Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8063–8067, September 1992
Medical Sciences

Single amino acid substitutions in "low-risk" human papillomavirus (HPV) type 6 E7 protein enhance features characteristic of the "high-risk" HPV E7 oncoproteins

BI-CHING SANG AND MIGUEL S. BARBOSA

Department of Microbiology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235-9048

Communicated by Jonathan W. Uhr, May 21, 1992

ABSTRACT **HPV types associated with genital disease are termed "high-risk" or "low-risk" viruses according to their prevalence in cancers. Two viral genes, *E6* and *E7*, are invariably expressed in cervical carcinomas. The *E7* gene product has been found to bind the retinoblastoma tumor suppressor protein and to be phosphorylated by casein kinase II. Although present in both high- and low-risk E7 proteins, these activities are diminished in the low-risk HPV-6 E7 polypeptide. To better understand the oncogenic potential of the HPV-6 E7 protein, we replaced four of its amino acids with HPV-16 E7 residues present in the analogous region of the N-terminal half of the protein. Replacement of the arginine at position 4 of the HPV-6 E7 protein with an aspartate present in HPV-16 E7 slowed the mobility of the protein when expressed *in vivo*. Replacement of the glycine at position 22 with an aspartate resulted in higher affinity for retinoblastoma protein binding. Replacement of valine residues at positions 30 and 37 with asparagine and aspartate, respectively, resulted in higher levels of casein kinase II phosphorylation. The substitution at position 22 was the only mutation that exhibited increased transforming activity, suggesting a correlation between the HPV E7 protein affinity for the retinoblastoma tumor suppressor protein and its ability to transform established cells. Our results show that subtle changes in sequence may result in marked differences in biological activity of HPV oncogenes.**

HPVs are small double-stranded DNA viruses that infect squamous epithelia. More than 60 genotypes have been identified and each is associated with distinct anatomic sites (1). A subset of these HPVs are found in genital and mucosal lesions, HPV types 6, 11, 16, and 18 being the most common. Within this subgroup some are most often found in benign lesions (HPV-6 and -11), whereas others are prevalent in malignant tumors (HPV-16 and -18). This distinct oncogenic association of the different genital types resulted in the naming of HPV-6 and -11 as "low-risk" and HPV-16 and -18 as "high-risk" types.

The carcinogenic association of high-risk HPV types is reflected in their ability to transform established cell lines and immortalize primary rodent cells and human keratinocytes (2–8). Analysis of HPV-associated cervical cancers revealed that two early genes, *E6* and *E7*, are invariably expressed (9–11). In accordance, it has been shown that both the E6 and E7 proteins of HPV-16 or -18 are necessary for efficient immortalization of human keratinocytes (12, 13). However, evidence suggests that the *E7* gene accounts for most of the transforming activity. HPV-16 and -18 E7 proteins transform established murine cells, complement activated *ras* in immortalizing rat embryo fibroblasts, and can alone immortalize human keratinocytes (14–19). In contrast, the HPV-6 E7 protein has low transforming activity in these assays (20, 21).

Structural characteristics of HPV E7 proteins suggest distinct features for the N- and C-terminal halves. The C-terminal 50 amino acids, conserved in high- and low-risk HPV E7 proteins, contains two Cys-Xaa-Xaa-Cys motifs separated by 29 residues that are thought to mediate zinc binding (22, 23). Mutations of the cysteine residues result in highly unstable proteins, suggesting an important structural role for the zinc-binding motif. The N-terminal 40 residues of HPV-16 E7 protein contains two independent domains, one that mediates binding of the retinoblastoma tumor suppressor protein (pRb) and another that serves as substrate for casein kinase II (CKII) phosphorylation (24, 25). This arrangement is similar to that found in other viral (adenovirus E1a and simian virus 40 large tumor antigen) and cellular (*myc*) oncogenes, all of which have been shown to bind pRb (26–28). Site-specific mutations disrupting the pRb binding domain abolish transforming activity (24). Mutations deleting the CKII site reduce E7 protein transforming activity (24, 29). When compared to HPV-16 E7, HPV-6 E7 polypeptide has lower affinity for pRb and is phosphorylated to a lower extent (24, 30). These biochemical characteristics correlate directly with the transforming activities of the proteins and may play a role in the observed different oncogenic association.

