Serum- and Calcium-Induced Differentiation of Human Keratinocytes Is Inhibited by the E6 Oncoprotein of Human Papillomavirus Type 16

L. SHERMAN† AND R. SCHLEGEL*

*Department of Pathology, Georgetown University Medical School,
Washington, D.C. 20007*

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Transfection of the E6 and E7 genes of the high-risk human papillomaviruses (HPVs) into primary genital keratinocytes generates colonies of proliferating cells which are resistant to calcium- and serum-induced terminal differentiation. To genetically map the HPV gene(s) responsible for this cellular phenotype, we cloned cDNAs for full-length E6, full-length E7, four truncated E6 splice variants (E6I to E6IV), and a series of E6 C-terminal truncation mutants into a simian virus 40 expression vector. The E6 proteins were tagged with the AU1 epitope at the C terminus to verify their expression in COS cells by immunoprecipitation and immunofluorescence. Transfection of the full-length E6 protein, either wild type or epitope tagged, induced calcium- and serum-resistant keratinocyte colonies, but the truncated E6 variants and full-length E7 protein did not. E6-induced colonies, while altered in response to serum and calcium, could not be established into cell lines without the combined presence of the E7 protein, further exemplifying the independent roles of E6 and E7 in cell differentiation and cell proliferation. The E6 C-terminal deletion mutants defined two distinct functional domains between amino acids 120 and 151. Amino acids 120 to 151 contained an apparent bipartite nuclear localization signal, whereas amino acids 132 to 141 were required for the induction of resistance to calcium- and serum-induced differentiation and for immortalization of human keratinocytes in conjunction with E7.

The natural host cells for human papillomavirus (HPV) are squamous epithelial cells, and the infection of such cells in the anogenital mucosa can induce epithelial neoplasia with the potential to progress to malignancy. HPV type 16 (HPV-16) is the prototype high-risk type which is most frequently associated with human cervical dysplasia and carcinoma (68), and the *in vivo* and *in vitro* transforming properties of this virus reside in two genes, E6 and E7. These genes are routinely retained and expressed in cervical cancers (2, 48, 49, 57), they cooperate to immortalize primary human genital keratinocytes (21, 46), they alter the differentiated phenotype of keratinocytes (20, 25, 66), and they inhibit keratinocytes terminal differentiation induced by serum and calcium (40, 46). In addition, each gene can independently transform established rodent cell lines (8, 28, 43, 50, 63).

The biological activity of E6 is dependent on the host cell used for assay. E6 cooperates with E7 in the efficient immortalization of human genital keratinocytes (21, 40) but cooperates with activated *ras* in the transformation and immortalization of baby mouse kidney cells (59) and baby rat kidney cells (36). Independently of E7 or *ras*, E6 can transform NIH 3T3 cells (50) and, more surprisingly, immortalize human mammary epithelial cells (5).

The ability of E6 and E7 proteins to form complexes with the cellular tumor suppressor proteins p53 (64) and Rb (17, 41) respectively, has been suggested as a mechanism by which the viral proteins interfere with cell growth and proliferation.

The HPV-16 E6 gene encodes 151 amino acids, many of

which are positively charged. Like E6 proteins of other papillomaviruses, it contains two pairs of the zinc finger motif Cys-X-X-Cys and has been shown to bind zinc directly (7). E6 localizes to the nucleus (29, 33), binds nonspecifically to DNA (37), and activates transcription from several viral gene promoters (11, 13, 50).

The ability of E6 to complex with p53 (64) and target its degradation through the ubiquitin-dependent pathway (45) is believed to be important for its immortalizing and transforming activities. This conclusion is supported by observations that mutated p53 is frequently found in cervical cancers lacking HPV DNA but not in those containing HPV (12, 44) and that a dominant-negative p53 mutant can substitute for E6 in the immortalization of human keratinocytes (51). In addition, p53 exhibits a three- to fivefold reduction in its half-life in HPV-immortalized epithelial cells (4, 24, 33), supporting the hypothesis that E6 mediates the degradation of p53 *in vivo* as well as *in vitro*.

The E6 gene of the high-risk HPVs also encodes several truncated transcripts which are formed as a result of differential splicing. Four such splice-variant open reading frames (ORFs) have been described for HPV-16 (16, 55, 56). The E6 variant transcripts E6I to E6IV have the potential to encode proteins of 43, 46, 48, and 64 amino acids, respectively, and all four splice variants share the N-terminal 41 residues. Whereas the full-length E6 proteins of HPV-16 and HPV-18 are detectable (at low levels) in cervical carcinoma cell lines (1, 47, 53) and cells transformed with HPV-16 DNA (1, 6, 21), only the E6I variant is made at sufficient levels to be detected in cervical carcinoma cells transplanted into nude mice (47). The other E6 variant proteins of HPV-16 have not yet been identified, and their functions are not known.

To date, there have been no genetic studies of the HPV genes which are required for inducing keratinocyte resistance

* Corresponding author. Mailing address: Department of Pathology, Georgetown University Medical School 3900 Reservoir Road, NW, Washington, DC 20007. Fax: (202) 687-5285.

† Present address: Department of Human Microbiology, Sackler School of Medicine Tel Aviv University, Tel Aviv 69978, Israel.

