoride and 0.005% aprotinin. Affinity-purified antiserum to E6 was used. Immune complexes were formed overnight at 4°C, and the complexes were bound to protein A-Sepharose CL-4B beads (Sigma Chemical Co.) for 2 h at 21°C. The beads were washed four times with radioimmunoprecipitation assay buffer and once with 62.5 mM Tris-HCl (pH 6.8) and suspended in sample buffer as described previously (19). Protein analysis was by SDS-10% polyacrylamide gel electrophoresis (PAGE), and the gels were fluorographed at -76°C (19).

Coprecipitation of E6 and p53. Extracts from F9 mouse embryonal carcinoma cells served as a source of p53. The cells, obtained from the American Type Culture Collection, were grown on gelatin-coated flasks in Dulbecco's modified Eagle medium supplemented with 15% fetal bovine serum and with penicillin (100 U/ml), streptomycin (100 μ g/ml), and amphotericin B (25 μ g/ml). The cells were harvested in lysis buffer containing 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.01% aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride. The cell lysate was centrifuged 10 min at 2,500 rpm, and the supernatant was aliquoted and stored at -76°C. The E6 proteins were generated by *in vitro* synthesis. RNA was prepared with an mRNA capping kit (Stratagene), and translation was performed in rabbit reticulocyte lysates (Promega) in the presence of L-[³⁵S]cysteine. The amount of protein synthesized was determined by trichloroacetic acid (TCA) precipitation, and a correction was made for the L-[³⁵S]cysteine incorporation into the reticulocyte lysate not programmed with RNA. Equal amounts of labeled E6 protein were supplemented with unprogrammed lysate, mixed with 230 μ l of F9 cell extract, and incubated 2 h at 4°C; 0.2 μ g of anti-p53 antibody (Ab-4 or Ab-1; Oncogene Science) was then added, and incubation continued at 4°C. After 1 h, 1.1 μ g of rabbit anti-mouse immunoglobulin G (Cappel) was added, and the complexes were bound to protein A-Sepharose CL-4B beads washed four times with ELB buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES [pH 7.0], 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 0.5 mM dithiothreitol) and once with 62.5 mM Tris-HCl (pH 6.8). The proteins were released by boiling in sample buffer as described for immunoprecipitations and analyzed by SDS-PAGE (13% gel), and the gels were fluorographed.

Binding of LE6 and SE6 to E6BP. *Escherichia coli* carrying the plasmids expressing the glutathione *S*-transferase (GST)-E6BP fusion protein and a control protein, GST-cpn10 (10), were kindly provided by J. J. Chen and E. J. Androphy. Bacteria were grown in LB broth, and expression of the GST fusion proteins was induced by the addition of 0.1 mM isopropylthiogalactopyranoside (IPTG). The bacteria were harvested 3 to 4 h after the addition of IPTG, washed in Tris-buffered saline (TBS; 0.01 M Tris-HCl [pH 7.6], 0.14 M NaCl), and broken by sonication in TBS containing 1% Triton X-100. Cellular debris was removed by centrifugation, and glutathione-Sepharose 4B beads (Pharmacia) were added to the supernatant. Binding of the GST fusion proteins to the beads was performed for 30 min at 21°C. The beads were washed twice in TBS-1% Triton X-100. To quantify the amount of fusion proteins bound, an aliquot of the beads was treated with sample buffer (19). The released protein was analyzed by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. The L-[³⁵S]cysteine-labeled E6 proteins, CRPV LE6, CRPV SE6, HPV-16 E6, and HPV-6 E6, were obtained by *in vitro* transcription and translation, using a coupled reticulocyte lysate system (Promega). The amount of protein synthesized was determined by TCA precipitation, and a correction was made for the L-[³⁵S]cysteine incorporation into reticulocyte lysate not containing DNA. Synthesis of the CRPV LE6 and SE6 proteins was further verified by immunopre-

FIG. 1. (A) Coprecipitation of E6 proteins with p53. HPV-6 E6, HPV-16 E6, and CRPV LE6 and SE6 were labeled with L-[³⁵S]cysteine *in vitro*. A total of 290,000 cpm of labeled E6 protein was mixed with p53-containing extracts from F9 mouse embryonal carcinoma cells. Binding of proteins and washing of the complexes were carried out at 4°C as described in Materials and Methods. Bound proteins were analyzed by SDS-PAGE (13% gel). A 2-day exposure of the fluorogram is shown. Lanes 1 and 3, HPV-6 E6; lanes 2 and 4, HPV-16 E6; lane 5, CRPV SE6; lane 6, CRPV LE6. The anti-p53 antibodies (Ab-1 [lanes 1 and 2] and Ab-4 [lanes 3 to 6]) were from Oncogene Science. Positions of E6 proteins and of molecular size markers (sizes are indicated in kilodaltons) are shown. (B) *In vitro*-translated LE6 and SE6 were immunoprecipitated with antibody to CRPV E6, and the proteins were analyzed by SDS-PAGE (13% gel). A 2-day exposure of the fluorogram is shown. The immunoprecipitates were prepared from 240,000 cpm of TCA-precipitable LE6 (lane 1) or from 110,000 cpm of TCA-precipitable SE6 (lane 2). Lane M, molecular size markers. Positions and sizes (in kilodaltons) of the markers are indicated on the right; positions of full-length LE6 and SE6 are shown on the left.

cipitation. Equivalent amounts of E6 protein were bound to fusion protein-loaded beads equilibrated with LSAB (10) (0.1 M NaCl, 0.1 M Tris-HCl [pH 8.0], 1% Nonidet P-40, 2 mM dithiothreitol, 0.1% nonfat dry milk, 1 mM phenylmethylsulfonyl fluoride). The binding reaction was performed at 4°C for 4 h with 50 μ l of beads in a total volume of 250 μ l. The beads were then washed four times with LSAB and with 62.5 mM Tris-HCl (pH 6.8) at 4°C. The bound protein was then released with sample buffer (19), analyzed by SDS-PAGE, and fluorographed at -76°C.

