

## **Adenovirus E1-transformed cells grow despite the continuous presence of transcriptionally active p53**

**Christian Löber, Claudia Lenz-Stöppler and Matthias Dobbelstein**

Institut für Virologie, Philipps-Universität Marburg, Robert Koch Str. 17, 35037 Marburg, Germany

**Author for correspondence:** Matthias Dobbelstein.

Fax +49 6421 28 68962. e-mail [dobbelst@mail.uni-marburg.de](mailto:dobbelst@mail.uni-marburg.de)

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### **Abstract**

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The E1 region of adenovirus (Ad) type 5 is capable of transforming cells. According to current concepts, the Ad E1B 55 kDa (E1B 55K) protein enables transformed cells to grow by constantly binding and inactivating the p53 tumour suppressor protein. To test this model, the transcriptional activity of p53 was determined in Ad E1-transformed cells. Surprisingly, it was found that a p53-responsive promoter is highly active in Ad E1-transformed cells and further activated only 3- to 4-fold (compared to 200-fold in *p53*<sup>-/-</sup> cells) by exogenously expressed p53 or p53mt24–28, a p53 mutant that is transcriptionally active but unable to bind the E1B 55K. On the other hand, the transient overexpression of E1B 55K led to a strong downregulation of a p53-responsive promoter relative to its baseline activity in Ad E1-transformed cells but not in *p53*<sup>-/-</sup> cells. COS-7 cells, transformed by simian virus 40 (SV40), also showed constitutive p53 activity, whereas HeLa cells, transformed with oncogenic human papillomavirus, did not. Upon stable transfection, Ad E1-transformed cells but not *p53*<sup>-/-</sup> cells gave rise to colonies that expressed exogenous p53 or p53mt24–28 but, nonetheless, grew at near-wild-type rates. It is

proposed that E1B 55K or the SV40 tumour antigen are saturated by the p53 protein, which accumulates in virus-transformed cells, leaving a proportion of active p53 molecules. The transformation of cells by the Ad E1 genes confers permissiveness for active p53, conceivably by inactivating the relevant products of p53 target genes that would otherwise prevent cell growth. Thus, Ad-transformed cells contain and tolerate active p53.

## Introduction

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The *p53* gene is subject to the most common genetic alteration in human malignancies and it plays a central role in tumour suppression and growth regulation (Levine, 1997; Vousden, 2000). The intracellular levels and activities of the p53 protein are upregulated by genotoxic stress (Schwartz & Rotter, 1998), consistent with a role as 'the guardian of the genome' (Lane, 1992). Even those tumours with wild-type *p53* frequently have defects in the regulation of p53 activity. p53 functions as a transcription factor, binding the DNA of various cellular promoters and stimulating transcription. In parallel, it induces cell-cycle arrest or apoptosis. Consistently, a growing number of p53-induced gene products was reported to prevent cell-cycle progression and/or to trigger cell death (Vousden, 2000).

Adenovirus and simian virus 40 (SV40) mediate the malignant transformation of cells and induce tumours in animals. The mechanisms underlying this phenomenon have been studied for decades, establishing many of the principles relevant to molecular tumorigenesis (Shenk, 1996). Understanding how tumour viruses induce and maintain malignancy has frequently elucidated virus and non-virus carcinogenesis in general. Recently, the investigation of virus-mediated transformation was intensified by the observation that human primary cells can be transformed by a limited set of overexpressed genes, i.e. the telomerase catalytic subunit, oncogenic Ras and SV40 large tumour (T) antigen. These oncogenes were found to be sufficient to transform human fibroblasts (Hahn *et al.*, 1999) and human mammary epithelial cells (Elenbaas *et al.*, 2001). While these studies may reveal a limited set of oncogenic changes needed for human carcinogenesis, the usefulness of the system depends on a full understanding of the effects caused by the continuous expression of the SV40 T antigen.

The Ad E1 region encodes the E1A proteins, which share common domains, and two entirely distinct E1B proteins, namely the E1B 55 kDa protein (E1B 55K) and the E1B 19 kDa protein (E1B 19K). These three protein entities co-operate to transform primary cells. Ad type 5 (Ad5) E1B 55K forms a specific complex with p53 (Sarnow *et al.*, 1982a) and, at least transiently, blocks p53-mediated transcription, an activity that co-segregates with the transforming potential of E1B 55K in mutational analysis (Yew & Berk, 1992). Ad5 E1B 55K binds to the

amino-terminal portion of p53 that is responsible for transcriptional activation (Lin *et al.*, 1994), actively represses p53-mediated transcription (Yew *et al.*, 1994) and sequesters p53 to characteristic perinuclear clusters (Blair Zajdel & Blair, 1988; Zantema *et al.*, 1985a). Given the close correlation between the ability of E1B 55K to bind p53 and its transforming potential (Yew & Berk, 1992), it is widely assumed that E1B 55K constantly inactivates p53 in Ad E1-transformed cells (Grand *et al.*, 1995). This view was further supported by the observation that peptides blocking the interaction between E1B 55K and p53 at least temporarily inhibit the growth of Ad E1-transformed cells (Hutton *et al.*, 2000).

A homologue of p53, termed p73, is expressed in mammalian cells (Kaghad *et al.*, 1997). Many activities are shared between p53 and p73, such as transcriptional activation and the induction of apoptosis (Jost *et al.*, 1997). However, unlike p53, p73 does not detectably interact with Ad E1B 55K (Higashino *et al.*, 1998; Marin *et al.*, 1998; Roth *et al.*, 1998; Steegenga *et al.*, 1999; Wienzek *et al.*, 2000). Based on this finding, a chimera of p53 and p73 was constructed, replacing the five residues at positions 24–28 of p53 with the corresponding amino acids from p73, to create the mutant p53mt24–28 (Roth *et al.*, 1998). This p53 mutant is fully active, inducing the transcriptional activation of p53-responsive promoters to the same extent as wild-type p53. However, it cannot be bound and inhibited by the E1B 55K proteins of Ad5 or Ad12 (Koch *et al.*, 2001; Roth *et al.*, 1998; Wienzek *et al.*, 2000). It also remains stable and active throughout an Ad infection (Koch *et al.*, 2001). Given these properties, p53mt24–28 represents a tool to analyse the behaviour of cells in the simultaneous presence of E1B 55K and transcriptionally active p53.

