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Mutant p53 Can Substitute for Human Papillomavirus Type 16 E6 in Immortalization of Human Keratinocytes but Does Not Have E6-Associated *trans*-Activation or Transforming Activity

SYLVIA A. SEDMAN, NANCY L. HUBBERT, WILLIAM C. VASS, DOUGLAS R. LOWY, AND JOHN T. SCHILLER*

Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, Maryland 20892

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Human papillomavirus type 16 (HPV16) E6 and E7 are selectively retained and expressed in HPV16-associated human genital tumors. E6 is active in several cell culture assays, including transformation of NIH 3T3 cells, *trans* activation of the adenovirus E2 promoter, and cooperation with E7 to immortalize normal human keratinocytes. Biochemically, the HPV16 E6 protein has been shown to bind to tumor suppressor protein p53 in vitro and induce its degradation in a rabbit reticulocyte lysate. To examine the relationship between the various biological activities of E6 and inactivation of p53, we tested the abilities of dominant negative mutants of p53 to substitute functionally for E6 in the three cell culture assays. While wild-type p53 inhibited keratinocyte proliferation, both mouse and human mutant p53s, in conjunction with E7, increased proliferation of the keratinocytes, resulting in generation of immortalized lines. However, in contrast to E6, mutant p53 was unable to induce transformation or *trans* activate the adenovirus E2 promoter in NIH 3T3 cells. These results suggest that inactivation of wild-type p53 is necessary for HPV-induced immortalization of human keratinocytes and that different or additional activities are required for E6-dependent transformation and *trans* activation of NIH 3T3 cells.

Infection with certain human papillomavirus (HPV) types, such as HPV16, appears to be a risk factor in the development of human cervical and other genital carcinomas (reviewed in reference 45). The E6 and E7 open reading frames are selectively retained and expressed in cancer cells, suggesting that the E6 and E7 proteins are involved in initiation and/or maintenance of human carcinomas (37, 39). The determination that E6 can cooperate with E7 to immortalize normal human foreskin keratinocytes and cervical epithelial cells (19, 27) has lent strong experimental support to the concept that the activity of E6 has pathogenic significance in the development of HPV-containing cancers. In addition to cooperating in immortalization of human epithelial cells, HPV16 E6 can independently induce anchorage-independent growth and *trans* activate the adenovirus E2 promoter in NIH 3T3 cells (38).

In contrast to the activities defined for the high-risk E6 proteins, the E6 gene from HPV6 (designated a low-risk type because it is only rarely detected in genital carcinomas) was inactive in immortalization of primary human keratinocytes and NIH 3T3 transformation assays (5, 38). However, both high- and low-risk E6 proteins were able to *trans* activate transcription of the adenovirus promoter (8, 38).

Biochemical characterization of E6 has been hampered by its low abundance in the cells in which it exerts a biological effect and by its tight association with nuclear complexes. However, it has recently been reported that E6 synthesized in a rabbit reticulocyte extract specifically binds wild-type p53 (43). The E6s of high-risk HPVs bind p53 more efficiently than do the E6s of low-risk viruses, and only the high-risk E6s induce ubiquitin-mediated degradation of wild-type p53 in these lysates (8, 35). Because p53 is frequently disrupted in human and animal cancers (2, 6, 28, 29) and the wild-type gene inhibits cellular transformation in experimental systems (3, 12, 16), it has been designated a tumor suppressor or antioncogene. Since the high-risk E6 proteins have immortalizing and transforming activities, while the low-risk E6 proteins do not, it is possible that inactivation of p53 plays an important role in these activities.

p53 genes isolated from tumors or cell lines frequently contain missense mutations in the coding sequences and act in a dominant fashion, in that cultured cells that express both the wild-type and mutant genes often have a greater proliferative capacity than cells that express only the wild-type gene (reviewed in reference 22). Inhibition of the growth suppression activity of wild-type p53 by the mutant protein is thought to occur, at least in some cases, through formation of inactive hetero-oligomers (11, 16, 33). Therefore, to investigate the relationship between E6 activity and p53 inactivation, we examined the abilities of dominant mutant p53 genes to substitute functionally for HPV16 E6 in the E6-dependent immortalization, transformation, and transcriptional *trans*-activation assays. If E6 activity in these assays were due to functional inactivation of wild-type p53, then one would expect the dominant mutant p53 genes to have a comparable activity. In previous studies using baby rat kidney cells, HPV16 E7, but not E6, was shown to cooperate with *ras* to induce colony formation efficiently (31, 41). A similar activity has been identified for mutant p53 (13, 20, 30). In this study, we determined that in contrast to the cooperation assay with rodent cells, mutant p53 can replace E6, but not E7, in the human keratinocyte immortalization assay but that it is inactive in transformation of NIH 3T3 cells and *trans* activation of the adenovirus E2 promoter.

