Identification of Three Transforming Proteins Encoded by Cottontail Rabbit Papillomavirus

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Cottontail rabbit papillomavirus (CRPV) provides an animal model for human papillomaviruses associated with a high risk of cancer development. So far, nothing is known about the transforming functions of CRPV genes because of the lack of an assay system. We have recently developed two systems to assay for CRPV transforming functions. One is based on the finding that transformation of NIH 3T3 cells by CRPV is considerably increased by deleting sequences in open reading frame L2. The second one is based on the use of a cottontail rabbit skin epithelial cell line, sf1Ep (C. Meyers and F. O. Wettstein, *Virology* 181:637-646, 1991). Mutations were introduced which abolished expression of the full-length E6 protein (LE6), the short E6 protein (SE6) initiated at the second ATG of E6, the E7 protein, or the E5 protein. Mutations affecting LE6 or E7, but not SE6, reduced transformation of NIH 3T3 and sf1Ep cells. Transformed NIH 3T3 cell lines with mutations in LE6 and E7 did not grow in soft agar, while those with mutations in SE6 and E5 grew with a reduced efficiency. The cell lines with mutations in LE6, SE6, or E7 still did induce tumors in nude mice. These mutations, however, abolished the ability to induce papillomas in rabbits. When expressed individually with a retroviral vector, LE6, SE6, or E7, but not E5, conferred anchorage-independent growth. The level of viral protein expression in these cell lines was generally low, and a comparison of the abundance of virus-specific mRNA showed that cell lines contained 20 to 50 times less mRNA than a cottontail rabbit papilloma. These data demonstrate that CRPV encodes at least three transforming proteins.

Cottontail rabbit papillomavirus (CRPV) was the first DNA tumor virus identified (50), and the finding that papillomas (warts) induced by the virus progressed at a high frequency to invasive and metastasizing cancers attracted considerable attention (53). However, interest in this virus waned since CRPV as well as other papillomaviruses could not be grown in tissue culture. The more recent findings that certain cutaneous human papillomaviruses (human papillomavirus types 5 and 8 [HPV-5 and -8]) and, more importantly, certain cervical HPV types (HPV-16, -18, -31, -33, -35, and others; reviewed in reference 14) have been implicated in cancer development has renewed an interest in CRPV as an animal model for papillomavirus-associated cancer development.

In general, the open reading frame (ORF) arrangement of CRPV (20) is very similar to that of other papillomaviruses (reviewed in reference 2), except that ORF E6 is longer than that of other papillomaviruses and contains additional Cys-X-X-Cys motifs. Furthermore, the location of ORF E5 is unusual; instead of being located just upstream of the early polyadenylation signal, the two early polyadenylation signals are located within ORF E5.

The state of the viral DNA and of transcripts in CRPV-induced papillomas and evolving carcinomas has been characterized. In papillomas as well as in many carcinomas, the viral DNA remains predominantly extrachromosomal (55), and in this regard CRPV resembles the cutaneous cancer-associated HPVs (40). Analysis of viral transcripts in papillomas and in carcinomas revealed no major qualitative or

quantitative differences, except that in the virus-producing cottontail rabbits, transcripts for the late proteins are detectable only in papillomas (42). In domestic rabbit tumors at either stage, late transcripts, if present, are of very low abundance (39). Of the two major early transcripts, one encodes an E6 protein, SE6, initiated at the second ATG of ORF E6 and one encodes the E7 protein (13, 54). Transcripts which are of about 10-fold lower abundance encode the full-length E6 protein, LE6, and a full-length E2 protein translated from a polycistronic mRNA encoding SE6 and E2 or E7 and E2 (7). No transcript encoding an E2 repressor protein initiated at an internal ATG could be detected (54). Furthermore, with transiently transfected COS-7 cells, the only E2 protein detected was the full-length E2. The two E6 proteins differ in several properties. LE6 is predominantly localized in the nucleus, with minor portions present in the cytoplasm and the membrane fraction (5), making it similar to E6 of bovine papillomavirus type 1 (BPV-1) (1). In contrast, SE6 is exclusively localized in the cytoplasm. Furthermore, SE6 is phosphorylated while LE6 is not (5). With respect to the E6-E7 region, CRPV shows some similarities to the oncogenic genital HPVs. First, two protein products can be generated from ORF E6, LE6 and SE6 in CRPV and E6 and E6* in the human viruses (15, 48, 51, 52), although the function of the E6* protein(s) has proven to be elusive (35, 45). Second, in both CRPV and genital HPVs, the E7 mRNA is much more abundant than the E6 mRNA, but the SE6 mRNA is as abundant as the E7 mRNA; similarly, the E6*-encoding mRNA is of high abundance.

So far, nothing is known about the transforming functions of CRPV proteins, since no efficient system for cell transformation has been available. Our recent finding (34) that the deletion of sequences in ORF L2 permits efficient transformation of NIH 3T3 cells, as well as the development of a

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rabbit epithelial cell system, has made such investigations possible. Furthermore, the extension of inducing papillomas in rabbits from viral DNA (27) to recombinant DNA (38) permitted the determination of a role for these proteins in the natural situation.

With the experiments reported here, we show that mutations in LE6 and E7 reduced the cell transformation efficiency of CRPV DNA and abolished growth in soft agar of transformed NIH 3T3 cells, while a mutation in SE6 only reduced growth in soft agar. Expressed individually in a retroviral vector, all three proteins—LE6, SE6, and E7—conferred anchorage-independent growth to NIH 3T3 cells, indicating that CRPV encoded at least three transforming proteins. Finally, expression of all three proteins was required to induce papillomas in rabbits.