RESULTS

Binding of E6 proteins to p53 and to E6BP. One of the characteristics of the E6 proteins of the high-cancer-risk genital HPVs is their ability to bind and degrade p53. To determine if the oncogenic properties of LE6 and SE6 were based on the same mechanism, coprecipitation experiments were performed. F9 mouse cells served as a source of p53. LE6 and SE6 were synthesized in a reticulocyte lysates programmed with RNA transcribed *in vitro*. Included in the experiment were HPV-16 E6 as a positive control and HPV-6 E6 as a negative control. To avoid potential problems with degradation mediated by enzymes in the reticulocyte lysates, all procedures were carried out at 4°C. The autoradiogram of the precipitated proteins shown in Fig. 1A represents a 2-day exposure. The only E6 protein coprecipitated with antibody to p53 was HPV-16 E6. The two different monoclonal antibody to p53, Ab-1 and Ab-4, were equally efficient in precipitating the p53-HPV-16 E6 complex. No coprecipitation was seen with HPV-6 E6 or with CRPV LE6 and SE6. Even after a five-times-longer exposure, no bands could be detected in the lanes containing HPV-6 E6 or CRPV LE6 or SE6. Since both LE6 and SE6 are larger proteins than HPV-6 or HPV-16 E6, it was important to show that the full-length proteins were synthesized. To demonstrate this, the CRPV E6 proteins were immunoprecipitated from translation reactions and analyzed by SDS-PAGE (Fig. 1B). As can be seen, a major band did mi-

FIG. 2. (A) Binding of GST-E6BP and GST-cpn10 to glutathione-Sepharose 4B. Binding of the fusion proteins was performed as described in Materials and Methods. The protein released from 15 μ l of beads was analyzed on an SDS-10% polyacrylamide minigel, and the gel was stained with Coomassie brilliant blue. Lane 1, GST-E6BP; lane 2, GST-cpn10; lane 3, no protein bound; lane M, prestained markers. Positions of the fusion proteins are shown on the right; those of the markers and their sizes (in kilodaltons) are shown on the left. (B) Binding of E6 proteins to GST-E6BP- or GST-cpn10-loaded Glutathione-Sepharose 4B. HPV-6 E6, HPV-16 E6, CRPV LE6, and CRPV SE6 were labeled with L-[35 S]cysteine in vitro in a coupled reticulocyte lysate transcription-translation system. A total of 10,000 cpm of labeled protein was bound to 50 μ l of GST-E6BP-loaded beads (lanes 1, 3, 5, and 7) or to GST-cpn10-loaded beads (lanes 2, 4, 6, and 8). HPV-6 E6 was present in lanes 1 and 2, HPV-16 E6 was present in lanes 3 and 4, CRPV LE6 was present in lanes 5 and 6, and CRPV SE6 was present in lanes 7 and 8. The immunoprecipitates of 10,000 cpm of LE6 and 11,000 cpm of SE6 were analyzed in lanes 9 and 10, respectively. Lane M, molecular size markers. The gel was processed as described in Materials and Methods. Exposure of the fluorogram shown was for 3 days. Positions of full-length CRPV LE6 and SE6 and of the markers (sizes are indicated in kilodaltons) are shown on the right; the position of HPV-16 E6 is shown on the left.

grate at the position of full-length LE6 and SE6, suggesting that these proteins do not form stable complexes with p53.

Recently, a second cellular protein, E6BP, has been identified (10); E6BP binds to the E6 proteins of the high-risk HPV-16 and -18 but not to those of the low-risk HPV-6 and -11. Furthermore, this protein also binds to BPV-1 E6, a transforming protein that does not bind p53 (55). A potential interaction of either CRPV E6 protein with E6BP was evaluated by analyzing binding of in vitro-translated LE6 and SE6 to the fusion protein GST-E6BP. Another fusion protein, GST-cpn10, served as a negative control. Figure 2A shows that similar amounts of the two fusion proteins were bound. Furthermore, HPV-16 E6 was included as a positive control and HPV-6 E6 was included as a negative control. The results of an experiment are shown in Fig. 2B. As can be seen, HPV-16 E6 is the only protein which bound to GST-E6BP-loaded glutathione-Sepharose 4B beads. None of the E6 proteins bound to beads loaded with the control fusion protein GST-cpn10. Included in the SDS-PAGE analysis were immunoprecipitations of LE6 and SE6, which show that the major products synthesized in the coupled reticulocyte system represented the full-length proteins. The results presented in Fig. 2B were obtained by using 10,000 cpm of each protein. The same result was obtained in an experiment with 80,000 cpm of protein except that the intensity of the HPV-16 E6 band seen after a 3-day exposure with 10,000 cpm of protein was observed after an 18-h exposure (data not shown). The results suggest that the

A) CRPV SE6 & E8 GENES

B) SE6 MUTANTS

FIG. 3. (A) Map of the CRPV segment cloned into the expression vector. The translation start sites for E8 and SE6 (arrows) and the stop sites (squares) are indicated. (B) Maps of the SE6 mutants. Positions of the six Cys-X-X-Cys motifs are indicated by crosses in the segments deleted, and the insertion of the simian virus 40 large T antigen (SV40NLS) is indicated by a triangle.

transforming properties of LE6 or SE6 are not dependent on binding to E6BP.

