The tumor suppressor p53 regulates transcription positively and negatively, depending on the target gene. Whereas p53 induces transcription through direct interaction with promoter DNA, the mechanism of p53-mediated transcriptional repression is less well understood. Early reports described the alleviation of p53-mediated repression by inhibitors of apoptosis, suggesting that negative regulation of transcription might occur only in conjunction with programmed cell death. More recently, it has been proposed that certain genes, such as survivin, are repressed by direct association of p53 with their promoters, followed by recruitment of a repressor complex. We show here that p53-mediated negative regulation of transcription could occur independently of apoptosis. In contrast, the amino-terminal transactivation domain of p53 was required for negative regulation of transcription. Similarly, the p53 homologue p73 diminished the expression of survivin and stathmin, depending on its transactivation domain. Mutation of the putative p53 binding site within the survivin promoter did not impair its repression. These observations raised the hypothesis that activation of an effector gene might be required for repression by p53. Strikingly, when the p53-inducible p21/CDKN1A gene was deleted, p53 no longer repressed any one among 11 genes that it down-regulates otherwise. Most of these genes were also repressed by ectopic p21 in the absence of p53. Overexpressed c-Myc reduced the transcription of p21/CDKN1A and impaired p53-mediated repression but did not abolish repression by ectopic p21. Taken together, these results strongly suggest that increased expression of p21/CDKN1A is necessary and sufficient for the negative regulation of gene expression by p53.

p53 is a key regulator of cell growth and apoptosis. Its central role in tumor suppression becomes evident by the fact that the p53 gene is mutated in about 50% of human malignancies. p53 acts as a transcription factor, modulating the expression of growth and death regulators. As a result, cell proliferation is suppressed, and/or programmed cell death is induced (1). It is generally accepted that p53 activates a number of promoters through direct interaction with the promoter DNA and the subsequent recruitment of the basal transcription machinery, e.g. the TFIID complex and the p300/CPB histone acetyl transferases. A tetramer of p53 molecules is assembled through the carboxyl-terminal oligomerization domains. This allows the central domains to interact directly with a consensus DNA element. As a consequence, the amino-terminal transactivation domains interact with basal transcription factors, resulting in increased gene expression (2, 3).

However, it has long been noticed that some genes are negatively regulated (referred to as “repressed” hereafter) by p53, and the list of those genes has been extended for almost a decade (see Table I). When analyzing p53 mutants, and p53 in combination with inhibitors of apoptosis, a striking correlation of p53-mediated transcriptional repression and p53-induced apoptosis was observed, and this raised the hypothesis that negative regulation of gene expression by p53 might be crucial for the induction of apoptosis (4–7).

In contrast to p53-mediated gene activation, the mechanism(s) of transcriptional down-regulation of genes by p53 remain controversial. Basically, three different scenarios can be envisioned.

Negative regulation of gene expression by p53 might merely represent an epiphenomenon of apoptosis, i.e. p53 down-regulates certain genes not directly but only through the onset of cell death. This model is supported by the observed correlation between the ability of p53 mutants to repress transcription in reporter assays and their potential to induce apoptosis. Such a correlation exists in the case of p53 mutants lacking the proline-rich domain within residues 62–91 (8, 9) or point mutants at residues 175 (10) and 246 (8). Further, early reports describe the down-regulation of reporter gene expression by p53, which was found to be reverted by inhibitors of apoptosis (4, 5). However, it should be noticed that in transient reporter assays, a large variety of promoters can be found repressed by p53 (5) and that this may not necessarily reflect the regulation of the corresponding cellular genes in all cases.