MATERIALS AND METHODS

Plasmids. The plasmid pdMMtneo (30), expressing the neomycin resistance gene under the control of the mouse metallothionein I promoter, was a gift from P. Howley. Plasmids pLAI-CRPV and pLAI-CRPV Δ XbaI were previously described (38). Plasmid pLAI-CRPVLE6 was constructed by partially digesting pLAI-CRPV with *Pst*I. The segment lacking the *Pst*I fragment extending from nucleotides (nt) 260 to 330 (fragment 260–330) was gel purified and religated. Deletion of fragment 260–330 was confirmed by digestion with *Nsi*I, *Bam*HI, and *Msp*I. The sequence from nt 260 to 330 is unique to the long E6 protein of CRPV, and the deletion also introduced a frameshift. Plasmids pLAI-CRPVSE6 and pLAI-CRPVE5 were constructed by site-specific mutagenesis, employing the Muta-Gene *In Vitro* Mutagenesis Kit (Bio-Rad, Richmond, Calif.) and oligonucleotides purchased from OCS Laboratories Inc. (Denton, Texas). For pLAI-CRPVSE6, a 21-mer oligonucleotide (5'-GC TGG TTC ATA CGT TGT ACA G-3') in which the initiator ATG for the short E6 was replaced by ATA was used. This mutation also created a new *Mae*II restriction site (ACGT) which facilitated screening. For pLAI-CRPVE5, a 22-mer oligonucleotide (5'-G GGA ATT TTT AAA GGC TTT AGT G-3') in which the initiator ATG for E5 was replaced by AAA was used. This mutation created a new *Dra*I restriction site (TTTAAA) which facilitated screening. A plasmid which cannot produce the long or short E6 protein, pLAI-CRPVE6, was constructed by deleting from pLAI-CRPVSE6 *Pst*I fragment 260–330. Plasmid pLAI-CRPVE7 was constructed by partially digesting pLAI-CRPV with *Hind*III. The ends were filled in and religated. This was followed by *Hind*III digestion to confirm that the desired *Hind*III site (nt 1093) was removed. Digestion with *Nhe*I confirmed that the fill-in was complete. The resulting plasmid, pLAI-CRPVE7, contained a frameshift very early in the E7 ORF. Plasmids pLAI-CRPV Δ XbaILE6, pLAI-CRPV Δ XbaISE6, pLAI-CRPV Δ XbaIE6, pLAI-CRPV Δ XbaIE7, and pLAI-CRPV Δ XbaIE5 were obtained by deleting *Xba*I fragment 4665–5775, within the L2 ORF, from plasmids pLAI-CRPVLE6, pLAI-CRPVSE6, pLAI-CRPVE6, pLAI-CRPVE7, and pLAI-CRPVE5, respectively. Plasmid pLAI-CRPVE5 Δ PvuII was constructed by partially digesting pLAI-CRPV with *Pvu*II. The segment lacking *Pvu*II fragment 4454–4793 was gel purified. *Pvu*II fragment 4454–4793 also deleted the plasmid vector as a result of the partial digest. The *Pvu*II segment was ligated back into the vector, pLAI, at the unique *Eco*RV site. The deletions were confirmed by restriction digests.

The pZipneo vector (11), kindly provided by T. Iftner, was

used to construct expression vectors for individual early proteins. *Dde*I fragments 144–1091, obtained from digestion of pLAI-CRPV and pLAI-CRPVSE6, were cloned first into the unique *Xho*I site of plasmid pMSGneo (Pharmacia). The fragments were released from these plasmids and methylated with *Bam*HI methylase (New England Biolabs); *Bam*HI linkers were then added, and the fragments were digested with *Bam*HI. The fragments were then inserted into the *Bam*HI-digested calf intestinal phosphatase-treated pZipneo vector, yielding pZipneoE6 and pZipneoLE6. The proper orientation was determined by *Eco*RI digestion; an *Eco*RI site is located at nt 1063, close to the 3' end of the inserts. The pZipneoSE6 plasmid was obtained in an analogous fashion, starting with the fragment extending from *Eae*I nt 316 to *Eco*RI nt 1063 originally inserted into the *Xho*I site of pMSGneo, yielding the plasmid pMSGneoSE6. The SE6-encoding fragment was then released from pMSGneoSE6 by *Xho*I digestion, provided with *Bam*HI linkers, and inserted into pZipneo. pZipneoE5 was constructed by cloning *Eco*0109 fragment 4230–4660 into the *Xho*I site of Bluescript (Stratagene). The fragment was reisolated from this plasmid, *Bam*HI linkers were added, and the fragment was inserted into pZipneo. The orientation was determined by *Nsi*I-*Eco*RI double digestion; the *Nsi*I site at nt 4297 is close to the 5' end of the insert, which does not contain an *Eco*RI site. The vector pZipneoE7 was constructed by releasing *Rsa*I fragment 1050–1373, originally cloned into the *Xho*I site of pMSGneo, by *Xho*I digestion; this was followed by the addition of *Bam*HI linkers and the insertion of the fragment into the *Bam*HI site of pZipneo. The orientation of the insert was determined by *Eco*RI digestion. An *Eco*RI site at nt 1063 is close to the 5' end of the insert.

Cell cultures and transfections. NIH 3T3 and sf1Ep cells were maintained and transfected by calcium phosphate precipitation (22) as previously described (34).

Anchorage independence assays and tumor formation in nude mice. Cell lines established from the expansion of individual colonies or from the combined G418-resistant colonies of one plate were assayed for their abilities to form colonies in soft agar and their abilities to form tumors in nude mice as previously described (34).

Papilloma induction in rabbits. Three- to four-month-old New Zealand White rabbits were injected intradermally with 15 μ g of recombinant DNA in 150 μ l of sterile Tris-EDTA, and the intradermal blister was punctured 200 times with a 26-gauge needle or a 3-cm² area on the rabbit's back was scarified with a scalpel and 15 μ g of recombinant DNA in 150 μ l of sterile Tris-EDTA was applied to the wound as previously described (38).