In this report, we assay HPV-6 E7 mutants carrying single amino acid substitutions. Each amino acid change was designed to replace an HPV-6 E7 amino acid with one from the homologous region from HPV-16. We replaced amino acids at positions 4 (valine; 4asp), 22 (glycine; 22asp), and 37 (valine; 37asp) with aspartate and position 30 (valine; 30asn) with asparagine. The replacement at position 4 (4asp) resulted in a protein with slower mobility on SDS/PAGE but no other high-risk features. *In vitro* binding assays showed that substitution at position 22 (22asp) resulted in increased affinity for pRb. Replacements at positions 30 (30asn) and 37 (37asp) increased the rate of phosphorylation by CKII. When tested for their ability to induce growth in agar upon transfection into NIH 3T3 cells, mutant 22asp protein showed an HPV-16-like transforming activity, whereas the others remained at wild-type HPV-6 levels. These results suggest that subtle amino acid changes in the HPV-6 E7 protein can result in a marked increase in biochemical and biological features some of which "activate" its transforming activity.

MATERIALS AND METHODS

Construction of Expression Clones and Mutagenesis. Isogenic constructs described previously (21) were modified to include the simian virus 40 poly(A) sequence contained within the *Rsa* I 635-base-pair fragment from the viral genome. The parent vector has the Harvey murine sarcoma virus long terminal repeat fragment inserted at the *Eco*RI site and the poly(A) signal sequence at the *Hin*dIII site of the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HPV, human papillomavirus; CKII, casein kinase II; pRb, retinoblastoma tumor suppressor protein; nt, nucleotide(s).

polylinker of pUC19. The HPV-16 E7 expression construct pHa.16E7a has the *Taq* I–*Ssp* I [nucleotides (nt) 505–1176] fragment, containing the coding region (nt 562–855) cloned into the *Bam*HI site. The E7 coding regions of HPV-18 (nt 590–905) and HPV-6 (nt 530–823) were inserted into the HPV-16 background by replacement of the *Nsi* I (present at the starting codon of all *E7* genes)–*Hin*cII fragment. All constructs share a 5′ untranslated 51-nt fragment from HPV-16 in addition to parent vector sequences.

Single amino acid substitutions were done by the polymerase chain reaction in the presence of two opposing oligonucleotides, one carrying the wanted mutation and the other encoding an adjoining sequence of the complementary strand as described (31). A clone containing the HPV-6 *Tha* I–*Ssp* I fragment (nt 473–996) in pUC18 served as template. The oligonucleotides were labeled according to the position and name of the amino acid introduced: 4asp, ^{529}CATGCATGGAGATCATGTTACCC^{551}/^{528}TTGTCCAGCAGTGTAGGCAG^{509}; 22asp, ^{584}GACCCTGTAGACTTACATTGCT^{605}/^{583}TGGAGGTTGCAGGTCTAATAC^{563}; 30asn, ^{607}TGAGCAATTAAACGACAGCTCAG^{629}/^{606}TAGCAATGTAACCCTACAGGG^{586}; 37asp, ^{628}AGAAGATGAGGACGACGAAGTGGA^{651}/^{627}GAGCTGTCTACTAATTGCTCA^{607}.

Each of the mutants was sequenced to identify clones carrying mutations (underlined nucleotides) and to ascertain fidelity of amplification for the rest of gene. The mutant open reading frames were then transferred into the Harvey murine sarcoma virus long terminal repeat expression vectors described above by replacement of the *Nsi* I–*Hin*dIII fragments. Therefore, mutants differ from wild-type HPV-6 E7 gene expression construct only in the mutated nucleotides.

***In Vitro* Association with pRb and CKII Phosphorylation Assays.** The above *E7* gene encoding fragments were cloned into pBluescript (Stratagene). E7 mRNA was synthesized using T7 polymerase and translated in the presence of [^{35}S]methionine in rabbit reticulocyte lysates according to the manufacturer's suggested conditions (Promega). The *in vitro* translation products were mixed and incubated on ice for 1 h in ELB buffer (250 mM NaCl/0.1% Nonidet P-40/50 mM Hepes, pH 7.0/0.5 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/5 mM EDTA). The mixtures were then immunoprecipitated with either polyclonal anti-E7 antibody (21) or monoclonal anti-Rb (Pharmingen). Complexes were analyzed by electrophoresis on SDS/15% polyacrylamide gels and autoradiography.