SV40 employs a similar strategy as Ad to inactivate p53. The large T antigen of SV40 binds p53 and prevents it from activating transcription (Jiang *et al.*, 1993; McCormick *et al.*, 1981; Sarnow *et al.*, 1982a) but does not decrease the stability of p53 (Zantema *et al.*, 1985b). In contrast, when cells were transformed by oncogenic human papillomaviruses (HPVs), p53 is destabilized and virtually eliminated. This effect is due to enhanced p53 ubiquitination in the presence of oncogenic HPV E6 proteins (Huibregtse *et al.*, 1991; Scheffner *et al.*, 1990, 1993). p53 induces the expression (Barak *et al.*, 1994; Juven *et al.*, 1993) of its cellular antagonist, Mdm2 (Oliner *et al.*, 1993), which mediates the degradation of p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997), thereby establishing a regulatory feedback loop (Wu *et al.*, 1993). In cells that express Ad E1 or SV40 T antigens, however, p53 is at least temporarily inactivated by either E1B 55K or SV40 T antigen and this can be expected to interrupt the activation of *mdm2* expression. Accordingly, p53 is stabilized in the presence of E1B 55K (Zantema *et al.*, 1985b) or SV40 T antigen (Reich *et al.*, 1983).

Since the stability of p53 is increased in Ad E1- and SV40-transformed cells, we hypothesized that p53 accumulates to an extent that will saturate E1B 55K molecules or T antigens present in these cells and, that beyond this point, any excess of p53 is transcriptionally active. This hypothesis was tested and, indeed, active p53 was constantly present in Ad E1-transformed cells and also in SV40-transformed cells. Furthermore, the stable overexpression of E1B-resistant, active p53mt24–28 was tolerated by Ad E1-transformed cells, strongly suggesting that all the relevant growth-preventing products of p53 target genes are effectively inactivated upon transformation of cells by Ad E1 proteins.

## Methods

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**Cell culture and transfections.** HEK293 cells, derived from human kidney cells by Ad-transformation (Graham *et al.*, 1977), and HeLa cells were from ATCC; H1299 cells and COS-7 cells were a gift from A. J. Levine, The Rockefeller University, New York, USA; HER911 cells, derived from human retina cells by Ad E1 transformation (Fallaux *et al.*, 1996), were from Introgene. HEK293 and HER911 cells express Ad E1 proteins but not E4 proteins and, therefore, destabilization of p53 cannot be mediated by the co-operation of E1 and E4 proteins (Marin *et al.*, 1998; Roth *et al.*, 1998; Steegenga *et al.*, 1999; Wienzek *et al.*, 2000) in these cells. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % foetal bovine serum (FBS). Transfections were carried out using Lipofectamine 2000 (Life Technologies). For colony-forming assays, transfected cells were split 48 h after transfection and further incubated in selection medium (DMEM containing 10 % FBS and 800 µg/ml G418) until colonies were detected (ca. 14 days). Cells were then harvested or stained with crystal violet.

**Plasmids.** Expression plasmids for p53 (Lin *et al.*, 1994), p53mt24–28 (Roth *et al.*, 1998) and Ad5 E1B 55K (Dobbelstein *et al.*, 1997) were described previously, as well as the reporter plasmids pGL2BP100 (Freedman *et al.*, 1997) and pGL2RSV (Ristea *et al.*, 2000). For stable expression of p53 and its mutants, the p53-encoding region was excised from pRcCMVp53 (Lin *et al.*, 1994) or pRcCMVp53R273H (Lin *et al.*, 1994) with *HindIII*/*XbaI*. The ends were filled with *Pfu* turbo DNA polymerase (Stratagene). The plasmid pCIN4, expressing a bicistronic mRNA encoding the gene of interest along with an enzyme conferring neomycin resistance (Rees *et al.*, 1996), was linearized with *EcoRV* and ligated to the p53-encoding region in the sense orientation. The plasmid pCINp53mt24–28 was made by site-directed mutagenesis using the plasmid pCINp53 and the same oligonucleotides as described previously (Roth *et al.*, 1998). All constructs were confirmed by sequencing.

**Luciferase assays.** For transfections,  $2 \times 10^5$  cells per assay were used. Luciferase activities were determined 24 h later using a pre-manufactured assay system (Promega) and an automated luminometer (Berthold).

**Immunoblots.** Proteins were separated by 10 % SDS-PAGE, transferred to nitrocellulose and then incubated with antibodies diluted in PBS containing 5 % milk powder and 0.1 % Tween 20. Detection was carried out by chemiluminescence (Pierce), using a peroxidase-coupled secondary antibody (Jackson). PAb1801, an antibody to p53 (Calbiochem), and another antibody against Lamin B (Zymed) were diluted 1:5000. Antibody 2A10 to Mdm2 was obtained from a hybridoma supernatant (gift from A. J. Levine) and diluted 1:50.

**PCR.** To determine the ratio of wild-type and mutant *p53* transcripts in a cell population, total RNA was prepared from these cells using Trizol (Life Technologies) and then treated with DNase. The RNA was reverse transcribed using the p53-specific primer 5' GGGAGCAGCCTCTGGCATTCTG and the RNA-dependent DNA polymerase Superscript II (Life Technologies). This cDNA was then used as a template for PCR amplification, using *Pfu* turbo DNA polymerase (Stratagene) and the primers 5' ATGGAGGAGCCGCAGTCAGATC and 5' GGGAGCAGCCTCTGGCATTCTG. The DNA obtained was then treated with the restriction enzyme *SacI*, which cut the PCR product originating from the mutant *p53* transcript but not that from the wild-type *p53* transcript.