DNA isolation and Southern blot analysis. DNA isolation and Southern blot analysis were performed by standard procedures, as described previously (34).

mRNA isolation and dot blot hybridization. mRNA was isolated by two different procedures. In one procedure, total RNA was isolated by the guanidinium-hydrochloride method and poly(A)⁺ RNA was selected by one cycle of oligo(dT) cellulose chromatography as described previously (51). Elution of mRNA was monitored by cochromatography of [³H]poly(A). On the basis of direct measurements with large-scale preparations, the amount of mRNA obtained was assumed to represent 1% of total RNA. Alternately, mRNA was isolated by the Fast Track method (Invitrogen). RNA was bound to Gene Screen Plus (DuPont) in a total volume of 45 μ l of 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 sodium citrate). One aliquot of each RNA preparation was treated briefly at room temperature with 1 M NaOH. The filters were

briefly soaked in formamide, washed with $3\times$ SSC, and baked for 2 h at 80°C.

Immunoprecipitations. Tissue cultures at 60 to 80% confluency were labeled for 2 h with the following per 60-mm-diameter dish: approximately 0.5 mCi of [35 S]cysteine (Amersham Corp.) in Dulbecco modified Eagle medium without cysteine, 1/10 the concentration of methionine, and 2% dialyzed fetal bovine serum. Cells were harvested in 0.5 ml of RIPA buffer (0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 mM Tris hydrochloride [pH 7.4]) containing 0.2 mM phenylmethylsulfonyl fluoride and 0.005% aprotinin. The antisera to E6 and E7 were characterized previously (4, 6). Immune complexes were formed overnight at 4°C with antisera diluted 1:100, 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 RIPA buffer and once with 62.5 mM Tris hydrochloride (pH 8.0) and suspended in sample buffer as described previously (19). Protein analysis was by 8, 12, or 13% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were fluorographed at -76°C (19).

Statistical analysis. A small-sample statistical test of $\mu_1 - \mu_2$ was used to analyze the transformation efficiency data (41). The test hypothesis was $\mu_1 - \mu_2 > 0$. *P* values were determined from reference 33.

RESULTS

Transformation of NIH 3T3 cells with mutant CRPV DNA.

Previously, we have shown that deletions of sequences in the L2 ORF increased the transformation efficiency of NIH 3T3 cells by CRPV DNA (34). Taking advantage of this finding, we used this system to identify potential viral transforming genes. Excluded from the analysis were E1, E2, and E4. E1 plays a critical role in BPV-1 DNA replication, and sequence homologies suggest that this will be true for other papillomaviruses, including CRPV (20). E2 of CRPV is a transactivating protein (21); thus, both E1 and E2 may have an indirect effect on transformation. For these reasons, we restricted the analysis to LE6, SE6, E7, and E5. The constructed mutants essentially eliminated the expression of the proteins. Three mutations affected the E6 proteins. The LE6 mutant had a deletion in the sequence unique to the LE6 protein and also introduced a frameshift, permitting expression of only the first 37 amino acids. In the SE6 mutant, the ATG for SE6 was eliminated, and the E6 mutant combined both the LE6 and the SE6 mutations (Fig. 1). The transformation efficiency of pLAI-CRPV and the E6 mutants was calculated as the percentage of colonies which had a transformed morphology (Table 1). As can be seen, the transformation efficiency with the LE6 and E6 mutants was about half that observed with the parental DNA, while mutation of SE6 had no effect, implicating LE6 as a possible transforming protein.

Mutations affecting both E6 proteins did not completely abolish the transformation potential of CRPV DNA. This suggested that CRPV may contain additional transforming genes. On the basis of observations with other papillomaviruses, E7 and E5 were prime candidates. In the E7 mutation, a translation termination signal was introduced after nine amino acids (Fig. 2B), and in the E5 mutation, the initiator ATG was converted to AAA (Fig. 2A). This mutation also introduced a stop codon in E2 three amino acids upstream of the normal termination signal. Both mutations resulted in reduced transformation of NIH 3T3 cells (Table 2) compared

with that of pLAI-CRPV Δ XbaI or of the SE6 mutant, pLAI-CRPV Δ XbaI SE6. This result provided evidence that E7 was involved in cell transformation. Because of the loss of the last three amino acids of E2 in the E5 mutant, the interpretation of the results with this mutant is ambiguous.

The effect of CRPV transformation of NIH 3T3 cells on cellular properties was further evaluated by testing individual cell lines for anchorage-independent growth (Table 3). The LE6- and the E7-mutant-transformed lines did not form foci above background, while the E5 and the SE6 mutant lines exhibited a low but significant level of anchorage-independent growth. Surprisingly, the E6 double mutation lines tended to have a higher growth potential than the parental DNA lines. Wild-type (pLAI-CRPV Δ XbaI) as well as E6- and E7-mutant-transformed lines were tested for tumor formation in nude mice (Table 3). All cell lines tested induced tumors in nude mice. It is noteworthy that the pLAI-CRPV Δ XbaI LE6 lines which cannot grow in soft agar still induced tumors in nude mice (pLAI-CRPV Δ XbaI LE6 line 1 was tested in nude mice). The results thus suggest that expression of LE6 and E7 was important for conferring anchorage-independent growth. In contrast to the absence of an effect on transformation efficiency, expression of SE6 appeared to augment anchorage-independent growth. Thus, potentially, CRPV may encode at least three different transforming proteins.