MATERIALS AND METHODS

Plasmids. pLTR, pSDE6, and pE7 have been described previously (38). Briefly, pLTR is a pUC19-based plasmid

* Corresponding author.

containing the Harvey murine sarcoma virus long terminal repeat (LTR) inserted into pUC19 at the *Eco*RI site. pSDE6 contains the Harvey murine sarcoma virus LTR and HPV16 nucleotides 24 to 654 such that the HPV16 sequences are transcribed from the LTR when the plasmid is transfected into mammalian cells. pSDE6 also contains a two-nucleotide substitution of the wild-type sequence that inactivates the splice donor site at nucleotide 226 (38). pE7 contains nucleotides 505 to 1176 of HPV16 downstream of the Harvey murine sarcoma virus LTR (5).

pEC113 contains the adenovirus E2 promoter, followed by the sequences that encode chloramphenicol acetyltransferase (CAT) (21).

The murine mutant p53 gene used in this study was originally cloned in Moshe Oren's laboratory (11) and designated pLTRp53cG. The wild-type equivalent, which contains an alanine in place of the valine residue at amino acid 135, was generated by Hinds et al. (20). The wild type and mutant clones have subsequently been designated p53-WT and p53-val 135, respectively (16). Both are transcribed from the Harvey murine sarcoma virus LTR. The human wild-type and mutant p53 clones were kindly provided by Bert Vogelstein. pC53-SN3 encodes the wild-type gene, and pC53-SCX3 encodes a mutant p53 protein containing a substitution of an alanine in place of the valine in the wild-type protein at amino acid 143. The human p53 genes are transcribed from a cytomegalovirus promoter.

Immortalization assay. Early-passage normal human foreskin keratinocytes were grown in KGM medium (Clonetics, San Diego, Calif.). Cells were transfected with 5 μg of SV2neo, which encodes the neomycin resistance gene, and 5 μg each of various combinations of plasmids that express either E6, p53, or E7. Cells were selected for G418 resistance as previously described (5).

Transformation assay. NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, Md.) and grown in Dulbecco's modified Eagle's medium plus 10% newborn calf serum. For transformation assays, cells were seeded at 3×10^5/35-mm-diameter dish at 24 h before transfection. The test plasmid (0.5 μg) plus 0.1 μg of pSV2neo were introduced into cells by calcium phosphate precipitation as described previously (38). Transfected cells were split at a ratio of 1:5 on the following day. Dulbecco's modified Eagle's medium containing 500 μg of G418 per ml was added 1 day later. Approximately 10 days after addition of G418, drug-resistant colonies were pooled and seeded into 0.4% agar at a density of 10^5/60-mm-diameter dish. Individual transfections were scored for colony formation after 3 to 4 weeks. Colonies composed of greater than 10 cells were scored as positive.

CAT assay. NIH 3T3 cells were also used for CAT assays. Cells were plated on 60-mm-diameter dishes and transfected 24 h later with 2 μg of pEC113 plus 8 μg of a plasmid that expresses HPV16 E6, wild-type p53, mutant p53, or the vector alone. At 72 h posttransfection, cells were scraped off of the plates in 100 mM Tris (pH 8.0) and subjected to five freeze-thaw cycles. Cell debris was removed by centrifugation. CAT assays were performed with [^{14}C]chloramphenicol and unlabeled acetyl coenzyme A as described previously (18). Amounts of acetylated and unacetylated chloramphenicol were quantified from thin-layer chromatography plates with an Ambis scanner.

Analysis of HPV proteins and p53 in NIH 3T3 cells. NIH 3T3 cells were cotransfected with pSV2neo alone or pSV2neo plus pSDE6 or murine mutant p53, selected with 500 μg of G418 per ml, and grown to confluence. Cells were then seeded in agar and maintained until transformed colonies appeared. Individual colonies transformed by E6 were picked from the agar and plated. Since cells transfected with the neomycin resistance gene or neomycin plus p53 do not grow in agar, mass cultures were used to analyze protein in these cells. Cells were replated and labeled as they approached confluence with [^{35}S]cysteine (1 mCi per T75 flask) and harvested 1 h later. E6 was immunoprecipitated, as described previously, with polyclonal rabbit antiserum raised against an E6 bacterial fusion protein (1). p53 was immunoprecipitated by using a monoclonal antibody, pAb421 (23), which recognizes human and mouse p53 (p53 Ab-1; Oncogene Science). p53 and E6 were separated by electrophoresis on 12 and 15% polyacrylamide gels, respectively. The gels were fixed, treated with fluor, dried, and exposed to X-ray film.