Alternatively, p53 may lead to the negative regulation of gene expression by virtue of a repressor function and by direct interaction with promoter DNA. Such a scenario has been proposed for the repression of stathmin and survivin by p53 (11, 12). p53 was found to interact with the mSin3a protein that is part of transcriptional repressor complex (11). Further, at least in the case of survivin, a p53 binding sequence was reported to exist within the promoter (12). However, the question remains how p53 would simultaneously activate some genes and repress other genes after binding to the respective promoters. Further, if p53 activates and represses by different mechanisms, these functions could be expected to be separable by mutational analysis. However, no p53 mutant has been described that represses but no longer transactivates. Instead, a mutation at residues 22/23 abolishes both transactivation and repression (7, 13), although the interaction of p53 with mSin3a was mapped to amino acids 61–75 within p53 (14). Unless a clear mode of distinction between activated and repressed promoters could be defined, the plausibility of a direct repression model remains to be questioned.

A third model that will be proposed in this work ascribes

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p21/CDKN1A Mediates Negative Regulation of Transcription by p53*
p53-mediated repression to the induction of a repressor. Thus, p53 would first increase the levels of another transcriptional regulator, which in turn would negatively affect the expression of downstream genes. The first p53-induced gene to be identified was \textit{p21/Cip1/Cip1/Cip1/waf1/CDKN1A} (15). As an inhibitor of cyclin-dependent kinases, p21 is known to prevent the phosphorylation of retinoblastoma (Rb) family proteins and hence lead to the accumulation of hypophosphorylated pRb (16). This protein species, in turn, binds to E2F family transcription factors and converts them from transcriptional activators to transcriptional repressors. Thus, it appears conceivable that p53 may negatively regulate the expression of genes through the induction of \textit{p21/CDKN1A} and the consecutive hypophosphorylation of pRb and its relatives. Indeed, certain cell cycle regulators responsible for G2 arrest, such as \textit{CHK1} (17) and \textit{cdc2} (18, 19), were found to be down-regulated at the mRNA and proteins levels by p53 and the p21/pRb/E2F pathway. However, the general dependence of p53-mediated negative gene regulation on the expression of \textit{p21/CDKN1A} remains to be assessed.

In this study we sought to discover the distinction between these three models. First, we provide evidence that, at least in the cases of \textit{survivin} and \textit{stathmin}, negative gene regulation by p53 is not a consequence of apoptosis. Second, p53 and the p53 homologue p73 were capable of down-regulating the expression of these genes, but each required a transactivation domain to do so. Analysis of the \textit{survivin} promoter suggested that se-

\footnote{The abbreviations used are: Rb, retinoblastoma; pRb, retinoblastoma protein; PARP, poly(ADP-ribose) polymerase; RT, reverse transcription; EMSA, electrophoretic mobility shift assay.}
Fig. 2. Influence of caspase activity on p53-mediated negative gene regulation. A, inhibition of p53-mediated apoptosis by a peptide inhibitor of caspases. H1299 cells were transduced to express p53 or β-galactosidase using adenovirus vectors (multiplicity of infection = 20). Immediately after transduction, the peptide ZVAD (100 μM) or the dimethylsulfoxazole (DMSO) solvent (1:1,000, v/v) were added to the cells. After 24 h, PARP cleavage was assessed as described in the legend to Fig. 1A. Note that in the presence of p53 and DMSO alone, neither full-length nor cleaved PARP was detected, presumably because of further degradation of the fragment. This was repeatedly observed when apoptosis had proceeded to a large extent, especially in the presence of DMSO. B, influence of caspase activity on p53-mediated reduction of survivin and stathmin expression. RNA was prepared and analyzed as described in the legend to Fig. 1B.

sequence elements other than the putative p53 binding motif are needed for repression by p53. Most strikingly, however, we show that the negative regulation of all p53-repressed genes analyzed entirely depends on the presence of p21/CDKN1A and that overexpressed p21 represses a similar set of genes. These results strongly argue that p53-mediated repression occurs mainly through the induction of the p21/CDKN1A gene.

Experimental Procedures

Cell Culture and Transfections—H1299 cells (p53−/−) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum. HCT116 cells with and without mutations disrupting p53 and p21/CDKN1A (20) were kindly provided by K. Roemer with generous permission by B. Vogelstein and were cultivated in McCoy's medium (Invitrogen) with 10% fetal calf serum. Transfections were done using FuGene6 (Roche Applied Science). Doxorubicin (Sigma) was added to the cell culture media where indicated at a concentration of 350 nM for 48 h. ZVAD (Calbiochem) was used at 100 μM.

