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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

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A comparative analysis of different properties of the E6 and E7 proteins of high-risk and low-risk cutaneous papillomaviruses was performed. The corresponding genomic regions of human papillomavirus types 1 and 8 (HPV1 and HPV8) and of the cottontail rabbit papillomavirus (CRPV) were cloned into the eucaryotic expression vector pZipNeo-SV(X)-1 and into vectors for in vitro transcription and translation. With the help of these vectors, the individual proteins were investigated for their ability to transform C127 and NIH 3T3 rodent fibroblasts, bind the *Rb* protein in vitro, transactivate the adenovirus E2 promoter, and cooperate in the immortalization of primary human keratinocytes. Expression vectors for HPV16 E6 and E7 were used as a positive control. A highly transformed phenotype could be observed with rodent cell lines expressing HPV8 E6, HPV16 E6 and E7, and, surprisingly, HPV1 E7. In contrast, no transformation was detected with CRPV long E6 and HPV8 E7, whereas cells expressing HPV1 E6 and CRPV short E6 exhibited a weakly transformed phenotype. Although neither CRPV E6 nor CRPV E7 caused morphological transformation of C127 cells, CRPV E6 was able to induce anchorage-independent growth in both rodent cell lines, whereas CRPV E7 led to high cloning efficiencies only in NIH 3T3 cells. The *in vitro Rb*-binding affinities relative to that of HPV 16 E7 were 66% for HPV1 E7, 34% for HPV8 E7, and 11% for CRPV E7. In spite of its high *Rb*-binding affinity, HPV1 E7 did not *trans* activate the adenovirus E2 promoter, whereas HPV8 E7 and CRPV E7 showed low activities. Complementation studies in primary human keratinocytes revealed a weak immortalizing potential for HPV8 E7 and indicated a low degree of cooperativity between CRPV E7 and CRPV or HPV16 E6.

A few distinct types of the large number of known papillomaviruses stand out because of their association with malignant tumors in their natural hosts. The first papillomavirus shown to be associated with the development of cancer was the cottontail rabbit papillomavirus (CRPV) (41), which induces epithelial tumors in both cottontail and domestic rabbits (47). The initially benign tumors develop within 8 to 14 months into invasive carcinomas in 25% of cottontail rabbits and in 66 to 80% of domestic rabbits (49, 53). The first human papillomaviruses (HPVs) suspected of involvement with malignant tumors represent a subgroup of cutaneous viruses specifically associated with the rare disease epidermodysplasia verruciformis (EV) (38). More than 18 different papillomavirus types have been isolated from benign EV-specific lesions, however, the carcinomas that developed in 30 to 60% of the patients contained a much more restricted spectrum of HPV types, most frequently HPV5 and HPV8 (38, 39). A similar situation was found with the second more prevalent subgroup of HPVs preferentially associated with neoplasias of the anogenital tract. DNA from the genital HPV types 16, 18, 45, and 56 is frequently present in invasive anogenital cancers, while DNA from types 6 and 11 is predominantly present in benign tumors and low-grade dysplasias but rarely in malignant lesions (32, 54). These observations led to the classification of papillomaviruses into viruses with a high risk and a low risk for malignant conversion of the induced lesions (58).

A number of studies support a role for the E6 and E7 genes during oncogenesis; however, the importance of the individual proteins is quite variable among different papillomaviruses. In the case of genital papillomaviruses, a correlation was recognized between the in vitro activities of the viral oncogenes and the carcinogenic potential of the respective viruses. Under the control of a strong heterologous promoter, the E7 gene of the high-risk HPV16 is sufficient to immortalize primary foreskin keratinocytes and to cause a cervical epithelial neoplasia-like histopathology during organotypic raft culture of transfected keratinocytes (15, 22). The E7 gene of the low-risk HPV6 reveals no immortalizing activity on its own but is able to cooperate with HPV16 E6 to immortalize primary human epithelial cells with low efficiency (16). Similarly, in rodent fibroblasts, HPV6 E7 led to a 10-fold-lower cloning efficiency in soft agar in comparison to the E7 protein of the high-risk type HPV16 (2). Because E6 by itself revealed no immortalizing activity (19), the E6 proteins of the genital papillomaviruses were initially regarded as auxiliary oncoproteins. Recently it was shown, however, that HPV16 E6 can independently induce anchorage-independent growth of NIH 3T3 cells (45), whereas the E6 genes of the low-risk genital HPV types express no comparable cell-transforming activities in rodent cell lines (2). Interestingly, human mammary epithelial cells can be immortalized by the E6 genes of both high-risk HPV16 and low-risk HPV6 (1).

An extremely important finding was that E6 and E7 interact

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with cellular proteins which are involved in the control of the cell cycle. The E6 proteins of HPV16 and -18 were shown to form complexes with p53, a multifunctional regulatory protein with tumor suppressor properties (31, 52). Complex formation leads to the enhanced degradation of p53 via the ubiquitin-dependent proteolytic pathway (42), which seems to be important for the efficient immortalization of human keratinocytes (1, 46). In contrast to the high-risk types, the E6 proteins of types 6 and 11 bind p53 with much lower affinity and do not mediate degradation (8, 42, 52). However, E6 harbors additional transforming domains that are not dependent on the interaction with p53, as demonstrated by the transformation of rodent fibroblasts in which HPV16 E6 could not be substituted for by mutant forms of p53 (46). Furthermore, mutational analysis of HPV16 E6 showed that *trans* activation of the adenovirus E2 (AdE2) promoter in NIH 3T3 cells was not dependent on the degradation of p53 (46).

The E7 protein of HPV16 can form complexes not only with *Rb* but also with other cellular proteins involved in the control of the cell cycle, like p107, p130, cyclin A, and the cyclin-associated kinase $p33^{cdk2}$ (9, 10, 50). The interaction of E7 with *Rb* leads to the activation of transcription factor E2F, which stimulates expression of cell cycle-regulated genes required for DNA synthesis (37). Because the E7 proteins of the low-risk genital types 6 and 11 bind to *Rb* with much lower affinity (12, 36), the respective carcinogenic potential of the individual types is believed to be reflected by the relative binding strength of E7 to the *Rb* protein.