Wild-type TrpE–E7 fusion proteins were expressed and isolated as described (24). HPV-6 E7 mutants were expressed by replacing the wild-type sequences within the *Nsi* I–*Hin*dIII fragment with the analogous fragment containing the mutated genes. TrpE–E7 proteins were separated by SDS/PAGE on 10% gels and transferred to Immobilon-P filters (Millipore), the appropriate bands were cut out, and the proteins were eluted with 50 mM Tris, pH 9/1% Triton X-100/2 mM dithiothreitol/0.1 mM $ZnCl_2$ for 20 min at room temperature as described by Szewczyk and Summers (32). CKII reactions were carried out with ≈500 ng of fusion protein, 1.4 μl of partially purified kinase (kindly provided by Melanie Cobb, University of Texas Southwestern Medical Center at Dallas), and 2.23 pmol of [γ-^{32}P]ATP in a total volume of 20 μl for 12 min (24).

Cell Culture and Transformation Assays. NIH 3T3 cells (provided by William C. Vass, Laboratory of Cellular Oncology, National Cancer Institute) were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% (vol/vol) newborn calf serum. Cells were seeded at 4×10^5 cells per 60-mm dish 24 h before transfection. Test plasmid (10 μg) and pSV2Neo (2 μg) were cotransfected by the calcium phosphate method according to manufacturer conditions (Promega). Cells from each transfection were split at a 1:5 ratio into two 100-mm dishes 24 h later and incubated in DMEM/10% newborn calf serum containing G418 (GIBCO; 500 μg/ml). After selection for 10–14 days, drug-resistant colonies were pooled and seeded into 0.4% agar at 10^5 cells per 60-mm dish. Colonies were evident after 5–6 weeks.

Metabolic Labeling and Immunoprecipitations. Approximately 2.5×10^5 cells from each pooled G418-selected culture were labeled for 1 h with 0.5 mCi of Tran^{35}S-label (1 Ci = 37 GBq). Lysates were prepared and immunoprecipitations were done as described (21). Immunoprecipitated complexes were analyzed by electrophoresis on SDS/15% polyacrylamide gels and autoradiography.

RESULTS

Design of HPV-6 E7 Mutants. Two independent domains have been identified in the HPV-16 E7 protein that are important for efficient transformation of established murine cells (Fig. 1) (21). One domain is centered around amino acids 24 and 26 (CYE) and is required for pRb binding. The second domain is located around Ser-31 and -32 (DSSEEEDE) and serves as an efficient substrate for CKII. These amino acids are present in the analogous region of the low-risk HPV-6 E7 protein (Fig. 1). Yet this protein shows lower affinity for binding pRb and is a poorer substrate for CKII (24, 33). More recently, it was shown that the histidine at position 2 of the HPV-16 E7 protein is also important for transforming activity (34).

The lower biochemical activity of the HPV-6 E7 protein is probably due to subtle differences in sequences adjoining these critical amino acid residues. Therefore, we substituted amino acids in the HPV-6 E7 protein with residues present in the analogous region of the HPV-16 E7 polypeptide (Fig. 1). The arginine at position 4 was replaced with an aspartate, the glycine at position 22 was replaced with an aspartate, and the valines at positions 30 and 37 were replaced with an asparagine and an aspartate, respectively. The secondary structure changes, as predicted by the Chou and Fasman algorithm (35), induced by the individual mutations indicate that each amino acid replacement results in local structural environment resembling that of the analogous region of the HPV-16 E7 protein.

Phosphorylation of HPV-6 E7 Mutant Proteins by CKII. HPV-16 and -6 E7 proteins have been shown to be phosphorylated by CKII (24, 36). However, the level of phosphorylation was lower in HPV-6 E7 protein. This difference in phosphorylation can be detected *in vitro* by using excess substrate and partially purified CKII (24). Therefore, we

HPV 16 E7
wt MHGDTPTLHE YMLDLQPETT DLYCYEQLND SSEEEDEIDG PAGQAEPDRA HYNIVTFCCK CDSTLRLCVQ STHVDIRTLE DLLMGTLGIV CPICSQKP

HPV 6 E7
mutants D_4 D_{22} N_{30} D_{37}
wt MHGRHVTLKD IVLDLQPPDP VGLHCYEQLV DSSEDEVDEV DGQDSQPLKQ HFQIVTCCCG CDSNVRLVVQ CTETDIREVQ QLLLGTLNIV CPICAPKT

FIG. 1. Amino acid sequence comparison of E7 proteins from HPV-16 and -6. Residues required for pRb binding and phosphorylation by CKII are identified by single and double underlines, respectively. Amino acids substituted are in boldface type in each of the wild-type sequences. Each of the HPV-6 E7 mutants is identified by the replacing amino acid name and position above the wild-type sequence.

