JOURNAL OF VIROLOGY, Oct. 1992, p. 6237–6241
0022-538X/92/106237-05$02.00/0

Vol. 66, No. 10

Human Papillomavirus Type 16 E6 Increases the Degradation Rate of p53 in Human Keratinocytes

NANCY L. HUBBERT, SYLVIA A. SEDMAN, AND JOHN T. SCHILLER*

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

Received 7 May 1992/Accepted 16 July 1992

The E6 proteins of the high-risk human papillomaviruses (HPVs) have been shown to form a complex with and induce the degradation of human p53 in vitro. To determine whether p53 is degraded more rapidly in cells expressing E6 in vivo, the half-life of p53 was determined by pulse-chase analysis in early-passage normal human keratinocytes and fibroblasts, human keratinocytes immortalized with HPV type 16 (HPV16) E6 plus E7, and nonimmortal keratinocytes transfected with E6. The results of these experiments indicate that (i) the half-life of newly synthesized p53 is relatively long (4 h) in early-passage human keratinocytes and fibroblasts but short in keratinocytes expressing E6 (15 to 30 min), (ii) a similar increased rate of p53 degradation was measured in lines immortalized with HPV16 E6 plus E7 and senescent cells expressing E6, indicating that this increase is not simply the result of selection in the immortalized lines, and (iii) very low levels of expression of E6 result in a greatly decreased half-life of p53, suggesting that E6 acts in a catalytic manner.

Infection with certain human papillomavirus (HPV) types, such as HPV type 16 (HPV16), appears to be a risk factor in the development of human cervical and other genital carcinomas (reviewed in reference 29). The E6 and E7 open reading frames are selectively retained and expressed in HPV-containing carcinoma cells, suggesting that the E6 and E7 proteins are involved in the initiation and/or maintenance of human carcinomas (23, 26). In addition, E6 can cooperate with E7 to immortalize normal human foreskin keratinocytes and cervical epithelial cells (13, 14, 18, 28), supporting the concept that the activity of E6 has pathogenic significance in the development of the HPV-associated cancers.

HPV E6 proteins synthesized in rabbit reticulocyte extracts form a complex with wild-type human p53 (27). This is of particular interest because p53 appears to be an important negative regulator of cell growth. Introduction of wild-type p53 into cultured cells has been shown to inhibit the growth of the transfectants (2, 8, 9). In addition, p53 is frequently mutated in a number of human cancers (5, 19), suggesting that inactivation of p53 is involved in the development of many human cancers. Cell lines derived from cervical tumors which lack HPV DNA express mutant forms of p53, while HPV-positive cell lines express wild-type p53, suggesting that inactivation of p53 is important in the development of cervical cancer and that an in vivo interaction between E6 and p53 may abrogate the antiproliferative activity of p53 (7, 21). Consistent with the idea that inactivation of wild-type p53 is critical to E6 immortalizing activity, we have found that dominant mutants of p53 can substitute for E6 in the keratinocyte immortalization assay (25).

The E6 proteins of the high-risk HPVs induce the degradation of p53 in the in vitro reticulocyte assay (22), apparently in a ubiquitin-dependent reaction. This result provided a possible mechanism by which E6 could inactivate p53 in vivo. In contrast to what would be predicted from the in vitro results, the same authors have reported that the steady-state levels of p53, as measured by Western blot (immunoblot) analysis, in cell lines derived from HPV-containing cervical tumors and in keratinocytes immortalized with HPV16 E6 plus E7 did not differ markedly from those in normal keratinocytes (22). In addition, E6 transformation of rodent cells does not appear to affect the levels or stability of p53 (16, 25). However, another recent study of HPV16 E6-immortalized breast epithelial cells reported that the steady-state levels of p53 were reduced (3). Given these seemingly conflicting reports and the fact that the rate of p53 degradation has not been measured directly in normal and E6-expressing human cells, we were interested in examining the effect that HPV-16 E6 has on the half-life of p53 in human foreskin keratinocytes, which are normal target cells for HPV16 infection.

