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Modulation of Immortalizing Properties of Human Papillomavirus Type 16 E7 by p53 Expression

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The E7 protein is one of the principle transforming proteins encoded by human papillomavirus type 16 (HPV16), a virus strongly associated with the development of cervical carcinoma. In the present study we show that cotransfection of wild-type human or murine p53 sequences with E7 and *ras* markedly reduces transformation in baby rat kidney cells, although no effect of p53 is seen on the ability of E7 to transform an established mouse line to anchorage independence. In contrast, expression of mutant p53 strongly potentiates the transforming function of E7 and confers marked growth factor independence to cells cotransformed by E7 and *ras*. These data suggest that E7 and p53 function in separate yet complementary biochemical pathways.

Human papillomavirus type 16 (HPV16) is the genital HPV type most frequently found associated with the development of cervical carcinoma (8, 19). Analysis of the viral genome has revealed that HPV16 encodes two transforming or immortalizing genes, E6 and E7 (22, 28, 32, 41). Expression of E7 alone can partially transform established mouse cells (41) and, in cooperation with *ras*, fully transform primary rat or mouse cells (6, 32). HPV16 E7 has been shown to bind the cellular RB protein in vitro and in vivo (9, 16, 29) and mutagenic analyses have shown that the ability to bind RB is essential for the transforming and immortalizing activities of the E7 protein in rodent cells (2, 3). It is therefore likely that E7 acts, at least in part, by interfering with the normal cellular function of RB. HPV16 E6 has recently also been shown to complex with another cellular antioncogene product, p53 (42). The ability to encode transforming proteins capable of binding RB and p53 is shared by two other DNA tumor viruses, adenovirus and simian virus 40 (7, 25, 33, 43), and it is possible that simultaneous interference with the function of both cellular proteins is important in transformation. In this study we have investigated the possibility that alterations within the p53-associated pathway will modulate transformation by the E7 protein, which apparently acts via an RB-associated pathway.

The cellular p53 gene shows characteristics of a tumor suppressor gene, in which loss of wild-type p53 expression is associated with the development of many different types of human malignancies (1, 31, 39). Although wild-type p53 does not appear to have any transforming activity (11, 15, 23), mutant murine p53 sequences have been shown to act as dominant transforming genes in assays such as cooperation with *ras* to transform primary rat cells (11, 15, 23, 30). Since activated mutant p53 sequences have been isolated from murine tumors (11, 30), it is possible that p53 can act as both an oncogene or an antioncogene, depending on the presence or absence of activating mutations (24). The mechanism by which mutant p53 transforms cells is unclear. One possibility is that the mutant p53 protein acts in a dominant negative fashion by binding to and inactivating wild-type p53 in the cell. However, there is also evidence that at least some mutant p53 proteins also exert a dominant positive transforming function (44) and that normal p53 may also play a positive, as well as a negative, regulatory role in allowing cells to enter S phase (27, 36). Previous studies have shown that wild-type p53 can inhibit the transforming activity of mutant p53, adenovirus E1a, and a number of other cellular immortalizing proteins (12, 14). We have investigated the effects of both wild-type and mutant p53 on the ability of E7 to cooperate with *ras* to transform primary baby rat kidney (BRK) epithelial cells.

Suppression of transformation by wild-type p53. Primary BRK cells were triple transfected with long terminal repeat-driven HPV16 E7 (pMoE7) (10), EJ-*ras* (pEJ6.6) (35), and plasmids encoding either murine or human wild-type p53 (MSVcL-ala or pJ4ΩArg53) (23, 40). BRK cell culture and transfections were carried out using the calcium phosphate coprecipitation method as previously described (5). In some experiments, pSV_2Neo (37) was included in order to compare efficiencies of drug-resistant colony formation with efficiencies of focus formation following transfection. In primary rat cells a stronger transforming activity appears necessary for focus formation than for colony formation, making this a more stringent assay for transformation (4). As positive controls for the transformation-suppressing ability of wild-type p53, EJ-*ras* was cotransfected with either E1a (pCE) (34) or a transforming murine p53 (pLTRp53cG) (13) together with the wild-type p53 (Table 1). Following cotransfection of wild-type p53 with E7 and *ras* a strong inhibition of transformation was seen in both colony formation and focus formation assays (Table 1). This inhibition was dependent on the amount of p53 plasmid, since cotransfection of an equal mass (5 μg) of either murine or human wild-type p53 with E7 produced a marked reduction in the number of G418-resistant colonies produced by E7 plus *ras* (Table 1), and increasing the mass of wild-type p53 to 10 μg resulted in virtually no G418-resistant colonies growing out by 4 weeks after transfection (data not shown).