FIG. 1. (A) Amino acid sequences of the major HPV-16 E6 gene, four E6 splice variants, and five E6 C-terminal deletion mutants. The complete 151-amino-acid E6 sequence is given in single-letter code. Cysteine residues in the metal binding motifs (Cys-X-X-Cys) are marked with bold letters and asterisks below. The position of the C-terminal amino acid of the region shared by the E6 variants (V-I-IV) and positions of the C-terminal amino acids of the deletion mutants (positions 86, 107, 120, 132, and 141) are indicated. The two short clusters of basic amino acids which constitute the apparent bipartite NLS are underlined. (B) Schematic representation of the E6 splice variants and C-terminal deletions. The positions of the four Cys-X-X-Cys motifs are shown at the top. The E6I and E6II variants are encoded by transcripts resulting from alternative splicing within the E6 gene at nucleotide 226 to 409 and 226 to 526, respectively (56). E6III is encoded by a transcript created from a splice between the E6 and the E4 genes (nucleotides 226 to 3357) (16). The E6IV variant represents a splice between the E6 and the E1 genes (nucleotides 226 to 2708) (55). The E6I to E6IV transcripts encode proteins of 43, 46, 48, and 64 amino acids, respectively. All four variants share the N-terminal 41 amino acids with the full-length E6. SV40 vectors expressing the full-length E6, E6 variant, and carboxy-terminal truncated mutants were constructed by PCR technology and were expressed as fusion proteins with the epitope HA1 (suffix "H") or AU1 (suffix "A") at the C terminus.

FIG. 2. Expression of epitope-tagged HPV-16 E6 splice-variant proteins (A) and deletion proteins (B) in COS cells. COS cells were plated into 10-cm-diameter dishes and transfected with the indicated plasmid DNAs. At 48 h posttransfection, cells were analyzed for expression of the fusion proteins. Cells were labeled for 4 h with 570 μ Ci of a commercial mixture of [35 S]methionine (90%) and [35 S]cysteine (10%) (Express; NEN) and then extracted in radioimmunoprecipitation assay buffer, and proteins were immunoprecipitated as previously described (10). Monoclonal antibodies raised against the epitope AU1 (35) or HA1 (65) were used to precipitate the epitope-tagged E6 proteins. Polyclonal antibodies raised against HPV-16 E6 bacterial fusion protein (1) were used to precipitate the wild-type E6 protein (data not shown). The immunoprecipitates were resolved on sodium dodecyl sulfate–14% polyacrylamide gels. Cells transfected with plasmid 6A65, which encodes the 12-kDa HPV-6 E5-AU1 fusion protein, were also immunoprecipitated with the AU1 antibody. Positions of molecular weight markers (indicated in kilodaltons) are shown on the left. Positions of the E6 and E6 variants are indicated on the right.

to terminal differentiation initiated by serum and calcium. To define the genes and genetic sequences required for the induction of resistance to differentiation stimuli, the activities of HPV-16 full-length E6, full-length E7, and E6 proteins encoded by splice variants and truncated mutants were evaluated in the keratinocyte colony formation assay (46). To facilitate the detection and analysis of proteins encoded by the E6 ORFs, proteins were tagged with epitopes recognized by highly specific monoclonal antibodies.

Expression of epitope-tagged forms of full-length, splice-variant, and carboxy-terminal deletion mutants of the HPV-16 E6 protein in COS cells. Expression plasmids encoding wild-type epitope-tagged and splice-variant E6 proteins of HPV-16 were constructed by the PCR procedure with oligonucleotides complementary to the translational start and termination sequences of each ORF containing *XhoI-HindIII* recognition sequences. SK vectors encoding the E6 and variant cDNAs (54) were used as templates. To generate the epitope-tagged E6 and E6 variant proteins (shown in Fig. 1), the sequence of the 6-amino-acid AU1 epitope (35) or 11-amino-acid HA1 epitope (65) was included, in frame, in the 3' primer immediately downstream of the sequence of the C terminus of each ORF followed by the sequence of the termination codon and the *HindIII* site. The 5' primer used to construct the E6 expression plasmids, with the exception of pE6, included the Kozak sequence (GCCACC) for optimal translation initiation (31) immediately upstream from the presumed initiation codon at position 104 (57). The 5' primer used to generate pE6 contained the authentic HPV-16 sequence from nucleotides 98 to 103 (52) upstream from the initiation codon in place of the Kozak sequence.

The full-length E6 ORF was tagged both with the AU1 and HA1 epitopes (E6A and E6H), as was the E6IV ORF (E6IVA and E6IVH). ORFs E6I to E6III (E6IA to E6IIIA) and the carboxy-terminal truncation mutants (M-141A to M-86A) were tagged with the AU1 epitope. The fusion ORFs were then cloned into a modified pSG5 vector (Stratagene), pJS55 (58), under the control of the early simian virus 40 (SV40) promoter.

To identify the recombinant proteins, plasmid DNAs were transfected into COS cells, which allow high levels of plasmid replication and expression. The cells were labeled with [35 S]methionine-cysteine and then extracted and immunoprecipitated with antibodies against the AU1 or HA1 epitope (Fig. 2). Full-length as well as variant E6 proteins could be detected, although the shorter E6 variants, E6I to E6III, were much less abundant (Fig. 2A). This difference was probably due to differences in protein stability, since isogenic constructs were used for protein expression. Electrophoretic mobilities of all of the E6 proteins correlated with their expected sizes. E6 and E6IV proteins tagged with AU1 and HA1 epitopes also showed different mobilities which were consistent with the relative sizes of their appended epitopes. Untagged (wild-type) E6 proteins were expressed at levels comparable to those of E6A from plasmids pKE6 and pE6 (data not shown). Finally, all of the E6 C-terminal deletion mutants were expressed at similar levels (Fig. 2B).