Transforming properties of SE6 mutant proteins. The p53 coprecipitation and the E6BP binding experiments suggested that the oncogenic properties of the two E6 proteins did not involve binding of p53 or E6BP. As a next step to elucidate potential transforming mechanisms of the E6 proteins, in-frame deletions covering the entire lengths of LE6 and SE6 were generated. It had been observed that some mutations of Cys in Cys-X-X-Cys motifs resulted in protein instabilities. Therefore, the strategy was to delete entire motifs rather than to disrupt motifs. To ascertain that no such motifs were affected, ORF E6 (nt 144 to 1091) of the Washington B strain (57) was sequenced first. Only two differences between the prototype sequence (20) and the Washington B strain sequence were detected. One at nt 394 (C→A) resulted in an amino acid change from proline to threonine, and a second at nt 569 (C→A) is a change from serine to tyrosine, but none of the Cys-X-X-Cys motifs were affected. Maps of the SE6 and LE6 mutants are shown in Fig. 3 and 4, respectively, and locations of deleted Cys-X-X-Cys motifs are indicated. One to three independent pools of G418-resistant cells transfected with individual mutants were established, and the cells were tested for growth in soft agar. The results are summarized in Table 1 for SE6 mutants and in Table 2 for LE6 mutants. As previously shown (40), SE6 was more efficient than LE6 in conferring anchorage-independent growth. Four of the five SE6 deletions affected the transformation efficiency. This was most pronounced for the three mutants with deletions in the amino-terminal half of SE6, SE6-in3GΔ4-28, SE6Δ29-79, and SE6Δ79-95. The number of colonies formed with these mutants was not significantly different from the background ob-

A) CRPV LE6 GENE

B) LE6 MUTANTS:

FIG. 4. (A) Map of the CRPV segment cloned into the expression vectors. The translation start sites for LE6 and E8 (arrows) and stop sites (squares) are indicated. The mutation (change [chg]) of the SE6 ATG is indicated by a vertical arrow. (B) Maps of the LE6 mutants. Positions of the eight Cys-X-X-Cys motifs are indicated by crosses in the segments deleted.

served with pools established from parental pZipNeo vector-transfected cells. The fourth mutant, SE6 Δ 124-176, exhibited a reduced, but still above background, number of colonies. Finally, insertion of an NLS behind the ATG of SE6 increased transformation above that observed with wild-type SE6. However, because of the considerable differences between different pools with the same mutant, this increase cannot be considered significant. These data indicated that integrity of the amino-terminal segment of SE6 was important to maintain transformation function. The loss of transformation function could also be the result of an extreme instability of the protein. When

wild-type SE6 (SE6 wt), SE6 Δ 79-95, SE6 Δ 96-139, and SE6 Δ 124-176 were labeled with L-[³⁵S]cysteine, an SE6 protein could be detected only in the SE6 wt-expressing cells (data not shown).

Transforming properties of the LE6 mutant proteins. The results of transformation with LE6 mutants are summarized in Table 2. Most strikingly, all of the mutants except LE6 Δ 193-236 transformed cells at least as efficiently as, and some more efficiently than, LE6 wt. There are multiple reasons which could lead to such a result. One possibility was a difference in the level of expression of the mutant proteins. To evaluate this possibility, NIH 3T3 cells transformed by the different mutants were labeled with L-[³⁵S]cysteine, and the E6 proteins were immunoprecipitated and analyzed by SDS-PAGE (Fig. 5). Most of the transformed cells expressed a protein of about the expected size. In general, the migration of the mutant proteins, including that of LE6 wt, was about 10% slower than expected on the basis of the calculated molecular size. Such a difference between calculated molecular size and migration on SDS-PAGE was previously observed for LE6 wt (5). In LE6 Δ 97-125-transformed cells, no band of the expected size was present. However, these cells contained a smaller protein of about 26 kDa. A band of similar size was also present as a second band in cells transformed with LE6 Δ 25-65 and LE6 Δ 66-96 and could even be detected in LE6 wt-transformed cells. Since premature translation termination is unusual in vivo and as the LE6 wt expression vector used contained only one ATG, it was likely that the smaller bands represented degradation products of the primary proteins. This interpretation is supported by pulse-chase experiments (Fig. 6). LE6 wt- or LE6 Δ 25-65-transformed cells were labeled with L-[³⁵S]cysteine, and the label was chased for 1.5 and 3 h. As can be seen, the full-length band for LE6 wt and LE6 Δ 25-65 had completely disappeared after a 1.5-h chase, while the 26-kDa band in LE6 Δ 25-65 persisted at least to the end of the 1.5-h chase. These data first indicate that the LE6 wt as well as the mutant LE6 protein had a short half-life of 0.5 h or less. Second, the data suggest that some of the mutant proteins were not randomly degraded but that an intermediate product of about 26 kDa persisted transiently. Overall, the data show that there was no simple correlation between the level of expression of the mutant proteins and their transformation efficiencies. This is clearly indicated by the weak or absent LE6 bands for mutants LE6 bands for LE6 Δ 176-192 and LE6 Δ 126-176, which both transformed NIH 3T3 cells more efficiently than LE6 wt.