Transformation of rabbit skin epithelial cells with mutant CRPV DNA. NIH 3T3 cells provide a unique assay system for viral and cellular oncogenes, but since CRPV, like most papillomaviruses, is strictly epitheliotropic, we also tested the effect of the CRPV mutants on the growth of the rabbit skin epithelial cell line sf1Ep. As previously shown, transformation of sf1Ep cells involves two steps, the loss of a cell density requirement for growth followed by a morphologic transformation (34). Since the sequences located within ORF L2 which are inhibitory to transformation of NIH 3T3 cells have little effect on the transformation of sf1Ep cells, the mutant DNAs employed here were constructed in pLAI-CRPV. The results of the transformation experiments are summarized in Table 4 and show that mutations of LE6, E7, and E5 (E2) reduce transformation while the SE6 mutation had no effect. The findings with sf1Ep cells were similar to those with NIH 3T3 cells. One interesting property of E7-mutant-transformed sf1Ep cell lines was that several maintained some of the CRPV DNA extrachromosomally (Fig. 3). The undigested DNA of the three lines analyzed in lanes 2, 4, and 6 (Fig. 3) shows a band migrating to the position of pLAI-CRPV Δ XbaI form I DNA (arrow). All three lines contained in addition DNA migrating more slowly than the 23.1-kb marker, and the lines analyzed in lanes 8 and 10 seemed to contain only DNA comigrating with cellular DNA. Although we have not established by two-dimensional analysis that the DNA comigrating with cellular DNA represented integrated DNA rather than concatemers or concatemers of viral DNA, we think the bulk of this DNA more likely represents integrated DNA. We favor this interpretation on the basis of previous one- and two-dimensional analyses of DNA from rabbit tumors with integrated and concatemeric DNA (55). These analyses showed that when large concatemers were present, multiple bands were resolved, and, more importantly, a disproportionate amount of viral DNA remained at the origin. After *Hind*III digestion, the predominant bands were those, found by *Hind*III digestion of the parental plasmid in gels stained with ethidium bromide (not shown), with the calculated sizes of 3996 and 3816 nt (double band) and 3536 nt. In some cell lines, notably

FIG. 1. Map of CRPV mutants. (A) Map of the ORFs of CRPV (20). The vertical dashed lines indicate the positions of the first ATG codon, except in E1, where none are shown, and in E6, where the positions of the first and second ATGs are indicated. In E2, the positions of the three internal ATGs are shown by short vertical lines. PE and PL denote the early and late promoters (13, 54). The vertical arrowheads indicate the positions of polyadenylation signals (AATAAA). (B) Map of the late region deletion. The map is aligned with the ORF map of panel A. wt, pLAI-CRPV; Δ Xba I, pLAI-CRPV Δ XbaI. (C) Top: expanded map of the amino-terminal segment of E6 and map of the E6 mutants; shown are the positions of the TATA boxes and the promoters for the minor 2.0-kb RNA (PE 95), encoding the long E6 protein initiated at the first ATG, and for the major 2.0-kb mRNA (PE 165), encoding the short E6 protein initiated at the second ATG (4, 5). Bottom: map of the E6 mutants. wt, wild-type CRPV; LE6, mutant not expressing the long E6 protein; SE6, mutant not expressing the short E6 protein; E6, mutant expressing neither of the E6 proteins.

those analyzed in lanes 5 and 9, additional strong bands suggest that there was some rearrangement. The presence of extrachromosomal DNA was not observed with any of the other mutants or with any NIH 3T3 cell lines (data not shown).

Papilloma induction with mutant CRPV DNA. The adaptation of the previously established procedure to induce papillomas on rabbits with viral DNA (27) to the use of recombinant CRPV DNA (38) has made it possible to test the ability of pLAI-CRPV mutants to induce papillomas (Table 5). In these experiments, a second E5 mutant (pLAI-CRPV Δ PvuII), in which the carboxy-terminal 40 amino acids of E5 were deleted, was also included (Fig. 2A). The results indicate that all three proteins (LE6, SE6, and E7) were required for tumorigenicity in rabbits. The requirement of an intact E7 protein for papilloma formation has recently been demonstrated (10). Although the number of test sites

TABLE 1. Transformation of NIH 3T3 cells with CRPV E6 mutants^a

Plasmid	Avg % of resistant colonies with transformed morphology	Normalized avg (%)
pLAI-CRPV	13	25 ^b
pLAI-CRPV Δ XbaI	52	100
pLAI-CRPV Δ XbaI/LE6	23	44 ^c
pLAI-CRPV Δ XbaI/SE6	50	96 ^d
pLAI-CRPV Δ XbaI/E6	25	48 ^c

^a The transformation assays were performed as described in Materials and Methods.

^b Value is significantly different from that for pLAI-CRPV Δ XbaI ($P < 0.0005$).

^c Value is significantly different from that for pLAI-CRPV Δ XbaI ($P < 0.005$).

^d Value is not significantly different from that for pLAI-CRPV Δ XbaI ($P < 0.9$).

FIG. 2. Map of the E5 and E7 mutants. (A) Map of CRPV E5 mutants. (Top) Expanded map of the E5 ORF. The positions of the E5 ATG codons, the two early polyadenylation signals, and pertinent restriction enzyme sites are shown. (Bottom) Map of E5 mutants: wt, wild-type CRPV; E5, mutant not expressing the E5 protein; E5ΔPvuII, mutant expressing an E5 protein lacking the carboxy-terminal two-fifths. The position of insertion of the plasmid vector pLAI is shown. (B) Map of CRPV E7 mutant. (Top) Expanded map of the carboxy-terminal segment of E6 and the E7 ORFs. The positions of the E7-specific TATA box, the ATG and TGA codons, and the HindIII restriction site within E7 are shown. (Bottom) Construction of the E7 mutant. The sequences of the HindIII restriction site before and after the fill-in reaction and the NheI restriction site are displayed.

with pLAI-CRPVE5ΔPvuII was relatively small, the finding that tumors did develop at two sites indicated that E5 was not absolutely required or that the amino-terminal three-fifths of the protein could substitute for the whole protein.