Full-length E6 and the E6IV variant have distinct intracellular localizations. High levels of expression of E6A and E6IVA fusion proteins in COS cells (Fig. 2A) allowed their intracellular localization by immunofluorescence staining. Cells were transfected with E6A plasmid DNA, and 48 h later they were fixed, permeabilized, and localized with AU1 monoclonal antibodies. E6A was predominantly localized in the nucleus, although a small number of cells (10%) showed cytoplasmic staining (Fig. 3A). This observation is consistent with previous studies showing the nuclear localization of the wild-type E6 in COS cells (29, 33) and demonstrates that the AU1 epitope did not interfere with the normal nuclear translocation of E6. In contrast, cells transfected with the E6IV splice variant showed strong cytoplasmic staining and complete exclusion of nuclear staining (Fig. 3A). Equivalent results were obtained with the HA1-tagged proteins (E6H and E6IVH), although background staining was higher with the HA1 antibody (data not shown). These results indicated that variant E6 proteins which lack the central and carboxyl domains are defective in their nuclear localization ability, consistent with the following results defining a putative nuclear localization signal (NLS) in the E6 C terminus (Fig. 3B).

Compatible with the immunoprecipitation data, COS cells transfected with the E6 deletion mutants showed strong immunofluorescent staining (Fig. 3B). Mutants M-141 and M-132 retained nuclear localization, although there was a slight reduction compared with full-length E6. In addition, some cells also displayed cytoplasmic staining, similar to that observed with full-length E6. Mutants M-120, M-107, and M-86 were completely defective for nuclear localization and showed only strong cytoplasmic staining. These results suggested the presence of an NLS between E6 residues 120 and 151.

Inspection of the amino acid sequence between these residues revealed two short clusters of basic residues at positions 121 to 124 and 144 to 147. NLSs have been identified in many nuclear proteins (18), but there is no single strict consensus sequence. However, the presence of two discrete clusters of the basic amino acids separated by a short spacer region of 10 amino acids (or more) is characteristic of a bipartite NLS (Fig. 4) (14). Such bipartite motifs are present in many nuclear proteins and have been suggested to be more reliable as indicators of nuclear localization *in vivo* (14). In some cases, both basic clusters are required for the nuclear targeting as in the *Xenopus* nucleoplasmin protein (15). They can also act additively as in the yeast ribosomal protein L29 (60).

Sequence comparison of several HPV E6 proteins revealed the presence of putative bipartite motifs in analogous regions

FIG. 3. Intracellular localization of epitope-tagged HPV-16 E6 splice-variant proteins (A) and deletion E6 proteins (B) in COS cells. COS cells were plated into 5-cm-diameter dishes and transfected with 5 μ g of the indicated plasmid DNAs. Cells were processed for immunofluorescence microscopy 48 h posttransfection as previously described (10). Monoclonal antibodies against the AU1 (35) epitope, diluted 1:125 in phosphate-buffered (PBS) containing 10% normal goat serum and 0.1% saponin, were used as primary antibodies. Incubation was carried out for 2 h at room temperature. The secondary antibody solution contained goat anti-mouse immunoglobulin G conjugated to trimethylrhodamine isothiocyanate diluted 1:50 in PBS with 10% normal goat serum and 0.1% saponin. Incubation with the secondary antibody was carried out for 1 h at room temperature. Cells were examined on a Zeiss ICM 405 UV microscope.

of the proteins (Fig. 4). Interestingly, the majority of HPVs with this bipartite signal belong to the high-risk (HPV-18, -45, and -56) and intermediate-risk (HPV-31, -33, -35, and -51) categories of the genital HPVs.

The domain shown by us to have a major importance for the nuclear homing of E6 (amino acids aa 121 to 132) was previously shown by Crook et al. (11) to be essential for the transcriptional activation activity of E6. Deletions within this region (amino acids 128 to 132 and 123 to 127) totally abolished transcriptional activity, evidently reflecting the inability of these proteins to translocate to the nucleus.

While our data and previous reports (29, 33) demonstrate the predominant nuclear location of HPV-16 E6 in COS cells, other investigators have localized E6 proteins of HPV-16 and HPV-18 in the cytoplasm (9, 34). E6 cytoplasmic immunofluorescence was observed in epithelial cells derived from cervical carcinomas and in mammary cells immortalized by HPV-16 (9, 34). One possible explanation for this difference may be the higher levels of E6 expression in COS cells and the increased abundance of p53 in these cells, which may affect E6 subcellular localization. Consistent with the apparent extranuclear localization, E6 has been shown to associate with an E6-binding protein (E6BP) located in the endoplasmic reticulum and perinuclear regions (9).

E6 protein, by itself, can induce keratinocyte resistance to calcium- and serum-induced differentiation, but the E6IV splice variant cannot. The transforming activities of the proteins encoded by the E6 ORF and E7 were evaluated in a quantitative human keratinocyte colony formation assay (46). In this assay, transformation is scored on the basis of resistance to signals of terminal differentiation. Normal human keratinocytes, when exposed to medium containing high levels of serum and calcium, terminally differentiate and stratify (22, 32, 67). When transfected with the E6 and E7 genes of the high-risk HPVs, keratinocytes resist these stimuli and continue to proliferate forming nonstratifying colonies which appear macroscopically as clear plaques on a background of the terminally differentiating normal keratinocytes.