Plasmids and Adenovirus Vectors—A reporter construct containing the survivin promoter (21) was kindly provided by D. C. Altieri. Mutants of this promoter were created in this plasmid background by the QuikChange methodology (Stratagene) for site-directed mutagenesis, using the following primers and their respective reverse complements:

mutant 1, GAG GGC GTG CGC TCC CGG GAT GCC CCG CGG CGC
mutant 2, CTA AGA GGG CGT ACG CTC CCG ACA TG
mutant 3, CTC CCG TGC CCA TGG GCC GAC ATT AAC
mutant 4 was obtained by sequential mutagenesis using the oligonucleotides corresponding to mutant 2 and mutant 3. All plasmids were sequenced by the sequencing service. The expression plasmid pcDNA3-based plasmid (27) and introduced into the same sites of the vector pAdEasy system (26). The p73TA dIII and XbaI, together with the 5′ untranslated region of human lamin mRNA, was excised with HindIII and XbaI from the corresponding pcDNA3-based plasmid (27) and introduced into the same sites of the plasmid pRcCMVp53mt22/23 (13) into pAdtrack-CMV using HindIII and XbaI, followed by recombination and transfection. Viruses were amplified, the titer was determined, and infections were carried out as described (22, 28).

Immunoblot—Proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose, followed by incubation with antibodies in phosphate-buffered saline containing 5% milk powder and 0.05% Tween 20. Peroxidase-coupled secondary antibodies (whole immunoglobulin G; Jackson Immunoresearch Laboratories) were then detected by chemiluminescence (Pierce). Antibody Pab1901 to p53, antibody Ab-1 to p21cip1/raf1, and antibody Ab-2 against polyADP-ribose polymerase (PARP) were from Calbiochem. Another monoclonal mouse antibody against actin (clone C-2) was from Santa Cruz Biotecology, Inc.

Semiquantitative RT-PCR—H1299 cells were transduced with adenovirus vectors, and HCT116 cells were treated with doxorubicin. After 48 h, total RNA was prepared (Trizol reagent; Invitrogen), followed by reverse transcription with Superscript II polymerase (Invitrogen) and PCR amplification with Expand HiFi DNA polymerase (Roche Applied Science). The PCR temperatures consisted of a 3-min denaturation step at 96 °C, followed by the indicated numbers of cycles at 96 °C for 30 s, 57 °C for 30 s, and 72 °C for 50 s. These temperature cycles were used for all amplifications, except that in the case of ubiquitin, the 57 °C step was omitted. The primers and numbers of PCR cycles used were as follows. For each gene, the reverse transcription was started with the first (RT) oligonucleotide, whereas the PCR was carried out using the second (forward) and third (reverse) oligonucleotide.