purified each of the E7 proteins expressed in bacteria as TrpE fusion proteins and used them for *in vitro* kinase assays. The CKII reaction products were examined by SDS/PAGE (Fig. 2). Similar amounts of E7 proteins were used in the kinase reaction as shown by Coomassie staining (Fig. 2 *Upper*). As previously reported (24), HPV-16 and -18 E7 proteins (Fig. 2 *Lower*, lanes 16 and 18) are phosphorylated to higher levels than the HPV-6 E7 one (Fig. 2 *Lower*, lane 6), whereas the bacterial portion of the fusion protein is not phosphorylated (Fig. 2 *Lower*, lane TrpE). Comparison of intensity of bands obtained for each of the HPV-6 E7 mutant proteins indicates that mutants 4asp and 22asp and wild type are phosphorylated to similar levels. However, mutants 30asn and 37asp show higher levels of phosphorylation, with 37asp being highest. Therefore, in this assay we found that charged amino acids near the CKII substrate site of the HPV-16 E7 protein when introduced into HPV-6 E7 polypeptide led to increased phosphorylation.

Binding of HPV-6 E7 Mutant Proteins to pRb. HPV-6 E7 protein has been shown to bind pRb with lower affinity when compared to high-risk HPV-16 and -18 E7 polypeptides (24, 30). Therefore, we tested the HPV-6 E7 mutant proteins for their ability to bind pRb *in vitro* (Fig. 3). Rabbit reticulocyte lysate-translated E7 and pRb were incubated together and immunoprecipitated with polyclonal antibodies anti-E7 (Fig. 3 *Upper*) or a monoclonal antibody specific for pRb (Fig. 3 *Lower*). pRb was consistently present when mixtures were immunoprecipitated with E7-specific antibodies. Quantitation was difficult because of the different titers of the individual E7 polyclonal antibodies used, as was found previously (21). The reciprocal experiment involving immunoprecipitation with a single monoclonal antibody specific for pRb allowed direct comparison of levels of E7 bound to pRb (Fig. 3 *Lower*). When normalized for levels of coimmunoprecipitated wild-type HPV-6 E7 protein, we found that HPV-18 and

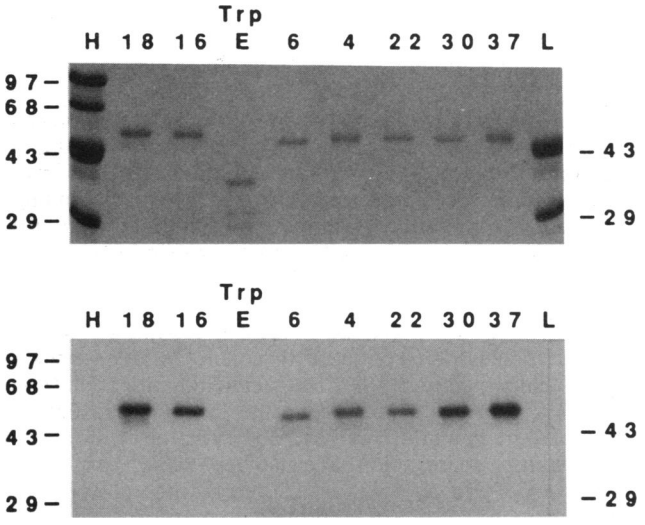

FIG. 2. Phosphorylation of HPV E7 protein by CKII. TrpE–E7 fusion proteins expressed in bacteria and isolated to single-band purity were phosphorylated *in vitro* with partially purified CKII and analyzed by electrophoresis on a SDS/12% polyacrylamide gel. (*Upper*) The gel containing phosphorylation reaction products was stained with Coomassie blue to determine the relative levels of protein in each reaction. Lanes contain the following fusion proteins. Lanes: 18, TrpE–HPV-18 E7; 16, TrpE–HPV-16 E7; TrpE, unfused TrpE; 6, TrpE–HPV-6 E7; 4, TrpE–4asp; 22, TrpE–22asp; 30, TrpE–30asn; 37, TrpE–37asp; H and L, prestained high and low molecular mass standards, respectively. The size of the molecular mass markers is indicated to the left (H) and right (L) in kDa. (*Lower*) The gel was then exposed to determine the level of ^{32}P incorporated into each of the fusion proteins. Lanes are as described for *Upper*. This figure is representative of four independent kinase assays.