Initially, plasmids expressing the AU1-tagged E6 and E6IV proteins were tested. Plasmids were transfected into primary human keratinocytes alone and in combination with pE7. Plasmid pE7 expresses the HPV-16 E7 genome fragment (nucleotides 505 to 863 [52]) from the early SV40 promoter (58). Plasmid 18CCB, which directs the expression of the E6 and E7 genes of HPV-18 from the viral locus control region, was also transfected as a positive control (64). Following transfection, cells were grown in keratinocyte serum-free medium (K-SFM) to allow the formation of monolayers and then selected in

FIG. 3—Continued.

HPV Type	
39	PAEKLRLHNS <u>KRRFH</u> KIAG. ...SYTGQCR RCWTTKREDB <u>RLTR</u> BRETQV
18	PAEKLRLHNE <u>KRRFH</u> NIAG. ...HYRGQCH SCCNRARQER <u>LQRR</u> BRETQV
45	PAEK <u>RRH</u> LKD <u>KRRFH</u> SIAG. ...QYRGQCN TCCDQARQER <u>LRRR</u> BRETQV
56	PEEKQLHCDR <u>KRRFH</u> LIAH. ...GWTGSCL GCW....RO <u>TSRE</u> PRESTV
26	PEEKQRIVDE <u>KRRFH</u> EIAG. ...QWGLCT NCW....R <u>PRROT</u> ETQV.
51	PEEKQLVDE <u>KRRFH</u> EIAG. ...RWTGQCA NCW....QR <u>TRQR</u> NETQV.
33	PQEK <u>KRH</u> VDL <u>NKR</u> FHNISG. ...RWAGRCA AC.....W <u>RSRR</u> RETAL.
58	PQEK <u>KRH</u> VDL <u>NKR</u> FHNISG. ...RWTGRCA VC.....W <u>RPRR</u> QETQV.
16	PEEKQRHLDK <u>KQRFH</u> NIAG. ...RWTGRCM SCC.....RS <u>SRTR</u> BETQL.
35	PVEKQRHLEE <u>KKRFH</u> NIAG. ...RWTGRCM SC.....W <u>KPTR</u> BETEVE.
31	PEEKQRHLDK <u>KKRFH</u> NIAG. ...RWTGRCI AC.....W <u>RRPR</u> TETQV.
SV40 Large T PKKKRKV	
Nucleoplasmin SPPKAVKRPAAATKKAGQAKKKLDKEDES	

FIG. 4. Amino acid sequences of representative HPV E6 C-terminal domains containing putative bipartite NLSs. Two separate clusters of positively charged amino acids are underlined in the C termini of 11 representative E6 proteins. The nuclear targeting sequences from *Xenopus* nucleoplasmin (15) and SV40 large T antigen (27) are also shown.

differentiation medium (Dulbecco modified Eagle medium [DMEM] containing 10% fetal calf serum [FCS] and 1 µg of hydrocortisone per ml). Colonies of proliferating cells started appearing 2 weeks after the initiation of selection and were allowed to expand for an additional week before being stained and counted (Fig. 5A). As anticipated from previous studies, differentiation-resistant colonies were produced in cells transfected with p18CCB (E6 and E7 together). In addition, colonies were also induced when cells were cotransfected with AU1-tagged E6 (E6A) and wild-type E7, indicating that E6A was biologically active. Similar results were obtained when wild-type E6 (expressed from pE6 and pKE6) and wild-type E7 (pE7) were cotransfected (data not shown).

Differentiation-resistant colonies also appeared in cells transfected with E6A alone, indicating that the E6 protein is sufficient to induce this phenotype. Untagged E6 proteins (pE6 and pKE6) also induced these colonies (data not shown). In contrast, E6IVA did not induce colonies, demonstrating that more than the E6 amino-terminal domain (amino acids 1 to 41) is required for inducing these cellular changes. E6A colony formation was slightly increased when E6A was cotransfected with E7, although E7 had no activity in combination with E6IV (Fig. 5) or by itself (data not shown). These studies have been

FIG. 5. Induction of differentiation-resistant colonies in human keratinocytes by HPV-16 E6 splice variants (A) and deletion mutants (B). Primary human keratinocytes cultured from neonatal foreskin explants were grown in K-SFM supplemented with 10 ng of epidermal growth factor and 50 ng bovine pituitary extract (Gibco-BRL, Gaithersburg, Md.) per ml. Cells were plated at passage 2 at 50% confluency in 10-cm-diameter dishes and transfected by using Lipofectin (Bethesda Research Laboratories) with 10 μ g of the indicated E6 plasmids together with 10 μ g of the vector DNA or DNA of the E7 plasmid. The second day after transfection, cells were plated into 162-cm² flasks in K-SFM and maintained until the cultures became confluent (4 to 5 days). The medium was then changed to a selection medium consisting of DMEM (containing 2 mM calcium) supplemented with 10% FCS and 1 μ g of hydrocortisone (Sigma) per ml. Selection in differentiation medium was carried out for 3 weeks. The macroscopic appearance of colonies in 162-cm² flasks stained with methylene blue is shown. The transformed, nonstratifying colonies appear clear against a dark background of stratified differentiated keratinocytes.

TABLE 1. Activities of HPV-16 E6 truncated mutants in the induction of serum- and Ca²⁺-resistant colonies and immortalization of human keratinocytes

Plasmid	Expt 1		Expt 2		Expt 3, ^c no. of colonies/ 10 µg of lipofected DNA
	No. of colonies/10 µg of lipofected DNA ^a	Immortalization ^b	No. of colonies/10 µg of lipofected DNA	Immortalization	
E6A	26	—	36		30
E6A+E7	25	+	29	+	
M-141A	23	—	49		20
M-141A+E7	21	+	36	+	
M-132A	0	—	0		
M-132A+E7	0	—	0	—	0
M-120A	0	—	0		
M-120A+E7	0	—	0	—	0
M-107A	0	—	0		
M-107A+E7	0	—	0	—	0
M-86A	0	—	0		
M-86A+E7	0	—	0	—	0
Vector	0	—	0		0

^a Colonies were counted without staining.^b Cells were pooled and further passaged in 3:1 medium. Lines that were passaged more than 20 times were considered immortal (+).^c Immortalization was not tested.

repeated independently at least twice with similar results (Table 1).