E8 is not a transforming protein. CRPV contains an ORF, designated ORF E8, which is colinear with ORF E6 (20). It could encode a protein of 50 amino acids. Furthermore, it contains a segment of 17 hydrophobic amino acids interrupted only by one threonine. With respect to size and the presence of a hydrophobic segment, this E8 protein resembles E5 of BPV-1, the major transforming protein of BPV-1 (reviewed in reference 26). Thus, it was possible that transforming functions assigned to LE6 or SE6 could be mediated by E8 (Fig. 2 and 3). To test a potential transforming function of E8, the CRPV segment from nt 316 to 523 was cloned into the pZipNeo vector. When G418-resistant cells transfected with this plasmid were tested in soft agar, no colonies above background were formed (Table 3). As an additional test of a biological function of E8, we constructed a pZipNeo vector expressing LE6 in which the ATG of E8 was changed to ACG. This mutation did not change the amino acid sequence of LE6. The soft agar assays of cells transfected with this vector, pZipNeo-LE6 Δ E8atg, revealed an increased transformation efficiency relative to that of pZipNeo-LE6 (Table 3). One explanation for this phenomenon could be that E8 modulated expression of

TABLE 1. Stimulation of anchorage-independent growth by SE6 and SE6 mutants^a

Cell line ^b	Avg no. of colonies/plate ^c
pZipNeo	108 $\pm$ 16, 43 $\pm$ 17, 384 $\pm$ 70
SE6	593 $\pm$ 170, 2,244 $\pm$ 444, 1,726 $\pm$ 798
SE6- <i>in3G</i> Δ 4-28	266 $\pm$ 80, 251 $\pm$ 99
SE6 Δ 29-79	97 $\pm$ 34, 209 $\pm$ 133, 20 $\pm$ 9
SE6 Δ 79-95	94 $\pm$ 10
SE6 Δ 96-139	2,453 $\pm$ 184, 657 $\pm$ 34, 280 $\pm$ 146
SE6 Δ 124-176	882 $\pm$ 108, 191 $\pm$ 62
SE6(NLS)	2,242 $\pm$ 278, 3,184 $\pm$ 60

^a One to three independently obtained pools of G418-resistant NIH 3T3 cells transfected with the same pZipNeo vector were assayed for anchorage-independent growth as described in Materials and Methods.

^b Nature of the insert in the pZipNeo vector used for transformation.

^c The different cell pools obtained by transformation with the same plasmid are indicated by different numbers, and the standard deviation for the four to six plates counted for each is given.

TABLE 2. Stimulation of anchorage-independent growth by LE6 and LE6 mutants^a

Cell line ^b	Avg no. of colonies/plates ^c
pZipNeo.....	108 ± 16, 43 ± 17, 384 ± 70
LE6.....	274 ± 6, 1,351 ± 187, 348 ± 95
LE6Δ3-24.....	1,723 ± 282, 2,145 ± 176, 1,338 ± 240
LE6Δ25-65.....	2,939 ± 50, 680 ± 79
LE6Δ66-96.....	2,424 ± 427, 2,187 ± 208, 3,554 ± 283
LE6Δ97-125.....	687 ± 114, 1,129 ± 151
LE6Δ126-176.....	3,037 ± 221, 1,045 ± 157
LE6Δ176-192.....	2,033 ± 151, 2,420 ± 611
LE6Δ193-236.....	396 ± 30, 336 ± 42
LE6Δ221-273.....	806 ± 72, 1,362 ± 204

^a Two to three independently obtained pools of G418-resistant NIH 3T3 cells transfected with the same pZipNeo vector were assayed for anchorage-independent growth as described in Materials and Methods.

^b Nature of the insert in the pZipNeo vector used for transformation.

^c The different cell pools obtained by transformation with the same plasmid are indicated by different numbers, and the standard deviation is given as indicated in Table 1, footnote c.

LE6. A direct test of LE6 expression in cells transfected with pZipNeo vectors expressing LE6 and with or without the potential to express E8 revealed that mutation of the E8 ATG had only a minor, reductive effect on LE6 synthesis (data not shown). Clearly, E8 does not have transforming functions of its own; however, it may modulate transformation by LE6.

DISCUSSION

We have constructed in-frame deletions of the two CRPV ORF E6-encoded oncoproteins LE6 and SE6 to identify segments important for transformation. Neither of the E6 proteins formed stable complexes with p53 or E6BP. Transformation by SE6 was dependent on the integrity of the amino-terminal segment of the protein, while all LE6 deletions except one exhibited a transforming potential equal to or higher than that of parental LE6.

