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# In Vitro Biological Activities of the E6 and E7 Genes Vary among Human Papillomaviruses of Different Oncogenic Potential

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**Human papillomavirus type 16 (HPV-16) and HPV-18 are often detected in cervical carcinomas, while HPV-6, although frequently present in benign genital lesions, is only rarely present in cancers of the cervix. Therefore, infections with HPV-16 and HPV-18 are considered high risk and infection with HPV-6 is considered low risk. We found, by using a heterologous promoter system, that expression of the E7 transforming protein differs between high- and low-risk HPVs. In high-risk HPV-16, E7 is expressed from constructs containing the complete upstream E6 open reading frame. In contrast, HPV-6 E7 was efficiently translated only when E6 was deleted. By using clones in which the coding regions of HPV-6, HPV-16, and HPV-18 E7s were preceded by identical leader sequences, we found that the ability of the E7 gene products to induce anchorage-independent growth in rodent fibroblasts correlated directly with the oncogenic association of the HPV types. By using an immortalization assay of normal human keratinocytes that requires complementation of E6 and E7, we found that both E6 and E7 of HPV-18 could complement the corresponding gene from HPV-16. However, neither E6 nor E7 from HPV-6 was able to substitute for the corresponding gene of HPV-16 or HPV-18. Our results suggest that multiple factors, including lower intrinsic biological activity of E6 and E7 and differences in the regulation of their expression, account for the low activity of HPV-6, in comparison with HPV-16 and HPV-18, in in vitro assays. These same factors may, in part, account for the apparent difference in oncogenic potential between these viruses.**

Human papillomaviruses (HPV) are small DNA viruses which infect squamous epithelia. Sixty different HPV genotypes have been identified, and all seem to be associated with distinct anatomic sites (8). A subset of these HPVs are found most commonly in genital and mucosal lesions, with HPV type (HPV-6), HPV-11, HPV-16, and HPV-18 being present most frequently. Within the genital-mucosal HPV types, some appear to have greater oncogenic potential. HPV-16 and HPV-18 are found in a majority of cervical cancers (36). HPV-6 and HPV-11 are the predominant types in benign condylomas but are only rarely found in carcinomas (36). The latter two HPVs are therefore considered low-risk types, while HPV-16 and HPV-18 are high-risk types. The putative oncogenicity of high-risk HPV types, such as HPV-16 and HPV-18, is reflected in vitro by their abilities to transform established cell lines and immortalize primary rodent cells and human keratinocytes (4, 9, 15, 17, 20, 22, 26, 34). Low-risk HPV types have been inactive in these assays (5, 20, 26, 34).

Further examination of cervical tumors and cell lines derived from them has suggested that the E6/E7 region of the HPV genome is important in cervical carcinogenesis, since tumors and cell lines preferentially transcribe the E6 and E7 genes (27, 29). The apparent significance of these findings has been further strengthened by analysis of the HPV genes responsible for in vitro transformation. The E6/E7 region of HPV-16 and HPV-18 has been found to be sufficient for immortalization of human keratinocytes (2, 15), with both genes being required under standard assay conditions (12, 13, 18). In bioassays that use murine cells, such as transformation of primary (7, 21) and established murine cells, the E7 open reading frame (ORF) seems to be primarily responsible for the transforming activity (32, 35).

A potentially important difference between low- and high-risk HPVs is the manner in which E6 and E7 transcription is regulated (6, 28). In high-risk HPVs, the two ORFs are transcribed from a single promoter; through differential splicing, this primary transcript gives rise to three mRNA species which together could encode full-length E6 and E7, as well as two truncated forms of E6. In HPV-6 and HPV-11, the E6/E7 region lacks such splicing; it is not clear whether the E7 ORF is translated from a polycistronic mRNA that also contains the upstream E6 ORF (6) or from a second mRNA initiated from an apparent promoter located within the E6 ORF (28). In addition, HPV-6 subtypes isolated from carcinomas invariably show alterations in the upstream regulatory region which may affect expression of the E6 and E7 genes (14, 23).

Therefore the greater biological activities observed for high-risk HPV types might be due to differences in expression of their E6 and/or E7 genes, differences in the intrinsic potency of their E6 and/or E7 protein products, or a combination of these factors. In the present study, we explored the relative importances of these factors. We conclude that each of these parameters contribute to the greater in vitro biological activity of high-risk HPV types.

## MATERIALS AND METHODS

**LTR-activated constructions.** pHa.16.E6/7 has been described by Vousden et al. (32), who named it p16HH. This clone has the HPV-16 *Hae*III fragment (nucleotides [nt] 7765 to 1952) inserted at the *Hin*dIII site of pML2 (16). It also contains the 600-bp permuted Harvey murine sarcoma virus long terminal repeat (LTR) inserted in the promoter-positive orientation at the *Eco*RI site. For construction of HPV-6 clones, the HPV-16 sequences were removed by digestion

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with *Cla*I and *Sph*I or *Nru*I. The *Nla*IV-to-*Sph*I (nt 34 to 1399), *Tha*I-to-*Sph*I (nt 473 to 1399), and *Nla*IV-to-*Dra*I (nt 34 to 672) fragments of HPV-6 were inserted to give pHa.6.E6/7, pHa.6.E7, and pHa.6.E6, respectively.