Previous studies using the colony formation assay to assess the activities of HPV-16 E6 and E7 failed to detect an independent effect of E6 on cell differentiation (40). In this earlier study, however, E6 and E7 were expressed from the actin gene promoter, and colony formation was dependent on the expression of both E6 and E7 proteins. In the current study, HPV-16 E6 was expressed from the stronger SV40 early promoter. This promoter proved to be highly efficient in directing the expression of E6 both in COS cells (Fig. 2) and human keratinocytes (data not shown). It is possible that high levels of protein expression by the SV40 vector not only blocked keratinocyte terminal differentiation but allowed the prolonged division of the undifferentiated cells during selection, thereby inducing colony formation. If expression from the actin promoter was lower, cells which became refractory to the differentiation signals may have entered a state of prolonged growth arrest and thus could not be detected unless enhanced proliferation was induced by coexpression of E7.

Transformation assays performed with the E6 deletion mutants showed differences in their abilities to induce colonies, depending on their lengths (Fig. 5B). Deletion of amino acids 141 to 151 (M-141A) from the carboxy end of E6 had no effect on the colony formation activity. The size, number, and appearance of colonies generated by M-141 were comparable to (if not slightly greater than) that of wild-type E6. The efficiency of colony formation by M-141 was not affected by cotransfection with E7 (data not shown). Further deletion to amino acid 132, which includes the Cys-X-X-Cys sequence in the second zinc finger, completely abolished colony formation. These results indicated that E6 biological activity was dependent on the presence of amino acids 132 to 141 but not on residues 141 to 151. As anticipated, further C-terminal deletions of E6 to residue 120, 107, or 86 were also biologically inactive despite the finding that these proteins were expressed in the COS cell system at levels equivalent to those of E6A (Fig. 2B). These findings are also compatible with our previous observation that E6IV, which lacks the C-terminal region of E6, is inactive.

While macroscopically there was no difference in appearance or number of colonies induced by E6A or E6A plus E7, we examined these colonies microscopically to determine if

they also exhibited similar histomorphologic properties (Fig. 6A). The two types of colonies were indistinguishable and contained small proliferating cells which seemed to push aside the terminally differentiating keratinocytes. These results indicated that E6 protein, by itself, induced early changes in keratinocyte growth properties which were equivalent to those observed to be capable of inducing resistance to signals of terminal differentiation.

The results described above demonstrated that the base of the second zinc finger was crucial for E6 differentiation resistance activity. From our data, it is difficult to evaluate the requirement of nuclear localization for colony formation (differentiation resistance) because sequences essential for the biological activity were located downstream of the nuclear targeting sequences (residues 120 to 132). However, deletion of the carboxy terminus of E6 (residues 141 to 151), while reducing nuclear localization somewhat, had no effect on the colony-forming activity. This result suggests that E6 biologic function may not be dependent on its nuclear localization.

TABLE 2. Extended life span of human keratinocytes isolated from serum- and Ca²⁺-resistant colonies induced by HPV-16 E6 alone and in combination with E7

Plasmid	Expt 1		Expt 2	
	Pool ^a	Clones ^b	Pool	Clones
E6A	—	0/6	—	0/6
E6A+E7	+	1/4	+	3/6
E6H	—	0/6		
E6H+E7	+	5/6		
KE6	—		—	0/6
KE6+E7	+		+	5/6
E6	—	0/6	—	
E6+E7	+	4/6	+	
E7	—		—	
Vector	—		—	
18CCB	+	4/7		

^a Cell lines established from pooled cell populations after serum-Ca²⁺ selection. +, line that was passaged more than 20 times.^b Number of colonies that continued to proliferate after more than seven passages/number of individual colonies isolated after serum-Ca²⁺ selection.

FIG. 6. Microscopic appearance of differentiation-resistant colonies induced by HPV-16 E6 alone or E6 plus E7 (A) and morphology of passed cultures of human keratinocytes established from such colonies (B). (A) Phase-contrast photographs of proliferating, differentiation-resistant keratinocytes at two magnifications ($\times 10$ and $\times 20$). Colonies were induced by plasmids expressing either tagged or untagged HPV-16 E6 alone or in combination with E7. Colonies induced by plasmids encoding E6A and E6A plus E7 are shown. The proliferating colonies are composed of small, poorly stratified cells which grow as a densely packaged monolayer and push aside the terminally differentiated cells. (B) Human keratinocytes transfected with the indicated plasmid DNAs were selected in high-calcium- and serum-containing medium, and eight individual colonies from each transfection were pooled and further passed in a 3:1 mixture of K-SFM and DMEM-10% FCS. Phase-contrast photographs of cell cultures at passage 3 are shown. Cultures originating from cells transfected with the E6A plasmid exhibit features of senescence. In contrast, cultures established from the E6A+E7 colonies show a homogenous population of small proliferating cells.