Transformation of NIH 3T3 and s1Ep cells with vectors expressing individual CRPV proteins. The results of experiments involving mutations in different ORFs strongly implicated LE6, SE6, and E7 in altering growth properties of NIH 3T3 and of rabbit epithelial cells. In order to distinguish between an indirect effect on transformation and a direct role of these proteins on transformation, we tested their abilities to transform cells. To accomplish this, the genes were individually cloned into pZipneo, where the proteins were expressed under the control of the Moloney murine leukemia virus long terminal repeat. This vector also confers G418 resistance (11). pZipneoLE6 contained the entire E6 ORF in

TABLE 2. Transformation of NIH 3T3 cells by CRPV E7 and E5 mutants^a

Plasmid	Avg % of resistant colonies with transformed morphology	Normalized avg (%)
pLAI-CRPVΔXbaI	78	100
pLAI-CRPVΔXbaISE6	75	96 ^b
pLAI-CRPVΔXbaIE7	33	43 ^c
pLAI-CRPVΔXbaIE5	26	35 ^c

^a The transformation assays were performed as described in Materials and Methods.

^b Value is not significantly different from that for pLAI-CRPVΔXbaI ($P < 0.9$).

^c Value is significantly different from that for pLAI-CRPVΔXbaI ($P < 0.0005$).

which the initiator ATG of SE6 was mutated. pZipneoE6 contained the wild-type E6 ORF. pZipneoSE6 contained only the sequences encoding SE6, and pZipneoE7 and pZipneoE5 contained only ORFs E7 and E5, respectively. In these experiments, transfected NIH 3T3 cells were selected for resistance to G418, and after 2 weeks the resistant cells were pooled and assayed for anchorage-independent growth. The numbers of colonies of cells expressing different proteins are compiled in Table 6 and show that all three proteins—LE6, SE6, and E7—individually conferred anchorage-independent growth. The finding that pZipneoE6, which potentially encodes LE6 and SE6, stimulated growth in soft agar only to the same level as did pZipneoLE6 is not surprising, since internal initiation at the second ATG is inefficient in the presence of the first ATG (4). This was shown directly by the analysis of immune precipitates from extracts of ³⁵S-labeled transiently transfected COS-7 cells

TABLE 3. Anchorage-independent growth of CRPV-transformed NIH 3T3 cells and tumor formation in nude mice^a

Cell line ^b	Avg no. of colonies/plate ^c	Overall avg no. of colonies/plate	No. of tumors/no. of animals inoculated
NIH 3T3	4, 1	3	0/3
pLAI-CRPVΔXbaI 1	253, 156	167	3/4
pLAI-CRPVΔXbaI 2	138, 121		
pLAI-CRPVΔXbaISE6 1	29, 31	30	4/6
pLAI-CRPVΔXbaISE6 2	30		
pLAI-CRPVΔXbaILE6 1	1, 0	1	3/3
pLAI-CRPVΔXbaILE6 2	0, 4		
pLAI-CRPVΔXbaIE6 1	278	352	3/6
pLAI-CRPVΔXbaIE6 2	427		
pLAI-CRPVΔXbaIE7 1	7	9	3/4
pLAI-CRPVΔXbaIE7 2	6		
pLAI-CRPVΔXbaIE7 3	10		
pLAI-CRPVΔXbaIE7 4	15		
pLAI-CRPVΔXbaIE5 1	33	31	ND ^d
pLAI-CRPVΔXbaIE5 2	20		
pLAI-CRPVΔXbaIE5 3	43		
pLAI-CRPVΔXbaIE5 4	26		

^a The assays for anchorage-independent growth were performed as described in Materials and Methods.

^b The plasmid used to transform the NIH 3T3 cells is indicated. The different cell lines obtained by transformation with the same plasmid are identified by different numbers.

^c In most cases, the average is from four plates; in some cases it is from three, five, or seven plates. Where two numbers are given, they represent repeat analysis of the same cell line.

^d ND, not done.

TABLE 4. Transformation of sf1Ep cells with CRPV mutants^a

Plasmid	No. of transformed colonies/total resistant colonies	% of transformed colonies normalized
pLAI	0/17	0
pLAI-CRPV	48/94	100
pLAI-CRPVΔXbaI	9/21	84
pLAI-CRPVLE6	5/38	26
pLAI-CRPVSE6	34/90	74
pLAI-CRPVE7	21/86	48
pLAI-CRPVE5	11/60	36

^a The transformation assays were performed as described in Materials and Methods.

expressing the entire ORF E6 under the control of the late simian virus 40 promoter. With these cells, SE6 was barely detectable (Fig. 4, lane 2). The synthesis of the E6 proteins in the transformed NIH 3T3 lines (Fig. 4, lanes 4 and 5) was lower than in transiently transfected COS-7 (Fig. 4, lanes 2 and 3), and the LE6 band was obscured by a background band (Fig. 4, lane 5). Separation of LE6 from the background band was possible by analysis on 8% gels (Fig. 4, lanes 9 to 16), and LE6 was positively identified by blocking the antiserum with the TrpE-E6 fusion protein (Fig. 4, lanes 9, 11, 14, and 16). In an analysis of the immune precipitates from the pZipneoE6-transformed lines on 13% gels, again, SE6 was barely detectable (data not shown). Surprisingly, in the pZipneoE6 pooled line, which must have also contained nontransformed G418-resistant cells, the level of E6 expression was higher than in the clonally derived line. This may indicate that in a pool, lines expressing a relatively high level of E6 have a growth advantage.