HPV E7 isogenic constructions were made by first replacing the Moloney murine sarcoma virus LTR with the Harvey murine sarcoma virus LTR in the previously described p16E7Mo (10). This clone consists of pUC19 carrying the Harvey murine sarcoma virus LTR at the *Eco*RI site and the *Taq*I-to-*Ssp*I (nt 505 to 1176) fragment of HPV-16 inserted at the *Bam*HI site via the appropriate linkers. The HPV-16 E7-coding sequences were removed by digestion with *Nsi*I (nt 566 of HPV-16) and *Hin*cII (nt 429 of pUC19) to give the pHa.16δ portion of the clones. The HPV-18 *Nsi*I-to-*Rsa*I (nt 594 to 931) and HPV-6 *Nsi*I-to-*Ssp*I (nt 534 to 996) fragments that encode the respective E7 proteins were inserted into pHa.16δ to give pHa.16δ.18E7 and pHa.16δ.6E7, respectively.

HPV E6 clones were constructed by replacing the *Bam*HI E7 fragments of pHa.16E7 with HPV-6 *Dra*I (nt 21 to 673), HPV-16 *Dde*I (nt 24 to 654), and HPV-18 *Ava*II (nt 57 to 597) fragments to give pHa.6E6, pHa.16E6, and pHa.18E6, respectively.

**Cell cultures and transformation assays.** NIH 3T3 cells obtained from the American Type Culture Collection (Rockville, Md.) were grown in Dulbecco modified Eagle medium and 10% newborn calf serum. Cells were seeded at $3 \times 10^5$/35-mm-diameter dish 24 h before transfection. Cotransfection of 0.5 μg of test plasmid and 0.1 μg of pSV2Neo was done by calcium phosphate precipitation for 6 h. The cells from each transfection were split at a 1:5 ratio into two 100-mm-diameter dishes 24 h later, and selective medium containing 500 μg of G418 per ml was added 24 h afterward. Upon selection for ~10 days, drug-resistant colonies were pooled and at passage 2 they were seeded into 0.4% agar at a density of $10^5$/60-mm-diameter dish. Individual transfections were scored for colony formation after 3 weeks. Colonies with more than 8 to 16 cells were counted.

**Immortalization of normal human foreskin keratinocytes.** Normal human foreskin keratinocytes (passage 3) were grown in KGM medium (Clonetics, San Diego, Calif.) and transfected in 60-mm-diameter dishes when 50% confluent. Plasmid DNA (10 to 15 μg) was added to 1.5 ml of KBM (Clonetics), followed by 50 μl of lipofectin (BRL, Bethesda, Md.). After 10 min of incubation at room temperature, the solution was added to the keratinocytes and the dishes were incubated at 37°C in a $CO_2$ incubator for 3 h. A 3-ml volume of KGM medium was then added, and the cells were incubated overnight. The medium was aspirated, and the cells were fed with 4 ml of KGM medium. The cultures were split into two 100-mm-diameter dishes when they approached confluence. Cells selected for G418 resistance were cotransfected with 5 μg of pSV2Neo and selected with 100 μg of G418 per ml for 2 to 3 days (22). Cultures were fed three times per week and split as they approached confluence. Selection for serum resistance was performed as previously described (26).

**Metabolic labeling and immunoprecipitations.** Pooled cultures of G418-selected cells were labeled for 1 h with 1 mCi of [$^{35}$S]cysteine. Samples of $10^7$ cpm for each lysate were immunoprecipitated as previously described (3). Antisera specific for each of the HPV E7 proteins were complexed with protein A-Sepharose and equal portions were used for immunoprecipitation. After the immunocomplexes were washed, samples were boiled in standard sodium dodecyl sulfate (SDS)–β-mercaptoethanol buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

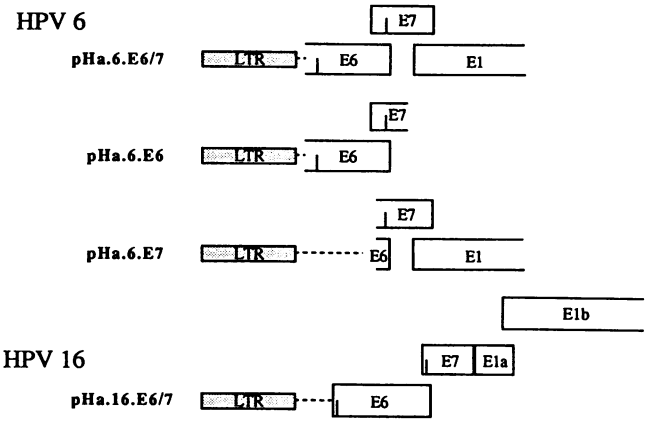


FIG. 1. HPV-6 and HPV-16 LTR-activated ORF constructs. HPV-6 E6/7 region (pHa.6.E6/7) and the individual E6 (pHa.6.E6) and E7 (pHa.6.E7) ORFs were cloned into a vector carrying the Harvey murine sarcoma virus LTR in the promoter configuration. The HPV-16 E6/7 region (pHa.16.E6/7) also cloned behind the Harvey murine sarcoma virus LTR has been previously described (32). ORFs are shown as open boxes, and vertical lines within the ORFs depict the locations of the first ATGs for E6 and E7.