p53 half-life in normal human cells. Before analyzing the possibility that E6 is associated with increased degradation of p53 in vivo, we first determined the half-life of p53 in early-passage normal human keratinocytes, grown in KGM (Clonetics, San Diego, Calif.) as previously described (13). First- or second-passage cells were plated in T25 flasks and, as they approached confluence, were starved for 30 min in Dulbecco's modified Eagle's medium (DMEM) lacking cysteine. Cells were then labeled with [^{35}S]cysteine (0.25 mCi/ml) for 20 min. One flask was harvested immediately by lysis in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate). The others were washed and incubated in DMEM for 15 min to 24 h. Equal numbers of trichloroacetic acid-precipitable counts from each time point were used to immunoprecipitate p53 by using PAb 1801 (Ab-2 from Oncogene Science, Mineola, N.Y.), a monoclonal antibody that binds human p53, and the immunoprecipitated proteins were separated by electrophoresis on 12% polyacrylamide gels. PAb 1801 was chosen because it recognizes wild-type, mutant, and denatured forms of human p53 and therefore will presumably immunoprecipitate the protein regardless of conformation. The results of the analysis of two independently derived cultures of normal early-passage keratinocytes are shown in Fig. 1A and B. The designated protein was identified as p53 on the basis of its immunoprecipitation by a second p53 monoclonal antibody (PAb 421; Oncogene Science) and its absence in lysates made from SAOS-2 cells, a human osteosarcoma line that does not express p53 (17) (data not shown). As quanti-

* Corresponding author.

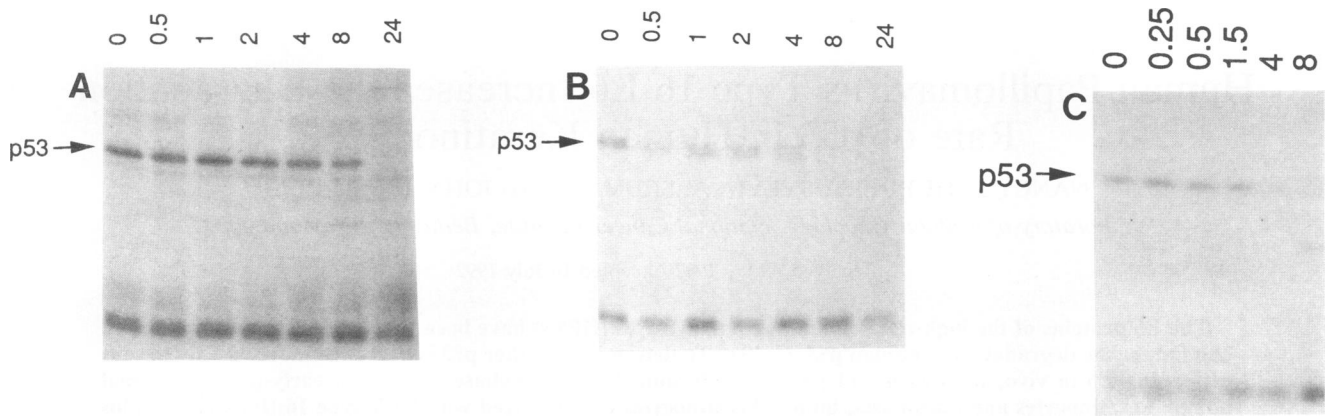

FIG. 1. Pulse-chase analysis of p53 in early-passage normal human cells. (A) Foreskin keratinocyte culture FS9; (B) foreskin keratinocyte culture FS20; (C) foreskin fibroblast culture. The length of the chase (in hours) is indicated above each lane.

fied with an Ambis Optical Imaging System, the half-life of p53 in the early-passage human keratinocytes was found to be approximately 4 to 5 h.

The half-life of p53 in early-passage rodent fibroblasts was shown previously to be between 15 and 30 min (10, 20). Since the half-life of p53 in normal human keratinocytes was much longer, we were interested in determining the half-life of p53 in early-passage human fibroblasts. Therefore, the rate of p53 degradation in third-passage fibroblasts that had been established from human foreskins in DMEM containing 10% fetal bovine serum was determined by the pulse-chase procedure outlined above. The results, shown in Fig. 1C, indicate that the half-life of p53 is also relatively long in human fibroblasts (4 h) compared with the half-life we (25) and others have measured in rodent fibroblasts expressing wild-type p53. These results suggest that the rate of p53 degradation may be more a species-specific than a cell-type-specific characteristic.