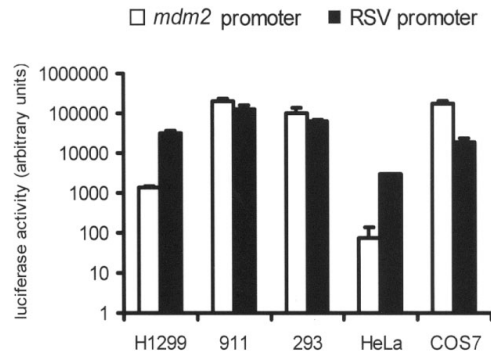
**Immunofluorescence.** Cells were seeded on plastic slides suitable for microscopy (Nunc) for 24 h, washed with PBS, fixed with paraformaldehyde (4 % in PBS, 15 min), permeabilized with Triton X-100 (0.2 % in PBS, 25 min), blocked (10 % FBS in PBS, 15 min) and incubated with antibody, as described previously (Dobbelstein *et al.*, 1992). To detect E1B 55K, the murine monoclonal antibody 2A6 (Sarnow *et al.*, 1982b) was used. The p53 protein was stained with a polyclonal rabbit antibody (FL-393, Santa Cruz). Primary mouse antibodies were visualized by secondary antibodies coupled to Alexa 488 (Molecular Probes). Primary rabbit antibodies were detected by an Alexa 594-labelled secondary antibody (Molecular Probes). Prior to mounting (Fluoroprep, bioMérieux), the cell nuclei were briefly stained using 4',6-diamidino-2-phenylindole (DAPI).

## Results

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## A p53-responsive promoter is active in Ad- and SV40-transformed cells

To assay for the transcriptional activity of p53 in Ad E1-transformed cells, HEK293 (Graham *et al.*, 1977) and HER911 (Fallaux *et al.*, 1996) cells were transfected with a reporter plasmid carrying the p53-responsive portion of the *mdm2* promoter, regulating the expression of luciferase (Freedman *et al.*, 1997). After incubation for 24 h, the activity of luciferase was determined. For comparison, parallel transfections were carried out with H1299 cells, which do not express endogenous p53 and do not contain Ad proteins either. In addition, HeLa and COS-7 cells were examined. These cells express wild-type p53 but also the HPV-18 E6 protein or the SV40 T antigen, respectively. In parallel, a reporter construct controlled by the Rous sarcoma virus (RSV) 3' long terminal repeat (LTR), a strong retrovirus promoter (Gorman *et al.*, 1982), was transfected. As shown in Fig. 1, the *mdm2* and the RSV promoter constructs yielded comparable reporter expression in Ad E1-transformed cells, whereas the *mdm2* promoter was much weaker in H1299 cells. *mdm2* promoter activity was also comparable to the RSV promoter in COS-7 cells but not in HeLa cells. This observation is compatible with the concept that constitutively active p53 is present in Ad- and SV40-transformed cells but not in HPV-transformed cells.



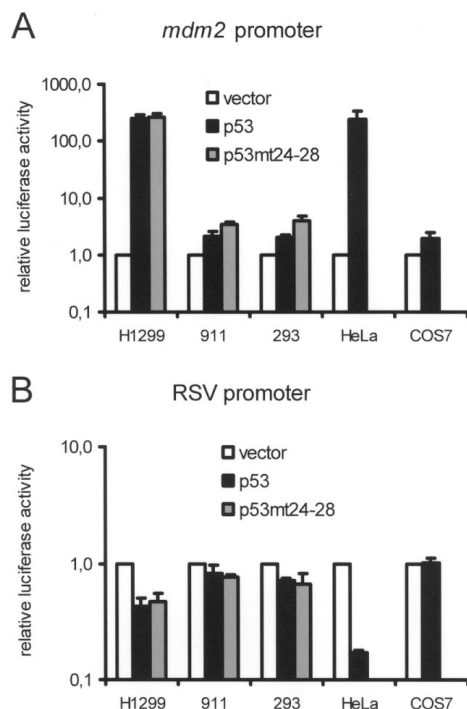
**Fig. 1.** Transcriptional activity of p53 in virus-transformed cells. H1299, HER911, HEK293, HeLa and COS-7 cells were each transfected with the reporter plasmid pGL2BP100, containing the p53-responsive portion of the *mdm2* promoter, or with pGL2RSV, containing the RSV 3' LTR. Samples of 100 ng of the reporter plasmids were transfected together with 2.2  $\mu$ g of a 'stuffer' plasmid (pcDNA3, Invitrogen). At 24 h after transfection, luciferase activity was measured in each case (arbitrary units as determined by the luminometer). The mean values of at least three

independent experiments are shown with the standard errors. Note that the luciferase activities are shown on a logarithmic scale in all diagrams.

## Exogenously expressed p53 or E1B 55K-resistant p53mt24–28 do not activate a p53-responsive promoter much beyond its baseline activity in Ad E1-transformed cells

Next, we tested whether the high baseline levels of p53 activity could be increased further by exogenous p53 in Ad E1- or SV40-transformed cells. H1299 (*p53*<sup>-/-</sup>), HEK293 and HER911 (Ad-transformed) cells, as well as HeLa (HPV-transformed) and COS-7 (SV40-transformed) cells, were transiently transfected with expression plasmids for p53, as well as a p53-responsive reporter plasmid. A luciferase assay was then conducted. In addition to wild-