In order to examine the expression of transfected plasmids, immunoprecipitations of proteins from independently isolated colonies of cells arising after transfection with various combinations of DNAs were carried out. Proteins were immunoprecipitated from [^{35}S]cysteine- and [^{35}S]methionine-labeled cells as previously described (10). Lysates were allowed to react with a polyclonal serum specific to HPV16 E7 (3), monoclonal antibody M73 specific for E1a

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TABLE 1. Effects of wild-type mouse and human p53 on transformation of BRK cells by E7, E1a, and mutant p53 in cooperation with *ras*

Transfected plasmid[a] (expressed protein)	No. of G418-resistant colonies[b] in expt:		No. of foci[c] in expt:	
	1	2	1	2
MSVcL-ala (wild-type mouse p53)	0	0	0	0
pJ4ΩArg53 (wild-type human p53)	0	0	0	0
pMoE7 (HPV16 E7)	41	52	32	55
pMoE7 + MSVcL-ala	5	3	2	3
pMoE7 + pJ4ΩArg53	3	1	0	0
pCE (E1a)	>100	>100	57	61
pCE + MSVcL-ala	14	9	10	7
pCE + pJ4ΩArg53	8	12	ND	ND
pLTRp53cG (mutant mouse p53)	10	11	3	3
pLTRp53cG + MSVcL-ala	0	0	0	0

[a] All plasmids were cotransfected with pEJ6.6 and pSV_2Neo.
[b] Colonies were counted 3 weeks after transfection.
[c] Foci were counted 4 weeks after transfection. ND, Not determined.

(21), or monoclonal antibody PAb421 which recognizes both normal and mutant forms of rat and mouse p53 (20). Three independently isolated cell lines obtained following transfection with E7, E1a, or mutant p53 sequences plus *ras* (Fig. 1A, B, and C) expressed E7, E1a, or mutant p53 proteins, respectively, as expected, although levels of expression were different between each of the three cell lines. p53 immunoprecipitations from the rare colonies of cells arising following transfection with pE7 plus *ras* plus wild-type p53 or E1a plus *ras* plus wild-type p53 show that the level of p53 synthesis in these lines is not significantly above the endogenous levels, although these lines did express E7 and E1a, respectively, as expected (data not shown). However, Southern blot analysis showed that all of these colonies contained the transfected wild-type p53 sequences. These data are in accordance with previous observations (12, 14) and support the notion that these cells are unable to tolerate high levels of wild-type p53 expression.

Although the cotransfection of wild-type p53 sequences clearly inhibited the appearance of transformed foci or colonies by E7 plus *ras*, it was not possible, by using this cell system, to determine whether this was because of transformation suppression by the p53 protein or simply a nonspecific toxic effect of overexpression of wild-type p53 in these cells. To address this question, we repeated the transfections by using NIH 3T3, an established murine fibroblast cell line, as a recipient for transfection as previously described (41).

