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Adenovirus E1A Proteins Inhibit Activation of Transcription by p53

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p53 stimulates the transcription of a number of genes, such as *MDM2*, *Waf1*, and *GADD45*. We and others have shown previously that this activity of p53 can be inhibited by adenovirus type 2 or 12 large E1B proteins. Here we show that the adenovirus E1A proteins also can repress the stimulation of transcription by p53, both in transient transfections and in stably transfected cell lines. The inhibition by E1A occurs without a significant effect on the DNA-binding capacity of p53. Furthermore, the activity of a fusion protein containing the N-terminal part of p53 linked to the GAL4 DNA-binding domain can be suppressed by E1A. This indicates that E1A affects the transcription activation domain of p53, although tryptic phosphopeptide mapping revealed that the level of phosphorylation of this domain does not change significantly in E1A-expressing cell lines. Gel filtration studies, however, showed p53 to be present in complexes of increased molecular weight as a result of E1A expression. Apparently, E1A can cause increased homo- or hetero-oligomerization of p53, which might result in the inactivation of the transcription activation domain of p53. Additionally, we found that transfectants stably expressing E1A have lost the ability to arrest in G_1 after DNA damage, indicating that E1A can abolish the normal biological function of p53.

Cell proliferation is a tightly regulated process in which the p53 tumor suppressor protein plays an important role. A possible mechanism by which p53 can, at least partially, fulfill its tumor suppressor function is by regulating the expression of a set of target genes. p53 is known to be able to down-regulate the expression of a number of genes (15, 33, 46, 53), probably by interacting with the basal transcription machinery (1, 41, 48). On the other hand, p53 has also been found to activate the transcription of genes containing a p53-responsive element (12, 43, 57). The list of p53-inducible genes at the moment features genes like *Waf1* (11), *MDM2* (2, 64), *GADD45* (19), the cyclin G gene (38, 67), *EGFR* (7), and *Bax* (36, 47), but probably many more p53-responsive genes will be identified in the near future. Most naturally occurring p53 mutants have lost their normal transcription-regulatory functions (20). In addition, a gain of function has been shown for certain p53 mutants (9, 24).

We have used the adenovirus transformation system to obtain more insight into the mechanism of regulation of transcription by p53 and to answer the question whether structural changes of p53 play a role in these modulations. It has been shown that the two major adenovirus E1B proteins have different effects on the transcription-regulatory functions of p53. The small E1B protein can inhibit only the transcription-inhibitory function of p53 (44, 49, 51), whereas the large E1B protein can inhibit the stimulation (66) as well as the repression of transcription by p53 (51). We have previously shown that in adenovirus type 12 (Ad12)-transformed cells, p53 is present in complexes of increased molecular weight compared with those in parental cells. We also found that Ad12-transformed cells or cells stably transfected with Ad5 E1A together with Ad12 E1B exhibit increased phosphorylation of the transcription activation domain of p53. This effect could not be observed in stable transfectants exclusively expressing either the small or the large E1B protein (50), suggesting that E1A is responsible for this effect.

The E1A proteins are known to affect p53 properties in a number of ways. In the presence of wild-type p53, the adenovirus E1A proteins can induce apoptotic cell death (8). Furthermore, E1A can cause stabilization of the p53 protein (26, 27). In addition, it has recently been found that the 13S splice variant of Ad5 E1A can prevent transcription inhibition by p53 (17). Stabilization of the p53 protein and p53-mediated apoptosis (28) have also been observed in response to DNA damage. The increased levels of p53 as a result of DNA damage cause increased expression of p53-inducible genes like *Waf1* and *GADD45*, which in turn results in G_1 cell cycle arrest (19, 21). Although stabilization of the p53 protein and p53-mediated apoptosis can occur as a result of DNA damage as well as by coexpression of E1A, the activation of transcription by p53 is not affected in the same way. Our results show that E1A has an inhibitory effect on transcription activation by p53, in contrast to DNA damage, which has been shown to cause an increase in the transcriptional activity of p53. We found that E1A does not affect the DNA-binding capacity of p53 but suppresses the transcription activation domain. This effect is not caused by a significant change in the phosphorylation of the transcription activation domain but might be the consequence of increased complex formation of p53 in stable cell lines expressing adenovirus E1A.

MATERIALS AND METHODS

Tissue culture and cell lines. The cell lines Saos-2 (31) and Hep3B (40) were grown in Dulbecco's modified Eagle's medium plus 8% fetal calf serum. The rhabdoid kidney tumor cell line G401 subclone G401.6TG.C6 (62) was grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and hypoxanthine (15 μg/ml)-thymidine (10 μg/ml). The stable transfectants G401-512RC3, G401-21C1, and G401-21C8 have been described previously (51). The G401 cell lines expressing Ad5 E1A and E1B (21 kDa) were obtained by cotransfection of pRSV-5E1A and pCMV21K (50) or by transfection of p5XT2.1, a genomic Ad5 E1 clone containing a mutation in the E1B gene such that only the 21-kDa E1B protein can be expressed (18). G401-5/54C2 and G401-5/54C10 were obtained by

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cotransfection of pRSV-5E1A and pCMV-54K (pCMV-12/54kDa [58]). All stable transfectants were cultured in the same medium as was the parental G401 cell line with the addition of G418 (300 μg/ml).

Plasmids, transient transfections, and luciferase assays. Saos-2 and Hep3B cells were transiently transfected by the calcium-phosphate precipitation method (59) on 30-mm-diameter petri dishes. A total volume of 500 μl of precipitate was made; 200 μl was added to cells. In the investigation of the effects of Ad5 and Ad12 E1A proteins on the activation of transcription by p53, each precipitate contained 2.5 μg of pOLXALuc reporter construct (51) and 0.001 μg of pCMV-neo or 0.001 μg of pCMV-p53. Cotransfections were performed with or without 0.5 or 0.05 μg of pRSV-5E1A or pRSV-12E1A. With the addition of pRSV-neo, the total amount of RSV-containing plasmid was adjusted to 0.5 μg in all precipitates.

To analyze the inhibitory effect on transcription stimulation in more detail, 2.5 μg of the mGAL4-luc construct was cotransfected with 1.0 μg of either mGAL4, mGAL4$_D$-p53(1-393), mGAL4$_D$-p53(1-73), or mGAL4$_D$-VP16 (12) in each precipitate. Cotransfections were performed with or without 0.5 or 0.05 μg of pRSV-5E1A, pRSV-13SE1A, pRSV-12SE1A, or pRSV-12E1A (37). With the addition of pRSV-neo, the total amount of RSV-containing plasmid was adjusted to 0.5 μg in all precipitates. Salmon sperm DNA was added as carrier DNA, and a total amount of 10 μg of DNA per 500-μl precipitate, of which 200 μl was added to cells, was used. The precipitates were made in triplicate, and the error bars (see Fig. 1 and 2) represent the fluctuations between precipitates. Exponentially growing cells were transfected overnight. The next morning, cells were washed twice with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9)-buffered saline, and 24 to 28 h later, lysates were made in cell culture lysis reagent (Promega, Madison, Wis.). After a 15-min incubation at room temperature, cell debris was removed by brief centrifugation. The protein concentrations of lysates were determined by the Bradford (Bio-Rad) assay. The luciferase activity in 10 μg of Saos-2 or Hep3B lysate in a 20-μl sample of lysate was determined by adding 100 μl of luciferase assay reagent (Promega). The produced light was measured for 10 s in a luminometer (Lumat LB9501; Berthold).