## RESULTS

**Transformation by HPV6 E7.** The intact E6/E7 region from low-risk HPV types, either fused to a heterologous promoter or in the context of its own upstream regulatory region, has yielded negative results in several bioassays, each of which requires E7 (20, 26, 30, 34). Given the differences in transcriptional regulation of E6 and E7 by low- and high-risk HPV types, it seemed possible that the apparent lack of E7 activity in bioassays is secondary to inefficient expression of E7. Two plasmids were constructed to explore this possibility (Fig. 1). In one, the intact HPV-6 E6/E7 region was inserted downstream from the Harvey murine sarcoma virus LTR (pHa.6.E6/7). In the second, the E7 ORF was placed adjacent to the Harvey murine sarcoma virus LTR by removing 439 bp from the 5′ end of the HPV-6 fragment in the first plasmid, which removed almost all of the E6 ORF (pHa.6.E7). Placing E7 next to the LTR in pHa.6.E7 should facilitate expression of HPV-6 E7 and serve as a comparison for E7 expression from the plasmid containing the intact E6/E7 region.

The biological activities of the two HPV-6 plasmids were examined by testing their abilities to induce anchorage-independent growth of NIH 3T3 cells. In HPV-16, the E7 ORF is necessary and sufficient for this activity (32). A construct in which the intact HPV-16 E6/E7 region was located downstream from the Harvey murine sarcoma virus LTR (pHa.16.E6/7), so it was structurally analogous to pHa.6.E6/7, was included as a positive control. Following cotransfection of each plasmid into NIH 3T3 cells with a vector carrying the neomycin resistance gene and selection with G418, the mass cultures were tested for the ability to grow in agar. As expected, cells transfected with pHa.16.E6/7 gave rise to numerous colonies (Fig. 2, bottom). By contrast, transfectants with pHa.6.E6/7 (Fig. 2, top) failed to grow; they were indistinguishable from cells transfected with the vector carrying the LTR alone. On the

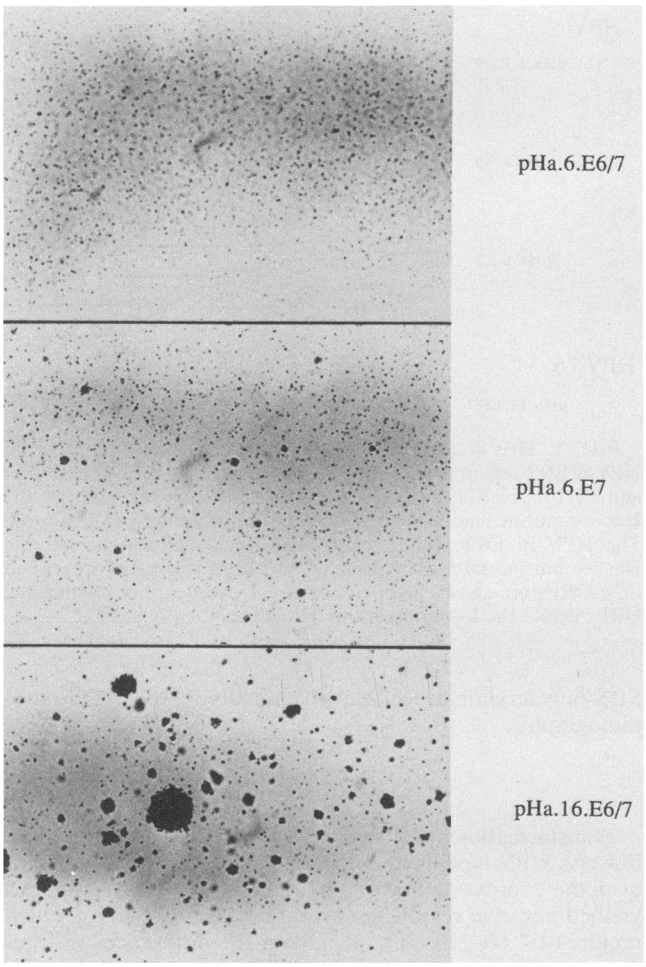


FIG. 2. Colony formation in agar. After cotransfection with pSV2Neo and selection in geneticin, mass cultures of geneticin-resistant cells were placed in agar. Photomicrographs were taken after 3 weeks. Colonies are shown for clones pHa.6.E7 and pHa.16.E6/7. As with cells transfected with pHa.6.E6 or pSV2Neo alone (data not shown), pHa.6.E6/7 showed no colonies. Clone pHa.16.E6/7 reproducibly gave rise to many more large colonies than did pHa.6.E7.