Analysis of protein in established keratinocyte lines. Analysis was done essentially as described above, except that cells were labeled for 20 min in cysteine-free KBM medium (Clonetics, San Diego, Calif.). p53 was immunoprecipitated with pAb421 or pAb1801 (4), which recognizes human p53 (p53 Ab-2; Oncogene Science). E7 was immunoprecipitated with antibodies raised against an E7 bacterial fusion protein (5) and separated by electrophoresis on 15% polyacrylamide gels.

TABLE 1. G418^r colonies in keratinocytes transfected with p53

Gene(s) transfected	No. of G418^r colonies	
	Mouse p53[a]	Human p53[a]
LTR alone	0	0
Neo + LTR	96	77
E7	78	
E6 + E7	93	
Wt[b] p53	12	15
Wt p53 + E6	32	
Wt p53 + E7	27	
Wt p53 + E6 + E7	35	
Mut[c] p53	165	95
Mut p53 + E6	146	
Mut p53 + E7	109	
Mut p53 + E6 + E7	145	

[a] Data for mouse and human p53 were obtained from independent experiments.
[b] Wt, wild type.
[c] Mut, mutant.

RESULTS

Wild-type p53 inhibits proliferation of normal human keratinocytes. Finlay et al. have shown that expression of wild-type p53 can inhibit the growth of normal rodent cells in culture (16). To determine whether p53 can influence the growth of normal human genital epithelial cells, wild-type and mutant p53 genes were cotransfected with the neomycin resistance gene into early-passage neonatal human foreskin keratinocytes and the transfected cells were selected for resistance to the antibiotic G418. In comparison with keratinocyte cultures transfected with the resistance gene alone, those cotransfected with either the wild-type mouse p53 or wild-type human p53 gene gave rise to 5- to 10-fold fewer G418-resistant colonies (Table 1). Cotransfection of cells with pSV2neo and HPV16 E6 or E7 did not affect the number of resistant colonies generated and did not appear to reverse the inhibitory activity of a cotransfected wild-type

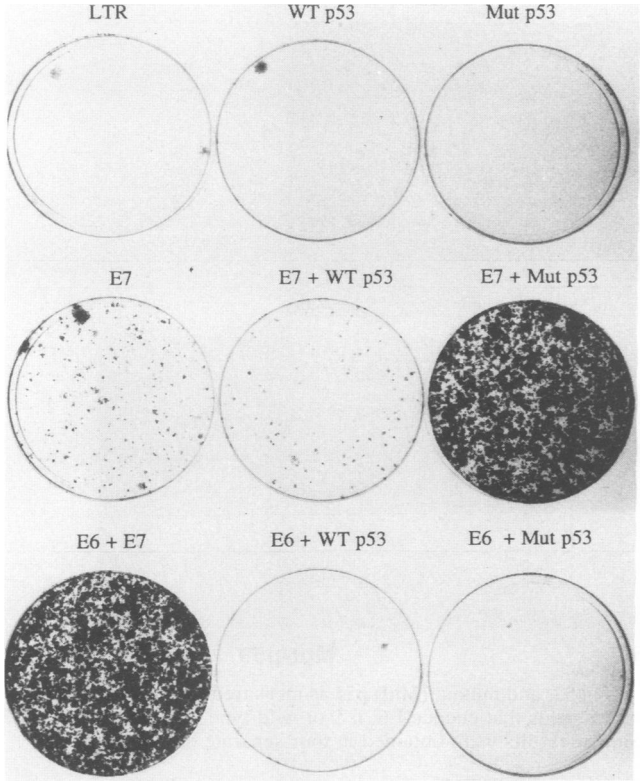

FIG. 1. Induction of proliferation in human foreskin keratinocytes. Early-passage normal keratinocytes were cotransfected with pSV2neo and plasmids that encode HPV16 E6, murine wild-type (WT) p53, murine mutant (Mut) p53, HPV16 E7, the vector alone (LTR), or combinations of the above. Cells were selected with G418, grown to confluence, and replated in duplicate. Cells on the plates were grown for 3 weeks and then stained; a parallel set was grown continuously in culture.

mouse p53 gene strongly. When the mutant mouse p53 or mutant human p53 gene was cotransfected with pSV2neo, a similar or slightly increased number of G418-resistant colonies was observed relative to that seen with the neomycin resistance gene alone. We conclude that increased expression of wild-type, but not mutant, p53 inhibits proliferation of human keratinocytes.