Nuclear extracts and electromobility shift assay. Exponentially growing cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested with a rubber policeman at 4°C. Cell pellets were resuspended in hypotonic buffer (10 mM Tris-HCl [pH 7.4], 3 mM $MgCl_2$, 10 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM trypsin inhibitor, 1 μg of pepstatin A per ml, 0.05 mM Microcystin-LR, 2 mM dithiothreitol [DTT]) and incubated for 10 min on ice. Nonidet P-40 (NP-40) (0.5%) was added, and nuclei were isolated by centrifugation, resuspended in 1 volume of lysis buffer I (20 mM HEPES [pH 7.9], 600 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM trypsin inhibitor, 0.05 mM Microcystin-LR, 2 mM DTT), and incubated on ice for 30 min. After centrifugation at 13,000 rpm in an Eppendorf centrifuge, the supernatant was subsequently diluted with 1 volume of lysis buffer II (lysis buffer I containing 20% glycerol instead of KCl). The p53 consensus present in the *Waf1* promoter was used as a probe (11). The probe was made by the annealing of oligonucleotides 5′-GAACATGTCCCAACATGTT-3′ (Isogen) and 5′-AACATGTTGGGA CATGTTC-3′ (Isogen), followed by labeling with [γ-^{32}P]dATP using T4 polynucleotide kinase (Biolabs). For the parental G401 cell line and 21C1 transfectants, 10 μg of nuclear protein was used, and for 5/21C3 and 5/21C24, 30 μg was used. Samples were preincubated for 10 min with 10 μl of 2× DNA-binding buffer (40% glycerol, 25 mM HEPES [pH 7.6], 50 mM KCl, 0.1% Triton X-100, 2 mM DTT), and for some of the samples (see the legend to Fig. 3B), 1 μl of monoclonal antibody PAb 421 and competitor DNA (250-fold excess of unlabeled probe) was added. After preincubation, 5 fmol of labeled oligonucleotide was added to each reaction mixture. Samples were incubated for 30 min at room temperature and subsequently loaded on a 5% polyacrylamide Tris-glycine gel. The gel was run for 3 h at 200 V and subsequently exposed to Kodak XAR-5 film at −80°C.

Phosphate labeling and immunoprecipitation. Exponentially growing cells were labeled for 20 h in normal tissue culture medium (Dulbecco's modified Eagle's medium plus 10% fetal calf serum and hypoxanthine [15 μg/ml]-thymidine [10 μg/ml]) with $^{32}P_i$ at a specific activity of 2 mCi/ml. After being labeled, cells were washed twice with ice-cold PBS and cell lysates were prepared in IPB 0.7 buffer (20 mM triethanolamine HCl [TEA; pH 7.8], 0.7 M NaCl, 0.5% NP-40, 0.2% deoxycholate, 0.5 mM PMSF, 0.5 mM trypsin inhibitor, 1 μg of pepstatin A per ml, 0.05 mM microcystin-LR, 1 mM Na_3VO_4, 1 mM NaF). Lysates were cleared by centrifugation at 13,000 rpm in an Eppendorf centrifuge for 15 min, and subsequent immunoprecipitations were performed with a mixture of PAb 122 and PAb 1801. Immunoprecipitates were washed three times with IPB 0.7 and once with PBS, resuspended in sample buffer (62.5 mM Tris [pH 8.6], 10% glycerol, 3% sodium dodecyl sulfate [SDS]), boiled for 5 min, and separated on SDS–10% polyacrylamide gels.

Two-dimensional tryptic phosphopeptide mapping. ^{32}P-labeled p53 was separated on an SDS–10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was exposed to X-ray film, and the p53-specific band was excised from the membrane and subjected to trypsin digestion as described by Boyle and coworkers (3). The generated peptides were separated on cellulose thin-layer plates at pH 1.9 for 40 min at 1 kV in the first dimension, with subsequent ascending chromatography in the second dimension by the method of Boyle et al. (3). The peptide maps were exposed to Kodak XAR-5 film at −80°C and to a PhosphorImager screen, which was analyzed by B&L Systems Molecular Dynamics software.

Gel filtrations. Exponentially growing cells were lysed in E1A buffer as modified by Giordano (50 mM Tris-HCl [pH 7.4], 0.25 M NaCl, 0.1% Triton X-100, 5 mM EDTA, 0.5 mM PMSF, 0.5 mM trypsin inhibitor, 0.05 mM microcystin-LR, 1 μg of pepstatin A per ml, 2 mM DTT). Then 0.2 ml of lysates was loaded on a Superose-6B HR 10/30 fast protein liquid chromatography column (Pharmacia) and eluted in Giordano buffer. Fractions of 0.3 ml were collected, and 60 μl of each fraction was separated on an SDS–10% polyacrylamide gel and blotted onto a polyvinylidene difluoride microporous membrane (Immobilon-PVDF; Millipore). The amount of p53 was detected by Western blotting (immunoblotting).

Western blotting. DO-1 and anti-p21 (SanverTECH) were used as first antibodies to detect p53 and Waf1, respectively. Monoclonal antibodies C1G11, M73, and 8A9 were used to detect E1B (21 kDa), Ad5 E1A, and Ad12 E1B (54 kDa), respectively. Anti-MDM2 (A2C10) was a kind gift from A. Levine (Department of Molecular Biology, Princeton University, Princeton, N.J.). After incubation with the first antibody, blots were subsequently incubated with horseradish peroxidase-conjugated anti-rat or anti-mouse antibodies (SanverTECH), after which bands were visualized by chemiluminiscence (Luminol; Sigma).

Northern (RNA) blot analysis. Exponentially growing cells were mock or X-ray irradiated (5 Gy), and RNAs were isolated at 2 and 5 h after irradiation. For RNA isolation, cells were harvested by trypsinization, washed twice with ice-cold PBS, and resuspended in 250 μl of STE (150 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 7.8]). After the addition of 20 μl of 10% NP-40, cells were lysed on ice for 2 min and centrifuged. Extraction buffer (250 μl; 350 mM NaCl, 20 mM Tris, 20 mM EDTA, 1% SDS [pH 7.8]) was added to the supernatant. After phenol-chloroform and chloroform-isoamyl alcohol extraction, RNA was ethanol precipitated. Twenty micrograms of total RNA was used for the Northern blotting procedure (45). To test for the expression of Waf1, a [^{32}P]dCTP-labeled *Not*I fragment from plasmid pCEP-WAF1 (a gift from B. Vogelstein, The Johns Hopkins University School of Medicine, Baltimore, Md.) was used. As a control, a [^{32}P]dCTP-labeled probe derived from rat glyceraldehyde-3-phosphate dehydrogenase cDNA was used. Northern blots were exposed to Kodak XAR-5 film at −80°C.