Deletion analysis of the HPV-16 E6 protein identified a segment which strongly affected in vitro binding of p53 (11). A segment of 10 amino acids, extending from amino acids 106 to 115 of LE6 or amino acids 9 to 17 of SE6, contains a perfect match at four positions with a 10-amino-acid segment of HPV-16 E6 centered on the deletions with strongly decreased p53 binding. In both proteins, the comparable segments are

FIG. 5. Detection of the E6 proteins in G418-resistant cells transfected with pZipNeo LE6 and LE6 mutants. Extracts were prepared from L-[³⁵S]cysteine-labeled cells, and the E6 proteins were precipitated with affinity-purified rabbit antiserum. The precipitates were analyzed by SDS-PAGE (10% gel) and fluorographed for 1 month. Positions of LE6 wt and of molecular size markers (sizes are indicated in kilodaltons) are shown.

FIG. 6. Pulse-chase of wild-type and mutant LE6 protein. Extracts were prepared from L-[³⁵S]cysteine-labeled G418-resistant cells, and the E6 proteins were immunoprecipitated and analyzed as described for Fig. 4. Extracts were from cells transfected with pZipNeo (lane 1 and 5), pZipNeo-LE6 wt (lanes 2 to 4), and pZipNeo-LE6Δ25-65 (lanes 6 to 8). Cells were labeled for 2 h (lanes 1, 2, 5, and 6), for 2 h followed by a 1.5-h chase with unlabeled medium (lanes 3 and 8), and for 2 h followed by a 3-h chase with unlabeled medium (lanes 4 and 8). Positions of LE6 wt (left) and LE6Δ25-65 (right) and of molecular size markers (sizes are indicated in kilodaltons) are shown.

located next to a Cys-X-X-Cys motif and could be located within a Zn finger. This potential Zn finger in CRPV, however, would be shorter than that of HPV-16, as previously noted (20). The direct test for in vitro binding of p53 to either of the CRPV E6 proteins revealed no binding. This was not entirely surprising since in HPV-6 E6, 6 of 10 amino acids match those in the critical segment of HPV-16 E6. Although HPV-6 E6 appears to bind to p53 under some experimental conditions (11), in other experiments no binding of HPV-6 E6 was detected (18, 55). In the experiments performed here with mouse cells as the source of p53, there was no binding to HPV-6 E6 or to LE6 or SE6. This result suggested that the oncogenic properties of the CRPV E6 proteins were not mediated by interference with p53 function.

In addition to p53, another protein, E6BP, which binds to E6 of the high-risk but not the low-risk genital HPVs, has been identified (10). Furthermore, the protein binds to BPV-1 E6, an E6 transforming protein which does not bind to p53 (55). Thus, it was possible that binding to the putative Ca²⁺-binding protein E6BP was a mechanism through which some E6 proteins exert their transforming function. Our results indicate that CRPV LE6 and SE6 did not bind to E6BP, suggesting that transformation by LE6 or SE6 was not dependent on binding to this protein. As a next step to understand the transforming functions of LE6 and SE6, deletions covering the entire length of the two proteins were constructed. The test of these deletions in NIH 3T3 cell transformation assays showed that in the segment common to both proteins, deletion of different segments affected transformation. In SE6, deleting either of the

TABLE 3. Stimulation of anchorage-independent growth by E8 and an E8 mutant^a

Cell line ^b	Avg no. of colonies/plate ^c
LE6.....	274 ± 6, 1,351 ± 187, 348 ± 95
LE6ΔE8atg.....	3,437 ± 97, 4,698 ± 2,539
E8.....	226 ± 40, 21 ± 8, 9 ± 10

^a Two and three independently obtained pools of G418-resistant NIH 3T3 cells transfected with pZipNeo-LE6ΔE8atg and pZipNeo-E8, respectively, were assayed for anchorage-independent growth as described in Materials and Methods.

^b Nature of the insert in the pZipNeo vector used for transformation.

^c The different cell pools obtained by transformation with the same plasmid are indicated by different numbers, and the standard deviation is given as indicated in Table 1, footnote c.

two closely spaced Cys-X-X-Cys motifs in SE6 Δ 4-28 and SE6 Δ 29-79, as well as short deletion in the middle of the protein, SE6 Δ 79-95, abolished transformation. The importance of the Cys-X-X-Cys motifs for transformation by a non-p53-binding E6 was previously demonstrated for BPV-1 E6, in which point mutations of Cys in all four motifs reduced or abolished transformation (54). In CRPV, deletions of either of the Cys-X-X-Cys motifs in the carboxy-terminal segment reduced, but did not abolish, transformation. Surprisingly, neither of the three deletions which abolished transformation by SE6 reduced transformation by LE6 (LE6 Δ 97-126, LE6 Δ 126-176, and LE6 Δ 176-192), and the only deletion reducing transformation, LE6 Δ 193-236, did not affect SE6 transformation (SE6 Δ 96-139). Considering LE6, it is clear that none of the Cys-X-X-Cys motifs or potential Zn fingers were essential. This finding could indicate that transformation by LE6 is based on structural features different from those of SE6 and BPV-1 E6. It is also possible that with eight Cys-X-X-Cys motifs in LE6 rather than four in BPV-1 E6, there is a redundancy in function. The notion that LE6 and SE6 perform different functions is also supported by the facts that they differ in subcellular distribution (6) and that both are required to induce papillomas in rabbits (40). The finding that several LE6 mutations transformed more efficiently than parental LE6 could indicate that a binding site for a cellular protein(s) may be more accessible in the mutant protein. Such an effect has been observed with HPV-16 E6: some mutant proteins bound p53 twice as efficiently as the wild-type protein (11). Finally, the effects of the LE6 mutants which increased the transformation efficiency were also reflected in a more vigorous growth of the cells in monolayer cultures.