In contrast to the genital papillomaviruses, only a limited number of investigations on the transforming potential of the E6 and E7 proteins of cutaneous papillomaviruses have been reported. The E6 gene product was identified for the first time as a transforming protein in the case of bovine papillomavirus type 1 (BPV1) (43). Both E6 and E7 of CRPV were found to be important for the transformation of 3T3 and sf1Ep cells (34), whereas the E7 genes of the high-risk EV-associated HPV8 and HPV47 did not exhibit any detectable transforming function (20, 24). Rather, E6 seems to be the major transforming protein of EV-associated HPVs when tested in rodent fibroblasts (20, 24, 29). Investigations of the low-risk cutaneous HPV1 revealed no transforming activity for the E6 protein, and although E7 had a weak effect on the morphology of 3Y1 cells, it did not lead to anchorage-independent growth of these cells (20, 51). In contrast to the genital papillomaviruses, the transforming protein E6 of BPV1, HPV8, and HPV47 does not form complexes with p53 when tested in reticulocyte lysates (20, 48, 52). No data have been reported so far on the in vitro *Rb*-binding affinities of the E7 proteins of cutaneous HPVs or on their abilities to immortalize human keratinocytes.

In this study, we compared the transforming activities of the E6 and E7 genes of low-risk and high-risk cutaneous viruses, using as a test assay the transformation of rodent fibroblasts and the immortalization of primary human keratinocytes, and we evaluated the relative *Rb*-binding affinities of the E7 proteins. Our results indicate that the carcinogenic potential of a papillomavirus is not necessarily reflected by the relative binding affinity of its E7 protein to *Rb*, although this property seems to correlate with transforming potential in rodent cells.

MATERIALS AND METHODS

Plasmids. The expression vector pZipNeo-SV(X)-1 (pZip) (7) was a gift from R. C. Mulligan. pZip-HPV1 recombinant plasmids containing the E6 and E7 open reading frames (ORFs) were constructed as follows. An *Eco*RI subfragment of HPV1 harboring the E6/E7 genomic region (nucleotides [nt] 7778 to 967 [13]) was first subcloned into pUC19, giving rise to clone pUC1-67. To construct pZ1-6 (a pZip recombinant clone containing the HPV1 E6 ORF), we cloned a *Bam*HI-*Bgl*II fragment of pUC1-67 containing the complete E6 ORF into the *Bam*HI site of pZip. For construction of pZ1-7, an *Rsa*I-*Rsa*I (nt 345 to 829) fragment of HPV1 was subcloned into the *Sma*I site of pIC19H (33), giving rise to pIC1-7. After digestion with *Bam*HI and partial digestion with *Bgl*II, a subfragment of 510 bp encompassing the complete E7 ORF was released and cloned into the *Bam*HI site of pZip. Expression vectors for HPV8 E6 and E7 (pI86 and pI87, respectively) (24), for HPV16 E6 and E7 (pWTE6 and pE7, respectively) (45), and for CRPV full-length E6 (pZipNeoE6), short E6 (pZipNeoSE6), long E6 (pZipNeoLE6), and E7 (pZipNeoE7), abbreviated here as pCE6, pCSE6, pCLE6, and pCE7, respectively, have been described before (34).

To generate RNA expression vectors for the different E6 and E7 genes of HPV1, HPV6, HPV8, HPV16, and CRPV, specific subfragments were subcloned into the Bluescribe M13+ vector (pBS; Vector Cloning System) or pBluescript II KS vector (BSKSII; Stratagene) as follows. To construct pBS1-6, an *Eco*RI-*Bgl*II (nt 7778 to 594) fragment of HPV1 was subcloned into pIC19H (33). The HPV1 genomic region encompassing E6 was released from the resulting plasmid through *Bam*HI-*Bgl*II digestion and cloned into the *Bam*HI site of pBS. The RNA expression vector pBS1-7 was obtained by cloning the 510-bp *Bam*HI-*Bgl*II fragment of pIC1-7 into the *Bam*HI site of pBS. To construct pBS8-6, an *Hpa*I-*Hin*cII (nt 1 to 725) restriction fragment of HPV8 was ligated to *Bam*HI linker molecules, cleaved with *Bam*HI, and cloned into the *Bam*HI site of pBS. The RNA expression vector pBS8-7 contains an *Hae*III-*Rsa*I (nt 586 to 970) fragment of HPV8, which was cloned into the *Bam*HI site of pBS after addition of *Bam*HI linkers. To construct BSKS/HPV6b-E7 (nt 466 to 2307), pBR322/HPV6b was cleaved with *Sty*I, filled in, and then digested with *Hin*dIII. The E7 fragment was gel purified and cloned into *Sma*I- and *Hin*dIII-digested BSKSII. BSKS/HPV16-E7 (nt 549 to 875) was constructed by cloning the *Sal*I-*Eco*RI fragment of pJ6Ω-HPV16-E7 (kindly provided by M. Nasseri) into the *Sal*I and *Eco*RI sites of BSKSII. The RNA expression vector BSKS/CRPV-E7 (nt 1050 to 1373) was created by cloning the *Xho*I fragment of pMSGneo/CRPV-E7 (34) into the *Xho*I site of BSKSII.

Plasmid pEC113 contains the AdE2 promoter followed by sequences encoding the chloramphenicol acetyltransferase (CAT) gene (27). pAd2-E1 contains the complete E1 region of adenovirus type 2 (28) and was a gift from W. Dörfler. Plasmids pWTE6, pE7, and pEC113 were a gift from J. T. Schiller, and plasmid pT24-ras was a gift from E. Ruley.