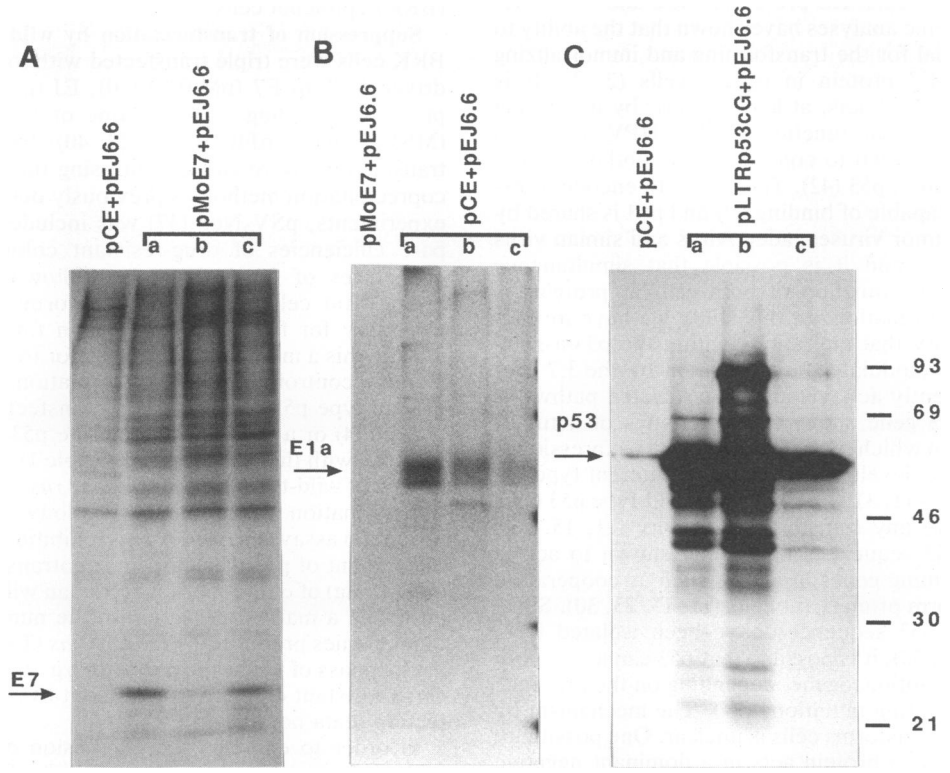

FIG. 1. Immunoprecipitations of E7, E1a, and p53 proteins from independently isolated colonies of transfected cells (lanes a, b, and c). p53 proteins were immunoprecipitated with antibody PAb421, which recognizes normal and mutant rat and mouse p53. Each panel shows immunoprecipitation of the protein indicated by the arrow. (A) E7 expression in cells isolated following transfection with E7 plus *ras*, with E1a plus *ras*-expressing cells as a negative control for E7 expression. (B) E1a expression in cells transfected with E1a plus *ras*, with E7 plus *ras* cells as a control. (C) p53 expression in mutant p53 plus *ras*-transfected cells. The low level of endogenous rat p53 expression is illustrated by the E1a plus *ras*-transfected control cell line; the endogenous rat p53 band runs slightly more slowly than the transfected mouse p53 band in the other lanes. This rat p53 band is masked by the high expression of mouse p53 in the other lanes. Molecular weight markers (10^3) are indicated.

TABLE 2. Effect of wild-type mouse and human p53 on colony formation and transformation of NIH 3T3 cells by E7 and E1a

Transfected plasmid[a] (expressed protein)	No. of G418-resistant colonies[b] in expt:		Growth[c] in agar (mass cultures)
	1	2	
pMo	28	34	−
pMoE7 (HPV16 E7)	34	21	+++
pCE (E1a)	38	11	++
MSVcL-ala (wild-type mouse p53)	40	34	−
pJ4ΩArg53 (wild-type human p53)	31	ND	−
pMoE7 + MSVcL-ala	53	29	++++
pMoE7 + pJ4ΩArg53	31	ND	+++
pCE + MSVcL-ala	29	20	+++
pCE + J4ΩArg53	20	ND	++++

[a] All plasmids were cotransfected with pSV_2Neo.
[b] Colonies were counted 2 weeks after transfection. ND, Not determined.
[c] Growth in agar was scored in terms of numbers and size of colonies ranging from −(0% growth) to ++++ (more than 20% growth).

Expression of E7 can induce anchorage-independent growth in these cells, but unlike the BRK cells, the growth of the cells is not dependent on transfected sequences. E7, E1a, and wild-type p53-containing plasmids were transfected alone or in combination (Table 2). All transfections also contained the plasmid pSV_2Neo as a selectable marker. There was no significant difference in the number of drug-resistant colonies obtained following transfection of wild-type p53 sequences compared with other transfections without these plasmids (Table 2). This indicates that transfection with these DNAs is not toxic to these cells. However, we were also unable to show an inhibition of transformation by wild-type p53 in these established cells as measured by colony formation after 3 weeks in 0.4% soft agar (Table 2). This suggests that the effect of p53 is specific to the immortalizing function of E7 in primary cells rather than the mechanism by which E7 transforms established cells. Interestingly, immunoprecipitations of p53 proteins from mass cultures of G418-resistant colonies again showed no elevated levels of p53 expression in cells which had been transfected with the p53-containing plasmids (data not shown). These results indicate that neither primary nor established rodent cells can tolerate high levels of wild-type p53 expression, although the observation that there is no decrease in numbers of G418-resistant colonies following wild-type p53 transfection suggests that transfection of these plasmid sequences is not toxic to the cells.

