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Human papillomavirus E6 proteins bind p53 *in vivo* and abrogate p53-mediated repression of transcription

Mark S.Lechner[1,2], David H.Mack[1,2], Angela B.Finicle[1], Tim Crook[4], Karen H.Vousden[4] and Laimonis A.Laimins[1,3]

[1]Department of Molecular Genetics and Cell Biology, [3]Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637, USA and [4]Ludwig Institute for Cancer Research, St Mary's Hospital Medical School, Norfolk Place, London W2 1PG, UK
[2]These authors contributed equally to this study

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The transforming proteins of DNA tumor viruses SV40, adenovirus and human papillomaviruses (HPV) bind the retinoblastoma and p53 cell cycle regulatory proteins. While the binding of SV40 large T antigen and the adenovirus E1B 55 kDa protein results in the stabilization of the p53 protein, the binding of HPV16 and 18 E6 results in enhanced degradation *in vitro*. To explore the effect of viral proteins on p53 stability *in vivo*, we have examined cell lines immortalized in tissue culture by HPV18 E6 and E7 or SV40 large T antigen, as well as cell lines derived from cervical neoplasias. The half-life of the p53 protein in non-transformed human foreskin keratinocytes in culture was found to be ~3 h while in cell lines immortalized by E6 and E7, p53 protein half-lives ranged from 2.8 h to <1 h. Since equivalent levels of E6 were found in these cells, the range in p53 levels observed was not a result of variability in amounts of E6. In keratinocyte lines immortalized by E7 alone, the p53 half-life was found to be similar to that in non-transformed cells; however, it decreased to ~1 h following supertransfection of an E6 gene. These observations are consistent with an interaction of E6 and p53 *in vivo* resulting in reductions in the stability of p53 ranging between 2- and 4-fold. We also observed that the expression of various TATA containing promoters was repressed in transient assays by co-transfection with plasmids expressing the wild-type p53 gene. The inclusion of human papillomavirus E6 or SV40 T antigen expression plasmids in these transfection assays abrogated p53-mediated repression, demonstrating that these oncoproteins can modulate p53 function *in vivo*. Mutant E6 proteins that bind p53 but fail to stimulate degradation retained the ability to relieve repression, while no such effect was observed with E6 mutants that were unable to bind p53. These studies demonstrate that E6 and p53 interact *in vivo* and suggest that E6 binding alone can modulate the transcriptional inhibitory effects of p53.

Key words: E6/HPV/p53/repression/transformation

Introduction

The p53 nuclear phosphoprotein is implicated in control of cell proliferation, and appears to play a central role in the development of human cancers as it is frequently mutated in numerous tumor types (Baker *et al.*, 1989; Nigro *et al.*, 1989; Takahashi *et al.*, 1989; Malkin *et al.*, 1990; Mulligan *et al.*, 1990). Several lines of evidence indicate that wild-type p53 functions to regulate normal cell growth, while mutant forms of p53 can alter this control. In cell culture, the wild-type mouse p53 gene can inhibit oncogene-mediated transformation and acts as a potent suppressor of cell growth (Finlay *et al.*, 1989; Michalovitz *et al.*, 1990) whereas mutant forms can efficiently transform rodent cells in cooperation with an activated *ras* gene (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984; Hinds *et al.*, 1989). Similar results have been reported with the human p53 gene (Baker *et al.*, 1990; Diller *et al.*, 1990; Hinds *et al.*, 1990; Mercer *et al.*, 1990a,b; Farrell *et al.*, 1991). Thus, p53 appears to play a critical role in cell cycle regulation and neoplastic transformation.

Recent studies have demonstrated that the transforming proteins of several DNA tumor viruses specifically interact with p53 protein. SV40 large T antigen, the adenovirus E1B 55 kDa protein and the human papillomavirus (HPV) E6 protein all bind p53 and presumably alter its regulation of cellular proliferation. (Lane and Crawford, 1979; Sarnow *et al.*, 1982; Werness *et al.*, 1990). The E6 proteins of HPV types 16 and 18, which are associated with a high risk of malignant progression, cooperate with the E7 gene product for efficient immortalization and transformation of primary human keratinocytes in culture (Schlegel *et al.*, 1988; Hawley-Nelson *et al.*, 1989; Hudson *et al.*, 1990; Barbosa *et al.*, 1991). In contrast, the corresponding proteins of the low risk HPV types 6 and 11 are only weakly transforming (Barbosa *et al.*, 1991). Although binding of SV40 T antigen and the adenovirus E1B 55 kDa protein leads to stabilization of p53 (Oren *et al.*, 1981; Mora *et al.*, 1982; Sarnow *et al.*, 1982), binding of E6 protein encoded by the high risk HPV types results in the rapid degradation of p53 through the ubiquitin-directed pathway (Scheffner *et al.*, 1990). E6 proteins of the low risk types bind p53 with reduced affinity but do not direct its degradation (Crook *et al.*, 1991a).

The importance of E6–p53 interaction in transformation is demonstrated by observations that mutant p53 genes are present in cervical tumor cell lines which are negative for HPV sequences, whereas only wild-type p53 is detected in HPV-immortalized lines (Crook *et al.*, 1991b; Scheffner *et al.*, 1991; Wrede *et al.*, 1991). Although this indicated that the E6 protein can alter wild-type activity of p53, the functional consequences of binding and degradation by E6 in HPV-induced cervical cancers remain unclear. To investigate further the role of E6–p53 interactions in transformation, we have examined the state of p53 in HPV-transformed human epithelial cells. We observe that E6 and p53 form a complex *in vivo*, and detect a reduction in p53 levels and half-lives in HPV-transformed cell lines, with several retaining appreciable amounts of p53 protein. Furthermore, we find that the presence of E6 can abrogate

p53-mediated transcriptional repression in transient transfection assays. This inhibition is dependent upon binding of E6 to p53 but does not require degradation of p53.

Results

A range of p53 protein levels is seen in HPV-transformed epithelial cells

Using Western blot analysis we determined the relative steady state levels of p53 protein in cell lines derived from biopsies of cervical neoplasias and in human keratinocytes immortalized by SV40 or HPV18 DNA in cell culture (Figure 1A). The steady-state levels of p53 observed in cells transformed by SV40 were at least 10-fold higher than those observed in non-transformed human keratinocytes (NHK cells) (Figure 1, lanes 1 and 2 respectively). This is consistent with previous observations of stabilized p53 in SV40 T antigen transformed mouse fibroblasts and human keratinocytes (Lane and Crawford, 1979; Scheffner *et al.*, 1991). In contrast, in HPV-positive cell lines derived from biopsies or in keratinocytes immortalized by HPV18 E6 and E7 in culture, we found a 2- to 4-fold reduction in the levels of p53 (Figure 1, lanes 4–11) which is similar to that previously reported by Scheffner *et al.* (1991). Interestingly, some lines such as the PE3 cell line, which was immortalized by both HPV18 E6 and E7 sequences, were found to contain levels of p53 similar to that seen in NHK cells (Figure 1). In addition, a cell line which was immortalized by HPV18 E7 alone (Bam3) contained levels of p53 nearly identical to that seen in NHK cells (Figure 1, lanes 3 and 2 respectively). These analyses show that while p53 protein levels are generally reduced in cell lines expressing E6 and E7, numerous HPV-positive cell lines retain significant amounts of p53. Northern blot analysis of all E6-expressing cell lines demonstrated that each expressed nearly equivalent levels of p53 mRNA (Figure 1B). We consistently found low levels of p53 mRNA in the Bam3 cell line but the reason for this is currently unknown. The reduced levels of p53 transcripts seen for NHK cells in Figure 1 were not observed in other Northern blots where expression was found to be equivalent to that in HPV-transformed cells. Therefore, the decrease in p53 protein levels in cells expressing HPV E6 was not due to a reduction in p53 mRNA.