E6-induced differentiation-resistant colonies do not progress to the immortalized state. Although E6 and E6 plus E7 colonies were morphologically indistinguishable, they differed dramatically in their abilities to establish into cell lines. For example, when E6 or E6 plus E7 DNAs were transfected into keratinocytes, selected in differentiation medium for 3 weeks, and then passaged as mass cultures in medium containing 3 parts of K-SFM and 1 part of DMEM with 10% FCS, only cultures receiving both E6 and E7 established into cell lines (Table 1). Thus, although cultures transfected with E6 or M-141 E6 gave rise to differentiation-resistant colonies, these cultures failed to generate cell lines upon continued passage and displayed morphological features of terminal differentiation (Fig. 6B). In contrast, when these same E6 constructs were cotransfected with E7, immortalized cell lines developed. As expected, the E6 mutants which were inactive for inducing differentiation-resistant colonies were also defective for cell immortalization in cooperation with E7. These results suggested that overlapping sequences contained within amino acids 132 to 141 are essential for both immortalization and resistance to differentiation. Similar results were obtained when isolated, cloned colonies, rather than mass populations of keratinocytes, were passaged (Table 2). Finally, E6 and the described mutants were also defective for immortalization when evaluated without prior exposure to serum and calcium (data not shown), which is consistent with previous reports (19, 21, 25).

In this study, we demonstrated that full-length E6, by itself, is capable of inducing resistance to serum- and calcium-induced differentiation and that this property is separable from the immortalizing activity of E6, which requires the cooperation of E7. The term calcium- and serum-induced terminal differentiation refers to a complex set of events including cessation of cell division, production of proteins specifically associated with the process of keratinization, and ultimately cell death (22, 23). Colony formation by E6 could result from inhibition of any of these processes.

The E6 and E7 proteins of the high-risk HPVs modulate both cell proliferation and differentiation (20, 21, 25, 40, 66). The roles of the individual viral proteins in these distinct processes are not completely understood. Studies in which immortalization was evaluated under conditions which did not stimulate differentiation (low serum and Ca^{2+}) showed that E6 alone had no effect on the life span (19, 20, 21, 25), while expression of E7 alone resulted in transient prolongation of life span or infrequent immortalization (25) which could be increased by using highly transducible retroviral vectors (19). The present study is the first to identify the exclusive role of E6 in the induction of resistance to serum- and calcium-induced terminal differentiation and to demonstrate that changes leading to such resistance do not necessarily result in immortalization.

Using raft culture systems as a model for morphological differentiation, Hudson et al. (25) showed that E7 of HPV-18 by itself had a limited effect on the morphological differentiation. However, introduction of E6 into keratinocytes immortalized by E7 resulted in their increased growth and altered differentiation (33), supporting the role of E6 in the alteration of the morphological differentiation of epidermal keratinocytes.

Inhibition of programmed cell death associated with the terminal differentiation of epidermal keratinocytes (38) could possibly also account for the observed activity of E6. HPV-16 E6 was shown to inhibit lens fiber cell differentiation which is accompanied by fiber cell denucleation and DNA degradation, characteristic to programmed cell death (42).

The mechanism by which HPV-16 E6 induces resistance to serum- and calcium-induced differentiation is a matter for further investigation. E6 could possibly exert its biologic activity either through transcriptional modulation of cellular genes participating in the differentiation process or by direct interaction with regulatory proteins. E6 modulates the transcriptional activity of certain viral gene promoters (11, 13) and physically interacts with several cellular proteins, including the p53 tumor suppressor protein (64) and the ubiquitin ligase E6A (26). The association of E6 with E6BP (9) is particularly intriguing, since E6BP is identical to the EF-hand putative calcium-binding protein ERC-55. Members of the EF superfamily of calcium-binding proteins act as regulators of epidermal differentiation (62).

The involvement of p53 in E6-induced resistance to differentiation stimuli needs further investigation. Our preliminary data indicate that plasmids encoding a dominant-negative mutant of p53 (3), human MDM-2 (an inhibitor of p53 [39]), and antisense p53 RNA fail to functionally replace E6 in the colony formation assay, implying that p53-independent events are necessary for inhibition of keratinocyte differentiation. E6BP (9) or several other cellular proteins (30) may represent the functional targets for E6 in altering keratinocyte responses to differentiation signals.

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REFERENCES

1. Androphy, E. J., N. L. Hubbert, J. T. Schiller, and D. R. Lowy. 1987. Identification of the HPV-16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. *EMBO J.* **6**:989-992.
2. Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley. 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J. Virol.* **61**:962-971.
3. Baker, S. J., S. Markowitz, E. R. Fearon, J. K. V. Willson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell line growth by wild type p53. *Science* **249**:1912-1915.
4. Band, V., S. Dalal, L. Delmolino, and E. J. Androphy. 1993. Enhanced degradation of p53 protein in HPV-6 and BPV-1 E6 immortalized human mammary epithelial cells. *EMBO J.* **12**:1847-1852.
5. Band, V., J. A. DeCaprio, L. Delmolino, V. Kulesa, and R. Sager. 1991. Loss of p53 protein in human papillomavirus type 16 E6-immortalized human mammary epithelial cells. *J. Virol.* **65**:6671-6676.
6. Banks, L., and L. Crawford. 1988. Analysis of human papillomavirus type 16 polypeptides in transformed primary cells. *Virology* **165**:326-328.
7. Barbosa, M. S., D. R. Lowy, and J. T. Schiller. 1989. Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *J. Virol.* **63**:1404-1407.
8. Bedell, M. A., K. H. Jones, S. R. Grossman, and L. A. Laimins. 1989. Identification of human papillomavirus type 18 transforming genes in immortalized and primary cells. *J. Virol.* **63**:1247-1255.
9. Chen, J. J., C. E. Reid, V. Band, and E. J. Androphy. 1995. Interaction of papillomavirus E6 oncoprotein with a putative calcium-binding protein. *Science* **269**:529-531.
10. Conrad, M., V. J. Bubbs, and R. Schlegel. 1993. The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. *J. Virol.* **67**:6170-6178.
11. Crook, T., J. A. Tidy, and K. H. Vousden. 1991. Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. *Cell* **67**:547-556.
12. Crook, T., D. Wrede, and K. H. Vousden. 1991. p53 point mutation in HPV negative human cervical carcinoma cell lines. *Oncogene* **6**:873-875.
13. Desaintes, C., S. Hallez, P. Van Alphen, and A. Burny. 1992. Transcriptional activation of several heterologous promoters by the E6 protein of human papillomavirus type 16. *J. Virol.* **66**:325-333.
14. Dingwell, C., and R. A. Laskey. 1991. Nuclear targeting sequences: a consensus? *Trends Biochem. Sci.* **16**:478-481.
15. Dingwell, C., J. Robbins, S. M. Dilworth, B. Roberts, and W. D. Richardson. 1988. The nucleoplasmic nuclear location sequence is larger and more complex than that of SV-40 large T antigen. *J. Cell Biol.* **107**:841-849.