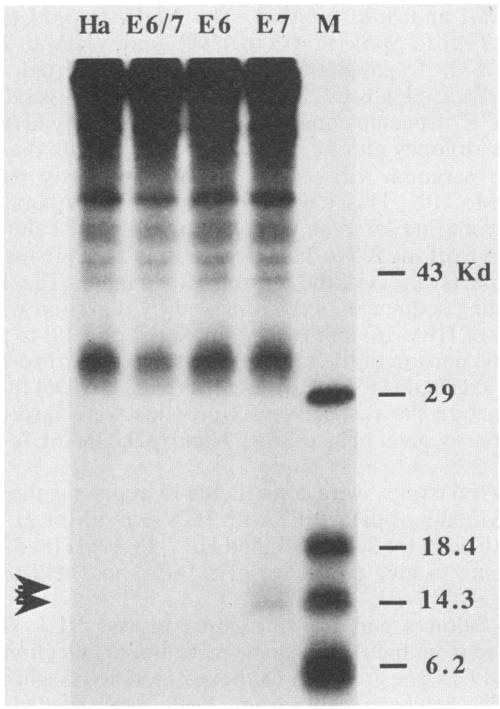


FIG. 3. Expression of HPV-6 E7 protein in neomycin-resistant cultures. Cells transfected with pHa (lane Ha), pHa.6.E6/7 (lane E6/7), pHa.6.E6 (lane E6), and pHa.6.E7 (lane E7) were metabolically labeled with [$^{35}$S]cysteine, and the lysates were immunoprecipitated with antibody raised against an HPV-6 E7 fusion protein expressed in bacteria (1). Lane M contained molecular mass standards; the sizes are indicated on the right (Kd, kilodaltons). The double band specific for HPV-6 E7 protein (arrowheads) was present only in the lane containing immunoprecipitation from the pHa.6.E7-transfected cell lysate.

other hand, pHa.6.E7 reproducibly gave rise to colonies in agar (Fig. 2, middle), although they were smaller and less numerous than colonies induced by pHa.16.E6/7.

These results indicated that HPV-6 E7 possessed some transforming activity. They also suggested that the failure of pHa.6.E6/7 to induce growth in agar was probably secondary to inefficient expression of E7. A less likely explanation for the negative results obtained with pHa.6.E6/7 might be that E6 suppressed the activity of E7 in *trans*. To eliminate the latter possibility, a plasmid that contained only the intact HPV-6 E6 ORF (pHa.6.E6) was constructed from pHa.6.E6/7 by deleting the 3'727 bp of HPV-6 sequences from pHa.6.E6/7. This plasmid expresses E6, as measured by its ability to *trans*-activate a heterologous promoter (27a), but does not induce growth in agar (data not shown). Cotransfection of pHa.6.E6 or pHa.6.E6/7 with pHa.6.E7 had no effect on the ability of pHa.6.E7 to induce growth in agar (data not shown).

Since E6 did not suppress E7 expression in *trans*, transfectants carrying the different plasmids were examined for the presence of the E7 gene product. Northern (RNA) blot analysis of RNAs extracted from cells transfected with the different clones, analyzed with an E7 probe, showed similar levels of HPV-6 E7 mRNAs (data not shown). To examine the transfectants for E7 protein, lysates from metabolically labeled cells carrying the different HPV-6 constructs were immunoprecipitated with antiserum raised against a TrpE–HPV-6 E7 bacterially expressed fusion protein (Fig. 3). An E7-specific double band (which represents phosphorylated and nonphosphorylated forms [1]) was found only in the lane containing the immunoprecipitate from cells transfected with the pHa.6.E7 clone (Fig. 3, lane E7). By contrast, no E7-specific bands were detected in lysates from cells carrying pHa.6.E6/7 (Fig. 3, lane E6/7). As expected, lysates from the analogous HPV-16 clone (pHa.16.E6/7), which was biologically active, gave a readily detectable E7-specific protein band when analyzed with an HPV-16 E7 antibody (data not shown). We conclude that the presence of the E6 ORF upstream of the E7 ORF in pHa.6.E6/7, but not in pHa.16.E6/7, severely reduced or completely abolished E7 translation, which implies that HPV-6 E7 is not efficiently translated from a polycistronic mRNA that also contains E6.

**Less activity by HPV-6 E7 than by E7s from HPV-16 and HPV-18.** The above-described biological experiments suggested that HPV-6 E7 was less active than HPV-16 E7. However, the non-E7 sequences unique to each of the


FIG. 4. Isogenic HPV E7 constructions. The HPV-16 E7-coding region under control of the Harvey murine sarcoma virus LTR was used as the prototype (pHa.16.E7). The HPV-16 E7-coding portion was replaced with HPV-18 E7 (pHa.16δ.18E7) or HPV-6 E7 (pHa.16δ.6E7), yielding clones capable of expressing the different E7 proteins from mRNAs with identical leader sequences. The *NsiI* site found in the ATGs of the E7s of all genital HPVs was used to fuse the E7-coding sequences of HPV-6 and HPV-18. Open boxes represent HPV-16 ORFs, vertical broken lines depict HPV-18 ORFs, and slanted broken lines illustrate HPV-6 sequences.

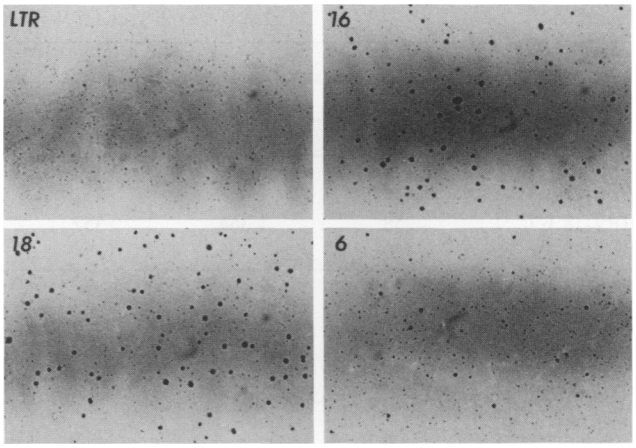


FIG. 5. Induction of growth in agar by isogenic constructs. Upon cotransfection with pSV2Neo and selection with geneticin, mass cultures were placed in agar at passage 2. Photomicrographs were taken after 3 weeks. No colonies were seen in plates with cells transfected with the Harvey murine sarcoma virus LTR-containing vector alone (panel LTR). Numerous colonies were seen in plates with cells transfected with pHa.16E7 (panel 16) or pHa.16δ.18E7 (panel 18). Colonies were reproducibly obtained with cells transfected with pHa.16δ.6E7 (panel 6), but there were consistently fewer than with the HPV-16 or HPV-18 E7 clone.