The immunoprecipitation of LE6 proteins from cells transformed with different mutants revealed in most bands of the appropriate sizes. There was some variation in the intensities of the bands, but it did not correlate with transformation efficiency. Indeed, in cell extracts transformed by LE6 Δ 97-125 and LE6 Δ 126-176, no band of the expected size could be detected, yet both transformed NIH 3T3 cells. A similar situation was also observed with one HPV-16 E6 mutant which mediated the *in vivo* degradation of p53, but the protein could not be detected in the transformed cells (18), which suggests that the oncogenes are biologically active at very low concentrations. Finally, the immunoprecipitations from cells subjected to a pulse-chase showed that the wild type as well as one mutant, LE6, had a short half-life of less than 0.5 h. This short half-life is not unique to the protein in NIH 3T3 cells but was also observed in CRPV-infected cells of the rabbit epithelial cell line sfiEp (data not shown). Similarly, HPV-16 E6 exhibited a biphasic decay with a half-life of less than 30 min for one component, while a portion was more stable (1). SE6 was detectable at a low level in wild-type-transformed cells but not in three mutant (SE6 Δ 79-95, SE6 Δ 96-139, and SE6 Δ 124-176) transformed cells (data not shown). Thus, for SE6 Δ 79-95, the stability of the protein rather than a certain structural feature may account for a loss of transforming activity.

E8 ORFs have been identified in BPV-1 as well as in CRPV (reviewed in reference 3). In BPV-1, the E8 ORF is colinear with ORF E1; in CRPV, it is colinear with E6. A CRPV E8 protein would be initiated in the segment unique to LE6 and extend just into SE6. The size of the protein, as well as the presence of a hydrophobic region, is the same as for BPV-1 E5. An E8 protein would most likely be translated from the SE6 mRNA, one of the two abundant mRNAs in CRPV tumors. The E8 ATG is the first ATG on this mRNA, which would be compatible with the translation of such a protein. The context of the ATG of E8 in the sequence CGTATGG corresponds

only in one nucleotide with the optimal sequence for eukaryotic ribosomes of ACCATGG (36). However, the sequences around the ATGs of SE6 and E7, the most abundant early proteins in CRPV-infected cells, do not correspond in any of the nucleotides with the optimal sequence. Thus, the match with the optimal sequence for eukaryotic ribosome translation initiation may not be a good indicator for translation of CRPV proteins. At present, we have no direct evidence that E8 is made. When directly tested for transforming function, E8 was not active. The E8 in the expression vector pZipNeo E8 was derived from LE6 in which the ATG of SE6 was mutated to ATA. This did not alter the amino acid sequence of LE6, but it changed a cysteine to a tyrosine in E8. Thus, there is still a possibility that E8 wt has transforming functions. Clearly, E8 is not responsible for cell transformation by LE6 and SE6 observed here since the LE6 mutants LE6 Δ 25-65, LE6 Δ 66-96, and LE6 Δ 97-125, which cannot synthesize an intact E8 protein, still transformed cells. Furthermore, two SE6 mutants which were deficient in transformation could synthesize E8. Surprisingly, mutation of the E8 ATG in the context of the LE6 wt resulted in an increased transformation efficiency, but it was not the result of increased LE6 expression. Evidence for a negative effect of E8 on LE6 transformation could also be adduced from the efficient transformation observed with LE6 Δ 66-96, which could not make an intact E8. Finally, the possibility that the E8 protein has biological effector properties is suggested from observations with a TrpE-E8 bacterial expression plasmid: when TrpE-E8 expression was induced in bacteria carrying this plasmid, growth was arrested.

Our results strongly suggest that the two E6 proteins transform by different mechanisms, both p53 and E6BP independent. Furthermore, ORF E8 colinear with ORF E6 could code for a protein with structural similarities to BPV-1 E5, but E8 is not a transforming protein. Thus, transformation by the CRPV E6 proteins most likely involves a yet unknown mechanism.

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REFERENCES

1. Androphy, E. J., N. L. Hubbert, J. T. Schiller, and D. R. Lowy. 1987. Identification of the HPV-16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. *EMBO J.* **6**:989-992.
2. Androphy, E. J., J. T. Schiller, and D. R. Lowy. 1985. Identification of the protein encoded by the E6 transforming gene of the bovine papillomavirus. *Science* **230**:442-445.
3. Baker, C. C. 1985. Sequence analysis of papillomavirus genomes, p. 321-385. In N. Salzman and P. M. Howley (ed.), *The Papovaviridae*. Plenum Press, New York.
4. Barbosa, M. S., D. R. Lowy, and J. T. Schiller. 1989. Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *J. Virol.* **63**:1404-1407.
5. Barbosa, M. S., and F. O. Wettstein. 1987. Transcription of the cottontail rabbit papillomavirus early region and identification of two E6 polypeptides in COS-7 cells. *J. Virol.* **61**:2938-2942.
6. Barbosa, M. S., and F. O. Wettstein. 1988. The two proteins encoded by the cottontail rabbit papillomavirus E6 open reading frame differ with respect to localization and phosphorylation. *J. Virol.* **62**:1088-1092.
7. Borresen, A. L., A. Helland, J. Nesland, R. Holm, C. Trope, and J. Kaern. 1992. Papillomaviruses, p53, and cervical cancer. *Lancet* **339**:1350-1351.
8. Busby-Earle, R. M., C. M. Steel, A. R. Williams, B. Cohen, and C. C. Bird. 1992. Papillomaviruses, p53, and cervical cancer. *Lancet* **339**:1350.