The initial analysis of p53 half-life in keratinocytes was performed with DMEM lacking cysteine, to maximize incorporation of radiolabeled cysteine. High concentrations of calcium stimulate the differentiation of keratinocytes (reviewed in reference 11), and proliferating cultures are therefore maintained in medium containing a low concentration of calcium. DMEM contains relatively high levels of calcium. Since it was formally possible that the analysis of p53 half-life in human keratinocytes was influenced by the media used during the labeling and subsequent chase, the pulse-chase experiment was repeated using keratinocyte basal growth medium lacking cysteine (KBM; Clonetics) in place of DMEM. The results were similar to those obtained with DMEM, indicating that the medium used for labeling did not measurably affect p53 metabolism during the course of the experiment (data not shown). The experiments described below were performed with DMEM unless stated otherwise.

p53 half-life in E6-plus-E7-immortalized keratinocytes. Since HPV-positive cervical tumors express both E6 and E7, the half-life of p53 was determined in keratinocyte lines immortalized with HPV16 E6 plus E7. The relative levels of E6 were determined for 13 independently derived lines that had been generated in two previous studies (4, 24). Cells were starved in DMEM lacking cysteine for 30 min prior to labeling for 20 min in the same medium containing [^{35}S]cysteine. Cells were harvested, and E6 was immunoprecipitated by using polyclonal antiserum raised against a bacterial E6 fusion protein as described previously (1). The amounts of E6 immunoprecipitated varied considerably (Table 1). Representative results of E6 expression from three lines expressing low, intermediate, and high levels of E6 are shown in Fig. 2A.

The half-life of p53 in six of the cell lines was determined with DMEM, and in two cases the determination was repeated with keratinocyte growth medium, as described above for normal keratinocytes. Representative results with DMEM are shown in Fig. 2B. The half-life of p53 is short (15 to 30 min) relative to that seen in the normal early-passage keratinocytes (Table 1). Similar results were obtained with KBM (data not shown). It is unlikely that the decrease in the amount of p53 immunoprecipitated during the chase periods of labeling experiments was due to an E6-induced change in p53 conformation that prevented recognition by the antibody, since the antibody used, PAb 1801, recognizes all known forms of human p53 and has been shown to efficiently immunoprecipitate p53-E6 complexes in vitro (6). We con-

TABLE 1. Summary of E6 levels, p53 levels, and p53 half-lives

Cell line	E6 level[a]	p53 level[b]	p53 half-life (h)
FS4[c]	−	1.0	ND[d]
FS9[c]	−	ND	5
FS20[c]	−	1.0	4
HFF[e]	−	ND	4
IK20	++++	0.2	0.2
IK8	+++	0.7	0.2
IK18	++	0.1	0.2
IK6	+	0.6	ND
IK5	+	0.7	0.5
IK11	+	0.1	ND
IK14	+	0.8	ND
IK16	+	0.5	ND
IK10	+/−	0.5	0.4
IK19	+/−	0.3	0.3
IK9	+/−	1.0	ND
IK7	−	0.9	ND
IK21	−	0.6	ND

[a] On a scale from − (not detectable) to ++++ (very high).
[b] Relative to that of normal early-passage keratinocytes.
[c] Early-passage human keratinocytes.
[d] ND, not determined.
[e] Early-passage human fibroblasts.

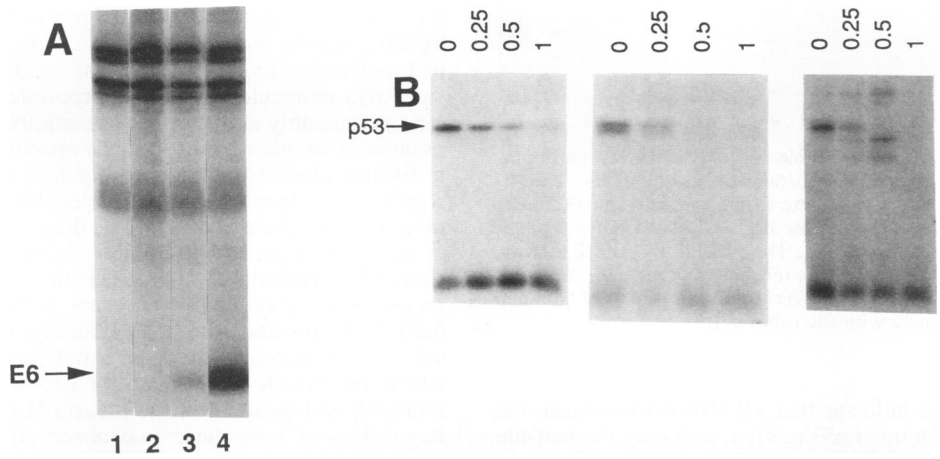

FIG. 2. E6 expression and p53 half-life in early-passage keratinocytes or human keratinocyte lines immortalized with HPV16 E6 plus E7. (A) Immunoprecipitation of E6. Lane 1, normal keratinocytes; lanes 2 to 4, immortalized keratinocyte lines IK19, IK8, and IK20, expressing very low, intermediate, or high levels of E6, respectively. (B) Pulse-chase analysis of p53 in lines IK19 (left), IK8 (middle), and IK20 (right). The length of the chase (in hours) is indicated above each lane.

clude that p53 is degraded more rapidly in cells immortalized with HPV16 E6 plus E7 than in normal keratinocytes, suggesting that E6 induces the degradation of p53 in vivo.