plasmids carrying HPV-6 and HPV-16 might have accounted for some of their differences in biological activity. To eliminate the possible contribution of different non-E7 sequences, E7 constructions that were more isogenic were generated by placing the E7 ORFs from HPV-6, HPV-16, and HPV-18 downstream from a common noncoding region (Fig. 4). Each of the isogenic constructs was transfected into NIH 3T3 cells, and the G418-selected mass cultures were tested for the ability to grow in agar (Fig. 5). The cells transfected with each of the E7-encoding constructs significantly increased the number of agar colonies over the background (Fig. 5, panels 16, 18, and 6). However, the HPV-16 and HPV-18 E7-expressing clones induced many more colonies (Fig. 5, panels 16 and 18, respectively) which were also larger, on average, than those induced by HPV-6 E7 (Fig. 5, panel 6). Similar results were obtained when a Moloney murine sarcoma virus LTR was used instead of the Harvey murine sarcoma virus LTR (Table 1).

To determine whether the individual E7 proteins were expressed at similar levels, lysates were prepared from metabolically labeled cultures from the passage used to test for growth in agar (Fig. 6). The cell extracts were normalized for level of [$^{35}$S]cysteine incorporation, and equal amounts of each lysate were immunoprecipitated with antisera specific for the individual E7 proteins. The results showed that the appropriate proteins were expressed in each line (Fig. 6). Scanning densitometric quantitation of each of the bands indicated approximately equal levels of expression; with normalization for HPV-6 E7 as 1, HPV-18 E7 had a value of 0.84 and HPV-16 E7 had a value of 1.21 (photographic reproduction resulted in greater contrast in band intensity than that observed in the initial autoradiograph). We assume that the levels of protein detected after the 1-h labeling period (equal to the half-life of HPV-16 E7) reflects the steady-state levels of the three E7 proteins. The apparently higher levels of HPV-16 E7 appear to be the result of a higher antibody titer. Successive immunoprecipitation of the same lysate completely removed 16.E7 in two rounds, while a minimum of three rounds was required to precipitate all of 18.E7 and 6.E7 (data not shown). Although absolute quantitation is difficult, since HPV-16 E7 and HPV-18 E7 appear as single bands while HPV-6 E7 separates into two bands, the data argue that the levels of the E7 proteins do not differ sufficiently to account for their differences in transforming activity. The levels of HPV-18 E7 and HPV-6 E7 were similar, yet HPV-6 E7-containing cells gave rise to about 10-fold fewer colonies in agar than did HPV-18 E7-expressing cells. Therefore, we conclude that the lower biological activity of the HPV-6 E7 ORF is intrinsic to the protein.

**Complementation of E6 and E7 to immortalize normal human foreskin keratinocytes.** Having determined that HPV-6 E7 was less active in NIH 3T3 cells than was E7 from HPV-16 or HPV-18, we turned to a second bioassay, immortalization of human foreskin keratinocytes. While the NIH 3T3 assay measures E7 activity, keratinocyte immortalization by HPV-16 has been found previously to require both E6 and E7 when these ORFs are under control of a murine retroviral LTR, although an LTR-activated HPV-16 E7 expression vector could transiently increase the growth potential of the keratinocytes. If the appropriate complementing gene is used, keratinocyte immortalization can therefore be used as an assay for E6 from different HPV types, as well as an assay for E7.

Previous experiments have shown that DNAs from low-risk HPV-6 and HPV-11 were unable to immortalize keratinocytes. The data presented in the first section of this report suggested that these negative immortalization results resulted in part from inefficient expression of E7. To eliminate this potential problem and enable us to examine the ability of E6 from one HPV type to cooperate with E7 from another HPV type, the E6 ORFs from HPV-6, HPV-11, HPV-16, and

TABLE 1. Ability of HPV E7 to induce anchorage-independent growth

| Plasmid | No. of colonies obtained with: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Moloney[a] LTR | | | | Harvey[b] LTR | | | |
| | Expt 1 | Expt 2 | Expt 3 | Mean | Expt 1 | Expt 2 | Expt 3 | Mean |
| LTR | 18 | 8 | 4 | 10 | 14 | 20 | 58 | 30 |
| HPV-16.E7 | 582 | 1,558 | 524 | 888 | 750 | 1,294 | 744 | 929 |
| HPV-18.E7 | 758 | 1,364 | 352 | 824 | 1,062 | 2,614 | 1,190 | 1,622 |
| HPV-6b.E7 | 146 | 84 | 64 | 98 | 124 | 226 | 150 | 166 |

[a] Moloney murine sarcoma virus.
[b] Harvey murine sarcoma virus.

HPV-18 were placed under control of the Harvey murine sarcoma virus LTR.