Rodent cell cultures and transformation assay. The maintenance and transfection of C127 and NIH 3T3 cells as well as the selection procedure by G418 were done as previously described (24). Pooled drug-resistant colonies were seeded into 0.3% agarose (type VII; Sigma) at a density of 10^5 cells per 60-mm dish, and colonies consisting of more than 16 cells were scored after 2 to 3 weeks by using photographic images of the cultures.

Immortalization assay. Human keratinocytes were cultured from newborn foreskin explants and grown in KGM medium (Clonetics, San Diego, Calif.). Cells were transfected at passage 3 with 10 to 15 μg of plasmid DNA in 0.5 ml of KBM (Clonetics) together with 50 μl of lipofectin (Bethesda Research Laboratories) (2). After approaching confluence, the cultures were split at a ratio of 1:2, and selection with 100 μg of G418 per ml was carried out for 3 days. Cultures were fed twice per week and split as they approached confluence. To

look for serum resistance, the transfected cells were split at a ratio of 1:8 and kept in KGM medium. After the appearance of large colonies, the medium was exchanged for KGM containing 10% fetal calf serum and 2.0 mM Ca^{2+} to select for proliferating dedifferentiated colonies (2). In a different approach, primary keratinocytes were transfected with 3 μg of E6 or E7 expression vector plasmid and 3 μg of an expression vector plasmid for an activated *ras* oncogene (pT24-ras) with lipofectin. After transfection, cells were grown in E medium (supplemented with epidermal growth factor at 5 ng/ml) together with mitomycin C-treated 3T3 feeders (40) and split at a ratio of 1:3 as they approached confluence.

Analysis of DNA and RNA. Cellular DNA extraction and Southern blot analysis were done as previously described (24). RNA was isolated from whole cells by the guanidium hydrochloride method. Detection of specific viral RNA was monitored by RNase protection analysis. To generate the respective antisense RNA probes, we used the following RNA expression vectors: pBS1-6, pBS1-7, pBS8-6, pBS8-7, pBSCE6 (nt 317 to 709 of CRPVE6), pBSCE7 (nt 1063 to 1186 of CRPVE7 [57]), and pBS16E6/E7 (nt 7766 to 1310 of HPV16 [5]). Briefly, 20 μg of total RNA was hybridized with 250,000 cpm of antisense ^{32}P-labeled riboprobes under the conditions described previously (26). The hybrids were digested with RNase A (6 μg/ml) and RNase T_1 (14 U/ml) for 1 h at 37°C. Digests corresponding to 50% of the hybridization mixture were analyzed on denaturing acrylamide gels. Autoradiography of the dried gels was carried out at −70°C with amplifying screens.

Preparation of HL-60 extracts. HL-60, a human promyelocytic cell line (ATCC CCL 240), was obtained from the American Type Culture Collection (Rockville, Md.) and maintained in RPMI supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), and amphotericin B (25 μg/ml). The cells do not express p53 protein (55) and can be induced to differentiate and express high levels of underphosphorylated *Rb* protein (35). Myeloid differentiation was induced according to a protocol from D. Smotkin (unpublished). Cells were split 1:2 in fresh medium, and 5 μM retinoic acid was added. The cells were harvested after 72 h and lysed in a buffer consisting of 0.25 M NaCl, 0.1% Nonidet P-40, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0), 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (1 μg/ml), and leupeptin (1 μg/ml) (11). Debris was removed by centrifugation at 10,000 × *g* for 10 min, and the extracts were stored at −70°C.

Transcription and translation of E7. The E7 ORFs were transcribed from the recombinant pBS or BSKSII vectors with T7 or T3 polymerase and capped by using the mRNA capping kit from Stratagene. After DNase digestion and phenol-chloroform extraction, the RNA was purified on 1-ml Sephadex G-50 spin columns. The RNAs were translated in a rabbit reticulocyte lysate system (Promega) with 4 μg of RNA per 100 μl of in vitro translation mixture containing [^{35}S]cysteine (Amersham). Incorporation of label into the E7 proteins was determined by trichloroacetic acid precipitation. After translation, aprotinin was added to 0.01% and PMSF was added to 0.4 mM, and the in vitro translation mixtures were stored at 4°C overnight.

Coprecipitation of E7 and *Rb*. Portions (250 μl) of extracts from differentiated HL-60 cells were mixed with 500,000 cpm of in vitro-translated E7 protein and incubated at 4°C. After 2 h, 0.2 μg of anti-*Rb* antibody (Ab 245; Bethesda) or 0.2 μg of anti-p53 antibody (Ab-4; Oncogene Science) was added, and incubation was continued for 1 h. This was followed by 1 h of incubation with 1.16 μg of rabbit anti-mouse immunoglobulin G (IgG; Cappel) and a 1-h incubation with swollen protein A-Sepharose CL-4B beads (Sigma Chemical Co.). The beads were washed, and the proteins were resolved on a 13% polyacrylamide–sodium dodecyl sulfate (SDS) gel and detected by fluorography. For quantitative analysis, the E7 bands were cut out from the gel and counted in a scintillation counter.

CAT assay. NIH 3T3 and CV1 cells were used for CAT assays. Cells were plated in 100-mm dishes and 24 h later were transfected with 20 μg of pEC113 plus 20 μg of the different E6 or E7 expression vectors or 10 μg of pAd2-E1 as a positive control. At 48 to 72 h posttransfection, cells were scraped off the plates, washed in 40 mM Tris-HCl (pH 7.5)–1 mM EDTA–150 mM NaCl, and lysed by repeated freezing and thawing in 0.25 M Tris-HCl (pH 8.0). CAT assays were performed with [^{14}C]chloramphenicol and unlabeled acetyl coenzyme A as described by Gorman et al. (14).