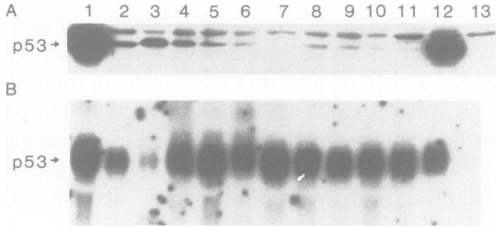

Fig. 1. p53 expression as determined by Western and Northern blot analyses. (**A**) Protein extracts from human cell lines probed with p53 monoclonal antibody PAb1801 in Western blot analysis. Lanes: 1, MK6–1BIII (SV40-immortalized); 2, non-transformed keratinocytes; 3, Bam3 (HPV18 E7-immortalized); 4, PE3 (HPV18 E7/E6-immortalized); 5, PE5 (HPV18 E7/E6-immortalized); 6, PE4 (HPV18 E7/E6-immortalized); 7, PEmass (HPV 18 E7/E6-immortalized); 8, CC-1 (HPV 18); 9, CC-2 (HPV 16); 10, CIN-612 (HPV 31b); 11, HeLa (HPV 18); 12, C33a (HPV-negative, p53 mutant); 13, STSAR-5 (p53 gene deletion). (**B**) Northern blot of total RNA isolated from the same cell lines in (A) probed with wild-type p53 probe.

The p53 protein is metabolically destabilized in cell lines containing HPV

We next performed pulse–chase experiments to determine if the stability of the p53 protein was altered in cells derived from HPV-positive biopsies and cells immortalized by E6 and E7 in culture. In these experiments, whole cell lysates were subjected to immunoprecipitation with either a pan-reactive p53 monoclonal antibody, PAb421 (Harlow *et al.*, 1981), or an antibody specific for a mutant p53 conformation, PAb240 (Gannon *et al.*, 1990). The results of these experiments are shown in Figure 2. In contrast to the levels observed in NHK cells, the p53 protein was rapidly turned over in CC-1, CC-2 and CIN-612 cell lines. Similar results were observed with HeLa cells which contain detectable yet short-lived p53. Previous studies have failed to detect p53 protein in HeLa cells unless a mutant p53 was introduced, despite the presence of translatable mRNA (May *et al.*, 1991). In the C33a cell line, which has been shown to contain mutant p53 (Scheffner *et al.*, 1991; Crook *et al.*, 1991b), we observed an increase in the stability of p53 proteins (Figure 2) similar to that seen with other mutant forms of p53 (Jenkins *et al.*, 1985; Finlay *et al.*, 1988; Halevy *et al.*, 1990). Although we were unable to precipitate the mutant p53 in these cells with the PAb240 antibody, previous studies showed that low levels of p53 could be immunoprecipitated with this antibody (Wrede *et al.*, 1991).

To quantify the changes in protein turnover, we determined the half-life of p53 in several HPV- and SV40-immortalized keratinocyte cell lines by pulse–chase experiments. Representative analyses are shown in Figure 3 and a summary of the data is found in Table I. We determined the half-life of p53 protein in monolayer cultures of NHK cells to be 3.3 h while in two SV40 T antigen immortalized keratinocyte cell lines the half-life increased to 5 h in one case and >10 h in the other. In contrast, the half-life of p53 in keratinocytes immortalized by HPV18 E6 and E7 was found to range from 0.8 to 2.8 h with an average value of 1.6 h. In the pulse–chase experiment shown in Figure 3 (top panel), an initial slight increase in p53 levels was seen prior to a reduction with extended chase periods. This has previously been observed in half-life determinations for mouse p53 and may be a result of incomplete removal of radioisotopic precursors and/or an intrinsic difference in

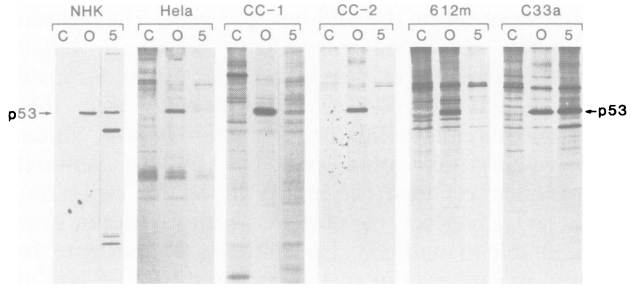

Fig. 2. Turnover of p53 in cell lines derived from biopsies of cervical neoplasias. Cells were pulse-labelled for 1 h and harvested or incubated with chase medium containing excess cold precursor for 5 h. Extracts were prepared in NP40 buffer. An autoradiogram is shown of an SDS–PAGE gel in which equal TCA-precipitable counts at 0 and 5 h chase were immunoprecipitated with pan-reactive p53 monoclonal antibody PAb421 (lanes 0 and 5). An equal amount of sample from the 0 h chase was also incubated with mutant conformation specific p53 monoclonal antibody, PAb240 (lanes labelled C).

the ability to degrade newly synthesized p53 (Reich *et al.*, 1983; Halevy *et al.*, 1989). The presence of E6 reduced half-lives on average by only 2-fold and in one case (PE3) only a minor reduction was observed. We examined the levels of E6 in these cell lines by immunoprecipitation and found that the amounts of E6 present in the PE3 and PE4 cell lines were similar (Figure 4), but that the steady-state levels of p53 (Figure 1) as well as its stability (Table I) were significantly greater in the PE3 cell line. These observations demonstrate that while the presence of E6 in HPV transformed cell lines consistently results in a decrease in p53 protein stability, significant levels of p53 protein are retained in the cells.

In the above studies, we examined the stability of p53 in cell lines transformed by co-electroporation of E6 and E7 which together are efficient immortalizing agents of keratinocytes. The E7 gene can immortalize cells in the absence of E6 but at a much reduced frequency (Hudson *et al.*, 1990; Halbert *et al.*, 1991). We performed pulse–chase experiments on one of these E7 immortalized cell lines (Bam 3) and found the p53 half-life to be >4 h. Similar values were obtained in two other cell lines which express only HPV18 E7 (unpublished data). To examine what effect E6 had on p53 levels in cells which were already immortalized, we supertransfected an HPV18 E6 expression plasmid (p18MTE6) into the Bam3 cell line and isolated stable lines. Immunoprecipitation confirmed the presence of the HPV18 E6 protein in the p18MTE6 transfected cell line (Bam3-E6) and not in the pSV2neo control transfected cell line (Bam3-neo; data not shown). The expression of E6 was found to reduce the p53 half-life to 1.2 h while no significant change in p53 stability was observed in the pSV2neo control cell line (Table I). This level of reduction is within the range of values we previously observed in E6/E7-immortalized cells.