Mutant p53 can substitute for E6 in keratinocyte immortalization. As previously shown, HPV16 E6 and E7 cooperated to induce proliferation in early-passage human foreskin keratinocytes (19, 27); transfection of E7 alone induced a transient increase in proliferation (Fig. 1), while E6-transfected keratinocytes grew no better than the negative controls (data not shown). Continuous passage of these cells indicated that only cultures transfected with both E6 and E7 become immortalized (Table 2). When wild-type or mutant p53 was transfected alone into keratinocytes, no increase in proliferation was detected relative to the vector-only negative control. Cotransfection of wild-type p53 with HPV16 E6 or E7, likewise, did not induce proliferation. In contrast, cotransfection of mutant p53 with E7 markedly stimulated proliferation to an extent that was initially indistinguishable from that induced by the E6-plus-E7 positive control (Fig. 1). Like E6-plus-E7-transfected cells, cells transfected with mutant p53 plus E7 continued to divide indefinitely (more than 30 passages). We noted that cells cotransfected with

TABLE 2. Immortalization of primary keratinocytes by HPV16 E7 and p53

Gene(s) transfected	No. of proliferating lines/no. of dishes transfected	
	Mouse p53	Human p53
Wt[a] p53	0/8	0/2
Wt p53 + E6	0/8	0/3
Wt p53 + E7	1/8	0/2
Mut[b] p53	0/8	0/2
Mut p53 + E6	0/8	0/3
Mut p53 + E7	8/10	2/3
E6 + E7	8/8	2/2

[a] Wt, wild type.
[b] Mut, mutant.

mouse or human mutant p53 and E7 generally became established more slowly than lines established with E6 plus E7; in one experiment, cells transfected with mutant p53 plus E7 were passaged four times within the first 8 weeks after transfection, compared with six passages for cells transfected with E6 plus E7. However, the differences in growth rate diminished upon further passage. Similar results were obtained in multiple transfections using a mouse mutant p53 gene and in a more limited number of transfections using a human p53 gene (Table 2).

Analysis of the expression of E7 and mutant p53 genes in the proliferating lines was performed by immunoprecipitation. The representative results in Fig. 2 indicate that both E7 and mutant mouse p53 are expressed at relatively high levels. The protein product of the transfected mouse p53

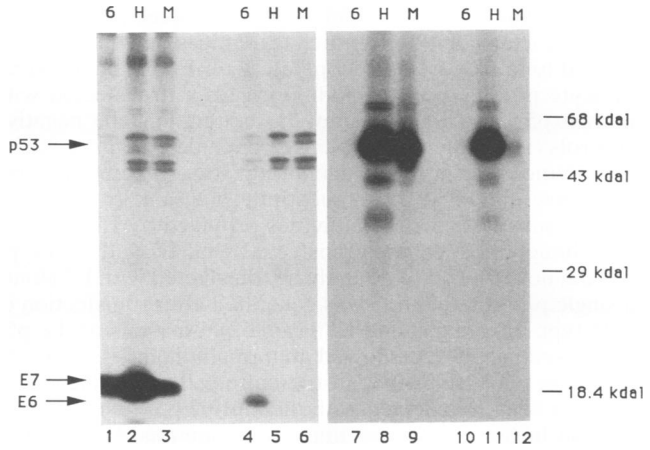

FIG. 2. Immunoprecipitation of E6, E7, and p53 from keratinocyte lines. Lines were generated by cotransfection with plasmids that encode neomycin resistance plus E7 and either SDE6 (6), human mutant p53 (H), or mouse mutant p53 (M) and selection with G418. Cells from lines which continued to proliferate in culture were pulse-labeled with [^{35}S]cysteine for 20 min. E6, E7, and p53 were immunoprecipitated from lysates containing 10^7 cpm. Cell lysates from lanes 1 to 3 were immunoprecipitated by using HPV16 E7-specific antisera, those from lanes 4 to 6 were immunoprecipitated by using HPV16 E6-specific antisera, those from lanes 7 to 9 were immunoprecipitated by using a monoclonal antibody which recognizes both mouse and human p53 proteins, and those from lanes 10 to 12 were immunoprecipitated by using a monoclonal antibody which recognizes only human p53. Exposure times used for immunoprecipitation of p53, E6, and E7 were 1, 3, and 3 days, respectively. Positions of molecular size standards are shown on the right.