RESULTS

Detection of transforming activity. To compare the transforming potential of the E6 and E7 genes of HPV1, HPV8, and CRPV, we constructed the respective expression vectors and transfected C127 cells with the recombinant plasmids. Cotransfections of the expression vectors pWTE6 and pE7 (for HPV16 E6 and E7, respectively) together with pSV2Neo served as a positive control. Three cell lines were established for each construct from individual transfection experiments by selecting for G418 resistance and pooling drug-resistant colonies. Southern blot analysis of DNA from all cell lines revealed the presence of integrated constructs at a low copy number. To test for the integrity of the pZip recombinant constructs, total DNA was cleaved with *Xba*I, which cuts in both long terminal repeats. This resulted in fragments consisting of the full-length insert linked to adjacent vector sequences. To examine the integrity of the inserted E6 and E7 sequences, the inserts were excised by cleavage with *Bam*HI for pCE7, pCE6, pCLE6, pCSE6, pWTE6, and pE7; with *Kpn*I for pZ1-6; and with *Eco*RI for pZ1-7. The detection of bands of the correct sizes indicated that both the transcription unit and the E6 or E7 insert had remained intact (data not shown). Transcription of the viral sequences in each cell line was evaluated by RNase protection analysis. Total cellular RNA was hybridized to ^{32}P-labeled antisense RNA probes derived from the respective E6 and E7 genomic regions. The autoradiogram (Fig. 1) shows bands of the expected sizes, and this demonstrated that E6 or E7 was being transcribed in the cell lines. The viral transcription pattern of the pI86- and pI87-bearing cell lines was described previously (24).

Phase contrast microscopy show that nonstained living C127 cell lines transfected with pZip, pI87 (data not shown), pCE6, and pCLE6 did not exhibit an altered morphology and showed the "cobblestone" appearance of the parental C127 cells (Fig. 2). All other cell lines exhibited typical signs of morphological transformation, with an irregular arrangement of the cells. Cell lines with constructs pCSE6, pCE7, and pZ1-6 appeared, however, less heavily transformed than cell lines containing expression vectors for HPV16 E6, HPV16 E7, HPV8 E6, and HPV1 E7 (Fig. 2 and Table 1). This morphological transformation of C127 cells could be reproduced in three independent assays.

To further characterize the transforming properties of the individual E6 and E7 proteins, they were tested for the induction of anchorage-independent growth in C127 and NIH 3T3 cells. Single cells of pooled drug-resistant colonies were seeded in 0.3% agarose, and the number of clones consisting of more than 16 cells was counted after 2 to 3 weeks. In general,

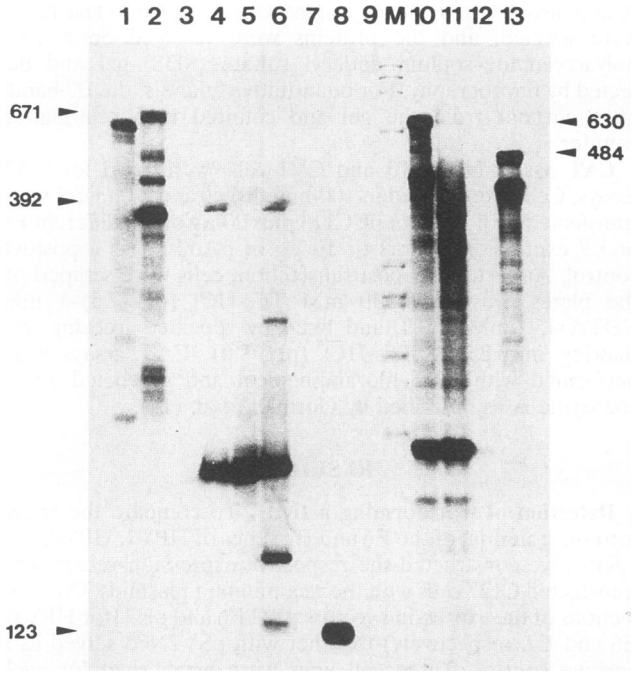

FIG. 1. RNase protection analysis of total RNA from C127 cell lines transfected with E6 and E7 expression vectors. Total RNA (40 μg) from C127 cell lines established with pWTE6 (lane 1), pE7 (lane 2), pCE6 (lane 4), pCSE6 (lane 5), pCLE6 (lane 6), pCE7 (lane 8), pZ1-6 (lanes 10 and 11), and pZ1-7 (lane 13) was hybridized to the ^{32}P-labeled antisense RNA probes pGEM16E6/7 (lanes 1 to 3), pBSCE6 (lanes 4 to 7), pBSCE7 (lanes 8 and 9), pBS1-6 (lanes 10 to 12), and pBS1-7 (lane 13). As a control, each probe was hybridized with *Escherichia coli* tRNA (lanes 3, 7, 9, and 12). The products of digestion with RNase A and RNase T_1 were analyzed on a 5% denaturing polyacrylamide gel. Arrows indicate the positions of the expected resulting fragment sizes. The sizes (in base pairs) of the labeled DNA molecular size markers (lane M) are as follows: 1,353, 1,078, 872, 603, 310, 281, 271, 234, and 194 kDa.

the expression of all E7 proteins, except for HPV8 E7, resulted in higher cloning efficiencies in NIH 3T3 cells than in C127 cells. No significant difference between the two cell lines could be noted, however, in their response to the different E6 proteins. All cells which expressed a full-length E6 protein revealed detectable colony-forming abilities in soft agarose (Fig. 3 and 4 and Table 1). The highest cloning efficiencies were exhibited by cells expressing HPV8 E6, HPV16 E6, and CRPV E6, and moderate efficiencies were exhibited by cells expressing HPV1 E6 and CRPV short E6. Cells harboring E7 expression vectors could be clearly divided into two groups. C127 and NIH 3T3 cells expressing HPV8 E7 did not produce significantly higher colony numbers than the parental cells transfected with the vector pZip alone. In contrast, C127 and NIH 3T3 cells with expression vectors for HPV16 E7 and HPV1 E7 revealed very high cloning efficiencies in soft agarose (Fig. 3 and 4). A dramatic difference could be noted in the susceptibility of the different rodent cell lines to transformation by CRPV E7. Although expression of CRPV E7 did not induce anchorage-independent growth in C127 cells, it led to a high percentage of colony formation in NIH 3T3 fibroblasts.