For BCC1A: 30 cycles, RT: oligo(dT), forward: CCA AAG CCA GCA AGA GAA TCC CAG; reverse: TCA GGT AGG TGTCCA GAC CTT CAG. For CDC2: 25 cycles, RT: oligo(dT), forward: CTT TGC CAG AGC TTT TGG AAT ACC; reverse: GAC ATG GGA TGC TAG CCT TCC TGG. For CDC25C: 25 cycles, RT: oligo(dT), forward: GTC TTT GCC AGG ACA CAT CCA GGG; reverse: CAA GTT GGT AGC CTT GTG ATT TG. For CDK1: 30 cycles, RT: oligo(dT), forward: CCT TGG TGA ACT GGC ACT TGG; reverse: CAT CTT GTT CAA CAA AGC CTC ACC. For Cyclin A2: 25 cycles, RT: oligo(dT), forward: AGC AGC CTT CAA ACT GCA AAG TTG; reverse: TGG TGG GTT GAG GAG AAG AAC ACC. For Cyclin B1: 30 cycles, RT: oligo(dT), forward: CCT CTA CCT
p53-mediated Repression Is Not Inhibited by a Viral Antagonist of Pro-apoptotic Mitochondrial Factors—We tested whether p53-mediated transcriptional repression might be a result of p53-induced apoptosis. p53 was expressed in the p53−/− cell line H1299 using an adenovirus expression vector. To block apoptosis, the adenovirus E1B-19-kDa protein was co-expressed with p53. E1B-19 kDa interferes with the proapoptotic functions of the mitochondrial bax and bak proteins (30) and inhibits p53-mediated cell death (31). Apoptosis was assessed by immunoblot detection of PARP. Cleavage of PARP, a characteristic of apoptotic cells (32), was detected when p53 was expressed alone but not when p53 and E1B-19 kDa were co-expressed (Fig. 1A). In a parallel experiment, identically treated cells were harvested to prepare RNA, and the levels of survivin and stathmin mRNA were determined by RT-PCR. Both genes were clearly down-regulated by p53, regardless of the presence or absence of E1B-19 kDa (Fig. 1B). The expression of a control gene, ubiquitin C, remained unchanged, and the transactivation of a p53-responsive gene, PIG3, was not affected by E1B-19 kDa. Finally, the repression of the survivin promoter by p53 in luciferase reporter assays was not detectably influenced by E1B-19 kDa (Fig. 1C). We concluded that p53-mediated negative regulation of transcription occurs independently of apoptosis.

p53-mediated Negative Regulation Is Not Inhibited by a Synthetic Inhibitor of Caspases—A similar set of experiments was
FIG. 5. A putative p53 binding element within the survivin promoter. A, p53 binding elements. A previously proposed p53 binding element (12) within the survivin promoter is depicted, along with a mutant that was used in the experiment shown in B. A p53-responsive element from the p21/CDKN1A promoter and its mutant were analyzed in parallel. The previously defined p53-binding consensus element (34, 35) is shown for comparison. Nucleotides not corresponding to the consensus are shown in lowercase, and mutated nucleotides are underlined. B, EMSA. Oligonucleotides corresponding to the sequence elements depicted in A were employed to generate a radioactively labeled, double stranded DNA probe. This probe was incubated with reticulocyte lysates that had been programmed for translation in vitro with a plasmid encoding p53 (+), or with an “empty” vector plasmid (−). Subsequently, the mixtures were separated on a native polyacrylamide gel, followed by autoradiography. The monoclonal antibody 421 binding near the carboxyl terminus of p53 activates the cryptic p53 DNA binding activity (36). Therefore, this antibody was added to the reactions as indicated. The positions of complexes containing p53 with or without the antibody, as well as a background band that occurred independently of p53 (*), are indicated by arrows. Note that the free DNA was allowed to run out at the bottom of the gel, to increase the resolution of the different complexes.

used to determine whether caspase inhibition would affect p53-mediated transcriptional repression. The peptide caspase inhibitor zVAD (33) effectively prevented p53-induced apoptosis, as determined by PARP detection (Fig. 2A), but did not affect the capability of p53 to repress the survivin and stathmin genes, as revealed by RT-PCR (Fig. 2B). Thus, p53-induced apoptosis can be prevented by caspase inhibition without compromising its ability to down-regulate gene expression.

p53 Requires a Functional Transactivation Domain to Mediate Negative Regulation of Gene Expression—To test whether the amino-terminal transactivation domain of p53 is necessary for the negative regulation of gene expression, we transduced H1299 cells to express p53, with or without a mutation near the amino terminus (L22Q/W23S) that abolishes the function of the transactivation domain (13). Unlike wild type p53, the mutant was not only defective with regard to the induction of PIG3 but also lost the capability of repressing the expression of survivin and stathmin, as determined by semiquantitative RT-PCR (Fig. 3). Thus, as in other assay systems (7, 13), the negative regulation of gene expression by p53 requires a functional transactivation domain.