Cell cycle analysis. Exponentially growing cells were irradiated with X rays (5 Gy). Fifteen hours after treatment, unirradiated and irradiated cells were labeled for 2 h with 20 μM 5-bromo-2′-deoxyuridine (BrdU). Cells were subsequently washed twice with PBS and harvested by trypsinization. Cells were washed again with PBS and fixed in 70% ethanol for at least 30 min at −20°C. Subsequently, cells were centrifuged and the pellet was resuspended in 0.1 M HCl–0.5% Triton X-100. After a 10-min incubation on ice, cells were washed with distilled water, resuspended in 2 ml of distilled water, boiled for 10 min, and chilled on ice. After centrifugation, cells were resuspended in 300 μl of PBS. Then 400 μl of a solution containing 3.4 mM trisodium citrate, 0.5 mM Tris, 0.1% NP-40, 3 mM spermine tetrahydrochloride, 50 μg of RNase A per ml, and 200 μg of propidium iodide per ml was added and cells were incubated for at least 30 min. Cells (10,000) were analyzed on a FACScan (Becton Dickinson) flow cytometer for DNA content and BrdU incorporation. Dot plots were prepared by using the lysis II program (version 1.1).

RESULTS

Ad5 and Ad12 E1A proteins inhibit transcription stimulation by p53. To examine whether the adenovirus E1A proteins have an effect on transcription activation by p53, we performed cotransfection experiments in p53-negative Hep3B cells. The results presented in Fig. 1 show that wild-type p53 stimulates the expression of the pOLXALuc reporter construct containing the p53 consensus sequence, as optimized by Halazonetis and coworkers (16), in front of the luciferase gene. However, cotransfection of increasing concentrations of either Ad5 E1A (Fig. 1A) or Ad12 E1A (Fig. 1B) inhibits transcription stimulation by p53. The same results were obtained when transfections were performed in p53-negative Saos-2 cells (data not shown).

Adenovirus E1A proteins inhibit the transcription activation domain of p53. Previous studies have shown that the transcription activation domain of p53 is located in the N-terminal part of the protein (12, 43). When fused to the GAL4 DNA-binding domain, the first 73 amino acids of the p53 protein are as powerful in activating transcription as the strong herpes simplex virus transcription activator VP16 (12). We used mGAL4$_D$-p53 fusion constructs to analyze which part of the p53 protein is the target domain for the repressive effect of the E1A proteins. As can be seen in Fig. 2A, cotransfection of

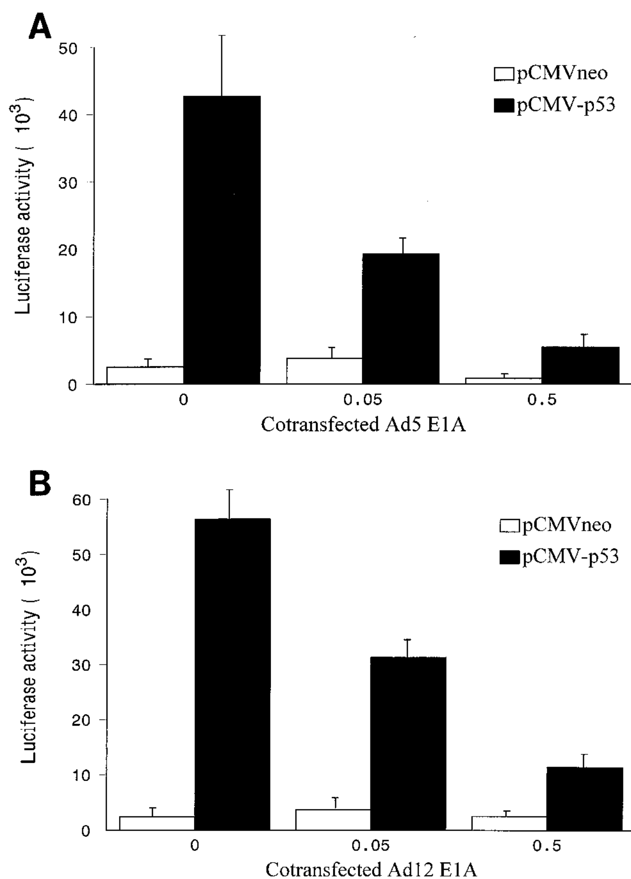

FIG. 1. Ad5 and Ad12 E1A proteins inhibit the activation of transcription by p53. pCMV-p53 was cotransfected with a luciferase reporter construct containing the p53 consensus sequence into p53-negative Hep3B cells. Cotransfection of increasing concentrations of either Ad5 E1A (A) or Ad12 E1A (B) resulted in inhibition of the transcription activation potential of p53. Transfections and luciferase assays were performed as described in Materials and Methods. The precipitates for transfection were made in triplicate, and error bars represent the fluctuations between the results of three precipitates.

Ad5 E1A in Saos-2 cells has no effect on transcription activation by the yeast transcription factor mGAL4. However, when the GAL4 transactivation domain was replaced by the complete p53 coding region, we found repression of transcription activation by cotransfection of increasing amounts of Ad5 E1A. We also observed repression of transcription stimulation when the $mGAL4_D$-p53(1-73) construct was used, indicating that the N-terminal part of p53, which contains the transcription activation domain, is the target domain for inhibition by Ad5 E1A. Recently, Horikoshi and coworkers (17) reported that only the 13S, not the 12S, splice variant of Ad5 E1A suppresses transcription inhibition by p53. We wondered whether the two splice variants of Ad5 E1A also have different effects on transcription activation by p53. Therefore, we cotransfected the three mGAL4 constructs mentioned above with increasing concentrations of either 13S or 12S Ad5 E1A. As can be seen in Fig. 2B and C, both 13S and 12S E1A proteins can repress transcription stimulation by both intact p53 and the truncated N-terminal transcription activation domain. No significant effect could be observed by cotransfecting each of the E1A splice variants with the mGAL4 construct. These observations indicate that the mechanisms by which E1A inhibits p53-mediated stimulation of transcription and p53-mediated repression of transcription are different.

The transcription activation domain of p53 is an acidic region (12). To examine whether E1A might function as a repressor of acidic transcription activation domains in general or specifically represses the stimulation of transcription by p53, we compared the effects of Ad5 E1A and Ad12 E1A on the $mGAL4_D$-p53 constructs with those on the $mGAL4_D$-VP16 construct. As can be seen in Fig. 2D, both types of E1A had no significant effect on transcription activation induced by VP16, while transcription activation by p53 was dramatically reduced. These results indicate that E1A specifically represses the transcription activation domain of p53, while it does not affect the acidic transcription activation of VP16.