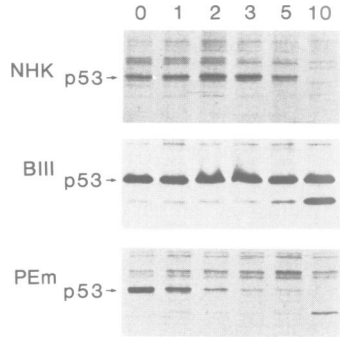

Fig. 3. Pulse–chase analysis of p53 from non-transformed and transformed human keratinocyte cell lines. Cells were pulse-labelled for 1 h and incubated with chase medium containing an excess of cold amino acid. An autoradiogram is shown of an SDS–PAGE gel in which equal amounts of protein (as determined by total TCA-precipitable counts) were immunoprecipitated with p53 monoclonal antibody PAb421 from cell extracts prepared in NP40 buffer after 0, 1, 2, 3, 5 and 10 h cold chase. Keratinocyte cell lines are NHK, non-transformed keratinocytes; BIII, MK6-1BIII (SV40-transformed) and PEm, PEmass (HPV18 E7/E6-transformed).

Table I. p53 half-life in human epithelial cell lines

Cell line	Transforming gene(s)	Half-life (hours)[d]
NHK[a]	–	3.3
MK6-1BIII	SV40 large and small T Ag	>10.0
JYMBI	SV40 large and small T Ag	5.0
PE3	HPV18 E6 and E7	2.8
PE5	HPV18 E6 and E7	1.6
PEmass	HPV18 E6 and E7	1.4
PE4	HPV18 E6 and E7	0.8
CC-1	HPV18	0.7
Bam3	HPV18 E7	>4.0
Bam3-neo	HPV18 E6 and E7	4.5
NHENF[b]	–	2.6
NHECF[c]	–	2.0

[a] Non-transformed human keratinocytes.
[b] Non-transformed human endocervical fibroblasts.
[c] Non-transformed human ectocervical fibroblasts.
[d] The majority of these determinations were performed at least twice.

Complexes of E6 and p53 found in Cos-7 cells transiently expressing HPV-18 E6

The association of p53 and HPV E6 has been demonstrated in *in vitro* translation systems but no such complex has been reported *in vivo*. To examine if we could isolate a complex of E6 and p53 from cells, we performed reciprocal coimmunoprecipitation experiments on labelled cell extracts. Using a monoclonal antibody to p53, we were able to coprecipitate a complex including SV40 large T antigen in SV40-transformed keratinocytes (Figure 4, lane 4). Similarly, a monoclonal antibody directed against T antigen was observed to coprecipitate the p53 protein (Figure 4, lane 5). We next used an identical methodology with a monoclonal antibody directed against HPV E6 to precipitate the E6 gene product from HeLa, CC-1 (data not shown) and HPV18 E6/E7-transformed cell lines, (Figure 4, lanes 7 and 9); however, no p53 protein was coprecipitated. Likewise, immunoprecipitation of p53 from HPV-immortalized cells failed to coprecipitate E6 (Figure 4, lanes 6 and 8).

The relative insolubility of E6 in mild detergents (Androphy *et al.*, 1987) may preclude the detection of a complex with p53 in transformed human keratinocytes where both proteins are present at low levels. We therefore used

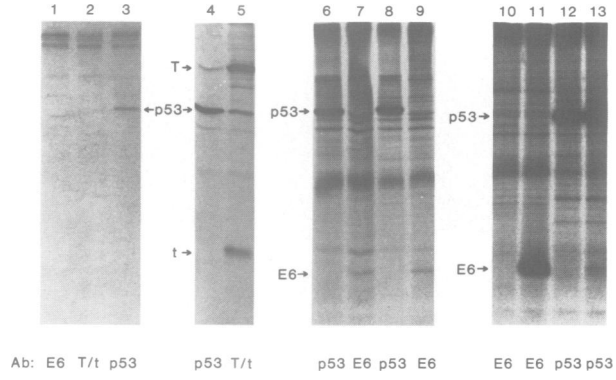

Fig. 4. Reciprocal immunoprecipitation of p53, SV40 T antigen and E6 proteins. Cells were pulse-labelled for 3 h with [^{35}S]translabel (lanes 1–5) or [^{35}S]cysteine (lanes 6–13) and harvested, and extracts (lanes 1–9 NP40 buffer and lanes 10–13 RIPA/SDS buffer) were subjected to immunoprecipitation with appropriate monoclonal antibodies: p53, PAb421; SV40 T antigens, PAb419; or E6, C1P5 (indicated below). An autoradiogram of SDS–PAGE analysis of the immunoprecipitated proteins is shown. Lanes 1–3, non-transformed keratinocytes; lanes 4 and 5, MK6–1BIII; lanes 6 and 7, PE3; lanes 8 and 9, PE4. Lanes 10 and 12, pSG5 electroporated Cos cells; lanes 11 and 13, pSG18E6 electroporated Cos cells. T indicates SV40 large T antigen and t indicates SV40 small T antigen.

an alternative system in which large amounts of HPV18 E6 protein were synthesized to try to detect a complex *in vivo*. Cos cells were transfected with an SV40 directed E6 expression vector (pSG18E6) and, at 48 h, cells were lysed in a buffer which efficiently extracts the E6 protein (Androphy *et al.*, 1987). As shown in Figure 4, lane 13, an anti-p53 monoclonal antibody was able to coprecipitate E6 specifically from Cos cells electroporated with pSG18E6. No bands comigrating with E6 were detected in immunoprecipitations using antisera against T antigen as a control (data not shown). The reciprocal experiment using an E6 antibody failed to coprecipitate p53 in this system (Figure 4, lane 11), consistent with *in vitro* studies which also failed to precipitate a complex with E6 antibodies probably as a result of blocked epitopes (Werness *et al.*, 1990). Inspection of E6 immunoprecipitations shown in Figure 4 as well as those performed in other experiments revealed no specific coprecipitation of a 100 kDa protein that was recently detected in association with E6 of HPV16 and 18 (Huibregtse *et al.*, 1991). We have also detected similar complexes of HPV-16 and 18 E6 proteins with p53 using transient assays in human keratinocyte MK6−1B cells (not shown) which are immortalized by SV40 T antigen (Lechner and Laimins, 1991).

p53 is localized to the nucleus in transformed and non-transformed human epithelial cell lines

The wild-type p53 protein is found in the cell nucleus, but mutant forms are often found in the cytoplasm (Sturzbecher *et al.*, 1987; Ginsberg *et al.*, 1991a; Martinez *et al.*, 1991). In association with the adenovirus 5 E1B 55 kDa protein, p53 is found in discrete clusters in the cytoplasm (Zantema *et al.*, 1985). In order to determine if the presence of E6 altered the subcellular localization of p53, we performed indirect immunofluorescence on NHK cells and on several transformed epithelial cell lines. In non-transformed keratinocytes p53 was localized in the nucleus and while the level of staining varied from cell to cell, newly divided cells often stained more intensely. In SV40-immortalized keratinocytes, strong nuclear staining of p53 was found in all cells, while in cell lines expressing E7 and E6, a dramatic decrease in the number of positively staining cells (<1%) was observed but staining remained localized to the nucleus.