High *Rb*-binding activity of HPV1 E7. To investigate whether the unexpectedly high transforming potential of HPV1 E7 is related to a high binding affinity for *Rb*, we compared the individual in vitro *Rb*-binding affinities of HPV1 E7, HPV6 E7, HPV8 E7, HPV16 E7, and CRPV E7. In vitro-synthesized RNA encoding the respective E7 proteins was translated in rabbit reticulocyte lysates in the presence of ^{35}S-labeled cysteine. To allow a quantitative measurement of the relative *Rb*-binding affinities, we incubated 500,000 cpm of labeled E7 proteins with 250 μl of *Rb*-containing protein extracts from HL-60 cells. *Rb*-E7 complexes were immunoprecipitated with an antiserum against *Rb*, denatured, electrophoresed by 13% PAGE–SDS, and fluorographed (Fig. 5). Immunoprecipitations with anti-p53 served as negative controls. The E7 protein bands were cut out from the gel, and a scintillation counter was used to quantitate the amount of E7 proteins complexed with *Rb*; the values obtained were normalized to the number of cysteine molecules present in each E7 protein. Relative to binding of HPV16 E7 (100%), *Rb* bound 66% of HPV1 E7, 34% of HPV8 E7, 22% of HPV6 E7, and 11% of CRPV E7.

Detection of *trans*-activating properties. The AdE2 promoter has been shown to be responsive to *trans* activation by several viral oncoproteins, including HPV16 E6 and E7. HPV16 E7 is thought to stimulate the E2 promoter by activating the transcription factor E2F sequestered in a complex with the *Rb* protein (37); the mechanism for E6 *trans* activation, however, is still unknown.

To determine the *trans*-activating properties of the different E6 and E7 proteins of HPV1, HPV8, and CRPV, we cotransfected NIH 3T3 and CV1 cells with the respective expression vectors and pEC113, which contains the AdE2 promoter 5′ of the CAT gene. As a positive control, we used HPV16 E6 and E7 expression vectors (pWTE6 and pE7, respectively) and a plasmid expressing the whole E1 region of Ad2 (pAd2-E1). A number of experiments performed with CV1 and NIH 3T3 cells revealed no differences in the transactivating properties of the constructs between the different cell lines. The results of the *trans* activation experiments in NIH 3T3 cells are compiled in Fig. 6. Cotransfections with expression vectors for HPV16 E6 and E7 and Ad2-E1 consistently resulted in higher levels of CAT activity than cotransfections with all other expression vectors (Fig. 6). Most surprisingly, in spite of a high *Rb*-binding affinity, HPV1 E7 did not significantly activate transcription from the AdE2 promoter. In contrast, the E7 proteins of HPV8 and CRPV, exhibiting lower in vitro binding affinities for *Rb*, *trans* activated the AdE2 promoter, although at a low rate. Among the E6 proteins, HPV16 E6 *trans* activated the AdE2 promoter most strongly, followed by CRPV long E6, but CRPV short E6 and HPV8 E6 revealed no *trans*-activating properties.

Immortalization of primary keratinocytes. Immortalization studies were carried out with human foreskin keratinocytes at passage 3. Primary cultures were established in KGM medium (Clonetics) and cotransfected pairwise with one of the expression vectors for E6 and one of the expression vectors for E7 (Table 2). The transfected cells were further selected for the expression of a cotransfected G418 resistance marker (Table 2) or the ability to resist serum-induced terminal differentiation (2). Both procedures yielded comparable results. In a different approach, we cotransfected keratinocytes with an expression vector for an activated *ras* oncogene (pT24-ras) and expression vectors for either E6 or E7. After transfection, these cultures were continuously passaged in E medium with mitomycin C-treated 3T3 feeder cells to look for the appearance of indefinitely growing cultures without selective pressure (Table 2).

In agreement with earlier findings, none of the E6 expression vectors, when transfected alone or together with pT24-ras, increased the proliferative capacity of the keratinocytes (2, 45).