E1A also represses transcription stimulation by endogenous p53. Intracellular p53 levels increase as a result of DNA damage (13, 29), and subsequently activation of p53-responsive genes takes place (19, 39, 68). We were interested whether induction of *Waf1* and *MDM2* by endogenous p53 would still be detectable after DNA damage in the presence of E1A proteins. To that end, we made a panel of stable transfectants of the G401 rhabdoid kidney tumor cell line (14). Immunoprecipitation experiments with PAb 1620 and PAb 240, as well as functional experiments, have shown that p53 is wild type in this cell line (51) (data not shown). Due to the apoptotic effect of E1A in cells expressing wild-type p53, we have not been able to establish cell lines expressing only Ad5 E1A. Because it has been reported that the small E1B protein inhibits apoptosis caused by E1A (42) and that this protein does not affect the stimulation of transcription by p53 (44, 51), the G401 cell line was cotransfected with the E1A and 21-kDa E1B genes, which resulted in several stable cell lines. As controls, we used the original G401 cell line, two cell lines transfected with only the 21-kDa E1B gene, two cell lines transfected with E1A and the large (54-kDa) Ad12 E1B gene, and two p53-negative cell lines, Hep3B and Saos-2. The expression of transfected proteins in the stable cell lines is shown in Fig. 3A.

The induction of *Waf1* mRNA in the parental G401 cell line and in stable transfectants was examined by Northern analysis (Fig. 3B). The results we obtained show that increased expression of *Waf1* was already detectable in G401 cells at 2 h after X-ray irradiation and persisted until at least 5 h after irradiation. The same induction pattern was found for the stable transfectant expressing only the small E1B protein (21C8). In contrast with these results, the lack of *Waf1* mRNA induction was observed in the two stable transfectants expressing Ad5 E1A (5/21C3 and 5/21C24), indicating that E1A can also repress transcription activation by endogenous p53.

In addition, we examined the protein expression levels of Waf1, MDM2, and p53 at different timepoints after X-ray treatment. In the parental G401 cell line and transfectants expressing only the small E1B protein (21C1 and 21C8), the induction of Waf1, MDM2, and p53 was detectable after X-ray irradiation (5, 10, and 24 h after irradiation), as can be seen in Fig. 3C. However, in cell lines expressing E1A plus the 21-kDa E1B (5/21C3 and 5/21C24), the induction of both p53 and Waf1 was completely inhibited, whereas the induction of MDM2 was considerably reduced (Fig. 3C). Neither Hep3B (Fig. 3C) nor Saos-2 (data not shown) cells exhibit any induction of Waf1 or MDM2. We have previously shown that the large E1B protein can inhibit transcription activation of Waf1 by endogenous p53 (51). Now we have found that when E1A and the 54-kDa E1B are both expressed, no induction of Waf1 was detectable, but there was still a slight increase in MDM2 expression (Fig. 3 and data not shown). These results indicate that the expression of adenovirus proteins in cells containing wild-type p53 completely inhibit the induction of Waf1 after

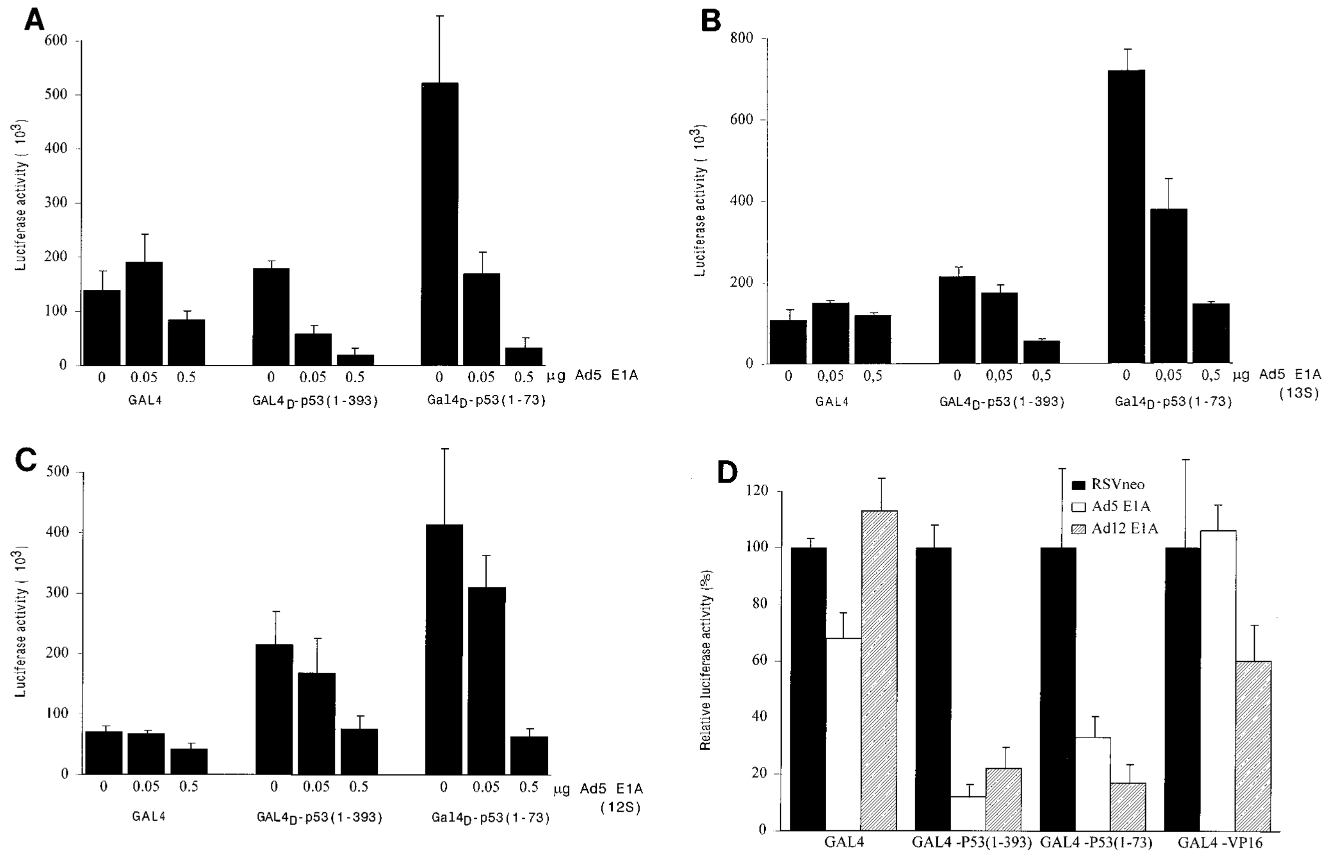

FIG. 2. Both 12S and 13S E1A proteins inhibit the activation of transcription by p53 via its transcription activation domain in p53-negative Saos-2 cells. mGAL4, $mGAL4_D$-p53(1-393), and $mGAL4_D$-p53(1-73) were cotransfected with a luciferase reporter construct containing 5× the GAL4-binding site in front of the luciferase gene together with Ad5 E1A (A), Ad5 13S E1A (B), or Ad5 12S E1A (C). (D) To examine whether E1A represses acidic transcription activation domains in general or affects only p53, the effects of Ad5 and Ad12 E1A proteins on p53 were compared with those on VP16 by using GAL4 fusion constructs. After the luciferase activities in the reaction mixtures were measured, the luciferase activity of all mGAL4 fusion constructs was set at 100% and the activity of each construct in the presence of either Ad5 or Ad12 E1A was calculated. Transfections and luciferase assays were performed as described in Materials and Methods. The precipitates for transfection were made in triplicate, and error bars represent the fluctuations between the results of three precipitates.

irradiation and severely repress the induction of MDM2 under these conditions.