In the Cos-7 cell expression system we were able to stain the nuclei of cells expressing E6 by indirect immunofluorescence and confirmed its nuclear localization (Figure 5). We noted that the staining for p53 was similar between cells expressing either pSG5 (expression construct alone) or pSG18E6 (Figure 5). In these studies, the presence of E6 did not alter the cellular localization of p53.

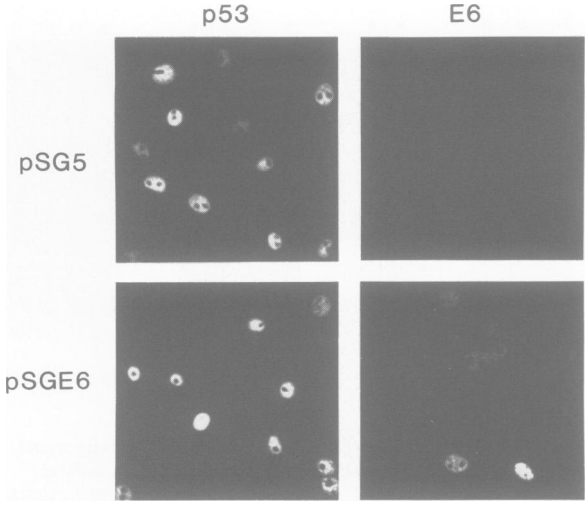

Fig. 5. Indirect immunofluorescence of p53 and E6 in electroporated Cos cells. Cos cells were electroporated with pSG5 or pSG18E6 and 48 h later they were fixed and incubated with E6 monoclonal antibody, C1P5 (vertical column labelled E6) or p53 monoclonal antibody, PAb122 (vertical column labelled p53). Top horizontal row (pSG5); pSG5 electroporated Cos cells. Bottom horizontal row (pSGE6); pSG18E6 electroporated Cos cells. Magnification: ×300.

Transcriptional repression by human p53 protein is abrogated by HPV E6 binding activity

Recently, evidence has been provided that p53 may act as a transcriptional regulator (Raycroft *et al.*, 1990; Fields and Jang, 1990; Weintraub *et al.*, 1991) and that this activity may play a role in transformation (Mercer *et al.*, 1991). We have observed that, in transient transfection assays, coexpression of wild-type human p53, but not of a mutant p53, results in dose-dependent repression of gene expression from multiple promoters, including the HPV18 URR (data not shown). To study in detail the inhibitory effects of wild-type p53 on gene expression, we chose as a reporter a promoter−CAT plasmid (pSV2CAT) which expresses at high levels in a variety of cell types. When C33a cells were cotransfected with a fixed amount of reporter plasmid together with increasing amounts of expression plasmid encoding wild-type human p53 (pC53-SN3), CAT activity was repressed by as much as 90% (Figure 6). In experiments in which the reporter was cotransfected with increasing amounts of a plasmid encoding a mutant p53 (pC53-SCX3), harboring a substitution of alanine for valine at amino acid 143, no significant effect on CAT expression was observed

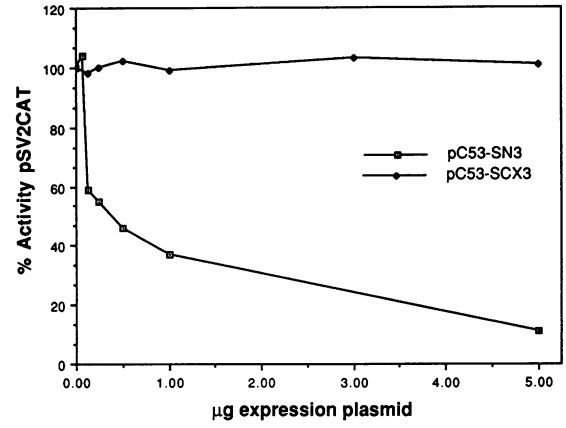

Fig. 6. Concentration dependent transcriptional repression of the SV40 early promoter by wild-type p53 expression plasmid. C33a cells were transiently transfected with 5 μg of pSV2CAT and increasing amounts of either wild-type (pC53-SN3) or a mutant (pC53-SCX3) human p53 encoding plasmid. The activity shown as 100% is that of pSV_2 CAT in the absence of any cotransfected p53 expression vector. The ordinate is the percent change in CAT activity of pSV2CAT due to cotransfection of the expression plasmids at the amounts indicated from a single experiment. Although the degree of change varied between experiments, the relative effects exerted by the p53 expression plasmids on reporter plasmids were consistent among six different experiments performed. Concentration of DNA in each transfection was kept constant by the addition of pSG5 to a total of 30 μg. Cells were harvested 40 h post-transfection.

even at 5 μg of effector plasmid (Figure 6). Similar results were obtained in HeLa as well as C33a cells using various promoter driven CAT constructs including the human c-*fos* promoter (Gius *et al.*, 1990), human β-actin promoter (Gunning *et al.*, 1987) and a minimal TATA element (data not shown). Of the promoters tested, wild-type p53 had no effect on the human proliferating cell nuclear antigen (PCNA) (Morris and Mathews, 1991) or human *ras* promoter (Ishii *et al.*, 1985) (data not shown). Interestingly, all promoters unaffected by p53 lack a characteristic TATA-box and direct transcription through initiator elements.

To determine if HPV18 E6 protein could influence the inhibitory effect of wild-type p53 on gene expression, we cotransfected increasing amounts of an HPV18 E6 expression vector (pSG18E6) with a fixed amount of pSV2CAT and pC53-SN3 plasmids into C33a cells (Figure 7A). Cotransfection with expression vector pSG11E7, expressing the HPV11 E7 protein, was used as a control for non-specific