16. Doorbar, J., A. Parton, K. Hartley, L. Banks, T. Crook, M. Stanley, and L. Crawford. 1990. Detection of novel splicing patterns in a HPV 16 containing keratinocyte cell lines. *Virology* **178**:254–262.
17. Dyson, N., P. M. Howley, K. Munger, and E. Harlow. 1989. The human papillomavirus 16 E7 oncoprotein is able to bind the retinoblastoma gene product. *Science* **243**:934–937.
18. Garcia-Bustos, J., J. Heitman, and M. N. Hall. 1991. Nuclear protein localization. *Biochim. Biophys. Acta* **107**:83–101.
19. Halbert, C. L., B. W. Demers, and D. A. Galloway. 1991. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J. Virol.* **65**:473–478.
20. Halbert, C. L., G. W. Demers, and D. A. Galloway. 1992. The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells. *J. Virol.* **66**:2125–2134.
21. 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 human foreskin keratinocytes. *EMBO J.* **8**:3905–3910.
22. Hennings, H., K. A. Holbrook, and S. H. Yuspa. 1983. Factors influencing calcium-induced terminal differentiation in cultured mouse epidermal cells. *J. Cell Physiol.* **116**:265–281.
23. Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S. H. Yuspa. 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19**:245–254.
24. Hubbert, N. L., S. A. Sedman, and J. T. Schiller. 1992. Human papillomavirus type 16 E6 increases the degradation rate of p53 in human keratinocytes. *J. Virol.* **66**:6237–6241.
25. Hudson, J. B., M. A. Bedell, D. J. McCance, and L. A. Laimins. 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.
26. Huibregtse, J. M., M. Scheffner, and P. M. Howley. 1991. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J.* **10**:4129–4135.
27. Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell* **39**:499–509.
28. Kanda, T., A. Furuno, and K. Yoshiike. 1988. Human papillomavirus type 16 open reading frame E7 encodes a transforming gene for rat 3Y1 cells. *J. Virol.* **62**:610–613.
29. Kanda, T., S. Watanabe, S. Zanma, H. Sato, A. Furuno, and K. Yoshiike. 1991. Human papillomavirus type 16 E6 protein with glycine substitution for cysteine in the metal-binding motif. *Virology* **185**:536–548.
30. Keen, N., R. Elston, and L. Crawford. 1994. Interaction of the E6 protein of human papillomavirus with cellular proteins. *Oncoprotein* **9**:1493–1499.
31. Kozak, M. 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J. Mol. Biol.* **196**:947–950.
32. Kulesz-Martin, M. F., B. Kochler, H. Hennings, and S. H. Yuspa. 1980. Quantitative assay for carcinogen altered differentiation in mouse epidermal cells. *Carcinogenesis* **1**:995–1006.
33. Lechner, M. S., D. H. Mack, A. B. Finicle, T. Crook, K. H. Vousden, and L. A. Laimins. 1992. Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53 mediated repression of transactivation. *EMBO J.* **11**:3045–3052.
34. Liang, X. H., M. Volkmann, R. Klein, B. Herman, and S. J. Lockett. 1993. Co-localization of the tumor suppressor protein p53 and human papillomavirus E6 protein in human cervical carcinoma cell lines. *Oncogene* **8**:2645–2652.
35. Lim, P. S., A. B. Jensen, L. Cowser, Y. Nakai, L. Y. Lim, X. W. Jin, and J. P. Sundberg. 1990. Distribution and specific identification of papillomavirus major capsid protein epitopes by immunocytochemistry and epitope scanning of synthetic peptides. *J. Infect. Dis.* **162**:1263–1269.
36. Liu, A., J. Ghai, R. S. Ostrow, R. C. McGlennen, and A. J. Faras. 1994. The E6 gene of human papillomavirus type 16 is sufficient for transformation of baby rat kidney cells in cotransfection with activated Ha-ras. *Virology* **201**:388–396.
37. Malion, R. G., D. Wojciechowicz, and V. Defendi. 1987. DNA binding activity of papillomavirus proteins. *J. Virol.* **61**:1655–1660.
38. McCall, C. A., and J. J. Cohen. 1991. Programmed cell death in terminally differentiating keratinocytes: role of endogenous endonuclease. *J. Invest. Dermatol.* **97**:111–114.
39. Momand, J., G. P. Zambetti, D. C. Olson, D. George, and A. J. Levine. 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation. *Cell* **69**:1237–1245.
40. 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.
41. Munger, K., B. A. Werness, N. Dyson, W. C. Phelps, and P. M. Howley. 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J.* **8**:4099–4105.
42. Pan, H., and A. E. Griep. 1994. Altered cell cycle regulation in the lens of the HPV16 E6 or E7 transgenic mice: implications for tumor suppressor gene function in development. *Genes Dev.* **8**:1285–1299.