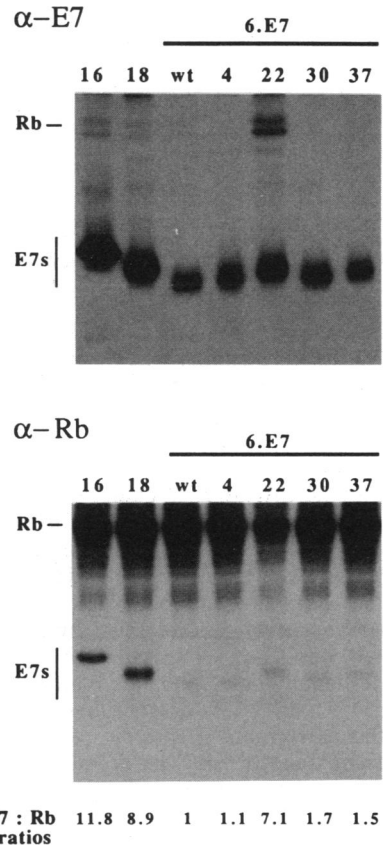

FIG. 3. Immunoprecipitation of HPV E7–pRb complexes. *In vitro*-translated radioactively labeled E7 and pRb were allowed to complex and then were immunoprecipitated with polyclonal antibody specific for E7 (α-E7) (*Upper*) or monoclonal antibody specific for Rb (α-Rb) (*Lower*). Immunoprecipitated complexes were separated by SDS/PAGE on 15% gels. All association mixtures contained equal amounts of pRb and equivalent amounts of E7. Lanes: 16, HPV-16 E7; 18, HPV-18 E7; wt, HPV-6 E7; 4, 4asp; 22, 22asp; 30, 30asn; 37, 37asp mutants. The pRb bands are denoted on the left [multiple bands obtained by *in vitro* translation of pRb have been described (37)]. The E7 protein bands are indicated by the bar on the left. Amounts of E7 and pRb present in each lane of the lower panel were quantified on a Molecular Dynamics scanner. The E7/pRb ratios normalized to wild-type values are given below the gel. This figure reflects results consistent for four experiments.

-16 E7 proteins were present at 11.8- and 8.9-fold higher levels, respectively. Similar comparison of HPV-6 E7 mutant proteins shows that only 22asp bound at significantly higher levels (7.1-fold) than wild type. These data show that although the amino acids (^{24}CYE^{26}) shown to be essential for pRb binding in the HPV-16 E7 protein are present in the low-risk HPV-6 E7 polypeptide, adjoining residues play an important role in this critical interaction.

Transformation of NIH 3T3 Cells by HPV-6 E7 Mutants. We have shown that HPV-6 E7 can transform NIH 3T3 cells, albeit at lower levels than HPV-16 and -18 E7 (21). This quantitative difference in transforming potential directly correlates with the two biochemical activities described above. Therefore, we tested each of the HPV-6 E7 mutant genes for their ability to induce growth in agar. NIH 3T3 cells were transfected with each of the E7 expression constructs and pSV2Neo in three experiments. G418-selected mass cultures were assayed for their ability to grow in agar (Table 1). Cells transfected with HPV-16 or -18 *E7* genes gave rise to colonies of >32 cells per colony, and those transfected with the parent vector developed none. Wild-type HPV-6 E7-containing cells showed a few small colonies (8–10 cells per colony). Colony

Table 1. Anchorage-independent growth of HPV E7-expressing NIH 3T3 cells

Exp.	Growth, colonies per dish							
	16E7	18E7	6E7	4asp	22asp	30asn	37asp	Ha
1	117	213	0	0	110	0	0	0
2	103	250	0	0	126	0	0	0
3	147	150	0	0	130	0	0	0

Only colonies of >32 cells per colony were counted. This is the average colony size of HPV-16 E7-expressing cells. 16E7, HPV-16 E7; 18E7, HPV-18 E7; 6E7, HPV-6 E7; Ha, vector alone.

formation in plates with cells transfected with mutants 4asp, 30asn, and 37asp were similar to wild-type HPV-6 E7, and 22asp had large colonies similar to those seen for high-risk E7 protein-expressing cells. As illustrated in Table 1, the 22asp mutation resulted in a protein with a transforming activity similar to that of the high-risk HPV-16 and -18 E7 proteins. Therefore, only the HPV-6 E7 mutant protein with increased affinity for pRb showed increased transforming activity.