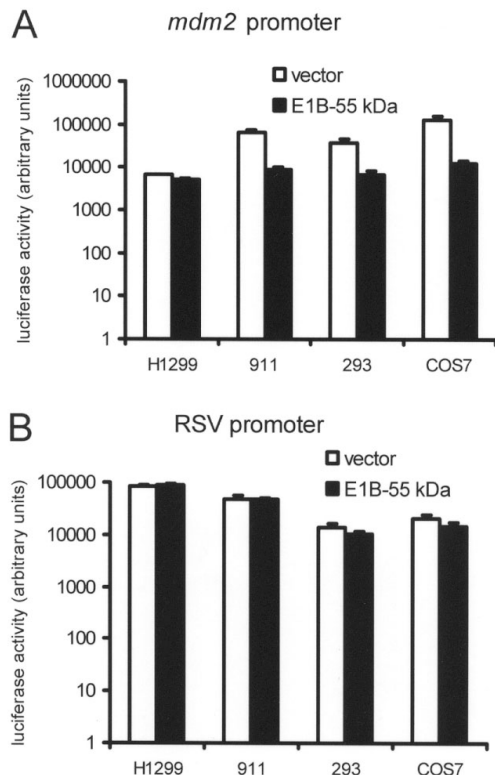
type p53, we also expressed a p53 mutant, p53mt24–28, in Ad E1-transformed cells and H1299 cells. This mutant is a chimera of p53 and the p53 homologue p73: five amino acid residues near the amino terminus of p53 (namely residues 24–28) were replaced with the corresponding residues of p73 to create the mutant p53mt24–28. The resulting chimera p53mt24–28 is transcriptionally as active, producing wild-type p53, but does not bind E1B 55K and is completely resistant to inhibition by E1B 55K (Koch *et al.*, 2001; Roth *et al.*, 1998). As shown in Fig. 2(A), p53 and p53mt24–28 each increased the activity of a p53-inducible promoter in Ad E1-transformed cells but even the E1B 55K-resistant mutant of p53 yielded a less than 5-fold increase in reporter activity. Similarly, exogenous p53 only weakly activated the *mdm2* promoter in COS-7 cells. In contrast, transient expression of p53 or p53mt24–28 activated the same promoter more than 200-fold in H1299 and HeLa cells. No comparable changes in activity were observed with the RSV promoter that was used as a control (Fig. 2B). Instead, this promoter was moderately repressed by p53 in H1299 and HeLa cells, as reported previously (Chen *et al.*, 1995; Shen & Shen, 1994), but not in Ad- or SV40-transformed cells, suggesting that, similar to *mdm2* promoter activation, RSV promoter suppression is not further enhanced by exogenously expressed p53 in Ad- or SV40-transformed cells. Taken together, these results argue that the transcriptional activities of p53 are already close to the achievable maximum in Ad E1- and SV40-transformed cells and are therefore not efficiently enhanced by exogenous p53.



**Fig. 2.** Transcriptional activation by exogenous p53 in virus-transformed cells. Cells of the indicated lines were transfected with the reporter plasmids pGL2BP100 (A) or pGL2RSV (B) (100 ng each), along with expression plasmids for p53 or p53mt24–28 (100 ng each) as indicated. In all experiments, the overall amount of plasmid was adjusted to 2.3  $\mu$ g with the empty vector plasmid pcDNA3. Luciferase activity was determined as described in the legend to Fig. 1 and the ratio of activity between p53 expression and vector control was calculated. The mean values of at least three independent experiments are shown with the standard errors.

### Transiently expressed E1B 55K inhibits p53 activity in Ad E1- and SV40-transformed cells

If a proportion of p53 molecules is active and not inhibited by endogenous E1B 55K in Ad E1-transformed cells, the transient overexpression of additional E1B 55K can be expected to reduce this activity. To test this, Ad E1- and SV40-transformed cells and H1299 cells were transiently transfected with an expression construct for E1B 55K, along with a p53-responsive reporter plasmid. HeLa cells were not analysed in this way because of their extremely low baseline p53 activity (Fig. 1). As shown in Fig. 3(A), exogenous E1B 55K reduced the activity of this reporter by about 10-fold in Ad- and SV40-transformed cells but not in H1299 cells. In control experiments, E1B 55K did not detectably influence the activity of the RSV promoter (Fig. 3B). We conclude that in Ad E1- or SV40-transformed cells, active p53 molecules that can be antagonized transiently by the expression of additional E1B 55K are present. Furthermore, since E1B 55K is an inhibitor of p53 but not of the p53 homologues p73 (Higashino *et al.*, 1998; Marin *et al.*, 1998; Roth *et al.*, 1998; Steegenga *et al.*, 1999; Wienzek *et al.*, 2000) or p63/p51 (Roth *et al.*, 1998; Wienzek *et al.*, 2000), it can be concluded that the majority of the p53-like transcriptional activity in Ad E1- or SV40-transformed cells is due to p53 itself rather than the p53 homologues.

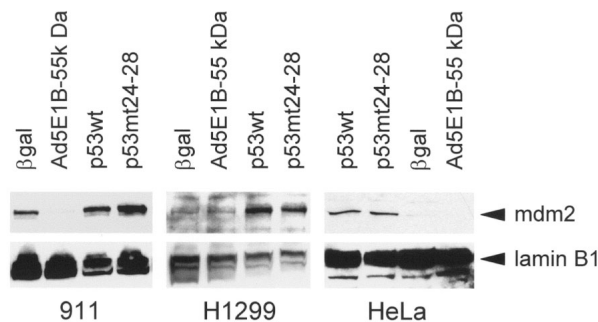


**Fig. 3.** Transiently expressed E1B 55K inactivates a p53-responsive promoter in Ad E1- and SV40-transformed cells. Virus-transformed and H1299 cells were transfected with the reporter plasmids pGL2BP100 (A) or pGL2RSV (B) (100 ng each), together with an expression plasmid for E1B 55K (pCGNE1B, 300 ng) (Dobbelstein *et al.*, 1997) as indicated. In all experiments, the total amount of plasmid DNA was adjusted to 2.3  $\mu$ g with the  $\beta$ -galactosidase expression plasmid pCGN $\beta$ gal (Roth & Dobbelstein, 1997). Luciferase activities were determined as described in the legend to Fig. 1.

**Levels of endogenous Mdm2 in virus-transformed cells and H1299 cells are consistent**

### with the expression of a p53-responsive reporter gene

The results of reporter assays are sometimes divergent from the regulation of cellular genes that are integrated into chromosomes. Therefore, we assessed the expression of a cellular p53-responsive gene, analysing the quantity of the endogenous Mdm2 protein by immunoblot. Cells with high transfection efficiencies were chosen for this experiment: HER911, H1299 and HeLa cells. These cells were transiently transfected to express p53, p53mt24–28 or E1B 55K. The expression of each protein was assessed by Western blot detection of Mdm2. The levels of Mdm2 were only moderately, if at all, upregulated by p53 and p53mt24–28 in Ad-transformed cells but clearly increased in H1299 and HeLa cells. In contrast, Mdm2 was downregulated by the transient overexpression of E1B 55K in HER911 cells (Fig. 4). Thus, the expression of the p53-responsive gene product Mdm2 exactly reflects the data obtained in reporter assays. Taken together, these results strongly suggest that a significant proportion of p53 molecules is not inhibited in Ad E1- and SV40-transformed cells, leading to continuous and strong transcriptional activity of p53.