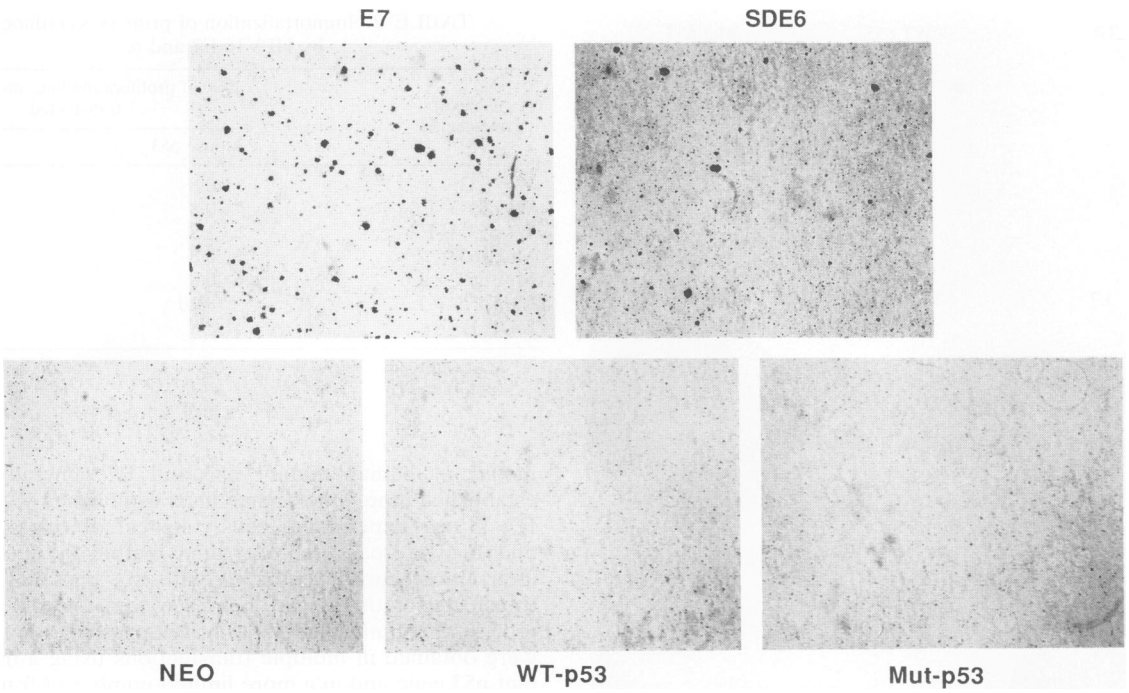

FIG. 3. Transforming activities of HPV16 E6, HPV16 E7, wild-type (WT) p53, and mutant (Mut) p53 as measured by growth of NIH 3T3 cells in agar. NIH 3T3 cells were cotransfected with pSV2neo (NEO) and plasmids that encode E6, E7, or wild-type or mutant mouse p53. Cells were selected with G418, and mass cultures were plated in agar. Similar results were obtained in four separate experiments.

gene can be distinguished from human p53 protein by its faster mobility when subjected to acrylamide gel electrophoresis (17) and by the specificity of immunoprecipitation by a p53 monoclonal antibody which recognizes only human p53.

Wild-type p53 did not cooperate with E6 to induce keratinocyte proliferation. In addition, cells cotransfected with mutant p53 plus E6 also grew no better than the negative controls (Fig. 1 and Table 2). We conclude that a specificity of activities is required for immortalization of keratinocytes, and while mutant p53 can substitute functionally for E6, it cannot substitute for the activities provided by E7.

Although most cells cotransfected with E7 and wild-type p53 did not grow better than those transfected with E7 alone, a single proliferating line was generated after transfection of wild-type mouse p53 and E7 (Table 2). Analysis of the p53 expressed in this line showed abnormally high levels of p53 (data not shown). Although the culture that produced this line had been transfected with the murine p53 gene, the p53 present in these cells was immunoprecipitated by a human p53-specific monoclonal antibody and migrated at a mobility characteristic of the human protein. Many mutant p53 proteins have an increased half-life relative to that of the wild-type protein (24). The increased level of p53 detected in this line suggests that it represents a mutated form of p53. A faster-migrating form was not detected by using an antibody that also recognizes the mouse protein. We therefore believe that the transfected mouse p53 gene is not expressed and that the increased proliferation of this culture may be due to the cooperative activities of E7 and a spontaneous mutation of the endogenous human p53 gene.