To determine the levels of E7 protein expressed in the transfected culture lysates were prepared from metabolically labeled cells. Extracts were normalized for the level of Tran^{35}S-label incorporation, and equal amounts were immunoprecipitated with antisera specific for the individual E7 proteins. The immunocomplexes were analyzed by electrophoresis through a SDS/15% polyacrylamide gel (Fig. 4). We found that the appropriate E7 proteins were present in cells transfected with each of the wild-type E7 gene expression constructs (Fig. 4, lanes 16, 18, and wt), but no HPV protein was present in lysates from cells transfected with the parent vector (Fig. 4, lanes Ha). The HPV-6 E7 mutant proteins were present in similar amounts in the respective immunoprecipitates (Fig. 4, lanes 4, 22, 30, and 37). Interestingly, each mutation resulted in a change in mobility of the protein. The 4asp mutant protein showed three bands in longer exposures. Although the wild-type, 4asp, 22asp, and 30asn proteins appeared as double bands, 37asp was seen as a single band, even in longer exposures, suggesting that phosphorylation of this mutant is virtually complete as observed for high-risk E7 proteins.

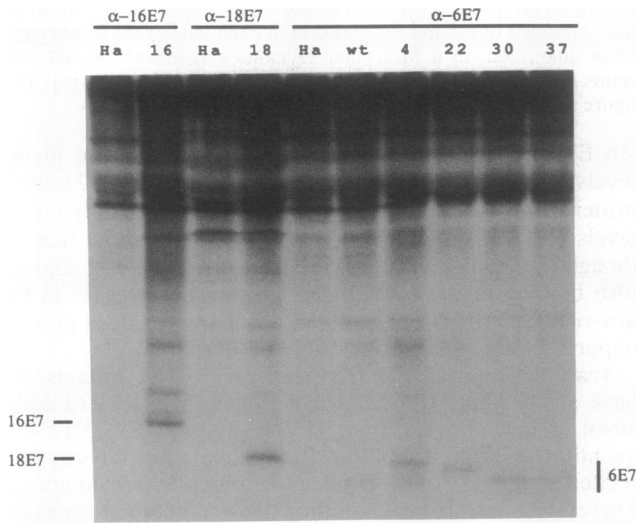

FIG. 4. Expression of individual E7 proteins in transfected NIH 3T3 cells. Cells transfected with pHa (lane Ha), HPV-16 E7 (lane 16), HPV-18 E7 (lane 18), HPV-6 E7 (lane wt), and HPV-6 E7 mutants 4asp (lane 4), 22asp (lane 22), 30asn (lane 30), and 37asp (lane 37) were metabolically labeled with Tran^{35}S-label and equal amounts of lysate were immunoprecipitated with antisera specific for HPV-16 E7 (α-16E7), HPV-18 E7 (α-18E7), or HPV-6 E7 (α-6E7). The individual E7 protein bands are identified at the sides.

DISCUSSION

The oncogenic association of certain HPV types is reflected in their ability to transform established cell lines and immortalize primary human keratinocytes. The *E7* gene has been shown to encode the strongest activity assayed in those experiments. Both high-risk and low-risk E7 proteins have been shown to bind pRB and be phosphorylated by CKII. Comparison of amino acid sequences shows that the critical residues for the two biochemical activities are present in both high- and low-risk E7 proteins. Therefore, our aim was to identify other amino acids responsible for the quantitative differences in pRb binding and CKII phosphorylation and determine which played a major role in the transforming activity of the protein.

Our experiments were targeted to the N-terminal half of the protein as this region contains the determinants for the high transforming potential of high-risk E7 (24, 25, 34). Mutations were designed to replace HPV-6 E7 amino acids with the residue present in the equivalent region of HPV-16 E7 protein. We found that each of the four amino acid substitutions introduced features of high-risk pE7 into the low-risk protein. The 4asp protein showed slower mobility on SDS/polyacrylamide gels a characteristic described for the HPV-16 E7 protein (10). Interestingly, we found that mobility of this mutant differed according to its method of synthesis; when translated in rabbit reticulocyte lysates, it migrated faster than when synthesized *in vivo* by transfection into NIH 3T3 cells. This could be a reflection of a post-translation modification elicited by the specific mutation that reticulocyte lysates are not capable of performing efficiently.

The mutation that affected both the biochemical and biological activity of the protein was the Gly-22 → Asp substitution. This mutant protein, 22asp, had greater binding affinity for pRb. The aspartate may allow an electrostatic interaction with some basic amino acid in pRb. A conformational change is also possible since presence of a glycine within a peptide precludes formation of an α-helix, whereas the aspartate would not. We also found that, when immunoprecipitating E7–pRb complexes with antibody specific for the viral protein, we consistently found more E7 protein when pRB binding was stronger. This observation could be due to a pRb-binding-induced conformational change in E7 making the antibody epitope more accessible for binding. Alternatively, it could be a reflection of multimerization of the complex that would result in more protein being immunoprecipitated than when the antibody and pE7 were present alone.