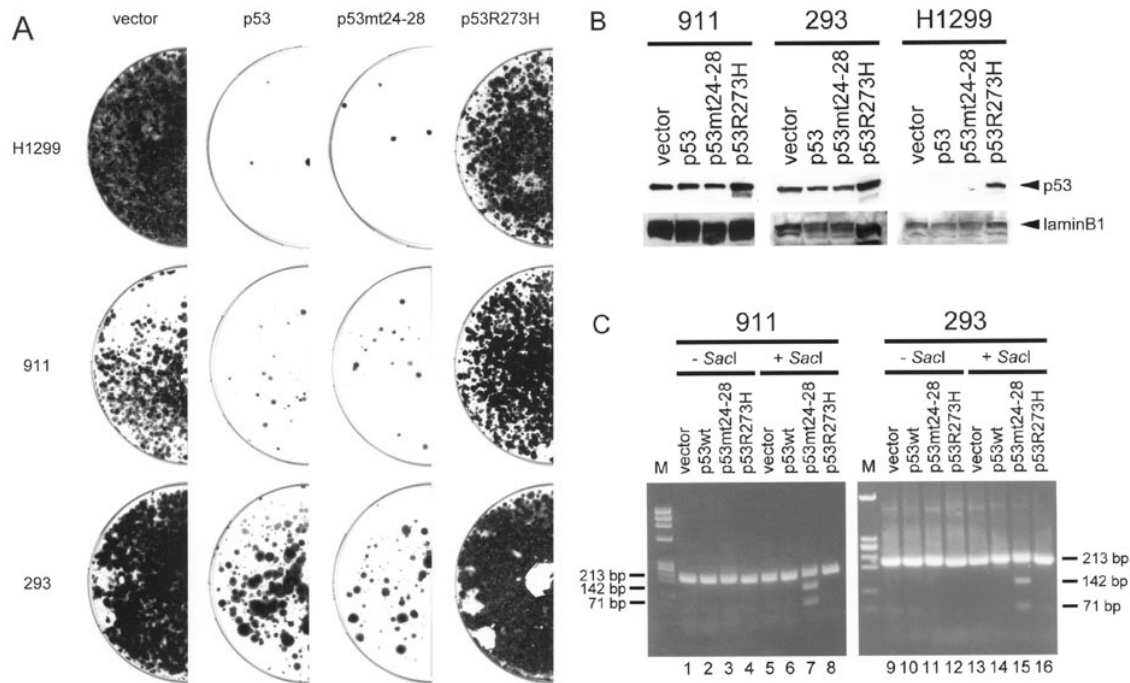


**Fig. 4.** Regulation of an endogenous p53-responsive gene in virus-transformed cells. HER911, H1299 and HeLa cells were transfected with expression plasmids for  $\beta$ -galactosidase, E1B 55K, p53 or p53mt24–28 (2.4  $\mu$ g each) as indicated. Subsequently, the levels of Mdm2 and Lamin B were determined by Western blot analysis.

### Ad E1-transformed cells tolerate the stable overexpression of the p53 mutant p53mt24–28, which is transcriptionally active but not inhibited by E1B 55K

The results described above suggest that p53 is constitutively active in Ad E1-transformed cells. Nonetheless, these cells grow rapidly and have a transformed phenotype, which is in contrast to the growth-inhibiting, death-inducing properties of p53. Therefore, we asked whether Ad E1-transformed cells might be tolerant for exogenous p53. To test this, expression plasmids for p53 or the E1B 55K-resistant p53mt24–28 were transfected into HER911 and HEK293 cells and, as a control, into H1299 cells; stable transfectants were selected using G418. To ensure tight coupling between the expression of p53 and the resistance gene, the two coding regions were combined on a bicistronic mRNA, separated only by an internal ribosomal entry site. This technology allows the selection of stable transfectants with a very low

background of cells that are drug-resistant but do not express the gene of interest (Rees *et al.*, 1996). Colony formation was compared between expression plasmids for p53 or p53mt24–28, the empty vector pCIN4 and an expression plasmid for the tumour-derived mutant p53R273H, which is not capable of suppressing cell growth. In all cells, p53 and p53mt24–28 reduced colony formation. However, colony suppression was less extensive in HER911 and HEK293 cells, whereas the difference between p53 or p53mt24–28 expression plasmids and the vector control was more pronounced in H1299 cells (Fig. 5A). The selected cells (pools of colonies in each case) were then probed for p53 expression by Western blot analysis (Fig. 5B). The selected H1299 cells expressed p53R273H but did not express any detectable wild-type p53 or p53mt24–28, indicating that the latter cell species belonged to the rare escape clones that became resistant to G418 without expressing p53. The levels of p53 in HER911 and HEK293 cells were largely unchanged by the stable transfection. This argues that clones expressing p53 at high levels were selected against. However, given the high baseline levels of endogenous p53 protein in these cells (Zantema *et al.*, 1985b), it remained possible that the exogenous p53mt24–28 was expressed in the selected clones without detectably increasing the overall amount of p53 protein. To establish whether p53mt24–28 was expressed in these cells, RNA was isolated and reverse transcribed. Then, the portion of the p53-encoding region that is distinct between p53 and p53mt24–28 was amplified by PCR. The PCR products derived from p53 or p53mt24–28 were distinguished by restriction digestion of the PCR product. As shown in Fig. 5(C, lanes 7 and 15), roughly 50 % of the p53 mRNA that originated from the stably transfected HER911 and HEK293 cells was derived from p53mt24–28, arguing that large amounts of E1B 55K-resistant p53mt24–28 are expressed and can be tolerated by these cells.