Enhancement of transformation by mutant p53. pMoE7 and pEJ6.6 were cotransfected into BRK cells together with a plasmid encoding murine mutant p53 (pLTRp53cG). In both focus-forming and G418-selected assays the effect of the mutant p53-encoding plasmid was two-fold. (i) The number of foci or G418-resistant colonies was consistently elevated by fivefold on dishes cotransfected with mutant p53. (ii) The rate of appearance of the colonies or foci was much greater on dishes triple transfected with the mutant p53 plasmids than on dishes transfected with E7 plus *ras* plus vector control plasmids. This potentiation of transformation was much more evident in the focus assay, in which the activity of E7 alone plus *ras* or mutant p53 alone plus *ras* is fairly modest (Table 3). Independent foci of transformed cells were isolated following transfection with E7 plus *ras* plus mutant p53 and each colony was shown to be expressing both E7 (data not shown) and mutant p53 proteins (Fig. 2) by immunoprecipitation. The p53 antibody PAb240 used in these immunoprecipitations is specific for the mutant conformation of p53 (18) and is therefore unable to detect the background of wild-type rat p53 normally expressed by the BRK cells.

TABLE 3. Effects of mutant p53 on transformation of BRK cells by E7 and E1a in cooperation with *ras*

Transfected plasmid[a] (expressed protein)	No. of foci[b] in expt:	
	1	2
pMoE7 (HPV16 E7)	12	8
pCE (E1a)	39	24
pLTRp53cG (mutant mouse p53)	2	0
pMoE7 + pLTRp53cG	62	50
pCE + pLTRp53cG	87	75

[a] All plasmids were cotransfected with pEJ6.6.
[b] Foci were counted 2 weeks after transfection.

Growth rates of independently isolated BRK lines expressing only E7 plus *ras*, mutant p53 plus *ras*, or E7 plus *ras* plus mutant p53 were examined in media containing 10% or 1% calf serum (Fig. 3). In 10% serum the three lines expressing both E7 and mutant p53 clearly grew much more rapidly than lines expressing only E7 or mutant p53 (Fig. 3A). The difference in growth potential of these cell lines was even more evident in 1% serum (Fig. 3B). Cell lines express-

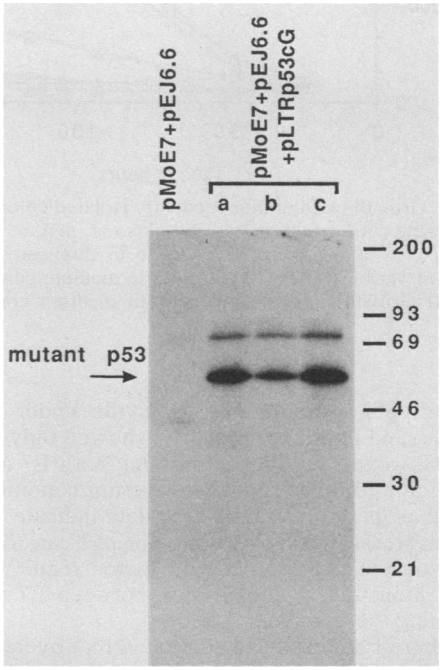

FIG. 2. Immunoprecipitation of mutant p53 from cells isolated following transfection with E7 plus *ras* plus mutant p53. The p53 antibody PAb240 used in these immunoprecipitations detects only mutant p53 protein. Immunoprecipitations from cells transfected with E7 plus *ras* are shown as a negative control for mutant p53 expression. Molecular weight markers (10^3) are indicated.