Activity of p53 in NIH 3T3 cells. HPV16 E6, as well as E7, can independently transform NIH 3T3 cells (38). To determine whether the mutant mouse p53 gene has a similar transforming activity, wild-type or mutant p53 was cotransfected with the neomycin resistance gene and selected for G418 resistance. Resistant colonies were pooled and analyzed for the ability to grow in soft agar. While cells transfected with either the E6 or the E7 gene formed colonies in agar, cells transfected with wild-type or mutant p53 were unable to grow in agar (Fig. 3). Cells transfected with mutant p53 contained elevated levels of p53 protein (Fig. 4), indicating that the lack of transforming activity associated with mutant p53 was not due to lack of expression of the protein. Therefore, it appears that inactivation of endogenous mouse p53 is not sufficient for E6-induced transformation in NIH 3T3 cells.

p53 metabolism in NIH 3T3 cells transformed by HPV16 E6. E6 has been shown to interact with and lead to the degradation of human p53 in reticulocyte extracts. From the results of the complementation experiments just described, it appears that p53 inactivation plays an important role in the immortalization of primary human keratinocytes. To determine whether E6 expression resulted in decreased stability of the endogenous p53 in NIH 3T3 cells, individual colonies of NIH 3T3 cells transformed with HPV16 E6 were isolated from agar and reseeded in liquid culture and the half-life of p53 was determined. Individual colonies were chosen for study rather than a mass culture because a significant percentage of cells transfected with E6 are not phenotypically transformed, and there was concern that metabolism of p53 in these two populations might be different. Transformed cells were grown, and E6 expression was verified by labeling the cells with [^{35}S]cysteine, followed by immunoprecipitation of E6 (Fig. 5A). The half-life of endogenous p53 was determined by pulse-labeling with [^{35}S]cysteine for 20 min and chasing with medium containing unlabeled cysteine for 15, 30, 90, and 240 min. Equal numbers of counts from each time point were immunoprecipitated by using a monoclonal

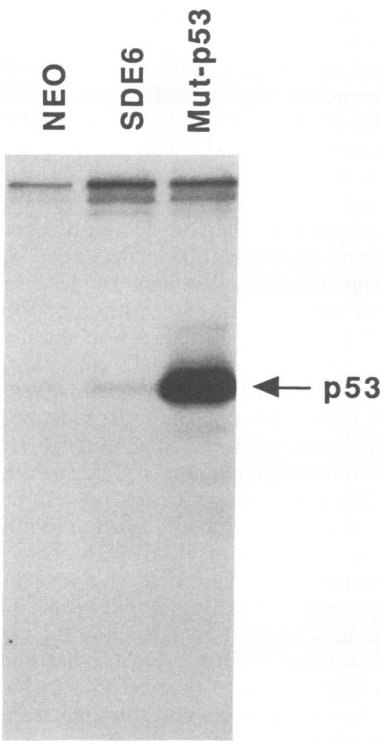

FIG. 4. Immunoprecipitation of p53 in NIH 3T3 cells transfected with pSV2neo (NEO) alone or pSV2neo plus plasmids that express HPV16 E6 (SDE6) or mouse mutant p53 (Mut-p53). Cells were selected with G418, replated, and pulse-labeled for 1 h with [^{35}S]cysteine. Immunoprecipitations were performed by using p53 Ab-1 as described in the legend to Fig. 2.

antibody which recognizes mammalian p53. The half-life of p53 in these cells was compared with that of p53 in a mass culture transfected with pSV2neo alone. The results obtained with one of the E6-transformed colonies analyzed are shown in Fig. 5B. The half-life of p53 was not appreciably

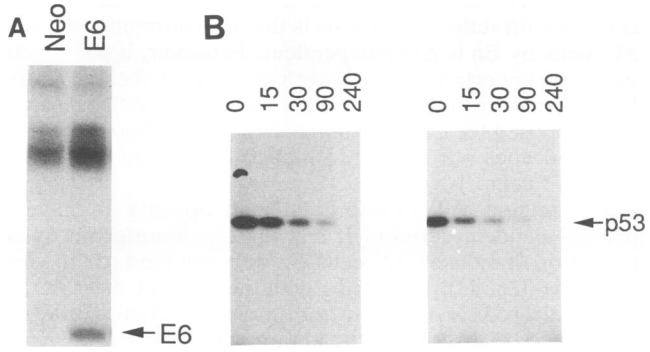

FIG. 5. (A) Immunoprecipitation of E6 from a colony of NIH 3T3 cells transformed with HPV 16 E6. Cells were pulse-labeled for 1 h with [^{35}S]cysteine and immunoprecipitated from lysates containing 1.5×10^7 cpm. (B) p53 metabolism in NIH 3T3 cells transfected with pSV2neo (left panel) or pSV2neo plus HPV16 E6 (right panel). Cells from the colonies analyzed in panel A were plated into T25 flasks. The cells were labeled with [^{35}S]cysteine for 20 min and chased with complete medium containing unlabeled cysteine for 0, 15, 30, 90, or 240 min. p53 was immunoprecipitated by using p53 Ab-1 from lysates containing 5×10^6 cpm from each time point.