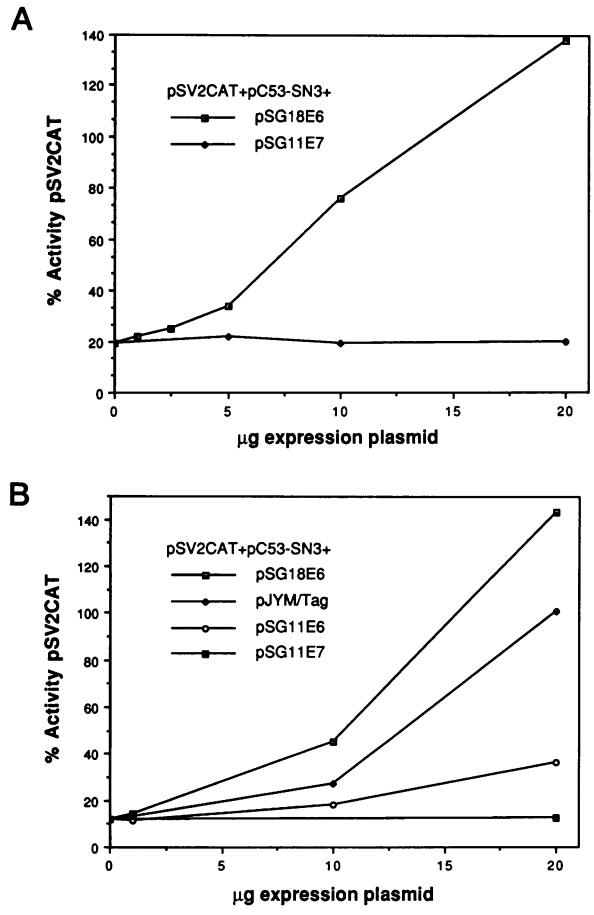

Fig. 7. Dose-dependent inhibition of p53-mediated transcriptional repression. (**A**) pSG18E6 construct (expressing the HPV18 E6 protein) or pSG11E7 construct (expressing the HPV11E7 protein) were separately transfected at increasing amounts in C33a cells with fixed quantities of pSV2CAT reporter plasmid (5 μg) and pC53-SN3 (5 μg) expression plasmid. (**B**) For comparative purposes SV40 T antigen (pJYM) and HPV11 E6 (pSG11E6) expression plasmids were assayed similarly for their effects on p53-mediated repression. The data presented are from single experiments. The activity shown as 100% is that of pSV_2 CAT in the absence of any cotransfected p53 or E6 expression vector. The ordinate is the percent change in CAT activity of pSV2CAT due to cotransfection of the expression plasmids at the amounts indicated from a single experiment. Although some variation in the degree of anti-repression was observed between individual experiments, the relative activities were consistent among three separate experiments performed.

effects. A clear abrogation of p53-mediated transcriptional repression was observed in a concentration dependent manner using pSG18E6 plasmid, whereas no effect was seen with pSG11E7. Similar effects were seen with an HPV16 E6 expression plasmid (Figure 8, pSG16E6). To examine whether proteins which physically interact with p53 may affect its functional activity in transient assays, we performed similar experiments using constructs expressing either SV40 large T antigen (pJYM) (Lusky and Botchan, 1981) or HPV11 E6 protein (pSG11E6). HPV11 E6 has recently been shown to bind wild-type p53, though with 3.5-fold lower affinity than E6s from the high risk types (Crook *et al.*, 1991a). We found that in both cases p53-dependent transcriptional repression was relieved dose-dependently, albeit to a significantly lower degree with pSG11E6 (Figure 7B: pSG11E6,32%; pJYM, 101%; or pSG18E6, 143%).

Since SV40 large T antigen and HPV11 E6 bind p53 but do not promote its degradation, we examined if binding activity alone could overcome the inhibitory effect of wild-type p53 on transcription (Figure 8). Cotransfection of plasmids expressing either HPV16 wild-type E6 protein (pSG16E6), or a mutant HPV16 E6 protein shown to bind strongly but not degrade p53 (Crook *et al.*, 1991a) resulted in efficient abrogation of p53-mediated repression. Since HPV E6 proteins possess transcriptional transactivating activity (Sedman *et al.*, 1991; Desaintes *et al.*, 1992) we determined whether this played a role in the observed derepression of CAT activity. Cotransfection of plasmid encoding a mutant HPV16 E6 (pSG16E6Δ106−110) which is severely reduced for p53 binding and degradation but retains significant transactivating activity (Crook *et al.*, 1991a) had little effect on p53-mediated repression of CAT expression (Figure 8). These experiments have been repeated several times and the results observed are qualitatively consistent. Taken together, these data demonstrate that binding of E6 to p53, but not its enhanced degradation, is sufficient to relieve the inhibitory effect on gene expression in transient assays.

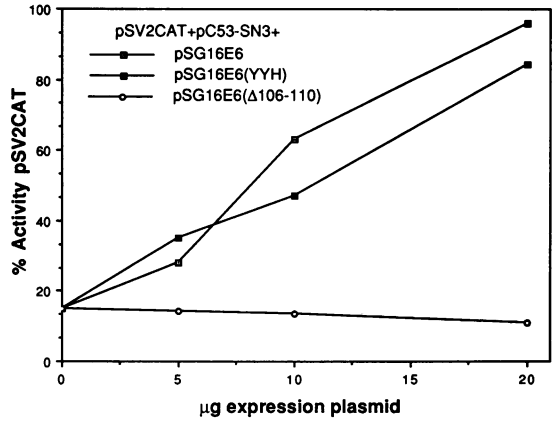

Fig. 8. Effect of E6 mutants on p53-mediated transcriptional repression. The ability of various E6 expressing plasmids to abrogate p53 inhibition of CAT expression was assayed for as described in Figure 7. pSG16E6 encodes the wild-type E6 protein of HPV16. pSG16E6(YYH) possesses three amino acid substitutions at residues 45, 47 and 49 of HPV16 E6 and binds p53 with high avidity, but does not direct its degradation (Crook *et al.*, 1991a). pSG16E6(Δ106−110) is deleted for amino acid residues 106−110 of HPV16 E6 and is severely reduced for p53 binding, though it retains wild-type transactivating activity (Crook *et al.*, 1991a).

Discussion

We have observed that cells derived from HPV-positive carcinoma biopsies and cells transformed *in vitro* by HPV18 E6/E7 contain lower steady-state levels of p53 protein than non-transformed human keratinocytes. The difference in p53 protein levels is not due to changes in p53 mRNA levels, but is a result of an increase in p53 protein turnover. Overall, the half-life of p53 in the cell lines studied, including human fibroblasts, was greater than that previously observed in mouse fibroblasts (Oren *et al.*, 1981; Mora *et al.*, 1982; Reich *et al.*, 1983); this may be a result of species and/or cell-type differences. Examination of human keratinocyte cell lines expressing HPV E6 revealed a range of reduced half-lives, including a cell line with a half-life nearly equal to that of non-transformed keratinocytes. While this one cell line may be unusual in its ability to maintain p53 levels, none of the cell lines we examined exhibited reductions in p53 half-life of >4-fold in contrast to the rapid degradation seen *in vitro*. It is possible that changes in p53 half-lives of only several-fold *in vivo* can severely alter cell growth properties. Alternatively, these observations raise the question of whether E6's association with p53 simply functions to direct it to degradation. The variability we observed in p53 half-lives was not a direct result of E6 levels because a wide range of steady-state p53 levels and stabilities were found in cells which contain equivalent amounts of E6. Other investigators have reported that different intracellular environments can affect p53 stability (Braithwaite and Jenkins, 1989; Halevy *et al.*, 1989). One possibility that may account for these observations is the presence of another cellular protein involved in E6−p53 binding, such as the recently identified 100 kDa E6AP factor (Huibregtse *et al.*, 1991); however, we have not detected this factor in our experiments. Nevertheless, our results suggest that a reduction in p53 level is not absolutely required for immortalization by HPV in tissue culture. In support of this idea, it has been shown that E7 alone can immortalize human keratinocytes and the level and stability of p53 in these cells is similar to non-transformed keratinocytes.