Pairwise cotransfections into keratinocytes of one E6 plasmid and one of the isogenic E7 plasmids were then carried out. Transfected keratinocytes were selected for either expression of a cotransfected G418 resistance marker (22) or the ability to resist serum-induced terminal differentiation (26). The two selection procedures gave identical results (Table 2). In agreement with the findings reported previously for HPV-16, none of the E6 or E7 genes alone could induce immortalization. A transient increase in proliferation was often seen with HPV-16 E7 and HPV-18 E7 but was never detected with HPV-6 E7 or any of the E6 genes (data not shown). On the basis of our experience with other HPV-16 E7- and HPV-18 E7-transfected cultures, it is our expectation that the two slow-growing cultures indicated in Table 2 will eventually senesce. When the E6 and E7 genes of HPV-16 and HPV-18 were assayed by using either the homologous or heterologous pair, almost all of the transfected cultures produced immortalized lines. In contrast, the E6 and E7 genes of HPV-6 were unable to induce immortalization. Negative results were obtained even when HPV-6 E6 was cotransfected with E7 from HPV-16 or HPV-18 or when HPV-6 E7 was cotransfected with E6 from HPV-16 or HPV-18. Similarly, HPV-11 E6 was unable to complement E7 from HPV-6, HPV-16, or HPV-18. In a limited number of assays, bovine papillomavirus E6, which can independently induce transformation of the mouse C127 cell line (25), was unable to complement HPV-6 E7 or HPV-16 E7.

The results show that E6 and E7 from the low-risk HPV are defective in the immortalization assay. They confirm in this second assay that HPV-6 E7 is less active than E7 from HPV-16 or HPV-18. They also show that E6 from HPV-6 and E6 from HPV-11 are less active than E6 from HPV-16 and E6 from HPV-18. The fact that the heterologous pairs involving HPV-16 and HPV-18 worked as well as the homologous pairs indicates the functional equivalence of E6 from HPV-16 and E6 from HPV-18 and of the E7s from these two HPV types. It is therefore likely that if either the E6 or E7 gene of HPV-6 were competent, it would functionally substitute for the corresponding HPV-16 or HPV-18 gene. Since all of the genes were controlled by the same LTR, the results suggest that the proteins encoded by both the E6 and E7 genes of the low-risk HPV types have much less immortalizing activity.

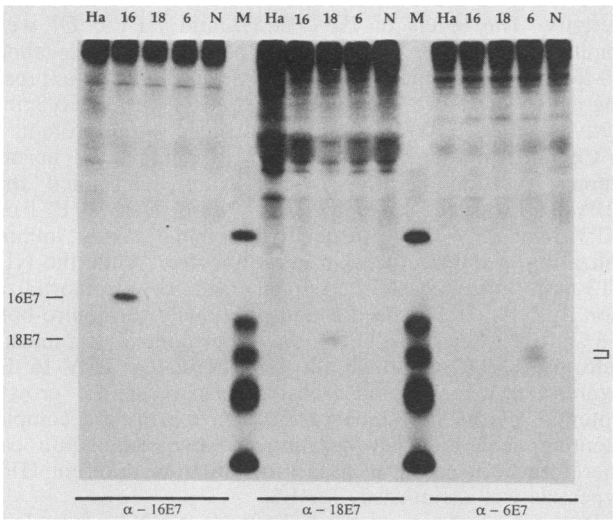


FIG. 6. Comparison of E7 protein levels expressed in transformed cells. Cells transfected with pHa (lanes Ha), pHa.16E7 (lanes 16), pHa.16δ.18E7 (lanes 18), and pHa.16δ.6E7 (lanes 6) were metabolically labeled with [$^{35}$S]cysteine, and the lysates were immunoprecipitated with antisera specific for the E7 proteins. The individual E7 protein bands are indicated at the sides. Lanes M contained molecular mass standards, with sizes of 29, 18.4, 14.3, 6.2, and 3 kilodaltons.

## DISCUSSION

The abilities of high-risk HPV types to immortalize human keratinocytes while low-risk types fail to do so have provided an important in vitro assay that correlates extremely well with the apparent oncogenic potentials of different genital HPV types. Perhaps because these cells are derived from the authentic cell type that these viruses infect in vivo, this in vitro assay depends upon both E6 and E7, which are the two viral genes that are preferentially expressed in cervical cancers. It has been unclear whether the ability of

TABLE 2. Human keratinocyte immortalization: complementation of HPV E6s and E7s

| Plasmid | No. of proliferating cultures/no. of transfections: | | | |
|---|---|---|---|---|
| | LTR | HPV-6 E7 | HPV-16 E7 | HPV-18 E7 |
| LTR | 0/8 | 0/6 | 1/6[a] | 0/4 |
| HPV-6 E6 | 0/4 | 0/9 | 0/9 | 1/5[a] |
| HPV-11 E6 | 0/2 | 0/6 | 0/7 | ND[b] |
| HPV-16 E6 | 0/4 | 0/9 | 9/9 | 3/4 |
| HPV-18 E6 | 0/4 | 0/9 | 9/10 | 3/4 |
| BPV[c] E6 | 0/3 | 0/2 | 0/3 | ND |

[a] *, slow growing.
[b] ND, not done.
[c] BPV, bovine papillomavirus.

high-risk HPV types to induce immortalization reflects a single important difference from low-risk HPV types, or whether multiple differences account for the positive results obtained with high-risk HPV types. The results presented here show that multiple differences from the low-risk HPV types participate in the ability of high-risk types to immortalize human keratinocytes. These differences include the mode in which E7 is expressed, as well as the intrinsic biological activities of the E6 and E7 protein products.