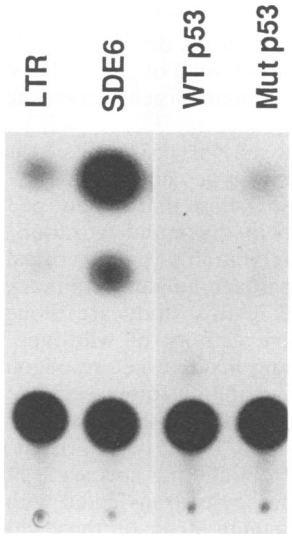

FIG. 6. *trans* activation of the adenovirus E2 promoter in NIH 3T3 cells. NIH 3T3 cells were cotransfected with pEC113 and either SDE6, mouse wild-type (WT) p53, mouse mutant (Mut) p53, or vector alone (LTR). Lysates were harvested at 72 h posttransfection and analyzed for CAT activity as described previously (18).

altered in NIH 3T3 cells transformed with HPV16 E6 relative to cells that express only the neomycin resistance gene.

p53 cannot *trans* activate the adenovirus E2 promoter. As previously reported, HPV16 E6 can *trans* activate the adenovirus E2 promoter in transient assays after transfection into NIH 3T3 cells. By using the CAT gene as a reporter, we compared *trans* activation of the adenovirus E2 promoter by E6 and p53. In parallel assays in which E6 demonstrated considerable *trans* activation (6- to 20-fold over the negative control), mutant mouse and mutant human p53s produced little or no activation of the adenovirus E2 promoter (Fig. 6 and Table 3). The wild-type mouse and human p53 genes appeared to repress transcription from the adenovirus E2 promoter weakly, but this may be an indirect effect since transfection of wild-type p53 into cells in culture has been shown to inhibit cell growth (3, 12, 16).

DISCUSSION

The results of this study, coupled with those of other recent studies, suggest that E6 is a multifunctional protein. Several lines of evidence have already suggested that functional inactivation of p53 is of importance for the activity of E6 in the immortalization of human epithelial cells and

TABLE 3. *trans* activation of the adenovirus E2 promoter by p53 in NIH 3T3 cells

Expt no.	Fold *trans* activation[a]				
	Murine			Human	
	SDE6	Wt p53	Mutant p53	Wt p53	Mutant p53
1	11	0.3	0.6	ND[b]	ND
2	6	0.3	0.1	ND	ND
3	10	0.2	3	0.2	0.2
4	21	0.2	1.5	0.3	0.3

[a] *trans* activation is tabulated relative to the activity of pEC113 alone.
[b] ND, not determined.

progression of HPV-induced tumors, including the following. (i) HPV16 E6 induces degradation of p53 in vitro (35). (ii) The oncogenic potential of genital HPVs and the immortalizing activities of their E6 genes correlate with their ability to degrade p53 in vitro (8, 35). (iii) HPV-negative cervical carcinomas have mutant p53 genes, while HPV-positive tumors express wild-type genes (9, 34).

In this study, we show that mutant p53 can functionally substitute for E6 in the immortalization assay, providing evidence that inactivation of p53 is critical in the immortalization of early-passage human keratinocytes. p53 mutants of the type used in this study are thought to inhibit the growth-suppressive activity of wild-type p53 specifically, perhaps by forming inactive hetero-oligomers (11, 16, 33). Mutant human and mutant mouse p53 genes were active, ruling out the possibility that the activity of mutant p53 in the immortalization assay might be the result of aberrant functioning of a p53 from another species in human cells.

Although keratinocytes transfected with mutant p53 plus E7 exhibit an indefinite life span, these cells generally grow more slowly than cells immortalized by E6 and E7, suggesting that while inactivation of wild-type p53 may be the E6 activity necessary and sufficient for immortalization, other E6 activities may play a facilitating role in promoting cell growth. Another possibility is that mutant p53 simply inactivates wild-type p53 less efficiently than E6.

While mutant p53 was able to complement HPV16 E7 to induce immortalization of human keratinocytes, it was clearly unable to complement HPV16 E6, supporting the notion that mutant p53 and E6 disrupt a common pathway that is distinct from that involving E7. These results contrast with those obtained by using early-passage rodent cells. In rat cells, mutant p53 or E7, but not E6, was able to complement *ras* to induce colony formation efficiently (13, 30, 31, 41). Possible explanations for the inactivity of HPV16 E6 in the rat cell immortalization assays include insufficient expression of E6 or inability of the HPV E6 protein to inactivate wild-type rat p53.