p73 Negatively Regulates Transcription Similarly to p53, Depending on a Transactivation Domain—Next, we asked whether the p53 homologue p73 can down-regulate the transcription of p53-repressible genes. We expressed the p73TA isoform (Fig. 4A) in H1299 cells and determined the levels of survivin and stathmin mRNA. p73 and p53 were equally capable of down-regulating survivin, and both reduced the expression of stathmin, albeit to a lesser extent in the case of p73 (Fig. 4B). Naturally occurring p73 isoforms that lack the transactivation domain (p73ΔNα and p73ΔNβ) were analyzed in parallel. These isoforms did not repress. We conclude that p73 has a similar capacity of down-regulating gene expression as p53 but that a transactivation domain is required to carry out this function in both cases.

A p53-binding Consensus Element within the survivin Promoter Interacts Inefficiently with p53, and Binding Is Further Impaired by Mutation—The repression of the survivin promoter by p53 was suggested previously (12) to be mediated by a direct interaction of p53 with a p53-binding consensus element. As a first step to understand the function of this element, we compared its capability of binding p53 with an established p53-responsive element derived from the p21/CDKN1A promoter, using EMSA. In each case, a mutated sequence element was used as a negative control. A comparison of these sequences with the previously defined p53-binding consensus (34, 35) is shown in Fig. 5A. When p53 alone was assayed for binding the different DNA elements, only the wild type p21/CDKN1A sequence yielded a band with decreased electrophoretic mobility, indicating a DNA-protein interaction (Fig.
5B, compare lanes 1 and 2). The interaction was strongly increased by the monoclonal antibody 421 against the carboxyterminal portion of p53, resulting in a supershifted and much more intense signal (Fig. 5B, compare lanes 9 and 10), as described previously (36). Under these circumstances, the wild type sequence derived from the survivin promoter was also found to interact with p53, albeit with far lower efficiency than the p53-binding consensus element (Fig. 5B, lanes 13 and 14). Even in the presence of antibody 421, the mutant sequence elements were not found to interact with p53 (Fig. 5B, lanes 11, 12, 15, and 16). Thus, the p53-binding consensus element of the survivin promoter can interact with p53 in a specific, mutation-sensitive manner, but with comparatively low efficiency, at least when assayed by EMSA.

A p53-binding Consensus Element within the survivin Promoter Is Disposable for p53-mediated Repression—The fact that a transactivation domain is needed to down-regulate survivin expression by p53 prompted us to analyze the survivin promoter with regard to its negative regulation. It was suggested previously (12) that p53 may directly interact with the promoter DNA through a consensus p53 binding element, and such an interaction was observed in vitro (Fig. 5). However, it should be noted that this promoter element is not conserved when comparing the human and the murine sequence (for alignment of the promoter sequences, see Ref. 21). The previously described p53 binding motif within the human survivin promoter (12) was mutated as in Fig. 5, at the site that is most conserved among p53 binding sequences, namely, the C residue within a RRRCW (R = purine, W = A or T) half site of the p53-responsive consensus element (34, 35). Moreover, mutations within two putative cell cycle-dependent element consensus motifs were introduced into the luciferase reporter construct, alone or in combination (Fig. 6A). These constructs were then tested for reporter expression in the presence or absence of a p53 expression plasmid (Fig. 6, B and C). It was found that mutations in the cell cycle-dependent element-like motifs resulted in reduced reporter expression but alleviated or abolished further down-regulation by p53. In contrast, when the putative p53 binding site was mutated, the promoter strength and susceptibility to repression by p53 essentially remained as in the wild type promoter. We conclude that the putative p53 binding site is not required to confer p53-mediated repression to the survivin promoter. Thus, the proposed direct binding by p53 (12) does not provide an appropriate explanation for the observed repression.