Studies have shown that the consensus substrate sequence for CKII consists of serine or threonine residues present within acidic peptides (38). An acidic amino acid three residues C-terminal from the serine/threonine residue is essential for recognition by CKII. However, the acidic nature of surrounding amino acids have also been shown to increase the efficacy of phosphorylation. Therefore, the Val-37 → Asp replacement in the HPV-6 E7 protein increases the acidity of the peptide and should affect the level of phosphorylation. Indeed we found that the 37asp mutation resulted in a marked increase in phosphorylation of the E7 protein. When the sequence N-terminal to Ser-32 and -33 was compared to the HPV-16 E7 protein, an asparagine was seen in the high-risk protein whereas a valine was present in the HPV-6 E7 polypeptide. Interestingly, the 30asn mutant also showed an increase in phosphorylation. Asparagine is polarizable and likely to engage in hydrogen bonding, two features that may enhance phosphorylation by CKII.

As discussed above, each of the four mutations resulted in biochemical characteristics resembling HPV-16 E7 protein. However, when tested for their ability to induce growth in agar only 22asp achieved the level of transformation obtained

with the high-risk E7 proteins. This result suggests that transformation of established murine cells correlates with the ability of pE7 to bind pRb. 30asn and 37asp mutant proteins although more efficiently phosphorylated by CKII did not give rise to larger colonies than wild-type HPV-6 E7 protein. This could be due to the stringency of our assay conditions. Nevertheless, our results suggest that pRb binding is a major factor involved in transformation of established murine cells by HPV E7 oncoproteins. However, an HPV-16 *E7* mutant incapable of binding pRb and transforming established murine cells (24) was still capable of immortalizing human keratinocytes when present in the context of the full-virus genome (39). Therefore, it is possible that mutants 4asp, 30asn, and 37asp may have an important role in some function not detectable in NIH 3T3 cells.

The high-risk E7 gene alone has been shown to immortalize primary human epithelial cells. If the difference between high-risk and low-risk immortalization activity is one of the quantitative differences described above, it is possible that overexpression of the low-risk protein would allow immortalization to take place. Recently, Halbert *et al.* (40) found that HPV-6 E7 could cooperate with HPV-16 *E6* gene to immortalize primary human keratinocytes, at low efficiency. Scheffner *et al.* (41) showed that human cervical carcinoma cells lines either have high-risk HPV *E6* and *E7* genes or mutated p53 and pRb genes. It is then possible that, in the rare HPV-6-containing tumors, a mutation in p53 may be complemented by an up-mutation in the HPV-6 *E7* gene, such as we made in 22asp. We believe that these observations warrant a closer look at the state of viral and tumor suppressor genes in HPV-6-associated carcinomas.

We thank Anne-Mette Snider for excellent technical assistance, William C. Vass for NIH 3T3 cells and helpful discussions, Melanie Cobb for CKII, and Robert Weinberg for the Rb clone. This work was supported by American Cancer Society Grant MV-513.