The biological consequence of the effect of E1A on p53. It has previously been shown that in response to DNA damage, intracellular p53 levels increase (13, 29), and subsequently activation of p53-responsive genes takes place (19, 39, 68). In cells containing wild-type p53, this leads to G_1 cell cycle arrest (19, 21). To examine the biological significance of the repression of p53-mediated transcription activation by E1A, we tested whether E1A-expressing cell lines could still be arrested in G_1 in response to DNA damage. Therefore, the parental G401 cell line and stable transfectants were either mock irradiated or irradiated by X ray (5 Gy) and 15 h later labeled for 2 h with BrdU. After being stained with anti-BrdU-fluorescein isothiocyanate (FITC) and propidium iodide, cells were analyzed by fluorescence-activated cell scanning. Exponentially growing cells can be divided in this way into three groups. Cells in G_1 have a 2 N DNA content and are stained with propidium iodide but are negative for BrdU incorporation because they do not synthesize DNA. Cells in G_2 or M also show no anti-BrdU-FITC fluorescence but have a 4 N DNA content, as indicated by propidium iodide staining. In between these two phases reside the cells in S phase, exhibiting intermediate DNA contents but being positive for BrdU-FITC staining because of DNA synthesis (Fig. 4A). As can be seen in Fig. 4B, after X-ray irradiation of the parental G401 cell line, a dramatic decrease in the number of cells in S phase, together with an accumulation of cells in G_1 and G_2/M, could be observed. The same results were obtained for the two control cell lines expressing only the small E1B protein (Fig. 4B and data not shown). In contrast with these results, the two cell lines stably expressing Ad5 E1A continued to progress from G_1 to S phase after X-ray treatment, indicating that p53-mediated G_1 cell cycle arrest is lost as a result of E1A expression.

E1A proteins do not affect the phosphorylation status of the p53 protein. It has been shown that p53 can be phosphorylated by a number of different kinases (32). We previously observed increased phosphorylation of the N-terminal part in adenovirus-transformed human embryonic retina cells and in G401 cells stably transfected with Ad5 E1A together with Ad12 E1B, but not in stable transfectants expressing only the large or the small E1B protein (50). To examine whether inactivation of the transcription activation domain of p53 was caused by changes in the phosphorylation of this part of the protein, we performed tryptic phosphopeptide mapping of p53 isolated from stable transfectants. Because the adenovirus proteins cause stabilization of the p53 protein and we previously observed that after a normal (3 to 4 h) labeling time some of the unlabeled phosphate groups of highly stabilized p53 were not replaced by radioactive phosphate groups (50), we labeled cells

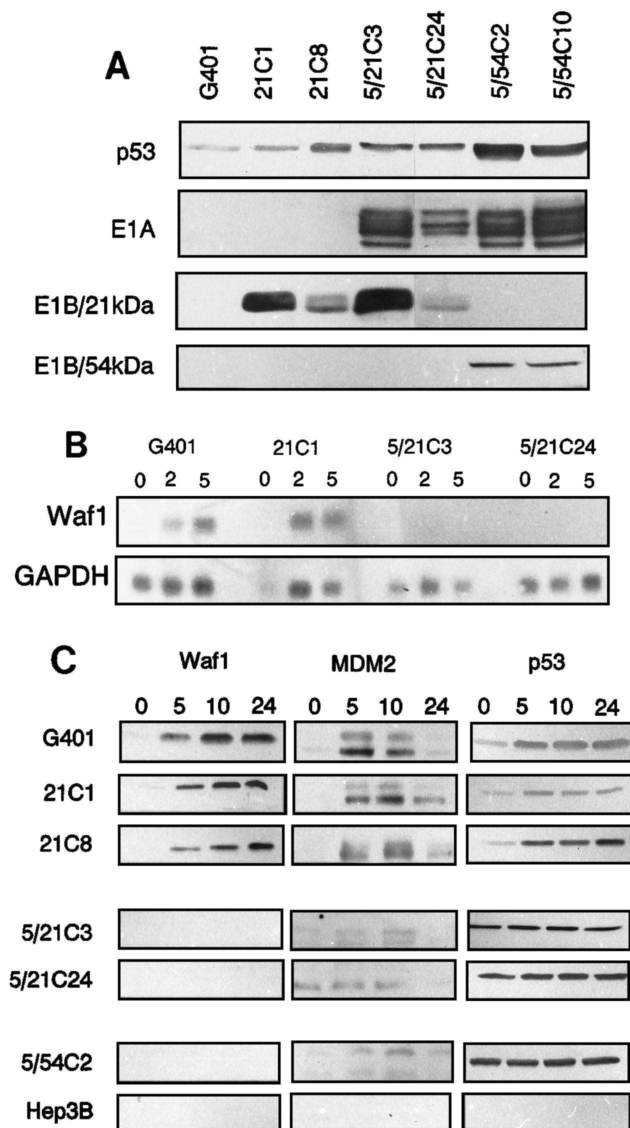

FIG. 3. (A) A panel of stable cell lines was made from the G401 cell line. The protein expression of transfected genes was checked by Western blotting. (B) The parental G401 cell line, control transfectant 21C1, and two transfectants expressing E1A (5/21C3 and 5/21C24) were irradiated by X ray (5 Gy) or mock irradiated. No induction of Waf1 mRNA was observed at 2 and 5 h after irradiation in E1A-expressing transfectants in contrast to the induction in control cell lines. (C) The parental G401 cell line and stable transfectants were irradiated by X ray (5 Gy) or mock irradiated and isolated at 5, 10, or 24 h after treatment to examine protein expression by Western analysis. The results show normal induction of Waf1 and MDM2 in the G401 cell line and the two transfectants expressing only the small E1B protein. No induction of Waf1 could be observed in all other cell lines. The induction of MDM2 was significantly less in E1A-expressing cell lines, while in p53-negative Hep3B cells, no induction of MDM2 was detectable. No enhancement of p53 expression after X-ray treatment was detectable in E1A-expressing cell lines. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