p53-mediated Negative Regulation Is Dependent on the Expression of p21/CDKN1A—The requirement of a transactivating domain to repress survivin and stathmin prompted us to test whether a p53-induced gene product might mediate repression. A candidate for such a mediator gene was p21/CDKN1A, because the product of this p53-responsive gene can be expected to repress genes by hypophosphorylation of Rb family members and the conversion of E2F transactivators into repressors (16). Therefore, we induced p53 accumulation and activity by treating HCT116 cells with doxorubicin, as verified by immunoblot detection of p53 and p21 (Fig. 7A, lanes 1 and 2). In addition, cells of the same line were used that carry targeted disruptions of the p21/CDKN1A and p53 genes (20) (Fig. 7A, lanes 3–6). All three cell lines were treated and analyzed in parallel. Eleven genes that were previously reported to be repressed by p53 (Table I) were found down-regulated in doxorubicin-treated wild type HCT116 cells (Fig. 7B). The known p53-inducible gene PIG3 (29, 37) was activated. Both repression and activation were defective in p53−/− cells, as expected. Surprisingly, however, in cells lacking p21/CDKN1A, p53 was no longer capable of down-regulating the expression of any gene that was repressed in wild type cells, while still being able to induce PIG3. Hence, expression of p21/CDKN1A is mandatory to allow p53-mediated negative regulation of all of these genes.

p21/CDKN1A Is Sufficient for Repression—The results described above indicate that p21 is required for repression of
transcription by p53. This raised the question whether the enhanced expression of p21 might be sufficient for this effect, even in the absence of p53. To test this, p21 was overexpressed in H1299 cells using an adenovirus vector. For comparison, p53 and β-galactosidase were expressed in parallel experiments. The amounts of p21 were comparable when endogenous expression was induced by p53 or when exogenous p21 was expressed by the virus, as determined by immunoblot analysis. Subsequently, the mRNA levels of p53-repressible genes were analyzed (Fig. 8B). In most cases, p21 alone was sufficient to repress these mRNA levels to an extent comparable with the effect of p53. In some cases (e.g. stathmin; see Fig. 8B), however, repression was less pronounced in the presence of exogenous p21, when compared with p53. In another case, the mRNA levels of LBR were not reduced by p53 or by p21, whereas they were repressed in HCT116 cells (see Fig. 7B), arguing that the susceptibility of this gene to repression depends on the cell type. Thus, in some cases, p53 appears to employ cellular factors other than p21 to efficiently repress gene expression. Nonetheless, p21 is not only uniformly required for repression but also sufficient for the negative regulation of the majority of

| Gene | References reporting negative regulation by p53 | References reporting induction by E2F proteins or repression by pRb | References reporting promoter association with E2F proteins |
|------|-----------------------------------------------|-------------------------------------------------|--------------------------------------------------|
| BRCA1| 60,61                                         | 62,63                                           | 64,65                                           |
| CDC2 | 46                                            |                                                 | 68                                              |
| CDC25C| 45,66,67                                      |                                                 | 64                                              |
| CHEK1| 17,69                                         |                                                 |                                                  |
| Cyclin A2 | 67                                               | 62                                          | 64,65                                           |
| Cyclin B1 | 45,67,70,71                                            | 62                                          |                                                  |
| LBR   | 72                                            |                                                 | 64                                              |
| POLD1 | 73                                            |                                                 |                                                  |
| Stathmin | 11,74,75                                       | 62                                          |                                                  |
| Survivin | 12,50                                          |                                                 |                                                  |
| Top 2a | 67,72                                         | 62,63                                           | 64                                              |

Fig. 7. Requirement for p21 to negatively regulate gene expression by p53. A, levels of p53 and p21 protein in doxorubicin-treated cells. HCT116 cells, wild type, or with targeted disruptions of the p21/CDKN1A or p53 genes were each treated with 350 nM doxorubicin. After 48 h, the protein levels of p53 and p21 were determined by immunoblot analysis. Staining of actin served as a loading control. Above each lane, the presence (+) or absence (−) of doxorubicin is indicated, as well as the genotype of the cells under study, with disruptions (−) or wild type copies (+) of the p53 and p21/CDKN1A genes. Note that p21 was not detected in cells lacking p53, despite the presence of the p21/CDKN1A gene in these cells (lanes 5 and 6). This was expected, because p53 appears as the principal inducer of p21/CDKN1A, and p21 levels are therefore below detectability in the absence of p53. B, impact of p53 and p21 on RNA levels. HCT116 cells were treated as in A. After 48 h, RNA was prepared, and the expression levels of the indicated genes were analyzed by RT-PCR as described in the legend to Fig. 1B.