Previous transcriptional mapping has shown that E6 and E7 expression in low-risk HPV types is regulated differently from that in high-risk HPV types. The latter HPV types contain a single promoter for expression of E6 and E7. The presence of donor and acceptor splice sites within E6 apparently results in efficient translation of E7 in high-risk HPV types. By contrast, low-risk HPV types lack the potential to undergo these splicing events. By using identical heterologous expression vectors for the E6/E7 regions from HPV-6 and HPV-16, we have shown here that HPV-16 E7 is translated much more efficiently in NIH 3T3 cells than is HPV-6 E7. The failure to detect HPV-6 E7 protein when the intact E6/E7 region was activated by the murine LTR, despite its transcription as measured by Northern blotting with an E7 probe, strongly suggests that the presence of the E6 ORF in the primary transcript inhibits translation of E7 of HPV-6. Evidence suggests that E7 in low-risk HPV types is translated from a promoter located within E6 (28), similar to what has been reported for cottontail rabbit papillomavirus (3). Our data suggest that this promoter is not utilized efficiently in murine cells. We do not know whether these results also apply to human cells in vitro or to the in vivo situation when the E6/E7 regions of low-risk HPV types are under control of their authentic regulator region. However, our findings do indicate that the different modes by which the low- and high-risk viruses regulate expression of E7 significantly influence the biological activities of the viruses and should be considered when interpreting results obtained with in vitro assays.

To avoid the possible confounding problem of differential transcription control between the high- and low-risk viruses, we made isogenic vectors that placed the E7 ORFs from HPV-6, HPV-16, and HPV-18 under control of the same promoter. By using these constructs, we found with the NIH 3T3 transformation assay that HPV-6 E7 possesses some biological activity. However, HPV-6 E7 was significantly less active than E7 from HPV-16 or HPV-18. Analogous results were obtained independently by Storey et al. (31), who found that E7s from HPV-6 and HPV-11 could, with very low efficiency, cooperate with *ras* to immortalize baby rat kidney cells. We have found that the lower activity of HPV-6 E7 is intrinsic to the protein, since the amount of HPV-6 E7 protein was roughly equivalent to that in transfectants with E7 from HPV-16 and HPV-18. These biological results correlate with previously reported biochemical differences between the E7 proteins of low- and high-risk HPVs and therefore support their probable biological relevance. The E7 proteins of the latter HPV types have been shown to bind the retinoblastoma tumor suppressor protein more avidly than do the low-risk HPV types (1a, 11, 19). The susceptibility of E7s to phosphorylation by casein kinase II also has been found to vary with the biological potential of the protein (1a). Mutational analysis of E7 has suggested that both of these properties contribute to the biological activity of the HPV-16 E7 protein (1a). We conclude that the relative hierarchy of biological activity of the E7 proteins is, at least in part, a reflection of the differences in these intrinsic biochemical properties of the protein.

Despite the biological activity of HPV-6 E7 in NIH 3T3 cells, this gene was unable to complement E6 from HPV-16 or HPV-18 in the keratinocyte immortalization assay, although E7 from HPV-16 could complement HPV-18 E6 and E7 from HPV-18 could complement HPV-16 E6. This result implies that the keratinocyte immortalization assay is a more stringent assay for E7.

Our data obtained with the keratinocyte immortalization assay also show that E6 from the low-risk HPV types is less active than E6 from the high-risk types, since E6 from HPV-6 or HPV-11 was unable to complement E7 from HPV-16 or HPV-18. The greater biological activity of E6 from the high-risk types does not seem to require the truncated forms of E6 protein, in view of preliminary results which suggest that a mutant from which the donor splice site in HPV-16 E6 has been removed can still provide E6 function in the keratinocyte complementation assay (27a). However, the biological differences do correlate with the recent biochemical finding that the p53 protein can bind in vitro to E6 proteins from high-risk HPV types, while p53 fails to bind to E6 proteins from low-risk HPV types (33) or bovine papillomavirus E6 (24, 33). Our results therefore provide biological support for the hypothesis that p53 binding itself is a biologically relevant intrinsic biochemical property of E6 or that it serves as a marker that correlates with the biological activity of E6.

In summary, the results presented here provide evidence that the greater biological activity of high-risk HPV types involves multiple qualitative and quantitative differences between E6 and E7, including their mode of transcription and translation, as well as the intrinsic biochemical properties of the two proteins.

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## REFERENCES

1. **Barbosa, M.** Unpublished data.
1a. **Barbosa, M. S., C. Edmonds, C. Fisher, J. T. Schiller, D. R. Lowy, and K. H. Vousden.** 1990. The region of HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. EMBO J. **9**:153–160.
2. **Barbosa, M. S., and R. Schlegel.** 1989. The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage *in vitro* transformation of human keratinocytes. Oncogene **4**:1529–1532.
3. **Barbosa, M. S., and F. O. Wettstein.** 1988. E2 of cottontail papillomavirus is a nuclear phosphoprotein translated from an mRNA encoding multiple open reading frames. J. Virol. **165**: 134–140.
4. **Bedell, M. A., K. H. Jones, and L. A. Laimins.** 1987. The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and Rat-1 cells. J. Virol. **61**:3635–3640.
5. **Chesters, P. M., and D. J. McCance.** 1989. Human papillomavirus types 6 and 16 in cooperation with Ha-*ras* transform secondary rat embryo fibroblasts. J. Gen. Virol. **70**:353–365.
6. **Chow, L. T., M. Nasseri, S. M. Wolinsky, and T. R. Broker.** 1987. Human papillomavirus types [sic] 6 and 11 mRNAs from genital condylomata acuminata. J. Virol. **61**:2581–2588.
7. **Crook, T., A. Storey, N. Almond, K. Osborn, and L. Crawford.** 1988. Human papillomavirus type 16 co-operates with activated *ras* and *fos* oncogenes in the hormone-dependent transformation of primary mouse cells. Proc. Natl. Acad. Sci. USA **85**:8820–8824.
8. **de Villiers, E. M.** 1989. Heterogeneity of the human papilloma-