Our results emphasize the apparent requirement for disruption of at least two growth-suppressing pathways for immortalization of human epithelial cells, in contrast to rodent cells, in which inactivation of either pathway can presumably induce immortalization. This difference correlates with the much higher rate of spontaneous immortalization of cultured rodent cells (10, 26, 42). Normal human keratinocytes transfected with mutant p53 alone did not grow better than those transfected with the negative control plasmid. This was also true for cells transfected only with E6. These results indicate that inactivation of the p53-dependent growth-inhibitory pathway is not sufficient to induce even transient mitogenesis in cultured keratinocytes. The ability of individuals with Li-Fraumeni syndrome, who are congenitally heterozygous for mutant p53 (25, 40), to develop normally suggests that this may generally be the case for all tissues in vivo, at least for certain types of p53 mutations. This is also consistent with the concept that inactivation of p53 is only one of the several genetic changes required for carcinoma development (14).

The fact that mutant p53 can substitute for E6 in the keratinocyte assay does not necessarily imply that inactivation of wild-type p53 is the only E6 activity required for immortalization. Since mutant p53 has been shown to increase the tumorigenic potential of a cell line lacking p53 (44), it is possible that these mutant proteins have activities, in addition to inactivation of p53, that contribute to immortalization. Analogous activities would, presumably, also be provided by E6.

Several lines of evidence imply that E6 also has activities that are independent of p53 inactivation. In this report, we present evidence that the same mutant p53 genes that substitute functionally for HPV16 E6 in the keratinocyte assay do not have the transforming and transcriptional *trans*-activating activities of E6 in NIH 3T3 cells, despite high-level expression of the transfected p53 gene.

Both wild-type and mutant p53s have been shown to have *trans*-activating activities, although not in the same assay (7, 15, 32). These activities must be different from that measured for HPV16 E6, since neither was able to activate the adenovirus E2 promoter in NIH 3T3 cells. The observation that HPV6 E6 and HPV16 E6 have similar *trans*-activation activities yet HPV6 E6 does not degrade p53 in in vitro assays is consistent with the idea that E6 transcriptional *trans* activation is independent of p53 degradation (8, 38). In support of this conclusion, Crook et al. have recently reported that p53 degradation and *trans* activation are genetically separable activities of HPV16 E6. They have shown that some E6 mutants that are defective for p53 degradation have wild-type *trans*-activating activity (8).

HPV16 E6 has been shown to interact with wild-type murine p53 in vitro (43). However, it is not known whether E6 promotes degradation of murine p53. In NIH 3T3 cells, p53 was detected in relatively low abundance and had a short half-life, suggesting that these cells express a wild-type p53 protein. In addition, the p53 protein expressed in NIH 3T3 cells does not react with a monoclonal antibody that specifically recognizes forms of mutant p53 commonly detected in spontaneous mouse fibroblast lines (7), also suggesting that the p53 in this cell line is the wild type. If an interaction between NIH 3T3 p53 and HPV16 E6 occurs in vivo, it does not appear to alter the half-life of p53 in E6-transformed cells appreciably, suggesting that E6 does not induce degradation of murine p53 in vivo. In contrast to this result, we have found that p53 is degraded at a higher rate in human keratinocytes that express HPV16 E6 relative to that seen in normal early-passage keratinocytes (unpublished data).

The observation that a mutant murine p53 does not transform NIH 3T3 cells leads to the conclusion that inactivation of p53 is not sufficient for transformation of these cells. An attractive hypothesis is that transformation of NIH 3T3 cells by E6 is p53 independent. However, if p53 inactivation is important in the transformation of these cells by E6, then inactivation occurs by a mechanism different from that proposed for E6 in the immortalization of keratinocytes, since E6 does not appear to induce degradation of p53 in NIH 3T3 cells.

The related BPV E6 protein also appears to have a p53-independent activity. It can induce tumorigenic transformation of mouse C127 cells yet does not bind p53 in vitro or in vivo (36, 43). In keeping with the concept that interaction with p53 is required for immortalization, BPV E6 appears to be inactive in the keratinocyte assay (5).

Regions of HPV16 E6 that are required for binding of p53 and inducing its degradation have been mapped (8), but the sequences required for E6 biological activity have not. Given the results discussed above, it is likely that E6 sequences required for immortalization include, but are not necessarily limited to, the domains that bind p53 and induce its degradation. However, the domains of E6 required for transformation cannot be predicted from the available data. It will be interesting to determine whether they are distinct

from or overlap with the domains involved in p53 interaction, immortalization, or *trans* activation.

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