It is noteworthy that in the context of the pLAI recombinant plasmids, mutations in SE6 had a relatively minor effect, whereas, expressed with the pZipneo vector, SE6 stimulated anchorage-independent growth the most (Table 6). Since we have not analyzed the E6 proteins expressed in

FIG. 3. Southern blot analysis of DNA from sf1Ep cells transformed with E7 mutant CRPV DNA. Southern blots were prepared as described in Materials and Methods. Odd-numbered lanes, *Hind*III-digested DNA; even-numbered lanes, undigested DNA. The arrow marks the position of supercoiled pLAI-CRPVE7 plasmid DNA (form I DNA). The sizes (in kilobases) and positions of lambda DNA *Hind*III-digested molecular size markers are indicated on the left.

TABLE 5. Rabbit papilloma induction with CRPV mutants^a

Plasmid	No. of papillomas/no. of sites inoculated	% of positive sites
pLAI	0/10	0
pLAI-CRPVΔXbaI	15/50	30
pLAI-CRPVLE6	0/19	0
pLAI-CRPVSE6	0/18	0
pLAI-CRPVE7	0/34	0
pLAI-CRPVE5	0/14	0
pLAI-CRPVE5ΔPvuII	2/16	13

^a Papillomas were induced as described in Materials and Methods.

cells transformed by pLAI recombinant plasmids, we do not know if this is due to a low level of SE6 expression. On the basis of transcription maps established for rabbit tumors, SE6 should be expressed at a high level in pLAI-CRPVΔXbaI and in pLAI-CRPVΔXbaILE6, since there are separate mRNAs for the two proteins (13, 39, 54). However, in NIH 3T3 cells with integrated DNA, the promoters for SE6 and E7 may not be functional. It has been shown previously that when the early region of CRPV is expressed under the control of the late simian virus 40 promoter, the CRPV-specific promoters are not functional (4, 7). The consequence is that with constructs encoding the entire early region, only LE6 is well expressed, while SE6 is poorly expressed (4) (Fig. 4, lane 2) and E7 could not be detected (7). If this is the situation, then SE6 would be poorly expressed in pLAI-CRPVΔXbaI and pLAI-CRPVΔXbaILE6. In the latter, a deletion-frameshift mutation is present upstream of the SE6 ATG, but reinitiation at this ATG would not be efficient since the fusion protein generated terminates downstream of the ATG. The effect on E7 expression would be similar, except that in pLAI-CRPVΔXbaILE6 and pLAI-CRPVΔXbaILE6, translation termination of the fusion protein would be well upstream of the ATG, and, on the basis of Kozak's rule (29), reinitiation of translation would be more likely. This would explain the high level of anchorage-independent growth of pLAI-CRPVΔXbaILE6-transformed lines.

Transformation of sf1Ep cells with the pZipneo vectors indicated that in these cells, LE6, SE6, and E7 also functioned as transforming proteins (Table 7). The expression of the proteins was verified by SDS-PAGE analysis (Fig. 5), and in cell lines established from transfections with pZipneoE6, LE6 was predominantly expressed (Fig. 5, lanes 1 and 2). Overall, in all lines analyzed, expression of the early

TABLE 6. Anchorage-independent growth of NIH 3T3 cells transfected with pZipneo vectors encoding different CRPV proteins^a

ORF cloned	No. of colonies/expt:					Avg no. of colonies
	1	2	3	4	5	
pZipneo (control)	6	23	26	25	25	21
LE6	102	188	128	ND ^b	ND	139
SE6	590	362	410	ND	ND	454
E6	98	92	186	ND	ND	125
E7	ND	432	307	300	353	348
E5	21	ND	ND	42	20	28

^a Anchorage-independent growth was assayed as described in Materials and Methods.

^b ND, not done.

FIG. 4. Expression of E6 proteins in transformed NIH 3T3 cells. Transformed NIH 3T3 cells and transiently transfected COS-7 cells were labeled with [³⁵S]cysteine, and the E6 proteins were immunoprecipitated and analyzed by SDS-PAGE as described in Materials and Methods. Left panel: COS-7 cells transiently transfected with the parental vector pSVLA4 (4) (lane 1), the LE6- and SE6-expressing vector pSVLA4S3 (4) (lane 2), and the SE6-expressing vector pSVLA4E4 (4) (lane 3) and clonally derived NIH 3T3 cell lines transfected with pZipneoSE6 (lane 4), pZipneoLE6 (lane 5), and parental pZipneo (lane 6). Middle panel: clonally derived NIH 3T3 cell line transformed with pZipneoSE6. Lane 7, antiserum blocked with TrpE-E6 fusion protein; lane 8, unblocked antiserum. Right panel: immunoprecipitates prepared from clonally derived cell lines (lanes 9, 10, 15, and 16) or pools of cells (lanes 11, 12, 13, and 14) transformed with pZipneoLE6 (lanes 9 to 12) or pZipneoE6 (lanes 13 to 16) by using TrpE-E6 blocked antiserum (lanes 9, 11, 14, and 16) or unblocked antiserum (lanes 10, 12, 13, and 15). The immunoprecipitates were prepared from labeled extracts containing ($\times 10^6$) 65, 60, 50, 43, 43, 49, 44, 50, 32, 32, 37, 39, 33, 28, 29 and 26 cpm for lanes 1 to 16, respectively. SDS-PAGE analysis was with 12, 13, or 8% gels, and fluorographic exposure was for 2 weeks, 8 days, and 8 days for the left, middle, and right panels, respectively. The position of size markers is indicated for each panel, and the sizes (in kilodaltons) are given on the left and right. The positions of LE6 and SE6, of SE6, and of LE6 are indicated by arrowheads at the left, middle, and right panels, respectively.

proteins was low when compared with expression in transiently transfected COS-7 cells. In transiently transfected cells, the amount of mRNA present was in the same general range as in papillomas or carcinomas, and analysis by Northern (RNA) blotting and S1 mapping could easily be accomplished (4, 6, 7). This was not possible with the cell lines established here. In an attempt to obtain some insight into the level of viral gene expression, dot blots of mRNA isolated from several sf1Ep lines were compared with dot blots of poly(A)⁺ RNA isolated from a cottontail rabbit papilloma which contained an average level of virus-specific RNA (Fig. 6). This comparison suggests that the transformed cells expressed 20 to 50 times less viral RNA and, presumably, viral proteins than did tumors in rabbits and indicates that even at low levels of expression, LE6, SE6, and E7 exhibit potent biological effects.