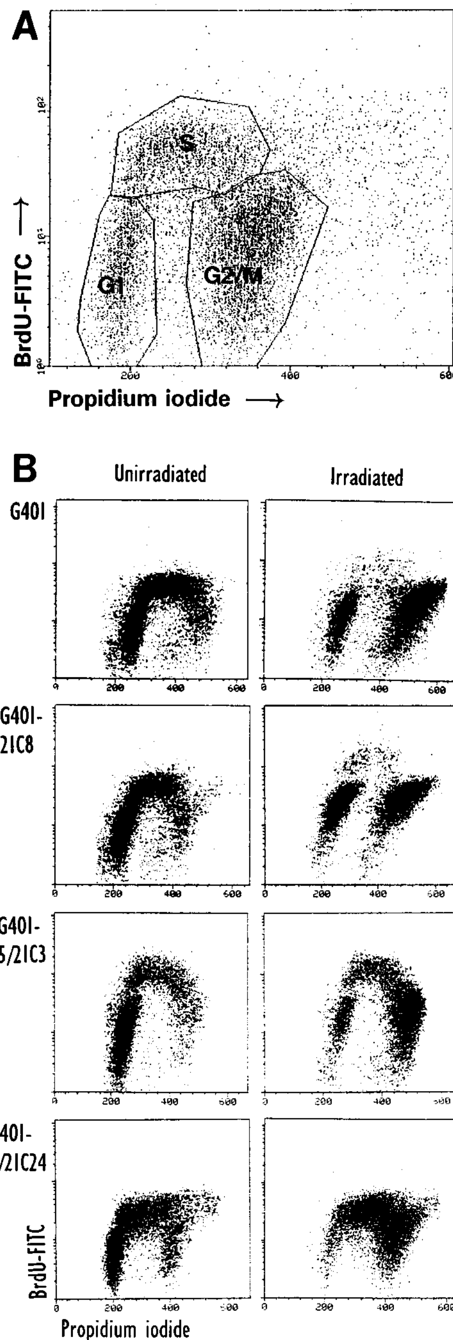

FIG. 4. The effect of E1A expression on the G_1 cell cycle arrest function of p53 was examined in the parental G401 cell line and stable transfectants. Exponentially growing cells were either mock irradiated or treated by X ray (5 Gy) and further grown for 15 h under normal conditions. Then cells were labeled for 2 h with BrdU, and samples were prepared for FACSscan analysis, as described in Materials and Methods. (A) Distribution of exponentially growing G401 cells over G_1, S, and G_2/M phases. (B) X-ray-induced G_1 cell cycle arrest was induced in G401 cells and the control transfectant expressing only the small E1B protein, while transfectants stably expressing E1A did not show G_1 cell cycle arrest.

for 20 h with $^{32}P_i$, after which p53 was isolated and tryptic phosphopeptide maps were made. The predicted mobility pattern calculated on the basis of molecular masses net charges, and hydrophobicities of these peptides, as described by Boyle and coworkers (3), is shown in Fig. 5A. Peptide M1 represents amino acids 386 to 393, M2 represents amino acids 307 to 320, M3-1 represents amino acids 1 to 24, and M3-2 and M3-3 are the double- and triple-phosphorylated forms of M3. The results shown in Fig. 5B indicate approximately equal phosphorylation of the mono- and double-phosphorylated forms of M3 in the parental cell line G401. In the two transfectants expressing only the small E1B protein (21C1 and 21C8), we did not

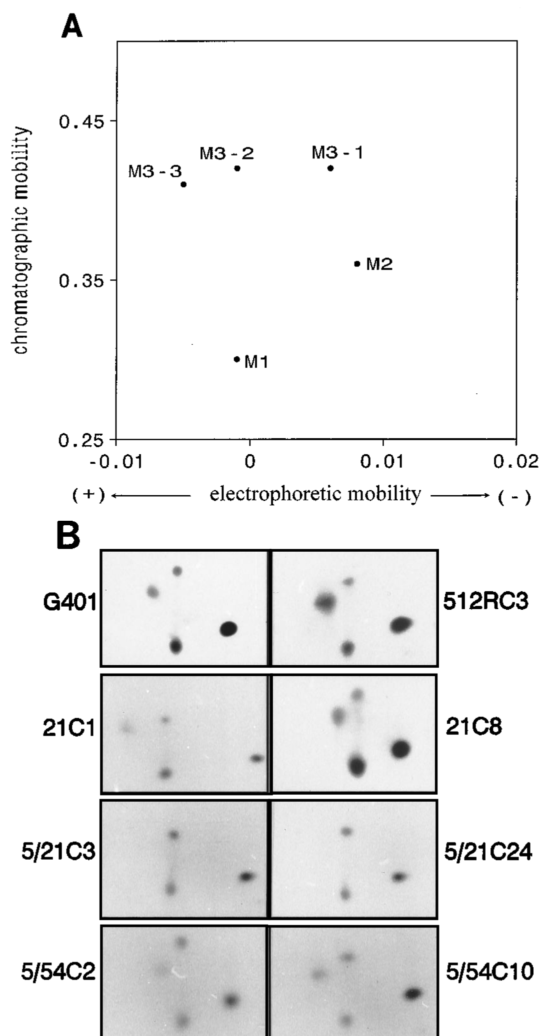

FIG. 5. E1A does not significantly change phosphorylation of the p53 protein. The parental G401 cell line and a panel of transfectants expressing various adenovirus E1 proteins were labeled overnight with $^{32}P_i$. Tryptic phosphopeptide maps were made as described in Materials and Methods. (A) The predicted mobility pattern of the tryptic phosphopeptides of human p53 was calculated as previously described (3). (B) Increased phosphorylation of the double-phosphorylated M3 peptide of p53 in a stable transfectant expressing Ad5 E1A together with all Ad12 E1B proteins (512RC3), compared with that of the parental G401 cell line. Slight decreases in phosphorylation were found in the transcription activation domains of the two transfectants expressing Ad5 E1A together with the 21-kDa E1B. No significant changes were detectable in the other transfectants.

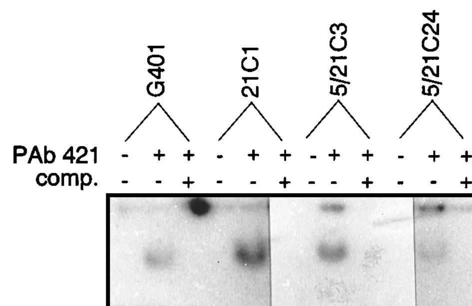

FIG. 6. E1A proteins do not significantly affect the binding of p53 to the DNA consensus sequence. Nuclear extracts were made of the parental G401 cell line and stable transfectants. The DNA-binding capacity of p53 was examined by electromobility shift assay, as described in Materials and Methods. The results show that p53 derived from all cell lines used can bind specifically to the p53 consensus sequence present in the Waf1 promoter. comp., competitor; +, present; −, absent.

observe a significant change in the phosphorylation pattern, while in the G401-512RC3 cell line, a shift from M3-1 to M3-2 could be observed, as we have shown before (50). However, this effect was not detectable either in the cell lines expressing Ad5 E1A together with the 54-kDa E1B or in those expressing Ad5 E1A together with the 21-kDa E1B. The latter transfectants even seem to display a decrease in the double phosphorylation of M3. Apparently, simultaneous expression of all adenovirus proteins is necessary for the increased phosphorylation observed in G401-512RC3 cells and Ad12-transformed human embryonic retina cells (50).