The half-life of p53 was similar in cell lines which express very low versus high levels of E6. Since very low levels of E6 can induce large changes in the half-life of p53, it is likely that, as suggested by the in vitro studies (22), E6 acts catalytically in inducing p53 degradation. The fact that higher levels of E6 only modestly increased the rate of p53 degradation suggests that cellular proteins involved in the process may become limiting. One candidate is the 100-kDa protein that appears to be required for the E6-p53 interaction (15). An alternative possibility is that degradation of p53 occurs only after it has been translocated to the nucleus and that this process is rate limiting. In any event, the finding that very low levels of E6 can induce efficient degradation of p53 may provide an explanation for the observation that E6 is expressed at very low levels in cell lines derived from cervical tumors (1).

p53 half-life in nonimmortal E6-expressing keratinocytes. It is possible that a reduction in p53 levels is required for the immortalization of human keratinocytes and therefore that selection for increased p53 degradation occurred through an E6-independent mechanism in the establishment of these lines. To rule out this possibility, we measured the half-life of p53 in keratinocytes expressing only E6, since these cells do not have increased growth potential and do not become immortal. Cells were transfected with the neomycin resistance gene alone or the neomycin resistance gene plus SDE6, a plasmid encoding HPV16 E6 (24), selected with G418 as previously described (4), and plated into T25 flasks, and the p53 half-life was analyzed. The half-life of p53 in cells transfected with the HPV16 E6 gene was found to be shorter than that in cells transfected with the neomycin resistance gene alone (0.5 versus 4 h; Fig. 3). These results indicate that induction of p53 degradation requires E6 but no other viral gene and does not require selection during the immortalization process.

The actual p53 half-life measured in the nonimmortal G418-selected cells may be even shorter than the measured half-life, because it is likely that some cells expressed the plasmid encoding the neomycin resistance gene but did not express the plasmid encoding E6. The half-life of p53 would be expected to be long in cells expressing only the neomycin resistance gene, and this may explain why the majority of p53 is degraded at early time points but a low level remains at the later time points. It appears that E7 does not influence the degradation of p53, since the half-life of p53 was similar in cells expressing E6 plus E7 and E6 only.

Although the population of E6-expressing neomycin-resistant cells which were used to analyze the half-life of p53 had senesced, p53 was degraded efficiently. This result suggests that p53 degradation does not require cell cycle-specific factors. Previous studies have shown that keratinocytes transfected with E6 plus the neomycin resistance gene grow no better than cells transfected with the neomycin resistance gene alone (4, 24). Taken together, these results indicate that inactivation of wild-type p53 through increased degradation is, by itself, insufficient to prevent senescence.

Although complexes between E6 and p53 could be detected in the in vitro assays (6, 27), preformed complexes, as indicated by coimmunoprecipitation of E6 and p53, were not detected in our study. This negative result may be due to rapid degradation of p53 in vivo after the complex forms. Alternative explanations include the possibility that the method used to make cell lysates destabilizes the complex and the possibility that a very small fraction of the total cellular p53 is complexed with E6 and therefore is not detectable in our assays.

Steady-state levels of p53 in normal and immortal kerati-

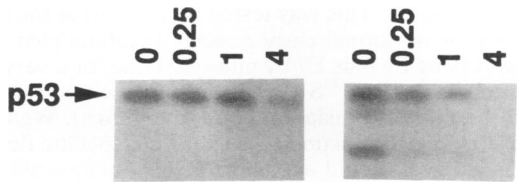

FIG. 3. Pulse-chase analysis of p53 half-life in early-passage keratinocytes transfected with the neomycin resistance gene (left panel) or the neomycin resistance gene plus HPV16 E6 (right panel). The length of the chase (in hours) is indicated above each lane.