Since the presence of E6 in a cell does not always lead to a significant reduction in p53 levels, it is possible that E6 may augment transformation through other routes. E6 and p53 both possess transcriptional transactivating activity in chimeric constructs (Fields and Jang, 1990; Lamberti *et al.*, 1990; Raycroft *et al.*, 1990) and HPV E6 can activate transcription from several eukaryotic promoters (Sedman *et al.*, 1991, Desaintes *et al.*, 1992). Vousden and coworkers have shown that the p53 binding domain of the E6 protein is separable from both the p53 degradation and transcriptional transactivation domains (Crook *et al.*, 1991a). Hence, p53 interaction is dispensable for transactivation and E6 can bind p53 *in vitro* without promoting its degradation. In this study, we have coprecipitated a complex of E6 and p53 from Cos-7 cells and SV40-transformed human keratinocytes, demonstrating that these proteins physically interact in cell extracts. Thus a E6−p53 complex may possess an additional function which could enhance cell transformation with or without the rapid degradation of p53.

A potential target for E6 action may be through an alteration of the transcriptional regulatory properties of p53. We have observed in transient expression experiments that wild-type p53 is capable of down-regulating transcription from a wide variety of eukaryotic promoters, while mutant p53 is unable to exert this effect. It is interesting to note that all promoters repressed by p53 in this study utilize a TATA box for transcriptional initiation, while those unaffected use initiator elements. TATA-less promoters are often characteristic of genes involved in cellular housekeeping functions and cell growth control. Since only a certain class of promoters appears to be repressed by p53, this may be an important clue in elucidating the mechanism by which p53 exerts its effect on transcription. In the course of this work several groups have also reported repression of transcription in transient assays by wild-type p53 (Chin *et al.*, 1992; Ginsberg *et al.*, 1991b).

We find that introduction of HPV18 or 16 E6 is capable of relieving transcriptional repression by wild-type p53, as is an E6 mutant protein which can bind p53 but is incapable of directing it to degradation. However, an E6 mutant deficient in p53 binding yet able to transactivate the Ad2 promoter is unable to exert this effect and demonstrates that E6 transactivation activity is not responsible for derepression. p53-mediated repression was also abrogated with overexpression of SV40 T antigen and, to a lesser extent, HPV11 E6. Both of these proteins are capable of binding p53, yet neither results in the rapid degradation of the p53 protein. The reduced ability of the E6 protein from HPV11 to block p53 repression is correlated with the less efficient transforming potential of low risk HPVs. Taken together, these data indicate that the physical interaction alone of HPV E6 with p53 is responsible for relieving p53-mediated transcriptional repression and that degradation is not required.

We have shown that the immortalization of human keratinocytes in culture by SV40 T antigen, HPV18 E7 alone, and in some instances by HPV18 E7 and E6 can occur without the destabilization of p53. It is worthwhile noting that keratinocytes immortalized by SV40 T antigen and HPV18 E7 alone retain the ability to differentiate *in vitro* but those immortalized in conjunction with E6 rapidly lose this capability (Hudson *et al.*, 1990; Lechner and Laimins, 1991). We have also observed that the introduction of HPV18 E6 into keratinocytes expressing E7 alone results in increased growth rate and altered epithelial differentiation of this cell line (unpublished data). It is tempting then to speculate that the role of HPV E6 is to disrupt the process of epithelial differentiation and that this is tied to the inactivation of cellular protein, p53. The association of high risk HPV types 16 and 18 with high-grade dedifferentiated epithelial lesions is correlated with the ability of their E6 proteins to efficiently bind and degrade p53. Future studies will help to define the role of the p53 binding and degrading activities of E6 in the disruption of epithelial differentiation.

Materials and methods

Plasmids

The pSG11E6, pSG18E6 and pSG18E7 plasmids were constructed by polymerase chain reaction with oligonucleotides complementary to the translational start and stop sequences of each open reading frame and *Bam*HI−*Bgl*II ends (or *Bgl*II only for HPV18 E6). The amplified sequences were cloned into pSG5 at the *Bam*HI site and confirmed by sequencing. pSG16E6, pSG16E6Δ106−110 and pSG16E6YYH plasmids were constructed by inserting the *Bam*HI fragment from plasmids pJ4Ω16E6, pJ4Ω16E6Δ106−110 and pJ4Ω16E6TYR/TYR/HIS45/47/49 (Crook *et al.*, 1991a) respectively into the *Bam*HI site of pSG5. The plasmids pC53-SN3 (Baker *et al.*, 1990), pC53-SCX3 (Baker *et al.*, 1990), PCNAcat (Morris

and Mathews, 1991), pRasCAT1 (Ishii *et al.*, 1985), pfos363 (Gius *et al.*, 1990), pJYM (Lusky and Botchan, 1981) and p18MTE6 (Bedell *et al.*, 1989) have been described previously.

Transient transfections and CAT assays

Transient transfection experiments were performed on ~500 000 cells per 10 cm dish by the calcium phosphate precipitation technique as described previously (Gorman, 1985). Concentration of DNA in each transfection was kept constant by the addition of pSG5 DNA to a total of 30 μg. Cells were harvested 40 h post-transfection and extracts prepared for CAT assays as described previously (Gorman, 1985). The total protein concentration in cell extracts was quantified by the Bio-Rad protein assay (Bio-Rad Laboratories) and equivalent amounts of protein were used in each CAT reaction. Assays for CAT enzyme activity were performed as described previously (Gorman, 1985).

Cell culture

Non-transformed human keratinocytes were obtained and cultured as described (Hudson *et al.*, 1990). SV40-immortalized: JYMB-I and MK6−1BIII, HPV18 E7 and E6-immortalized: PEmass, PE3, PE4, PE5 and HPV18 E7 immortalized: Bam3, human keratinocyte cell lines have been described previously (Hudson *et al.*, 1990; Lechner and Laimins, 1991). Isolation and characterization of the CIN-612 (HPV31b-positive) cell line has been described (Bedell *et al.*, 1991) as have the CC-1 (HPV18-positive) and CC-2 (HPV16-positive) carcinoma cell lines (Rader *et al.*, 1990). The Bam3-neo and the Bam3-E6 cell lines were produced by electroporating Bam3 cells with pSV2neo or pSV2neo and p18MTE6 respectively. Mass cell lines were expanded after drug selection and expression of E6 was confirmed by immunoprecipitation. The cell lines above were cultured in E medium and maintained as described by Hudson *et al.* (1990). HeLa (HPV18-positive) and C33a (HPV-negative) cell lines were maintained in DMEM/10% calf serum (Gibco, Grand Island, NY) and the STSAR-5 cell line, a human histiocytoma cell line carrying a p53 gene deletion (Brachman *et al.*, 1991), was maintained in DMEF12/20% fetal bovine serum (Gibco, Grand Island, NY). Human cervical fibroblasts (kindly supplied by M.Turyk, Rush-Presbyterian St Luke's Hospital, Chicago, IL) were isolated from normal cervical biopsies and maintained in DMEM/10% calf serum.