9. Cepko, C. L., B. E. Roberts, and R. C. Mulligan. 1984. Construction and application of a highly transmissible murine retrovirus shuttle vector. *Cell* **37**:1055–1062.
10. Chen, J. J., C. E. Reid, V. Band, and E. J. Androphy. 1995. Interaction of papillomavirus E6 oncoproteins with a putative calcium-binding protein. *Science* **269**:529–531.
11. Crook, T., J. A. Tidy, and K. H. Vousden. 1991. Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. *Cell* **67**:547–556.
12. Crook, T., and K. H. Vousden. 1992. Properties of p53 mutations detected in primary and secondary cervical cancers suggest mechanisms of metastasis and involvement of environmental carcinogens. *EMBO J.* **11**:3935–3940.
13. Crook, T., D. Wrede, J. A. Tidy, W. P. Mason, D. J. Evans, and K. H. Vousden. 1992. Clonal p53 mutation in primary cervical cancer: association with human-papillomavirus-negative tumours. *Lancet* **339**:1070–1073.
14. Crook, T., D. Wrede, and K. H. Vousden. 1991. p53 point mutation in HPV negative human cervical carcinoma cell lines. *Oncogene* **6**:873–875.
15. Danos, O., E. Georges, G. Orth, and M. Yaniv. 1985. Fine structure of the cottontail rabbit papillomavirus mRNAs expressed in the transplantable VX2 carcinoma. *J. Virol.* **53**:735–741.
16. Defeo-Jones, D., G. A. Vuocolo, K. M. Haskell, M. G. Hanobik, D. M. Kiefer, E. M. McAvoy, M. Ivey-Hoyle, J. L. Brandsma, A. Oliff, and R. E. Jones. 1993. Papillomavirus E7 protein binding to the retinoblastoma protein is not required for viral induction of warts. *J. Virol.* **67**:716–725.
17. DiMaio, D., D. Guralski, and J. T. Schiller. 1986. Translation of open reading frame E5 of bovine papillomavirus is required for its transforming activity. *Proc. Natl. Acad. Sci. USA* **83**:1797–1801.
18. Foster, S. A., G. W. Demers, B. G. Etscheid, and D. A. Galloway. 1994. The ability of human papillomavirus E6 proteins to target p53 for degradation in vivo correlates with their ability to abrogate actinomycin D-induced growth arrest. *J. Virol.* **68**:5698–5705.
19. Gage, J. R., C. Meyers, and F. O. Wettstein. 1990. The E7 proteins of the nononcogenic human papillomavirus type 6b (HPV- 6b) and of the oncogenic HPV-16 differ in retinoblastoma protein binding and other properties. *J. Virol.* **64**:723–730.
20. Giri, L., O. Danos, and M. Yaniv. 1985. Genomic structure of the cottontail rabbit (Shope) papillomavirus. *Proc. Natl. Acad. Sci. USA* **82**:1580–1584.
21. Gorman, C. M., and B. H. Howard. 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* **11**:7631–7648.
22. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456–467.
23. Grossman, S., and L. A. Laimins. 1989. E6 protein of human papillomavirus type 18 binds zinc. *Oncogene* **4**:1089–1093.
24. Haskell, K. M., G. A. Vuocolo, D. Defeo-Jones, R. E. Jones, and M. Ivey-Hoyle. 1993. Comparison of the binding of the human papillomavirus type 16 and cottontail rabbit papillomavirus E7 proteins to the retinoblastoma gene product. *J. Virol.* **74**:115–119.
25. Hiraiwa, A., T. Kiyono, K. Segawa, K. R. Utsumi, M. Ohashi, and M. Ishibashi. 1993. Comparative study on E6 and E7 genes of some cutaneous and genital papillomaviruses of human origin for their ability to transform 3Y1 cells. *Virology* **192**:102–111.
26. Howley, P. M., and R. Schlegel. 1987. Papillomavirus transformation, p. 141–166. *In* N. Salzman and P. M. Howley (ed.), *The Papovaviridae*. Plenum Press, New York.
27. Huibregtse, J. M., M. Scheffner, and P. M. Howley. 1991. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J.* **10**:4129–4135.
28. Huibregtse, J. M., M. Scheffner, and P. M. Howley. 1993. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol. Cell. Biol.* **13**:775–784.
29. Huibregtse, J. M., M. Scheffner, and P. M. Howley. 1993. Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. *Mol. Cell. Biol.* **13**:4918–4927.
30. Iftner, T. 1990. Papillomavirus genomes: sequence analysis related to functional aspects, p. 182–199. *In* Pfister (ed.), *Papillomaviruses and human cancer—1990*. CRC Press, Boca Raton, Fla.
31. Iftner, T., S. Bierfelder, Z. Csapo, and H. Pfister. 1988. Involvement of human papillomavirus type 8 genes E6 and E7 in transformation and replication. *J. Virol.* **62**:3655–3661.
32. Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell* **39**:499–509.
33. Kanda, T., S. Watanabe, S. Zanma, H. Sato, A. Furuno, and K. Yoshiike. 1991. Human papillomavirus type 16 E6 proteins with glycine substitution for cysteine in the metal-binding motif. *Virology* **185**:536–543.