DISCUSSION

The finding that at least three proteins play a direct role in transformation is a novel one for papillomaviruses. For most papillomaviruses, two viral proteins which play a direct role in transformation have been identified. Among genital HPVs associated with a high risk of cancer, HPV-16 and HPV-18 E6 and E7 have been implicated in transformation. In these viruses, E7 represents the major transforming protein, since under some experimental conditions only this protein, not E6, is required for immortalization of primary keratinocytes (23, 25). HPV-16 and -18 E6, however, can transform NIH 3T3 cells independently (8, 47). In the cutaneous HPV-8, a transforming function has been assigned so far to E6 (26), and in HPV-6, E5a has been identified as a transforming protein (12). In BPV-1, E6 and E5 represent transforming proteins (reviewed in references 24 and 31).

The major interest in CRPV as an animal model for HPVs derives from the fact that papillomas induced by this virus have a high rate of progression to cancers, particularly in domestic rabbits (53). Furthermore, as directly shown with CRPV (28) and strongly suggested for epidermodysplasia verruciformis- and genital cancer-associated HPVs, cofactors play a role in the progression to cancer (40, 58). Thus, although the cancer-associated papillomaviruses do not induce cancer directly, the expression of their early proteins presumably greatly facilitates the subsequent development of cancer; this is not the case for a large number of HPV types or for BPV-1. In addition, in the genital HPVs, integration of viral DNA observed with the majority of cancers may also contribute to cancer development (57). A

TABLE 7. Transformation of sf1Ep cells with pZipneo vector encoding different CRPV proteins^a

ORF cloned	No. of transformed colonies/total resistant colonies	% of colonies transformed
pZipneo (control)	1/8	13
LE6	4/8	50
SE6	5/8	63
E6	3/8	38
E7	4/9	44
E5	1/9	11

^a Transformations were carried out as described in Materials and Methods.

FIG. 5. Expression of LE6, SE6, and E7 in transformed sf1Ep rabbit epithelial cells. Immunoprecipitates were prepared, as described in Materials and Methods, from clonally derived cell lines transformed with pZipneoE6 (lanes 1 and 2), pZipneoLE6 (lane 3), pZipneoSE6 (lanes 4 and 5), and pZipneoE7 (lanes 7 and 8) and from parental sfEp cells (lanes 6, 9, and 10). Anti-E6 serum was used in lanes 1 to 6, and anti-E7 serum was used in lanes 7 to 10. The antiserum was blocked with the TrpE-E7 fusion protein (lanes 7 and 9). The immunoprecipitates were prepared from labeled extracts containing ($\times 10^6$) 24, 19, 16, 17, 21, 43, 43, 44, and 44 cpm, respectively, for lanes 1 to 10. SDS-PAGE analyses were performed as described in Materials and Methods. The left panel represents the 6-week fluorogram of a 12% gel, and the right panel represents a 4-week fluorogram of a 13% gel. The sizes (in kilodaltons) and positions of size markers are indicated, and the positions of LE6, SE6, and E7 are marked by arrowheads.

comparison of the properties of the high-cancer-risk transforming proteins with those of the low-cancer-risk genital HPVs shows differences in their interaction with the RB protein, the product of the retinoblastoma susceptibility gene (19, 36) and p53 (47). The finding that HPV-negative cervical cancer-derived cell lines contain mutations in RB and p53 (46) suggests that these interactions are necessary for progression to cancer. The E7 proteins of the high-risk viruses bind the RB protein more efficiently than do the low-risk types. The CRPV E7 protein contains sequences similar to those considered important for RB binding, and, in addition, it also contains a casein kinase II phosphorylation site. Phosphorylation at this site in HPV-16 E7 or replacing the serine residues with negatively charged amino acids contributes to the transforming function of E7 (18). The RB binding of CRPV E7 has not been directly tested yet; however, its sedimentation behavior on nondenaturing glycerol gradients more resembles that of HPV-6 E7 than that of HPV-16 E7 (6, 19). CRPV E7 and HPV-6 E7 sediment in a biphasic pattern, with a major peak at 22 kDa and a minor one at 88 kDa (19), while the majority of HPV-16 E7 sediments faster, and this fast-sedimenting E7 can be immunoprecipitated with anti-RB serum (19). On the basis of this criterion, CRPV E7 seems not to bind to RB as strongly as HPV-16 E7. If RB does bind to CRPV E7, it is similar to HPV-6 E7. One possibility, however, is that there are critical