Northern and Western blot analyses

Total RNA was isolated and 10 μg hybridized in RNA (Northern) blots as described by Bedell *et al.* (1989). All Northern blots were stripped and reprobed with the glyceraldehyde-3-phosphate dehydrogenase gene to confirm equal loading of RNA. Cellular proteins were extracted in modified RIPA/SDS buffer: 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 100 mM NaF, 200 μM sodium orthovanadate, 0.5 mM DTT, 1.0 mM PMSF, 5 mM benzamidine HCl, 1 μg/ml pepstatin and 1 μg/ml leupeptin (in ND buffer), 1.0% Triton X-100, 1.0% deoxycholate (in RIPA) and 1.0% SDS or NP40 buffer: ND buffer plus 0.5% NP40 as indicated. Extracts were clarified on ice for 20 min and centrifuged for an additional 20 min and either frozen at −80°C or used immediately. 100 μg of extract was applied to 12.5% SDS−PAGE and transferred to PVDF membrane (Immobilon P, Millipore Corp., Bedford, MA). Primary antibody, monoclonal antibody PAb1801 (Banks *et al.*, 1986) (Ab-2, Oncogene Science Inc., Manhasset, NY), was applied to the membrane and p53 was visualized using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

Cell labelling and immunoprecipitation

Cells were metabolically labelled at 70−80% confluency with either 0.5 mCi/ml [^{35}S]cysteine or [^{35}S]tran^{35}Slabel (ICN, Irvine, CA) for 3 h at 37°C in a humidified 5% CO_2 atmosphere after 1 h starvation in cysteine- or methionine-free DMEM (Gibco, Grand Island, NY). For pulse−chase analysis cells were labelled for 30 min or 1 h, washed in phosphate-buffered saline (PBS) and either harvested or incubated in chase medium containing an excess of cold cysteine and/or methionine. Cells were then harvested after cold chase times of 1, 2, 3, 5 and 10 h and lysed in NP40 buffer. Clarified extracts of harvested cells were precleared with protein A−Sepharose (Pharmacia, Piscataway, NJ) and then used for immunoprecipitation. Samples were normalized using an equal amount of TCA-precipitable counts for each time point. Antigen−antibody complexes were collected with protein A−Sepharose and washed in extraction buffer. A secondary rabbit anti-mouse antibody (PharMingen, San Diego, CA) was included with anti-p53 monoclonal antibody PAb240 (Ab3, Oncogene Science Inc. Manhasset, NY) (Gannon *et al.*, 1990) and the anti-E6 monoclonal antibody C1P5 (Androphy *et al.*, 1985). Samples were then separated on a 12.5% SDS−PAGE gel and gels were fixed, fluorographed in Amplify (Amersham, Arlington Heights, IL), dried and exposed to Kodak XAR-5 film at −80°C. Quantification of radioactive proteins was performed by Betascope analysis (Betagen, Waltham, MA). After correction for background counts, the initial amount of radioactivity (0 h chase) in the p53 band was assigned a value of 100%. The half-life was determined from a plot of radioactivity in the p53 band versus time and expressed as the time at which p53 levels had decreased to 50%.

Immunofluorescence and Cos cell expression

Cells were seeded onto chamber slides (Nunc, Naperville, IL) and after 24 h fixed in PLP buffer (8% paraformaldehyde:0.1 M lysine, 0.05 M monobasic sodium phosphate, 0.05 M dibasic sodium phosphate, 0.01 M sodium *meta*-periodate in a 1:3 ratio). After washing in PBS, the slides were incubated with either p53 monoclonal antibody PAb122 (ATCC, Rockville, MD) (Gurney *et al.*, 1980) at a 1:500 dilution of ascites fluid, SV40 large T antigen monoclonal antibody PAb419 (Ab-1, Oncogene Science Inc., Manhasset, NY) (Harlow *et al.*, 1981) at a 1:1000 dilution, or E6 monoclonal antibody C1P5 at a 1:1000 dilution of ascites fluid for 1 h. Excess antibody was washed off and slides were incubated with a fluorescein isothiocyanate-conjugated secondary antibody (Amersham, Arlington Heights, IL) for 30 min, washed with PBS and mounted before viewing. For transient expression of HPV18 E6, 1 × 10^6 Cos-7 cells (Gluzman, 1981) were electroporated with 10 μg pSG18E6. Electroporation of expression plasmid alone, pSG5, served as a negative control. Cells were seeded into chamber slides for immunofluorescence or 100 mm dishes for protein analysis. After 48−72 h, cells were labelled with 0.5 mCi/ml [^{35}S]cysteine for 3 h. Proteins were extracted as described (Banks *et al.*, 1987) in RIPA, and SDS was added to a concentration of 0.5%. After adjusting the volume to give a final SDS concentration of 0.1%, samples were sheared through a 0.22 gauge needle and prepared for immunoprecipitation as described above. Immunofluorescence of Cos-7 cells was carried out as described above, 48−72 h after electroporation.

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References

Androphy,E.J., Schiller,J.T. and Lowy,D.R. (1985) *Science*, **230**, 442−446.

Androphy,E.J., Hubbert,N.L., Schiller,J.T. and Lowy,D.R. (1987) *EMBO J.*, **6**, 989−992.

Baker,S.J. *et al.* (1989) *Science*, **244**, 217−221.

Baker,S.J., Markowitz,K., Fearon,E.R., Willson,J.K.V. and Vogelstein,B. (1990) *Science*, **249**, 1912−1915.

Banks,L., Matlashewski,G. and Crawford,L. (1986) *Eur. J. Biochem.*, **159**, 529−534.

Banks,L., Spence,P., Androphy,E., Hubbert,N., Matlashewski,G., Murray,A. and Crawford,L. (1987) *J. Gen. Virol.*, **68**, 1351−1359.

Barbosa,M.S., Vass,W.C., Lowy,D.R. and Schiller,J.T. (1991) *J. Virol.*, **65**, 292−298.

Bedell,M.A., Jones,K.H., Grossman,S.G. and Laimins,L.A. (1989) *J. Virol*, **63**, 1247−1255.

Bedell,M.A., Hudson,J.B., Golub,T.R., Turyk,M.E., Hosken,M., Wilbanks,G.D. and Laimins,L.A. (1991) *J. Virol.*, **65**, 2254−2260.

Brachman,D.G., Hallahan,D.E., Beckett,M.A., Yandell,D.W. and Weichselbaum,R.R. (1991) *Cancer Res.*, **51**, 6393−6396.

Braithwaite,A.W. and Jenkins,J.R. (1989) *J. Virol.*, **63**, 1792−1799.

Chin,K.-V., Ueda,K., Pastan,I. and Gottesman,M.M. (1992) *Science*, **255**, 459−4462.

Crook,T., Tidy,J.A. and Vousden,K.H. (1991a) *Cell*, **67**, 547−556.

Crook,T., Wrede,D. and Vousden,K.H. (1991b) *Oncogene*, **6**, 873−875.

Desaintes,C., Hallez,S., Van Alphen,P. and Burny,A. (1992) *J. Virol.*, **66**, 325−333.