34. Keen, N., R. Elston, and L. Crawford. 1994. Interaction of the E6 protein of human papillomavirus with cellular proteins. *Oncogene* **9**:1493–1499.
35. Kiyono, T., A. Hiraiwa, and M. Ishibashi. 1992. Differences in transforming activity and coded amino acid sequence among E6 genes of several papillomaviruses associated with epidermodysplasia verruciformis. *Virology* **186**:628–639.
36. Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**:283–292.
37. Kurvinen, K., A. Tervahauta, S. Syrjanen, F. Chang, and K. Syrjanen. 1994. The state of the p53 gene in human papillomavirus (HPV)-positive and HPV-negative genital precancer lesions and carcinomas as determined by single-strand conformation polymorphism analysis and sequencing. *Anticancer Res.* **14**:177–181.
38. Lee, Y. Y., S. P. Wilczynski, A. Chumakov, D. Chih, and H. P. Koeffler. 1994. Carcinoma of the vulva: HPV and p53 mutations. *Oncogene* **9**:1655–1659.
39. Lin, Y. L., L. A. Borenstein, R. Selvakumar, R. Ahmed, and F. O. Wettstein. 1993. Progression from papilloma to carcinoma is accompanied by changes in antibody response to papillomavirus proteins. *J. Virol.* **67**:382–389.
40. Meyers, C., J. Harry, Y. L. Lin, and F. O. Wettstein. 1992. Identification of three transforming proteins encoded by cottontail rabbit papillomavirus. *J. Virol.* **66**:1655–1664.
41. Meyers, C., and F. O. Wettstein. 1991. The late region differentially regulates the in vitro transformation by cottontail rabbit papillomavirus DNA in different cell types. *Virology* **181**:637–646.
42. Nasser, M., and F. O. Wettstein. 1984. Differences exist between viral transcripts in cottontail rabbit papillomavirus-induced benign and malignant tumors as well as non-virus-producing and virus-producing tumors. *J. Virol.* **51**:706–712.
43. Orth, G. 1987. Epidermodysplasia verruciformis, p. 199–235. *In* N. Salzman and P. M. Howley (ed.), *The Papovaviridae*, vol. 2. Plenum Press, New York.
44. Park, D. J., S. P. Wilczynski, R. L. Paquette, C. W. Miller, and H. P. Koeffler. 1994. p53 mutations in HPV-negative cervical carcinoma. *Oncogene* **9**:205–210.
45. Pfister, H. 1992. Human papillomaviruses and skin cancer. *Semin. Cancer Biol.* **3**:263–271.
46. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 60–62. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
48. Scheffner, M., K. Munger, J. C. Byrne, and P. M. Howley. 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc. Natl. Acad. Sci. USA* **88**:5523–5527.
49. Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**:1129–1136.
50. Schmitt, A., J. B. Harry, B. Rapp, F. O. Wettstein, and T. Iftner. 1994. Comparison of the properties of the E6 and E7 genes of low- and high-risk cutaneous papillomaviruses reveals strongly transforming and high Rb-binding activity for the E7 protein of the low-risk human papillomavirus type 1. *J. Virol.* **68**:7051–7059.
51. Steger, G., and H. Pfister. 1992. In vitro expressed HPV 8 E6 protein does not bind p53. *Arch. Virol.* **125**:355–360.
52. Stoeppler, H., M. Conrad Stoeppler, and R. Schlegel. 1994. Transforming proteins of the papillomaviruses. *Intervirology* **37**:168–179.
53. Syverton, J. T. 1952. The pathogenesis of the rabbit papilloma-to-carcinoma sequence. *Ann. N.Y. Acad. Sci.* **54**:1126–1140.
54. Vousden, K. H., E. J. Androphy, J. T. Schiller, and D. R. Lowy. 1989. Mutational analysis of bovine papillomavirus E6 gene. *J. Virol.* **63**:2340–2342.
55. 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.
56. Wettstein, F. O., M. S. Barbosa, and M. Nasser. 1987. Identification of the major cottontail rabbit papillomavirus late RNA cap site and mapping and quantitation of an E2 and minor E6 coding mRNA in papillomas and carcinomas. *Virology* **159**:321–328.
57. Wettstein, F. O., and J. G. Stevens. 1980. Distribution and state of viral nucleic acid in tumors induced by Shope papilloma virus. *Cold Spring Harbor Conf. Cell Proliferation* **7**:301–307.
58. Wrede, D., J. A. Tidy, T. Crook, D. Lane, and K. H. Vousden. 1991. Expression of RB and p53 proteins in HPV-positive and HPV-negative cervical carcinoma cell lines. *Mol. Carcinog.* **4**:171–175.
59. Wu, X., W. Xiao, and J. L. Brandsma. 1994. Papilloma formation by cottontail rabbit papillomavirus requires E1 and E2 regulatory genes in addition to E6 and E7 transforming genes. *J. Virol.* **68**:6097–6102.
60. zur Hausen, H. 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* **184**:9–13.