These results also indicate that the inhibitory effect of adenovirus E1A proteins on the stimulation of transcription by p53 in transient transfections, as well as in cell lines stably expressing Ad5 E1A, is not caused by a significant change in the phosphorylation of the N-terminal part of p53.

Ad5 E1A has no effect on the DNA-binding capacity of p53. We subsequently checked whether the inhibition of transcription activation of p53 by E1A is mediated only via the transcription activation domain or whether it also affects the DNA-binding capacity of the p53 protein. The results shown in Fig. 6 indicate binding of p53 proteins from the parental G401 cell line and the control 21-kDa E1B-expressing transfectant (21C1) to the p53 consensus sequence present in the Waf1 promoter (11). Because we found two- to threefold decreases in the p53 concentrations in the nuclear extracts of transfectants expressing E1A plus the 21-kDa E1B (data not shown), we used three times more protein to examine the DNA-binding capacity of p53 in nuclear extracts made from these transfectants. Our results show that p53 is still capable of binding to the consensus sequence in the presence of E1A. These results confirm our previous observation that in a stable transfectant expressing Ad5 E1A together with Ad12 E1B, no change in the DNA-binding capacity could be observed (51).

E1A proteins cause shifts in the molecular weights of complexes containing p53. We previously found that in Ad12-transformed cells, p53 is contained in complexes of increased molecular weight compared with those in untransformed cells (50). Here we examined whether the expression of E1A proteins in stable transfectants of the G401 cell line could change the complexes in which p53 is present in cells or whether other adenovirus proteins are responsible for this effect. To that end, we made lysates of the G401 cell line and different transfectants under mild conditions, loaded these lysates on a Superose-6B column, and fractionated the proteins on the basis of molecular weight. Equal parts of each fraction were separated on an SDS–10% polyacrylamide gel, and the amount of p53 in each fraction was visualized by Western blotting. As can be seen in Fig. 7A, in the parental G401 cell line p53 is present in complexes with molecular masses of about 440 kDa. The same result was observed for two transfectants expressing only the small E1B protein (21C1 and 21C8), indicating that expression of the 21-kDa E1B protein has no effect on the complex formation of p53. However, p53 proteins isolated from transfectants expressing the 21-kDa E1B together with Ad5 E1A (5/21C3 and 5/21C24) show shifts into complexes with increased molecular masses, ranging between 440 and 800 kDa, suggesting that E1A can influence the intracellular complex formation of p53. The results also show that in the parental G401 cell line and in small-E1B-expressing transfectants, both polymorphic

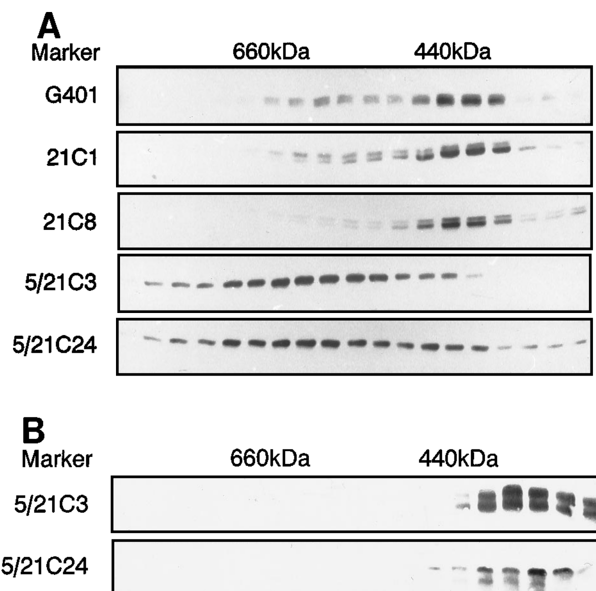

FIG. 7. Increases in the molecular masses of p53 complexes in E1A-expressing cell lines. Lysates were made under mild conditions, with the intention of leaving intracellular protein complexes intact. Lysates were size fractionated on a Superose-6B column. (A) The amounts of p53 in various fractions were examined by Western immunoblot analysis. In the G401 cell line and in the two transfectants expressing the small E1B protein, p53 is present in complexes with molecular masses of about 440 kDa, while in the transfectants expressing Ad5 E1A, a shift to complexes with molecular masses of up to 800 kDa was observed. (B) In stable transfectants 5/21C3 and 5/21C24, E1A is present in complexes with molecular masses of 300 to 400 kDa, which differ from the fractions in which p53 is present.

forms of wild-type p53 are present, while in both E1A-expressing transfectants, only one of the two forms is detectable. Immunoprecipitation experiments with PAb 1620 and PAb 240 indicated that there was no change in the wild-type characteristics of p53 in stable transfectants compared with those in the G401 cell line (data not shown), indicating that the observed changes in the molecular masses of p53 complexes are due to coexpression of E1A, not mutation of the p53 gene.

We also analyzed the same lysates for the presence of E1A and found E1A to be present in both 5/21C3 and 5/21C24 transfectants in complexes with molecular masses of less than 440 kDa (Fig. 7B). The results in Fig. 7 show clearly that in E1A-expressing cell lines, p53 is present in different fractions than is E1A, indicating that inactivation of p53 by E1A is not caused by direct interaction.

DISCUSSION

In the present study we have shown that the Ad5 and Ad12 E1A proteins inhibit the activation of transcription by p53 in transient transfections. In addition, we found that in stable transfectants expressing Ad5 E1A together with the small E1B protein, no induction of Waf1 and hardly any induction of MDM2 was detectable after X-ray irradiation, in contrast to the results for the parental cell line and stable transfectants expressing only the small E1B protein. The levels of p53 induction after X-ray treatment were also affected in E1A-expressing cell lines. While an increase in the p53 expression level was found in the parental cell line and in 21-kDa E1B-expressing control transfectants, in the presence of E1A, no induction of p53 was detectable as a result of DNA damage. On the basis of these observations, we can conclude that also in stably transfected cell lines, E1A inhibits the activation of transcription by endogenously expressed p53. This effect might be caused by the lack of induction of p53 after X-ray treatment when E1A is coexpressed, by repression of the transcriptional activity of p53 under these circumstances, or by a combination of both these effects.