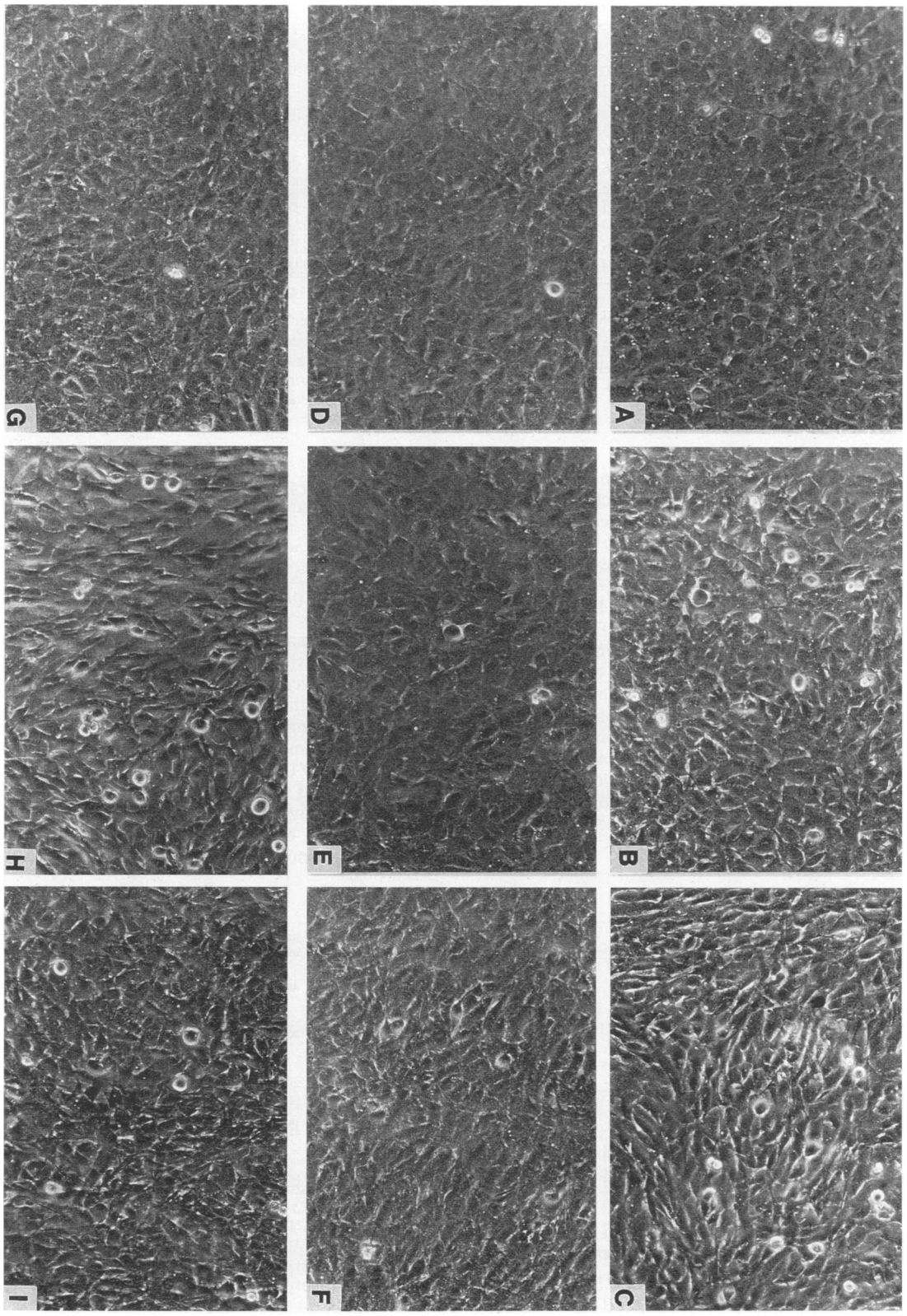

FIG. 2. Morphology of C127 cell lines. C127 cell lines established after transfection with pZip (A), pZ1-6 (B), pZ1-7 (C), pCE6 (D), pCLE6 (E), pCSE6 (F), pCE7 (G), pWTE6 (H), and pE7 (I) observed by phase contrast microscopy.

TABLE 1. Transformation of rodent cell lines

Plasmid	Transformation of C127 cells[a]	Anchorage-independent growth induction[b] (%)	
		C127 cells	3T3 cells
HPV1 E6	++	10.7	13.8
HPV1 E7	++++	22.4	32.9
HPV8 E6	++++	17.3	17.2
HPV8 E7	−	3.3	3.2
HPV16 E6	++++	16.3	15.6
HPV16 E7	++++	26.4	32.6
CRPV E6	−	15.8	14.2
CRPV long E6	−	4.3	ND[c]
CRPV short E6	+	9.7	ND
CRPV E7	+/−	6.4	31.3
pZip	−	3.2	4.5

[a] The morphological transformation of C127 cells was assessed by phase contrast microscopy. +, ++, +++, and ++++, weakly to strongly positive; +/−, marginal; −, negative.

[b] The cloning efficiency is given as the average value for at least six individual experiments and represents the percentage of seeded cells that formed colonies of more than 16 cells in soft agarose after 2 to 3 weeks.

[c] ND, not determined.

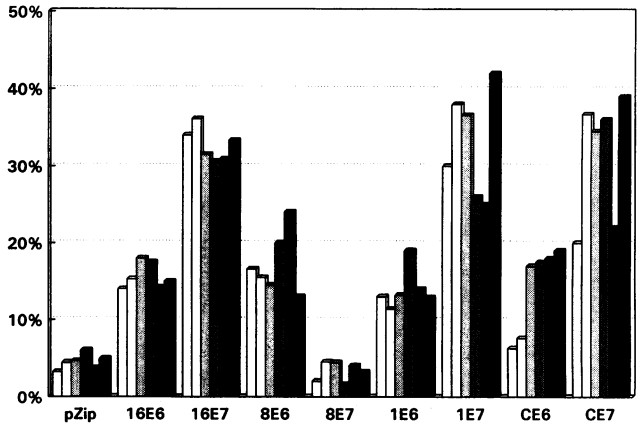

FIG. 4. Anchorage-independent growth of NIH 3T3 cells transfected with the different E6 and E7 expression vectors. The cloning efficiency is given as the percentage of seeded cells that formed colonies of more than 16 cells after 2 to 3 weeks. Each bar represents the average value from two culture dishes analyzed in the same experiment.

All of the cultures transfected with the E6 and E7 genes of HPV16 and half of the cultures cotransfected with pT24-ras and HPV16 E7 produced immortalized lines. In contrast, the E6 and E7 genes of HPV1, which showed remarkable transforming activities in rodent cell lines, were unable to induce immortalization of human keratinocytes. A weak induction of proliferation could be observed with HPV8 E7 and HPV16 E7 alone, but not with CRPV E7 and HPV1 E7. However, only HPV16 E7 was able to efficiently complement the homologous E6 gene in the immortalization assay. Cells cotransfected with pT24-ras and the different expression vectors for E6 and E7 showed no differences in their growth capacities versus cells transfected with the E6 or E7 expression plasmids alone. HPV16 E7 cotransfection with an activated *ras* oncogene resulted in four lines of keratinocytes, which have been cultured for 8 months, up to passage number 32. Karyotype analysis confirmed the human male origin of these immortalized cultures, and Southern blot analysis with total DNA revealed the presence of the integrated expression vectors for HPV16 E7 at a low copy number (data not shown).