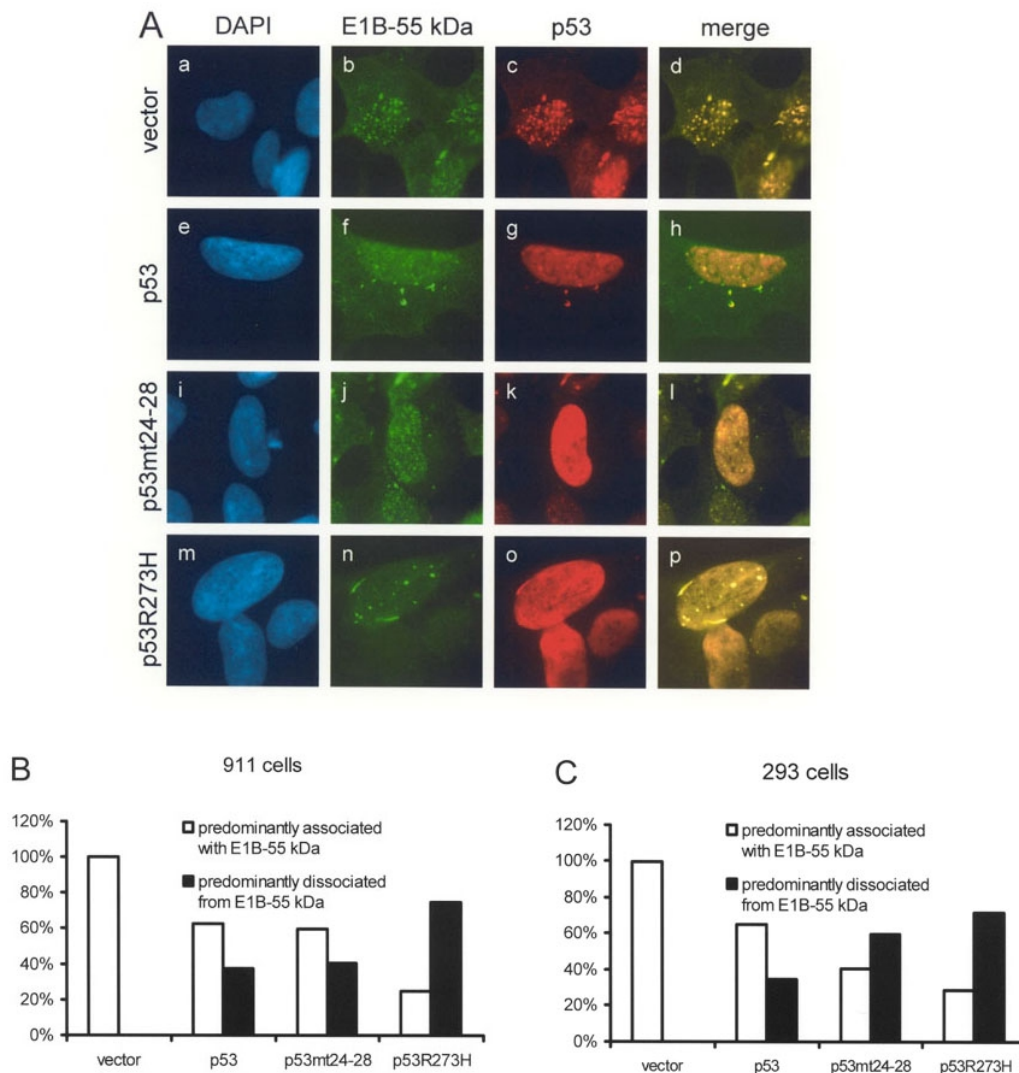


**Fig. 5.** Stable expression of active p53 in Ad E1-transformed cells. (A) The cell lines HER911, HEK293 and H1299 were transfected with 2.4  $\mu$ g of pCIN4-based expression plasmids that encode a bicistronic mRNA comprising the p53-encoding region, an internal ribosomal entry site and a neomycin resistance gene. Transfections were carried out either with the empty vector construct or with plasmids encoding p53, p53mt24–28 or p53R273H. Colonies were selected by addition of G418 to the cell culture media. After removing the cells from one-half of the dish for further analysis (see below), the remaining colonies were stained with crystal violet. (B) The selected cells were pooled and equal amounts of cellular proteins were analysed by SDS–PAGE and immunoblot using antibodies against p53 (upper panel) or Lamin B (lower panel, loading control). (C) RT–PCR assay to distinguish endogenous p53 from exogenously expressed p53mt24–28. Total RNA was prepared from the stable transfectants of HER911 and HEK293 cells described in (A). The mRNA portion encoding the amino-terminal part of p53 was reverse transcribed and amplified by PCR. PCR products were treated with the restriction enzyme *SacI*, which cuts only the DNA derived from the coding region of p53mt24–28. Fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

To test whether p53 was retained by E1B 55K in the perinuclear clusters or released into its normal diffusely nuclear localization, p53 was immunostained in the G418-selected cells. The association of p53 and E1B 55K in clusters, which is typical for Ad5 E1B 55K-transformed cells, was observed in vector-transfected HER911 (Fig. 6A, panels a–d) and HEK293 cells (data not shown). In cells stably transfected to express p53, the amount of nuclear p53 was increased (Fig. 6A, panels e–h). Stable expression of p53mt24–28 further enhanced nuclear p53 staining (Fig. 6A, panels i–l), as did the expression of p53R273H (Fig. 6A, panels m–p). Statistical analysis confirmed these observations in HER911 and HEK293 cells (Fig. 6B, C). Thus, a substantial proportion of exogenously expressed p53 does not associate with E1B 55K in Ad E1-

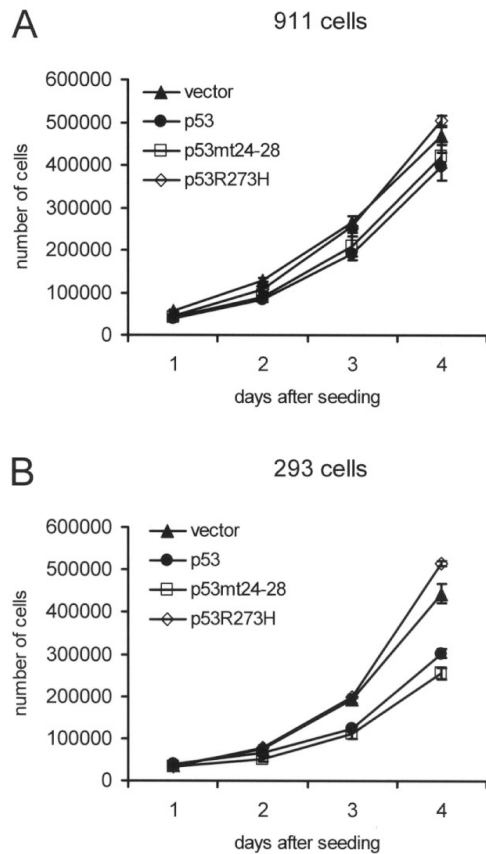
transformed cells.