In stable transfectants expressing E1A plus the 21-kDa E1B, we did not observe an increase in the p53 concentration compared with that in the parental G401 cell line (Fig. 3A). Previously, it has been shown that E1A caused stabilization of the p53 protein in rat embryonic fibroblasts (REF52) as well as in mouse embryonic fibroblasts (26, 27). A possible explanation for the discrepancy in results might be that the half-life of p53 in G401 cells (approximately 4 h) is already prolonged, compared with that in primary human cells (approximately 1 h) (50), and cannot be extended by E1A.

We found that the activity of the mGAL4$_D$-p53(1-73) fusion construct could be suppressed by E1A in cotransfection experiments, strongly indicating that the repressive effect by Ad5 E1A is mediated via the transcription activation domain of the p53 protein.

Apart from activating transcription, p53 can also repress transcription via the basal transcription machinery in transient assays (1, 41, 48). Recently, Horikoshi and coworkers (17) have shown that E1A can counteract this repressive function of the p53 protein. The data they presented suggest that E1A can dissociate the complex between the C-terminal part of p53 and the TATA-binding protein (TBP). The repression of transcription was relieved only by 13S E1A, not by 12S E1A, probably because the latter protein is known to interact less efficiently with the TBP (23).

TBP not only binds to the C-terminal part of the p53 protein but also interacts with its N-terminal region (25, 56). We found that both the 12S- and 13S-encoded proteins of Ad5 E1A can repress the function of the transcription activation domain of p53. Thus, E1A-induced repression of the transcription activation potential of p53 is most likely not caused by the disruption of TBP binding to the transcription activation domain of p53. Thut et al. (55) have shown that the human coactivators TAF$_{II}$32 and TAF$_{II}$70, two subunits of the TFIID complex, can also bind to the transcription activation domain of p53 in vitro. These factors are important for stimulation of the basal transcription initiation complex. Moreover, interaction between p53 and different subunits of the TFIIH complex has been shown. However, only the p62 subunit was suggested to bind to the transcription activation domain (65), while XPB and XPD were found to bind to the C-terminal part of p53 (61).

We found that in stable transfectants derived from the G401 cell line and expressing Ad5 E1A together with the 21-kDa E1B, p53 is present in complexes with molecular masses of around 660 kDa. In contrast, in the parental G401 cell and stable transfectants expressing only the small E1B protein, p53 occurs mostly in complexes with molecular masses of about 440 kDa. Apparently, expression of Ad5 E1A drives p53 into higher-order complexes. This increased complex formation of p53 is most probably an indirect effect, since no direct interaction between p53 and the E1A proteins has been detected (10, 27, 63) and since we found p53 and E1A to be present in different fractions in E1A-expressing stable transfectants. The appearance of higher-order complexes might be due to increased homo-oligomerization of the p53 protein via its oligomerization domain (52) but also might involve binding to other proteins. Inhibition of transcription activation is mediated via the N-terminal part of the p53 protein, which suggests that E1A inhibits the p53 activity via modulation of the activity or the level of expression of a protein which binds the N-terminal part

of the p53 protein. As a result of this interaction, binding of p53 to subunits of the TFIID or TFIIH complex may no longer take place, resulting in the inhibition of transcriptional activity. Since we observed that E1A specifically represses transcription activation by p53 and no significant effect was found on transcription activation by VP16, we can conclude that E1A does not repress the stimulation of transcription by acidic transcription factors in general. Because in the presence of E1A stimulation of transcription by VP16 can still take place, we assume that the inhibition of transcription activation by p53 is not caused by the sequestration of transcription activators by E1A, which indirectly results in transcription inhibition by p53. Because of the fact that different cofactors seem to be involved in stimulation of the transcription activation domain of p53, it also does not seem likely that $TAF_{II}32$, $TAF_{II}70$, and p62 would all be squelched by E1A. Furthermore, when repression of transcription is indeed caused by binding of an inhibiting protein to the transcription activation domain of p53, this protein does not bind to the acidic transcription activation of VP16. The existence of such a p53-binding protein in E1A-expressing cell lines is under current investigation.

An important biological function of the p53 protein is the maintenance of genomic stability after DNA damage. It has been shown that as a result of DNA damage, p53 levels increase, with subsequent enhanced expression of p53 target genes like *Waf1*. p53-proficient cells have been found to have radiation-induced G_1 cell cycle arrest (19, 21) as well as mitotic cell cycle arrest (6), thereby providing time for a cell to repair its DNA before the next round of replication takes place (22). We found that in E1A-expressing cell lines the G_1 cell cycle arrest function of p53 is lost. The consequence of the loss of this biological function is an increase of changes in the genome.

In normal cells in the case of irreparable DNA damage, the expression of wild-type p53 is necessary to lead cells into apoptosis. It has been shown that p53 can stimulate the transcription of a stimulator of apoptosis *Bax* (36, 47). On the other hand, it was found that p53 is able to repress the transcription of *Bcl-2* (34, 35), which is an inhibitor of apoptosis. These observations indicate that p53 can cause apoptosis via regulation of the transcription of genes involved in this process. E1A-induced apoptosis also depends on the expression of wild-type p53 (8, 27) and can be counteracted by the adenovirus small and large E1B proteins (5, 42). It is known that these E1B proteins can influence the transcription-regulatory properties of p53. The large E1B protein inhibits both transcription stimulation (66) and the repression of transcription by p53 (51). The observation that the small E1B protein has no effect on the stimulation of transcription by p53 but only inhibits its repression (44, 49, 51) suggests that the repression of transcription is involved in p53-dependent apoptosis (49). Another option is that the small E1B protein inhibits the apoptotic pathway further downstream and that this inhibition is independent of its effect on transcription inhibition by p53 (30, 54).

The observation that the adenovirus E1A proteins inhibit transcription stimulation by p53 is surprising. At the level of transcription regulation by p53, the effects of the various adenovirus E1 proteins do not counteract each other but rather seem to enhance each other's effect. The observation that E1A-induced p53-dependent apoptosis is correlated with inhibition of transcription stimulation indicates that the apoptotic process can occur independently of the induction of p53 target genes. In fact, this is in agreement with the results of a number of other studies. Caelles and coworkers have shown that DNA-damage-induced apoptosis is strictly p53 dependent but is independent of the synthesis of new RNA or protein (4). Furthermore, Wagner and coworkers have shown that wild-type p53 is essential for myc-mediated apoptosis, but the apoptotic response occurs in the absence of induction of p53 target genes like *Waf1* (60). The data published by Teodoro et al. also suggest that transcription activation by p53 is not strictly essential for the apoptotic process since they found that E1A can induce apoptosis by both p53-dependent and p53-independent mechanisms (54). The results of these studies indicate that the induction of p53 target genes is essential for p53-mediated growth arrest but is not strictly necessary for p53-mediated apoptosis.

We have now shown that apart from inhibiting the repression of transcription by p53, E1A can inhibit the activation of transcription by p53. These observations, together with previously published results for E1B proteins in terms of transcription stimulation and repression by p53, suggest that inactivation of the transcription-regulatory functions of p53 is very important for cellular transformation.

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