43. 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.
44. Scheffner, M., K. Munger, J. C. Byrne, and P. M. Howley. 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc. Natl. Acad. Sci. USA* **88**:5523–5527.
45. Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley. 1990. The E6 oncoprotein encoded by human papillomavirus type 16 and 18 promotes the degradation of p53. *Cell* **63**:1129–1136.
46. Schlegel, R., W. C. Phelps, Y.-L. Zhang, and M. Barbosa. 1988. Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. *EMBO J.* **7**:3181–3187.
47. Schneider-Gadicke, A., S. Kaul, E. Schwarz, H. Gausepohl, R. Frank, and G. Bastert. 1988. Identification of the human papillomavirus type 18 E6* and E6 proteins in nuclear protein fractions from human cervical carcinoma cells grown in the nude mouse or in-vitro. *Cancer Res.* **48**:2969–2974.
48. Schneider-Gadicke, A., and E. Schwarz. 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. *EMBO J.* **5**:2285–2292.
49. Schwarz, E., U. K. Freese, L. Gissman, W. Mayer, B. Roggenbuck, A. Strem-lau, and H. zur Hausen. 1985. Structure and transcription of human papillomavirus virus sequences in cervical carcinoma cells. *Nature (London)* **314**:111–114.
50. Sedman, S. A., M. S. Barbosa, W. C. Vass, N. L. Hubbert, J. A. Haas, D. R. Lowy, and J. T. Schiller. 1991. The full-length human herpesvirus E6 protein has transforming and transactivating activities and cooperates with E7 to immortalize keratinocytes in culture. *J. Virol.* **65**:4860–4866.
51. Sedman, S. A., N. L. Hubbert, W. C. Vass, D. R. Lowy, and J. T. Schiller. 1992. Mutant p53 can substitute for human papillomavirus type 16 E6 in immortalization of human keratinocytes but does not have E6-associated transactivation or transforming activity. *J. Virol.* **66**:4201–4208.
52. Seedorf, K., G. Krammer, M. Durst, S. Suhai, and W. G. Rowekamp. 1985. Human papillomavirus type 16 DNA sequence. *Virology* **145**:181–185.
53. Seedorf, K., T. Oltersdorf, G. Krammer, and W. Rowekamp. 1987. Identification of early proteins of the human papillomaviruses type 16 (HPV16) and type 18 (HPV18) in cervical carcinoma cells. *EMBO J.* **6**:139–144.
54. Shally, M., N. Alloul, M. Muller, L. Gissman, and L. Sherman. Submitted for publication.
55. Sherman, L., and N. Alloul. 1992. Human papillomavirus type 16 expresses a variety of alternatively spliced mRNAs putatively encoding the E2 protein. *Virology* **191**:953–959.
56. Smotkin, D., H. Prokoph, and F. O. Wettstein. 1989. Oncogenic and non-oncogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. *J. Virol.* **63**:1441–1447.
57. Smotkin, D., and F. O. Wettstein. 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. USA* **83**:4680–4684.
58. Sparkowski, J., J. Anders, and R. Schlegel. 1994. Mutation of the bovine papillomavirus E5 oncoprotein at amino acid 17 generates both high- and low-transforming variants. *J. Virol.* **68**:6120–6123.
59. Storey, A., and L. Banks. 1993. Human papillomavirus type 16 E6 gene cooperates with EJ-ras to immortalize primary mouse cells. *Oncogene* **8**:919–924.
60. Underwood, M. R., and H. M. Fried. 1990. Characterization of nuclear localization sequences from yeast ribosomal protein L29. *EMBO J.* **9**:91–99.
61. Villa, L. L., and R. Schlegel. 1991. Differences in transformation activity between HPV18 and HPV16 map to the viral LCR E6-E7 region. *Virology* **181**:374–377.
62. Voiz, A., B. P. Korge, J. G. Compton, A. Ziegler, P. M. Steinert, and D. Mischke. 1993. Physical mapping of a functional cluster of epidermal differentiation genes on chromosome 1q21. *Genomics* **18**:92–99.
63. 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.
64. 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.
65. Wilson, I. A., H. L. Niman, R. A. Houghten, A. R. Cherenon, M. L. Connolly, and R. A. Lerner. 1984. The structure of an antigenic determinant in a protein. *Cell* **37**:767–778.
66. Woodworth, C. D., S. Cheng, S. Simpson, L. Hamacher, L. T. Chow, T. R. Broker, and J. A. DiPaolo. 1992. Recombinant retroviruses encoding human papillomavirus type 18 E6 and E7 stimulate proliferation and delay differentiation of human keratinocytes early after infection. *Oncogene* **7**:619–626.
67. Yuspa, S. H., and D. L. Morgan. 1981. Mouse skin cells resistant to terminal differentiation associated with initiation of carcinogenesis. *Nature (London)* **293**:72–74.
68. zur Hausen, H. 1989. Papillomaviruses as carcinomaviruses. *Adv. Viral Oncol.* **8**:1–26.