Finally, we analysed the growth characteristics of the stably transfected cells. HER911 cells grew at the same rate regardless of the stable expression of p53 or its mutants (Fig. 7A) and HEK 293 cells were growth-inhibited by less than 2-fold in the presence of p53 or p53mt24–28, as compared with vector-transfected cells (Fig. 7B). Thus, Ad E1-transformed cells can tolerate the continuous expression of active p53. To our knowledge, this is the first example of cells that grow despite the stable overexpression of transcriptionally active p53.



**Fig. 6.** Intracellular location of p53 in stable transfectants. (A) HER911 cells that were stably transfected to express the resistance gene alone (vector), p53, p53mt24–28 or p53R273H were fixed and stained with antibodies to E1B 55K (green) and p53 (red). The nuclei were counterstained with DAPI (blue). Colocalization is shown by merging the p53 and E1B 55K-staining patterns (yellow). Statistical analysis of these results was performed for HER911 (B) and HEK293 (C) cells. The cells of the experiment shown in (A) were categorized with respect to the p53-staining pattern. The categories were either ‘predominantly within the perinuclear clusters associated with the E1B 55K protein’ or ‘predominantly dissociated from E1B 55K’. The evaluation was carried out by visual inspection of at least

100 stained cells per experiment by a person who was unaware of the identity of the samples. The percentage of cells in each category is indicated by columns.

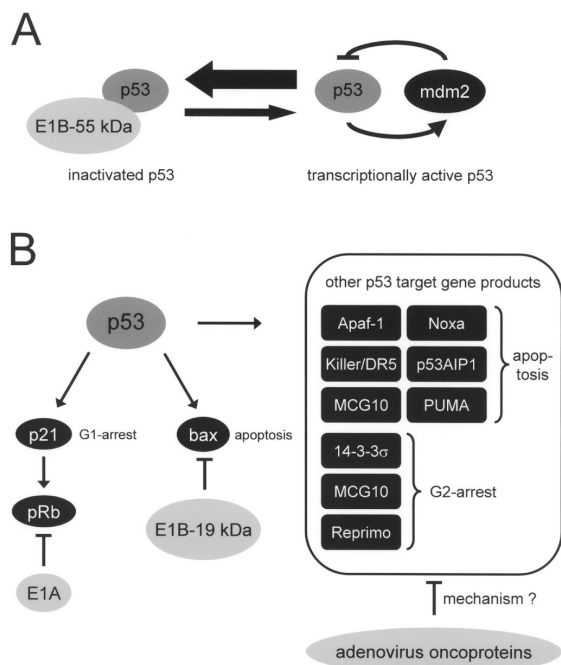


**Fig. 7.** Growth of Ad E1-transformed cells stably overexpressing p53. The indicated stable transfectants of HER911 and HEK293 cells were seeded in quadruplicate on 6-well dishes at day 0. At 4 consecutive days after plating, the cells were trypsinized from one well and counted in a Neubauer chamber. The results of four independent experiments are shown with the standard error.

## Discussion

Our results strongly suggest that p53 is constitutively active in cells transformed with the Ad E1 region or with the SV40 T antigen. Among the virus p53 antagonists, only HPV E6 proteins, which not only form a complex with but also destabilize p53, appear to continuously eliminate p53 activity. This is in contrast with the previous concept that the contribution of Ad E1B 55K to malignant growth consists in the continuous inactivation of p53 (Grand *et al.*, 1995; Hutton *et al.*, 2000) and that the SV40 T antigen contributes to tumorigenesis, at least in part, by permanently blocking p53 (Pipas & Levine, 2001). They are, however, in agreement with recent studies showing that an SV40 T antigen mutant unable to bind p53 was nonetheless capable of relieving contact inhibition, a hallmark of transformation (Sachsenmeier & Pipas, 2001). We propose that, in Ad E1- and SV40-transformed cells, an equilibrium exists between p53 bound to oncoproteins and free, transcriptionally active p53 (Fig. 8A). Such an equilibrium is reached because p53, when bound to Ad E1B 55K or SV40 T antigen, is not degraded but stabilized. As a consequence, p53 accumulates and saturates the viral oncoprotein, leaving some

non-bound p53 molecules. This free form of p53 is limited by the regulatory feedback loop between p53 and Mdm2. According to this model, the inactivation of p53 by E1B 55K or the T antigen can only be transient. In the long run, an equilibrium is reached when E1B 55K or the T antigen become saturated by accumulating p53. In agreement, the transient overexpression of E1B 55K blocked p53 activity, whereas the constant presence of E1B 55K in Ad-transformed cells did not (Figs 1–4).



**Fig. 8.** Model describing the role of p53 in Ad E1-transformed cells. (A) Regulation of p53 in Ad E1-transformed cells. In these cells, p53 is bound and inactivated by E1B 55K. We hypothesize that, as a result of p53 stabilization, the p53 protein accumulates until the binding sites on E1B 55K are saturated. Then, a fraction of p53 becomes active again, inducing *mdm2* and thereby limiting further accumulation of p53 through Mdm2-mediated degradation. The net result consists in an equilibrium comprising a proportion of active p53 molecules. (B) p53 target genes and their regulation by Ad E1 gene products. Since Ad E1-transformed cells contain and tolerate active p53, it is proposed that p53 target gene products are inactivated, directly or indirectly, by E1 gene products. Known examples for this

include the direct inactivation of Bax by Ad E1B 19K or the indirect impairment of p21<sup>WAF1/CIP1</sup> function by complex formation of the retinoblastoma protein pRb and Ad E1A proteins. The latter mechanism overcomes cell growth arrest, which would otherwise occur as a result of cyclin-dependent kinase inhibition by p21 and underphosphorylation of pRb. We hypothesize that, in cells transformed by the Ad E1 region, the activities of other p53 target gene products, at least as far as they induce cell death or prevent cell growth, are also inhibited by Ad E1 proteins through as yet unknown mechanisms.