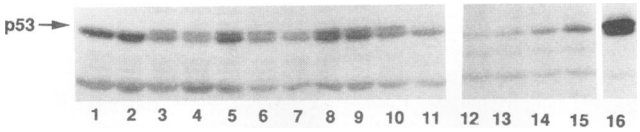

FIG. 4. p53 immunoblot of whole-cell extracts from early-passage normal and E6-plus-E7-immortalized keratinocytes. The arrows indicate the two electrophoretic variants of human p53. Lanes 1 and 2, early-passage keratinocytes FS4 and FS20; lanes 3 to 15, immortalized lines IK5, IK6, IK7, IK8, IK10, IK11, IK9, IK14, IK16, IK18, IK20, IK19, and IK21, respectively; lane 16, 293 cells. Lanes 12 to 16 were run concurrently on a separate gel and processed simultaneously with the other gel.

nocytes. Our results indicate that HPV16 E6 increases the degradation rate of human p53 in vivo, reducing the half-life of p53 at least eightfold in human keratinocytes. Therefore, one might have expected considerably lower steady-state levels of this protein in E6-expressing versus normal keratinocytes. To determine whether this was the case, the steady-state p53 levels were examined in two cultures of normal keratinocytes and 13 independently derived E6-plus-E7-immortalized lines. The monoclonal antibody that recognizes human p53 (PAb 1801) was used to probe Western blots of whole-cell extracts in radioimmunoprecipitation assay buffer (150 μg of total protein). ^{125}I-labeled sheep anti-mouse immunoglobulin G (Amersham) was used for detection (21), and the signals were quantitated with an Ambis Optical Imaging System. Extracts from SAOS-2 cells (150 μg), which do not contain p53 (17), and human 293 cells (30 μg), which have high levels of p53 (due to the presence of adenovirus type 5 E1b protein), were used as negative and positive controls to unambiguously identify p53 protein on the blots. In many of the keratinocyte lines and in 293 cells, both of the allelic variants of human p53 (12) were detected (Fig. 4). Relative to the normal keratinocytes, all but one of the lines contained less p53, although most were reduced less than 50%. The relative p53 levels in this study varied from 0.1 to 1.0 (Table 1), in agreement with the levels reported in the previous study of four E6-plus-E7-immortalized lines, which varied from 0.4 to 1.2.

We have tested several hypotheses to explain the apparent discrepancy between the large E6-induced change in p53 half-life, as measured by immunoprecipitation, and the generally small change in steady-state p53 levels, as measured by Western blot, but none have provided a satisfactory explanation. For instance, transcription of the p53 gene may have been increased to offset the decreased stability of the p53 protein in lines expressing E6. However, the steady-state level of p53 mRNA is indistinguishable in normal keratinocytes and keratinocytes immortalized with E6 plus E7 as measured by Northern (RNA) blot (data not shown). Another possibility is that cells which express E6 compensate for the decreased stability of p53 by increased translation of p53 mRNA. This was tested by measuring the rate of p53 synthesis in normal early-passage keratinocytes and in cells expressing E6 plus E7 by pulse-labeling for a very short interval (5 min) with [^{35}S]cysteine. We observed no difference in the rate of translation (data not shown). We do not favor a previous suggestion that cell cycle-specific degradation of p53 by E6 could account for this unexpected result (21), since nondividing keratinocyte cultures expressing E6 had rates of p53 degradation similar to those of the proliferating lines.

The observations that, in general, the apparent half-life, but not the steady-state level, of p53 is markedly decreased by E6 could be reconciled if there were two populations of p53 molecules. One would consist mainly of newly synthesized p53 molecules that are susceptible to E6 degradation (and presumably active in growth suppression). The second population would be a more stable species that is not subject to E6 degradation (and presumably does not act as a negative regulator). Assuming that the molecules in the second population are derived from the first, they might not be detected in our immunoprecipitation assay because of the relatively short labeling period employed. If the amount of p53 in the second population, which appears to be large, is actually limited, for instance by the availability of a specific binding site or complexing protein, it could produce a situation in which the steady-state levels of p53 would appear to be relatively independent of the measured rate of degradation. Regardless of how these two observations are eventually reconciled, the results of our study strongly support the conclusion that the degradation rate of newly synthesized p53 is increased by E6 in keratinocytes and suggest that studies of the mechanism of E6-induced p53 degradation in the in vitro assay system could yield biologically relevant results.

We thank Douglas Lowy for his support, advice, and critical reading of the manuscript.

S.A.S. is a National Institutes of Health fellow (IRTA grant TA-CA-B032).