FIG. 6. Detection of CRPV RNA in pZipneoE6- and pZipneoSE6-transformed sf1Ep rabbit epithelial cells. Poly(A)⁺ RNA was isolated from a cottontail rabbit papilloma by oligo(dT) cellulose chromatography, treated with RNase-free DNase, and spotted onto Gene Screen Plus (DuPont) (row 1). The amount spotted (in micrograms) is indicated on the left. The first spot (NaOH) contained RNA treated with NaOH. Poly(A)⁺ RNA from cell lines was isolated by the Fast Track method (Invitrogen) (rows 2 to 6) and was not treated with DNase. The estimated amount of poly(A)⁺ RNA spotted (in micrograms) is indicated on the right. RNA was from two pZipneoSE6-transformed lines (rows 2 and 3), from two pZipneoE6-transformed lines (rows 4 and 5), and from sf1Ep cells (row 6). The filters were hybridized with a ³²P-labeled probe obtained by nick translation of the CRPV *Bgl*II fragment 2123–7660. The specific activity of the probe was about 2×10^8 cpm/ μ g. Hybridization conditions were as described for RNA elsewhere (51). Both filters were hybridized with the same hybridization mixture and exposed for 40 h with an amplifying screen at -76°C .

differences between rabbit and monkey (COS-7 cell) RB proteins, with only the former strongly binding to CRPV E7. Although possible, this seems less likely, since mouse and human RB show a high (91%) sequence homology, indicating that the protein is highly conserved (9). A second property in which CRPV E7 more resembles HPV-6 E7 than HPV-16 E7 is its state of phosphorylation. Both HPV-16 and HPV-6 E7 can be phosphorylated *in vitro* by casein kinase II (3, 17), but HPV-16 E7 is more efficiently phosphorylated (3). Expressed *in vivo* in CaSki cells (17, 52) or in COS-7 cells (18), HPV-16 E7 is highly phosphorylated, while HPV-6 E7 expressed in COS-7 cells is only weakly phosphorylated (19) and CRPV E7 expressed in these cells is not phosphorylated (6). Thus, in two properties important for the transforming function of HPV-16 E7, CRPV E7 appears more to resemble HPV-6 E7. This finding is surprising, since cervical keratinocytes can be immortalized with CRPV DNA (37). It is possible that other properties of E7 common to CRPV and HPV-16 E7 could play a role. One common property is transactivation by HPV-16 E7 (43, 44) and by CRPV E7 (23a). At present, however, this function does not appear to be important for transformation with HPV-16 E7 (16). Finally, the finding that in some sf1Ep cell lines transformed by the CRPV E7 mutant some of the DNA is maintained extrachromosomally suggests yet another function mediated by E7. No comparable observations have been made with HPV-16. E7 mutants of BPV-1 maintain the viral DNA at a reduced copy number (32), a condition which can be partly relieved by HPV-8 E7 (26). Results with CRPV suggest instead an opposite function for E7, as extrachromosomal maintenance is rather facilitated by an E7 mutation.

Two additional transforming proteins are both generated from ORF E6. LE6 is predominantly located in the nucleus and shows the subcellular distribution of BPV-1 E6, a transforming protein (1). Nothing is known about the subcellular distribution of HPV-16 E6. Its interaction with p53 (46) suggests that it too is a nuclear protein. Originally, it appeared

that HPV-16 E6 in itself had no transforming functions but rather augmented the effect of E7. However, it has recently been shown that HPV-16 E6 can independently transform and confer anchorage-independent growth to NIH 3T3 cells (49), as previously shown for BPV-1 E6 (56) and now also for CRPV LE6. Evidence strongly suggests that the critical function of HPV-16 E6 is its binding to p53 and the subsequent stimulation of p53 degradation (46). So far, nothing is known about how CRPV LE6 or BPV-1 E6 stimulates transformation. p53, in contrast to the RB-binding site, has not been narrowly defined; therefore, predictions based on sequence data are not possible. Again, the fact that CRPV can immortalize human cervical keratinocytes (37) and that HPV-16 E6 and E7 are generally required for this process could indicate that CRPV LE6 and HPV-16 E6 exert their influence by similar mechanisms.

The second E6-encoded transforming protein, SE6, differs biochemically as well as biologically from LE6. SE6 is a phosphorylated cytoplasmic protein which appears less important for morphologic transformation of NIH 3T3 cells (Table 1 and 2) or transformation of sflEp cells (Table 4) than LE6; however, it is more efficient than LE6 in stimulating anchorage-independent growth in a direct assay (Table 6). The difference in the biochemical and the biological properties suggests that the two proteins probably differ in their interaction with cellular mechanisms. In contrast to the case with CRPV SE6, no biological functions for the second protein encoded by ORF E6 of HPV-16, E6*, have been identified; it cannot substitute for E6, nor does it augment the activity of E6 (8, 35). Of course, the biological test in which the requirement for SE6 is most prominent, the induction of papillomas in rabbits, cannot be performed with E6*. Thus, it is still possible that in the most stringent test for function, in the natural host *in vivo*, E6* may be required. Alternately, as suggested originally (52) and now shown directly (49), the intron in E6 of HPV-16 and related HPV types may serve only to obtain a high level of E7 expression and not to express a second ORF E6-encoded protein.

The interpretation of our data related to E5 has to remain open. E5 is the major transforming protein of BPV-1, and E5a is also a transforming protein of HPV-6. The sequence of CRPV E5, however, has no resemblance to that of BPV-1 (2) or HPV-6 E5a, and if it were a transforming protein, its mode of action most likely would be different.

There are aspects common to both genital HPV types associated with a high risk of cancer and CRPV. Most importantly, as far as has been investigated, these are the only papillomaviruses in which E6 and E7 are transforming proteins. It will have to be determined whether the mechanisms of transformation are also similar. To be sure, there are also differences; DNA is usually not integrated in CRPV-induced cancers, while it usually is in genital cancers (57). Furthermore, although ORF E6 of CRPV and the HPVs encode two proteins, only in CRPV do both proteins have transforming functions. Most importantly, in an animal model, the critical factors needed for the development of cancer can be investigated, and our data have shown that simultaneous expression of all three transforming proteins is required for papilloma formation. Finally, the introduction of a new, less time-consuming method to induce tumors in rabbits with DNA (10) makes a fine *in vivo* genetic analysis possible.

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