Diller,L. *et al.* (1990) *Mol. Cell. Biol.*, **10**, 5772–5781.
Eliyahu,D., Raz,A., Gruss,P., Givol,D. and Oren,M. (1984) *Nature*, **312**, 646–649.
Farrell,P.J., Allan,G.J., Shanahan,F., Vousden,K.H. and Crook,T. (1991) *EMBO J.*, **10**, 2879–2887.
Fields,S. and Jang,S.K. (1990) *Science*, **249**, 1046–1049.
Finlay,C.A., Hinds,P.W., Tan,T.H., Eliyahu,D., Oren,M. and Levine,A.J. (1988) *Mol. Cell. Biol.*, **8**, 531–539.
Finlay,C.A., Hinds,P.W. and Levine,A.J. (1989) *Cell*, **57**, 1083–1093.
Gannon,J.V., Greaves,R., Iggo,R. and Lane,D.P. (1990) *EMBO J.*, **9**, 1595–1602.
Ginsberg,D., Michael-Michalovitz,D., Ginsberg,D. and Oren,M. (1991a) *Mol. Cell. Biol.*, **11**, 582–585.
Ginsberg,D., Mechta,F., Yaniv,M. and Oren,M. (1991b) *Proc. Natl. Acad. Sci. USA*, **88**, 9979–9983.
Gius,D., Cao,X., Rauscher,F.J., Cohen,D.R., Curran,T. and Sukhatme,V.P. (1990) *Mol. Cell. Biol.*, **10**, 4243–4255.
Gluzman,Y. (1981) *Cell*, **23**, 175–182.
Gorman,C. (1985) In Glover,D.M. (ed.), *DNA Cloning: A Practical Approach*. IRL Press, Oxford, Vol. II, pp. 143–164.
Gunning,P., Leavitt,J., Muscat,G., Ng,S-Y. and Kedes,L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4831–4835.
Gurney,E.G., Harrison,R.O. and Fenno,J. (1980) *J. Virol.*, **34**, 752–763.
Halbert,C.L., Demers,G.W. and Galloway,D.A. (1991) *J. Virol.*, **65**, 473–478.
Halevy,O., Hall,A. and Oren,M. (1989) *Mol. Cell. Biol.*, **9**, 3385–3392.
Halevy,O., Michalovitz,D. and Oren,M. (1990) *Science*, **250**, 113–116.
Harlow,E., Crawford,L.V., Pim,D.C. and Williamson,N.M. (1981) *J. Virol.*, **39**, 861–869.
Hawley-Nelson,P., Vousden,K. H., Hubbert,N.L., Lowy,D.R. and Schiller,J.T. (1989) *EMBO J.*, **8**, 3905–3910.
Hinds,P., Finlay,C. and Levine,A.J. (1989) *J. Virol.*, **63**, 739–746.
Hinds,P.W., Finlay,C.A., Quartin,R.S., Baker,S.A., Fearon,E.R., Vogelstein,B. and Levine,A.J. (1990) *Cell Growth Diff.*, **1**, 571–580.
Hudson,J. B., Bedell,M. A., McCance,D.J. and Laimins,L.A. (1990) *J. Virol.*, **64**, 519–526.
Huibregtse,J.M., Scheffner,M. and Howley,P.M. (1991) *EMBO J.*, **10**, 4129–4135.
Ishii,S., Merlino,G.T. and Pastan,I. (1985) *Science*, **230**, 1378–1381.
Jenkins,J.R., Rudge,K. and Currie,G.A. (1984) *Nature*, **312**, 651–653.
Jenkins,J.R., Rudge,K., Chumakov,P. and Currie,G.A. (1985) *Nature*, **317**, 816–817.
Lamberti,C., Morrissey,L.C., Grossman,S.R. and Androphy,E.J. (1990) *EMBO J.*, **9**, 1907–1913.
Lane,D.P. and Crawford,L.V. (1979) *Nature*, **278**, 261–263.
Lechner,M.S. and Laimins,L.A. (1991) *Virology*, **185**, 563–571.
Lusky,M. and Botchan,M. (1981) *Nature*, **293**, 79–81.
Malkin,D. *et al.* (1990) *Science*, **250**, 1233–1238.
Martinez,J., Georgoff,I., Martinez,J. and Levine,A.J. (1991) *Genes Dev.*, **5**, 151–159.
May,E., Jenkins,J.R. and May,P. (1991) *Oncogene*, **6**, 1363–1365.
Mercer,W.E., Shields,M.T., Amin,M., Sauve,G.J., Appella,E., Romano,J.W. and Ullrich,S.J. (1990a) *Proc. Natl. Acad. Sci. USA*, **87**, 6166–6170.
Mercer,W.E., Amin,M., Sauve,G.J., Apella,E., Ullrich,S.J. and Romano,J.W. (1990b) *Oncogene*, **5**, 973–980.
Mercer,W.E., Shields,M.T., Lin,D., Appella,E. and Ullrich,S.J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 1958–1962.
Michalovitz,D., Halevy,O. and Oren,M. (1990) *Cell*, **62**, 671–680.
Mora,P.T., Chandrasekaran,K., Hoffman,J.C. and McFarland,V.W. (1982) *Mol. Cell. Biol.*, **2**, 763–771.
Morris,G.F. and Mathews,M.B. (1991) *J. Virol.*, **65**, 6397–6406.
Mulligan,L.M., Matlashewski,G., Scrable,H.J. and Cavenee,W.K. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5863–5867.
Nigro,J.M. *et al.* (1989) *Nature*, **342**, 705–708.
Oren,M., Maltzman,W. and Levine,A.J. (1981) *Mol. Cell. Biol.*, **1**, 101–110.
Parada,L.F., Land,H., Weinberg,R.A., Wolf,D. and Rotter,V. (1984) *Nature*, **312**, 649–651.
Rader,J.S., Golub,T.S., Hudson,J.B., Patel,D., Bedell,M.A. and Laimins,L.A. (1990) *Oncogene*, **5**, 571–576.
Raycroft,L., Wu,H. and Lozano,G. (1990) *Science*, **249**, 1049–1051.
Reich,N.C., Oren,M. and Levine,A.J. (1983) *Mol. Cell. Biol.*, **3**, 2143–2150.
Sarnow,P., Ho,Y.S., Williams,J. and Levine,A.J. (1982) *Cell*, **28**, 387–394.
Scheffner,M., Werness,B.A., Huibregtse,J.M., Levine,A.J. and Howley,P.M. (1990) *Cell*, **63**, 1129–1136.
Scheffner,M., Munger,K., Byrne,J.C. and Howley,P.M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5523–5527.
Schlegel,R., Phelpls,W.C., Zhang,Y.-L. and Barbosa,M. (1988) *EMBO J.*, **7**, 3181–3187.
Sedman,S.A., Barbosa,M.S., Vass,W.C., Hubbert,N.L., Haas,J.A., Lowy,D.R. and Schiller,J.T. (1991) *J. Virol.*, **65**, 4860–4866.
Sturzbecher,H.W., Chumakov,P., Welch,W.J. and Jenkins,J.R. (1987) *Oncogene*, **1**, 201–211.
Takahashi,T. *et al.* (1989) *Science*, **246**, 491–494.
Weintraub,H., Hauschka,S. and Tapscott,S.J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4570–4571.
Werness,B.A., Levine,A.J. and Howley,P.M. (1990) *Science*, **248**, 76–79.
Wrede,D., Tidy,J.A., Crook,T., Lane,D. and Vousden,K.H. (1991) *Mol. Carcinogen.*, **4**, 171–175.
Zantema,A., Schrier,P.I., Davis-Olivier,A., van Laar,T., Vaessen,R.T.M.J. and van der Eb,A.J. (1985) *Mol. Cell. Biol.*, **5**, 3084–3091.

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