REFERENCES

1. **Androphy, E. J., N. L. Hubbert, J. T. Schiller, and D. R. Lowy.** 1987. Identification of the HPV16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. EMBO J. **6**:989–992.
2. **Baker, S. J., S. Markowitz, E. R. Fearon, J. K. U. Willson, and B. Volgelstein.** 1990. Suppression of human colorectal carcinorma cell growth by wild-type p53. Science **249**:912–915.
3. **Band, V., J. A. De Caprio, 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.
4. **Barbosa, M. S., W. C. Vass, D. R. Lowy, and J. T. Schiller.** 1991. In vitro biological activities of the E6 and E7 genes vary among human papillomaviruses of different oncogenic potential. J. Virol. **65**:292–298.
5. **Bartek, J., J. Bartkova, B. Vojtesek, Z. Staskova, J. Lukas, A. Rejthar, J. Kovarik, C. A. Midgley, J. V. Gannon, and D. P. Lane.** 1991. Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. Oncogene **6**:1699–1703.
6. **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.
7. **Crook, T., D. Wrede, and K. H. Vousden.** 1991. p53 point mutation in HPV negative human cervical carcinoma cell lines. Oncogene **6**:873–875.
8. **Eliyahu, D., D. Michalovitz, S. Eliyahu, O. Pinhasi-Kimhi, and M. Oren.** 1989. Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Natl. Acad. Sci. USA **86**:8763–8767.
9. **Finlay, C. A., P. W. Hinds, and A. J. Levine.** 1989. The p53 proto-oncogene can act as a suppressor of transformation. Cell **57**:1083–1093.
10. **Finlay, C. A., P. W. Hinds, T.-H. Tan, D. Eliyahu, M. Oren, and A. J. Levine.** 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol. Cell. Biol. **8**:531–539.
11. **Fuchs, E.** 1990. Epidermal differentiation: the bare essentials. J. Cell Biol. **111**:28007–2814.
12. **Harris, N., E. Brill, O. Shohat, M. Prokocimer, D. Wolf, N. Arai, and V. Rotter.** 1986. Molecular basis for heterogeneity of

the human p53 protein. Mol. Cell. Biol. **6**:4650–4656.
13. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV16 E6 and E7 proteins cooperate to immortalize primary human foreskin keratinocytes. EMBO J. **8**:3905–3910.
14. **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.
15. **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 and 18. EMBO J. **10**:4129–4135.
16. **Inoue, H., G. Kondoh, M. Sudiro, and A. Hakura.** 1992. Stability of p53 protein in rat cells transformed by various viral transforming genes. Virology **187**:343–347.
17. **Masuda, H., C. Miller, H. P. Koeffler, H. Battifora, and M. J. Cline.** 1987. Rearrangement of the p53 gene in human osteogenic sarcomas. Proc. Natl. Acad. Sci. USA **84**:7716–7719.
18. **Munger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63**:4417–4421.
19. **Nigro, J. M., S. J. Baker, A. C. Preisinger, J. M. Jessup, R. Hostetter, K. Cleary, S. H. Bigner, N. Davidson, S. Baylin, P. Devillee, T. Glover, F. S. Collins, A. Weston, R. Modali, C. C. Harris, and B. Vogelstein.** 1989. Mutations in the p53 gene occur in diverse human tumour types. Nature (London) **342**:705–708.
20. **Oren, M., W. Maltzman, and A. J. Levine.** 1981. Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. Mol. Cell. Biol. **1**:101–110.
21. **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.
22. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes degradation of p53. Cell **63**:1129–1136.
23. **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.
24. **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 E6 protein of human papillomavirus type 16 has transforming and *trans*-activating activities and cooperates with E7 to immortalize keratinocytes in culture. J. Virol. **65**:4860–4866.
25. **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 in immortalization of human keratinocytes but does not have E6-associated *trans*-activation or transforming activity. J. Virol. **66**:4201–4208.
26. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83**:4680–4684.
27. **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.
28. **Woodworth, C. D., P. E. Bowden, J. Doniger, L. Pirisi, W. Barnes, W. D. Lancaster, and J. A. DiPaolo.** 1988. Characterization of normal human exocervical epithelial cells immortalized in vitro by papillomavirus types 16 and 18 DNA. Cancer Res. **48**:4620–4628.
29. **zur Hausen, H., and A. Schneider.** 1987. The role of papillomaviruses in anogenital cancer, p. 245–263. *In* N. P. Salzman and P. M. Howley (ed.), The papillomaviruses. Plenum Publishing Corp., New York.