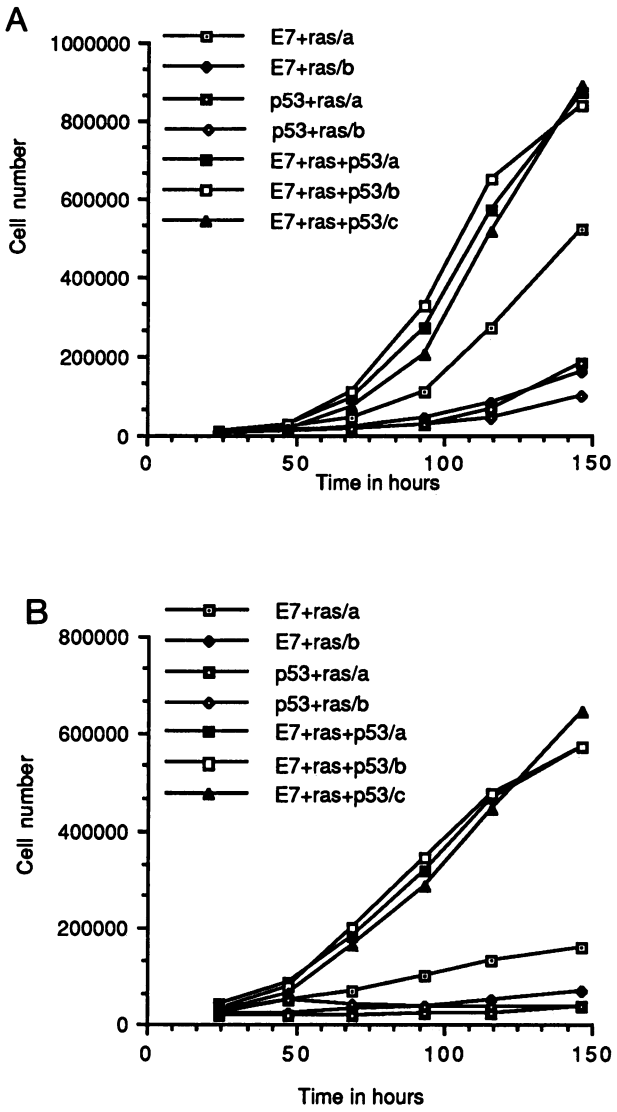

FIG. 3. Growth rates of independently isolated colonies of BRK cells following cotransfection with E7 plus *ras*, mutant p53 plus *ras* or E7 plus *ras* plus mutant p53. Cells in duplicate wells were harvested at various times. (A) Growth in medium containing 10% serum. (B) Growth of the same cells in medium containing 1% serum.

ing mutant p53 were unable to divide under low-serum conditions, and lines expressing E7 showed only very slight growth. However, all lines expressing both E7 and mutant p53 were able to divide under low-serum conditions almost as rapidly as in 10% serum. These data indicate that simultaneous expression of E7 and mutant p53 can relieve BRK cells of some exogenous growth factor requirements and clearly demonstrate a cooperation between E7 and p53 in transforming these cells.

A number of studies have suggested that overexpresssion of wild-type p53 has a transformation-suppressing effect in rodent systems or is incompatible with normal cell growth or both (12, 14, 26). Insomuch as cotransfection of wild-type p53 virtually eliminated the transforming effects of E7 in cooperation with *ras*, our data are consistent with these reports. Although we were unable to determine directly whether the lack of transformation by E7 following cotransfection with wild-type p53 was due to a specific effect of p53 on the mechanism of transformation by E7 (as previously suggested for E1a and *myc*) or simply a nonspecific cytotoxic effect of wild-type p53 expression on these cells, by demonstrating a potent transformation-promoting effect of mutant p53, we provide direct evidence that p53 expression can indeed modulate the biological effects of E7. In previous work, expression of mutant p53 has been shown to modestly enhance the transformation of cells by E1a (12, 14), but in our study we observed a much stronger potentiation of both foci number and rate of appearance, with at least a fivefold increase in numbers of foci. Furthermore, the growth rate of the cloned cell lines expressing E7 and mutant p53 was markedly faster than that of cell lines expressing only E7 plus *ras* or p53 plus *ras* in both high- and low-serum conditions. It has previously been noted that NIH 3T3 cells expressing mutant p53 show a reduced requirement for platelet-derived growth factor (17). The observation that coexpression of E7 and mutant p53 can relieve cells of growth factor requirements raises the possibility that the two proteins function within complementary signal transduction pathways. It is clear from the results that the transforming activity of E7 in the BRK assay is strongly affected by expression of p53. Since E7 is thought to act, at least in part, by binding to cellular RB, these results suggest that simultaneous interference with p53 and RB can cooperate to enhance cellular transformation and indicate that these two proteins function in complementary but independent pathways.

The possible biological relevance of our data to cervical carcinoma is underlined by the recent observation that the E6 protein from the malignant HPV types is able to complex with p53 (42). Other p53 binding proteins such as LT and E1b have been suggested to act by binding to and inactivating endogenous cellular p53. This seems a less likely mechanism of action for E6, since transformed cells express only very low levels of E6 and since there is no evidence for the stabilization of p53 proteins in E6-transformed cells such as that seen in LT-transformed cells. It has been suggested that binding to E6 induces a more rapid degradation of p53, depleting the cell of functional wild-type p53 in this way (42). However, the function of mutant p53 in this system is clearly different from the effect of E6-p53 binding, since expression of E6 in the E7-transfected BRKs does not enhance transformation (38). This may be a reflection of dominant positive transforming activity encoded by the mutant p53 protein in addition to the negative function (presumably shared by E6) of inactivation of normal p53. It will clearly be important to determine the effects of mutant and wild-type p53 expression on the biology of HPV-transformed human epithelial cells and to ascertain the frequency of activating p53 mutations in HPV-positive and -negative cervical cancers.

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