1. de Villiers, E. M. (1989) *J. Virol.* **63,** 4898–4903.
2. Bedell, M. A., Jones, K. H. & Laimins, L. A. (1987) *J. Virol.* **61,** 3635–3640.
3. Durst, M., Dzarlieva-Petrusevska, T., Boukamp, P., Fusenig, N. E. & Gissmann, L. (1987) *Oncogene* **1,** 251–256.
4. Matlashewski, G., Schneider, J., Banks, L., Jones, N., Murray, A. & Crawford, L. (1987) *EMBO J.* **6,** 1741–1746.
5. Pirisi, L., Yasumoto, S., Feller, M., Doniger, J. & DiPaolo, J. A. (1987) *J. Virol.* **61,** 1061–1066.
6. Schlegel, R., Phelps, W. C., Zhang, Y. L. & Barbosa, M. (1988) *EMBO J.* **7,** 3181–3187.
7. Kaur, P. & McDougall, J. K. (1988) *J. Virol.* **62,** 1917–1924.
8. Woodworth, C. D., Doniger, J. & DiPaolo, J. A. (1989) *J. Virol.* **63,** 159–164.
9. Schneider-Gadicke, A. & Schwarz, E. (1986) *EMBO J.* **5,** 2285–2292.
10. Smotkin, D. & Wettstein, F. O. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4680–4684.
11. Baker, C. C., Phelps, W. C., Lindgreen, V., Braun, M. J., Gonda, M. A. & Howley, P. M. (1987) *J. Virol.* **61,** 962–971.
12. Hawley-Nelson, P., Vousden, K. H., Hubbert, N. L., Lowy, D. R. & Schiller, J. T. (1989) *EMBO J.* **8,** 3905–3910.
13. Munger, K., Phelps, W. C., Bubb, V., Howley, P. M. & Schlegel, R. (1989) *J. Virol.* **63,** 4417–4421.
14. Phelps, W. C., Yee, C. L., Munger, K. & Howley, P. M. (1988) *Cell* **53,** 539–547.
15. Vousden, K. H., Doniger, J., DiPaolo, J. A. & Lowy, D. R. (1988) *Oncogene Res.* **3,** 167–175.
16. Yutsudo, M., Okamoto, Y. & Hakura, A. (1988) *Virology* **166,** 594–597.
17. Bedell, M. A., Jones, K. H., Grossman, S. R. & Laimins, L. A. (1989) *J. Virol.* **63,** 1247–1255.
18. Hudson, J. B., Bedell, M. A., McCance, D. J. & Laimins, L. A. (1990) *J. Virol.* **64,** 519–526.
19. Halbert, C. L., Demers, G. W. & Galloway, D. A. (1991) *J. Virol.* **65,** 473–478.
20. Storey, A., Osborn, K. & Crawford, L. (1990) *J. Gen. Virol.* **71,** 165–171.
21. Barbosa, M. S., Vass, W. C., Lowy, D. L. & Schiller, J. T. (1991) *J. Virol.* **65,** 292–298.
22. Barbosa, M. S., Lowy, D. R. & Schiller, J. T. (1989) *J. Virol.* **63,** 1404–1407.
23. Grossman, S. R. & Laimins, L. A. (1989) *Oncogene* **4,** 1089–1093.
24. Barbosa, M. S., Edmonds, C., Fisher, C., Schiller, J. T., Lowy, D. R. & Vousden, K. H. (1990) *EMBO J.* **9,** 153–160.
25. Munger, K., Yee, C. L., Phelps, W. C., Pietenpol, J. A., Moses, H. L. & Howley, P. M. (1991) *J. Virol.* **65,** 3943–3948.
26. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A. & Harlow, E. (1988) *Nature (London)* **334,** 124–129.
27. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J.-Y., Huang, C.-M., Lee, W.-H., Marsilio, E., Paucha, E. & Livingston, D. M. (1988) *Cell* **54,** 275–283.
28. Rustgi, A. K., Dyson, N. & Bernards, R. (1991) *Nature (London)* **352,** 541–544.
29. Firzlaff, J. M., Luscher, B. & Eisenman, R. N. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5187–5191.
30. Munger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E. & Howley, P. M. (1989) *EMBO J.* **8,** 4099–4105.
31. Hemsley, A., Arnheim, N., Toney, M. D., Cortopassi, G. & Galas, D. J. (1989) *Nucleic Acids Res.* **17,** 6545–6551.
32. Szewczyk, B. & Summers, D. F. (1987) *Anal. Biochem.* **164,** 48–53.
33. Gage, J. R., Meyers, C. & Wettstein, F. O. (1990) *J. Virol.* **64,** 723–730.
34. Watanabe, S., Kanda, T., Sato, H., Furuno, A. & Yoshiike, K. (1990) *J. Virol.* **64,** 207–214.
35. Chou, P. Y. & Fasman, G. D. (1978) *Adv. Enzymol.* **47,** 145–148.
36. Firzlaff, J. M., Galloway, D. A., Eisenman, R. N. & Luscher, B. (1989) *New Biol.* **1,** 44–53.
37. Dyson, N., Howley, P. M., Munger, K. & Harlow, E. (1989) *Science* **243,** 934–937.
38. Kuenzel, E. A., Mulligan, J. A., Sommercorn, J. & Krebs, E. G. (1987) *J. Biol. Chem.* **262,** 9136–9140.
39. Jewers, R. J., Hildebrandt, P., Ludlow, J. W., Kell, B. & McCance, D. J. (1992) *J. Virol.* **66,** 1329–1335.
40. Halbert, C. L., Demers, G. W. & Galloway, D. A. (1992) *J. Virol.* **66,** 2125–2134.
41. Scheffner, M., Munger, K., Byrne, J. C. & Howley, P. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5523–5527.