DISCUSSION

Different properties of the E6 and E7 proteins of high-risk and low-risk cutaneous papillomaviruses, affecting preferentially the nonmucosal epithelium, were investigated by expressing the individual proteins in cultured cells with a retroviral expression vector or with an in vitro translation system. The analysis of the capacity to transform C127 cells and induce anchorage-independent growth in NIH 3T3 and C127 cells revealed transforming potential for the E6 proteins of HPV1, HPV8, HPV16, and CRPV and for the E7 proteins of HPV1, HPV16, and CRPV. Morphologic transformation, however, did not always correlate with anchorage-independent growth. Thus, C127 cells expressing CRPV E6 exhibited no transformed phenotype, but 16% of the cells seeded in soft agarose

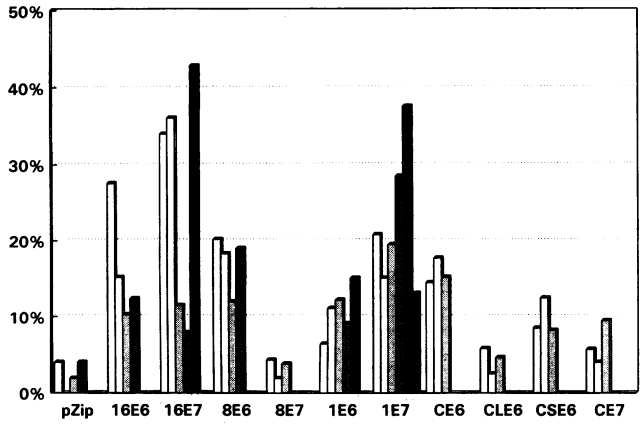

FIG. 3. Anchorage-independent growth of C127 cells transfected with the different E6 and E7 expression vectors. The cloning efficiency is given as the percentage of seeded cells that formed colonies of more than 16 cells after 2 to 3 weeks. Each bar represents the average value from three culture dishes analyzed in the same experiment.

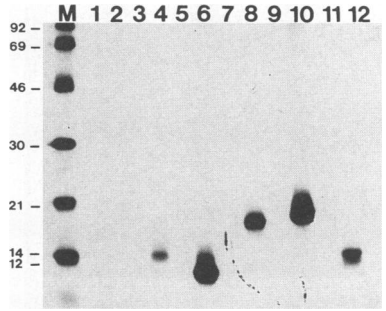

FIG. 5. Immunoprecipitation of in vitro-translated E7 proteins with an anti-*Rb* antibody. A total of 500,000 cpm of the ^{35}S-labeled E7 proteins of CRPV (lanes 3 and 4), HPV1 (lanes 5 and 6), HPV8 (lanes 7 and 8), HPV16 (lanes 9 and 10), and HPV6 (lanes 11 and 12) were incubated with extracts of differentiation-induced HL-60 cells containing underphosphorylated *Rb* protein and immunoprecipitated with antibodies against p53 (lanes 1, 3, 5, 7, 9, and 11) or *Rb* (lanes 2, 4, 6, 8, 10, and 12). CRPV long E6 (lanes 1 and 2) served as a negative control. The sizes of the labeled molecular size markers in lane M are indicated (in kilodaltons) on the left.

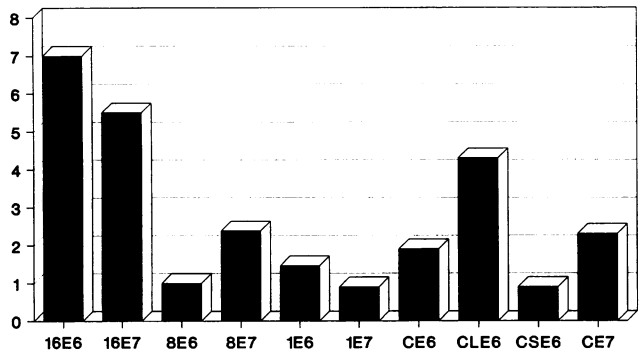

FIG. 6. *trans* activation of the AdE2 promoter by E6 and E7 expression vectors. Fold transactivation is calculated relative to the activity of pEC113 alone. Each bar represents the average value for 10 independent transfection experiments with NIH 3T3 cells. Typical *trans* activation values for the positive control pAd2-E1 were in the range of 21- to 34-fold. The standard deviations (σ) for each average value were as follows: 16E6, $\sigma = 0.6$; 16E7, $\sigma = 1.5$; 8E6, $\sigma = 0.1$; 8E7, $\sigma = 0.4$; 1E6, $\sigma = 0.3$; 1E7, $\sigma = 0.1$; CE6, $\sigma = 0.5$; CLE6, $\sigma = 1.2$; CSE6, $\sigma = 0.3$; and CE7, $\sigma = 0.3$.

gave rise to growing colonies. Because ORF E6 of CRPV encodes a long E6 protein and a short E6 protein, which differ in several properties (4), the use of specific expression vectors for long E6 (pCLE6) and short E6 (pCSE6) (34) indicated that the short E6 protein was the more important one conferring anchorage-independent growth. This result is in agreement with those of Meyers et al. (34), who used NIH 3T3 cells and also identified short E6 as the stronger transforming protein.