These observations argue that recent reports about the creation of human tumour cells with defined genetic elements (Elenbaas *et al.*, 2001; Hahn *et al.*, 1999) may require re-interpretation. It was found that the catalytic subunit of telomerase hTERT, in combination with oncogenic H-ras and SV40 T antigen, is able to convert normal human cells into tumourigenic cells. Furthermore, it was suggested that the T antigen contributes to this transformation process through the perturbation of p53 and pRb functions (Hahn *et al.*, 1999; Weitzman & Yaniv, 1999). In contrast, our results argue that, at least in the Ad E1- and SV40-transformed cells under study, p53 is not permanently inactivated. This raises the possibility that transient

but not permanent inactivation of p53 is compatible with the malignant transformation of human cells. However, it should be taken into consideration that the T antigen is a highly multifunctional protein that acts on cellular growth regulation by various known and unknown pathways (Ali & DeCaprio, 2001). Investigating these pathways further can be expected to clarify the requirements for the transformation of human cells.

If p53 binding by E1B 55K is dispensable for the growth of Ad E1-transformed cells, what is the role of E1B 55K in transformation? One possible explanation is that p53 binding may be required only during the initial steps of cell transformation, as described by a 'hit-and-run' scenario. However, this would not explain the maintenance of E1B 55K expression in the majority of E1-transformed cells. We therefore favour the hypothesis that E1B 55K contributes to malignant transformation by mechanisms in addition to p53 binding. Even though mutational analysis revealed a close association between p53 binding and transformation by E1B 55K (Yew & Berk, 1992), it should be considered that most mutants of E1B 55K have lost several functions at a time (Kao *et al.*, 1990; Rubenwolf *et al.*, 1997; Yew & Berk, 1992; Yew *et al.*, 1990), presumably due to a labile conformation of the protein, which is easily disrupted even by small mutations. Therefore, mutational analysis cannot exclude the presence of at least one more, as yet unknown, biochemical activity of E1B 55K contributing to malignant transformation. A specific E1B 55K mutant that selectively abolishes p53 binding recently became available (Shen *et al.*, 2001). Testing this mutant in transformation assays may clarify whether E1B 55K contributes to transformation by mechanisms independent of p53 binding.

Although Ad E1-transformed cells can apparently tolerate p53, this tolerance is not without limitations, as is evident from the considerable colony suppression by p53mt24–28 (Fig. 5A). Also it is clear that some, but not all, p53 molecules in these cells are inactivated by association with E1B 55K. This can explain the fact that the addition of a p53-derived peptide to Ad E1-transformed cells, and the consecutive massive activation of p53, led to a cell-cycle arrest (Hutton *et al.*, 2000). Nonetheless, it remains to be stated that Ad E1-transformed cells grew rapidly despite a considerable level of exogenous nuclear p53, whereas *p53*<sup>-/-</sup> cells did not.

To our knowledge, this is the first example of cell lines that grow despite the stable overexpression of active p53 from the cytomegalovirus major immediate early promoter – one of the strongest known promoters (Boshart *et al.*, 1985). This tolerance for active p53 suggests that p53 target gene products, in addition to p53 itself, are inactivated, directly or indirectly, in Ad E1-transformed cells. For some p53 targets, this was indeed found to be the case (Fig. 8B). p53 induces the p21<sup>CIP1/WAF1</sup> gene product (el-Deiry *et al.*, 1993), which inhibits the phosphorylation of the retinoblastoma protein pRb and thereby the transition from the G<sub>1</sub> to the

S phase of the cell cycle (Bartek & Lukas, 2001). However, the Ad E1A gene products bind and inactivate pRb (Whyte *et al.*, 1988), thereby antagonizing p21. Likewise, p53 induces the expression of Bax, which induces apoptosis (Miyashita & Reed, 1995). On the other hand, the E1B 19K protein interacts with Bax and inhibits cell death (Chen *et al.*, 1996; Han *et al.*, 1996). The induction of apoptosis by p53-induced Fas CD95 (Muller *et al.*, 1998) may be prevented by E1B 19K as well (Hashimoto *et al.*, 1991; Perez & White, 1998). However, many more p53 target gene products were shown to block cell growth. These gene products include inhibitors of G<sub>2</sub>-M transition, such as 14-3-3 $\sigma$  (Hermeking *et al.*, 1997), MCG10 (Zhu & Chen, 2000) and Reprimo (Ohki *et al.*, 2000), as well as inducers of apoptosis, such as Apaf-1 (Kannan *et al.*, 2001; Moroni *et al.*, 2001), Killer/DR5 (Wu *et al.*, 1997), MCG10 (Zhu & Chen, 2000), Noxa (Oda *et al.*, 2000a), p53AIP1 (Oda *et al.*, 2000b), PUMA (Nakano & Vousden, 2001) and many others (Vogelstein *et al.*, 2000; Vousden, 2000). If Ad E1-transformed cells grow despite ongoing p53 activity, this could be explained in three ways. Firstly, the expression of most p53 target genes does not inhibit cell growth, which would be in contradiction with their previous functional analysis in many cases. Secondly, the p53 target genes might be mutated in Ad E1-transformed cells. However, it appears unlikely that such a large number of mutations should have occurred during the process of *in vitro* transformation. Therefore, we favour the third possibility: at least a growth-limiting subset of the p53 target gene products may be regulated, by as yet unknown mechanisms, through the action of Ad E1 proteins at levels downstream from p53 itself. This hypothesis implies that Ad E1 proteins may promote cell growth by a considerably wider variety of mechanisms than previously anticipated.

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