virus group. J. Virol. 63:4898–4903.
9. **Durst, M., T. Dzarlieva-Petrusevska, P. Boukamp, N. E. Fusenig, and L. Gissman.** 1987. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene 1:251–256.
10. **Edmonds, C., and K. H. Vousden.** 1989. A point mutational analysis of human papillomavirus type 16 E7 protein. J. Virol. 63:2650–2656.
11. **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.
12. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV 16 E6 and E7 proteins cooperate to immortalize primary human foreskin keratinocytes. EMBO J. 8:3905–3910.
13. **Hudson, J. B., M. A. Bedell, D. J. McCance, and L. A. Laiminis.** 1990. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. J. Virol. 64:519–526.
14. **Kasher, M. S., and A. Roman.** 1988. Characterization of human papillomavirus type 6b DNA isolated from an invasive squamous carcinoma of the vulva. Virology 165:225–233.
15. **Kaur, P., J. K. McDougall, and R. Cone.** 1989. Immortalization of primary human epithelial cells by cloned cervical carcinoma DNA containing human papillomavirus type 16 E6/E7 open reading frames. J. Gen. Virol. 70:1261–1266.
16. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature (London) 293:79–81.
17. **Matlashewski, G., J. Schneider, L. Banks, N. Jones, A. Murray, and L. Crawford.** 1987. Human papillomavirus type 16 DNA cooperates with activated *ras* in transforming primary cells. EMBO J. 6:1741–1746.
18. **Munger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. 63:4417–4421.
19. **Munger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley.** 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. 8:4099–4105.
20. **Pecoraro, G., D. Morgan, and V. Defendi.** 1989. Differential effects of human papillomavirus type 6, 16, and 18 DNAs on immortalization and transformation of human cervical epithelial cells. Proc. Natl. Acad. Sci. USA 86:563–567.
21. **Phelps, W. C., C. L. Yee, K. Munger, and P. M. Howley.** 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1a. Cell 53:539–547.
22. **Pirisi, L., S. Yasumoto, M. Feller, J. Doniger, and J. A. DiPaolo.** 1987. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J. Virol. 61:1061–1066.
23. **Rando, R. F., D. E. Groff, J. G. Chirikijian, and W. D. Lancaster.** 1986. Isolation and characterization of a novel human papillomavirus type 6 DNA from an invasive vulvar carcinoma. J. Virol. 57:353–356.
24. **Schiller, J. T., E. J. Androphy, W. C. Vass, and D. R. Lowy.** 1986. Cellular transformation by bovine papillomavirus, p. 571–579. *In* M. Botchan, T. Grodzicker, and P. A. Sharp (ed.), Cancer cells 4, DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Schiller, J. T., W. C. Vass, and D. R. Lowy.** 1984. Identification of a second transforming region in bovine papillomavirus DNA. Proc. Natl. Acad. Sci. USA 81:7880–7884.
26. **Schlegel, R., W. C. Phelps, Y. L. Zhang, and M. Barbosa.** 1988. Quantitative keratinocyte assay identifies papillomavirus types associated with human cervical carcinoma and detects two distinct viral activities. EMBO J. 7:3181–3187.
27. **Schwarz, E., U. K. Freese, L. Gissman, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) 314:111–114.
27a. **Sedman, S., M. Barbosa, D. Lowy, and J. Schiller.** Unpublished data.
28. **Smotkin, D., H. Prokoph, and F. O. Wettstein.** 1989. Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. 63:1441–1447.
29. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA 83:4680–4684.
30. **Storey, A., K. Osborn, and L. Crawford.** 1990. Co-transformation by human papillomavirus types 6 and 11. J. Gen. Virol. 71:165–171.
31. **Storey, A., D. Pim, A. Murray, K. Osborn, L. Banks, and L. Crawford.** 1988. Comparison of the *in vitro* transforming activities of human papillomavirus types. EMBO J. 7:1815–1820.
32. **Vousden, K. H., J. Doniger, J. A. DiPaolo, and D. R. Lowy.** 1988. The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. Oncogene Res. 3:167–175.
33. **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.
34. **Woodworth, C. D., J. Doniger, and J. A. DiPaolo.** 1989. Immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. J. Virol. 63:159–164.
35. **Yutsudo, M., Y. Okamoto, and A. Hakura.** 1988. Functional dissociation of transforming genes of human papillomavirus type 16. Virology 166:594–597.
36. **zur Hausen, H., and A. Schneider.** 1987. The role of papillomaviruses in anogenital cancer, p. 245–263. *In* N. P. Salzman and P. M. Howley (ed.), The papillomaviruses, vol. 2. The Papovaviridae. Plenum Publishing Corp., New York.