In summary, the transforming activity of the different E6 genes for rodent fibroblasts, measured by the cloning efficiencies in soft agarose, correlated with the postulated risk of malignant behavior of the parent papillomaviruses. Clearly, it did not correlate with the ability to bind p53; HPV8 E6, CRPV E6, and the transforming E6 protein of BPV1 do not bind p53 in vitro (17, 48, 52). The results thus show that the transforming activity of the E6 proteins is independent of the ability to bind p53. A separation of the two functions, binding of p53 and transformation of cells, appears also to exist for HPV16 E6. This is indicated by the finding that mutant p53 can substitute for HPV16 E6 in the immortalization of human keratinocytes but not in the transformation of fibroblasts (46). In this regard, transformation of established cell lines differs from immortalization of primary human keratinocytes, where the ability of E6 to bind to and degrade p53 appears to be important. In contrast to the cooperativity of E6 with E7 in the immortalization of primary human keratinocytes, which depends on the ability to bind to and degrade p53 (19, 46), the transforming potential in rodent cells thus could represent a common p53-independent function of the E6 proteins conserved among distantly related papillomaviruses.

TABLE 2. Immortalization of primary human keratinocytes: cooperation between expression vectors

Plasmid	No. of proliferating[a] cultures/no. of transfections					
	pZip	HPV1 E7	HPV8 E7	HPV16 E7	CRPV E7	pT24-ras
pZip	0/4	0/6	1/6 (s)	2/6 (s)	0/6	ND[b]
HPV1 E6	0/6	0/6	0/6	1/6 (s)	0/6	0/6
HPV8 E6	0/6	0/6	1/6 (s)	2/6 (s)	0/6	0/6
HPV16 E6	0/6	0/6	1/6 (s)	6/6	1/6	0/6
CRPV E6	0/6	0/6	0/5	1/6 (s)	1/6	0/6
pT24-ras	0/6	0/6	1/6	4/10 (h)	0/6	0/5

[a] s, slowly proliferating; h, highly proliferating.
[b] ND, not done.

The transforming activity of the different E7 proteins tested in C127 cells correlated with their ability to efficiently form a complex with the *Rb* protein in vitro. HPV1 E7 and HPV16 E7 bound *Rb* with high affinity, strongly transformed C127 cells, and greatly enhanced anchorage-independent growth. In contrast, the E7 proteins of CRPV and HPV8, which bound to *Rb* with 11 and 34% of the affinity of HPV16 E7, respectively, had little or no effect on C127 cells. The absence of an effect of CRPV E7 on C127 cells is in contrast to our results with NIH 3T3 cells and to a study reported previously (34). Although CRPV E7 did not induce morphological transformation or anchorage-independent growth in C127 cells, it was highly transforming in NIH 3T3 cells. Most likely, this reflects a difference between the two rodent cell lines in their response to the expression of CRPV E7. Such differences in the degree of transformation between NIH 3T3 and C127 cells in response to transforming proteins of other papillomaviruses have already been reported (24, 43).

Earlier experiments with COS 7 cells did not allow the detection of stable complexes of HPV8 E7 with the *Rb* protein (26). The high sedimentation rate of HPV8 E7 in nondenaturing glycerol gradients, which was similar to that of CRPV E7 and HPV6 E7 (3, 12), pointed to an interaction with other proteins. One possibility would be that in COS 7 cells, these E7 proteins preferentially bind to p107 rather than *Rb*.

In spite of its high *Rb*-binding affinity, E7 of HPV1 did not activate transcription from the AdE2 promoter when tested in cotransfection experiments in NIH 3T3 and CV1 cells. Recently published findings show that the transcription factor E2F and HPV16 E7 bind to separate sites on the *Rb* protein and that peptides that bind to *Rb* do not necessarily disrupt the *Rb*-E2F complex (21, 56). This suggests that HPV1 E7 bound to *Rb* does not overlap or only minimally overlaps the E2F binding site. Some overlap might be suggested by experiments in which HPV1 E7 was highly overexpressed and did stimulate the AdE2 promoter in 3Y1 cells (51). The situation with the E7 proteins of high-risk HPV8 and CRPV is different. These proteins did not bind to *Rb* as well as HPV1 E7 but still *trans* activated the AdE2 promoter to a low level. This may indicate that these proteins, when bound to *Rb*, interfere with E2F binding more extensively than HPV1 E7 does. Alternatively, activation of the AdE2 promoter could be mediated through disruption of the p107-E2F complex (6, 44). The high *Rb*-binding affinity of CRPV E7 observed by others with a truncated in vitro-translated *Rb* protein (18) additionally suggests that sequences outside the core E7 binding site on *Rb* influence *Rb*-E7 interactions.

There was no correlation between the *trans*-activating properties of the E6 genes and the carcinogenic potential of the corresponding papillomavirus types. The long E6 protein of CRPV and E6 of HPV16 significantly stimulated the AdE2 promoter, whereas HPV1 and HPV8 E6 showed no such activity. The absence of *trans* activation function in the short E6 protein of CRPV suggests that this function may be encoded by the amino-terminal domain of the long E6 protein of CRPV. This domain contains a 36-amino-acid-long sequence with a central histidine, a structure which is conserved in the long E6 protein of CRPV and E6 of HPV16 but not in HPV8 E6 (23).

Interestingly, none of the E6 or E7 proteins of the cutaneous papillomaviruses revealed true immortalizing abilities in primary human keratinocytes. Only a weak induction of prolifer-

ation could be observed with HPV8 E7 alone, and complementation assays identified at best a very low degree of cooperativity between CRPV E6 and CRPV E7. This suggests that immortalization by E7 does not correlate with a high risk for cancer development for all papillomaviruses, as it does for the genital human papillomaviruses. Clearly, the pathway toward malignancy differs for the different high-cancer-risk papillomaviruses. This notion is also supported by the finding that E7 of HPV8 does not exhibit transforming properties; however, this virus contains another, not yet clearly defined transforming activity located within the 3′ part of the early region of the HPV8 genome (25, 39).

In summary, this investigation has shown that the transforming activity of different E7 proteins in rodent cell lines and the *Rb*-binding affinity in vitro do not correlate with the carcinogenic potential of the corresponding papillomaviruses. The observed differences in p53 and *Rb* binding and in immortalizing potential between high-risk genital and high-risk cutaneous papillomaviruses suggest different strategies of oncogenesis for these two virus groups.

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