Fig. 8. p21 as a negative regulator of gene expression. A, levels of p21 protein in transduced cells. H1299 cells were transduced with adenovirus vectors to express β-galactosidase, p53, or p21 as indicated. After 48 h, the protein levels of p21 were determined by immunoblot analysis. Staining of actin served as a loading control. B, impact of p53 and p21 on RNA levels. H1299 cells were transduced as in A. After 48 h, RNA was prepared, and the expression levels of the indicated genes were analyzed by RT-PCR as described in the legend to Fig. 1B.
It is tempting to speculate that p21 carries out its function in p53-mediated repression by inactivating cyclin-dependent kinases. This may lead to the hypophosphorylation of retinoblastoma family proteins and to the repression of E2F-responsive promoters. Retinoblastoma proteins can convert E2F proteins from transcriptional activators to transcriptional repressors (40). Strikingly, a large proportion of the known p53-repressed genes have also been reported to be regulated by E2F proteins, and in some cases, association of E2Fs with their promoters has been detected by chromatin immunoprecipitation or EMSA (Table I). The concept that p53 converts E2Fs into transcriptional repressors through p21 is also supported by the finding that the p53 antagonist Mdm2 induces transactivation by E2F1 (41). Finally, a recent study shows that the repression of stathmin in response to genotoxic stress can be inhibited by a dominant negative E2F mutant, as well as by the E7 protein from human papillomavirus type 16, which binds and inactivates Rb family proteins (42). Besides E2Fs, however, Rb family proteins may also affect the activity of other transcription factors, among them the ID2 protein (43). Possibly, Rb family proteins might also regulate cell cycle-dependent element/cell cycle genes homology element binding factors (44) or the transcription factor NF-Y that was shown to bind some p53-repressed promoters (45, 46), and each of these factors could fail to activate transcription or even act as a transcriptional repressor in the presence of hypophosphorylated Rb family proteins. The dependence of a particular promoter on several of these factors might further increase its repression by p53 and p21. This may explain why not all E2F-responsive promoters are equally susceptible to suppression by p53. Indeed, the first promoter (TA-promoter) of the p73 gene is strongly induced by E2F (47–49). Nonetheless, active p53 does not detectably influence the expression of p73TA (23). We propose that some promoters may be inducible by E2F while being only marginally susceptible to repression by the E2F/pRb complex, perhaps depending on the position of the E2F binding DNA element(s) and/or the activity of different transcription factors on these promoters.

Our analysis of the survivin promoter (Fig. 6) strongly suggests that no consensus p53 binding element is required to mediate repression. On the other hand, it was reported that removing the three spacer nucleotides between the two consensus sites of the putative p53 binding element turned transcriptional repression of this promoter into activation by p53. Therefore, it was suggested that the spacer nucleotides might render a p53 binding element susceptible to transcriptional repression by p53 (12). In contrast, it was proposed by others that p53 binding is not required to repress survivin transcription (50). Based on the results shown here (Fig. 6), we suggest that removing the spacer nucleotides in the previous report (12) newly created a p53-inducible element that overrides any repressing effect of p53 on the survivin promoter, whereas within the wild type sequence, the putative p53 binding site functions poorly and does not significantly alter transcription in any direction.

Because p53 induces apoptosis readily in cells lacking p21/CDKN1A (20, 51), without repressing the genes analyzed (this study), we propose that repression is not a requirement for p53-induced apoptosis, at least in the systems studied here. This is in contrast to the previously suggested proapoptotic role of p53-mediated repression (4–7). survivin was shown to inhibit apoptosis when overexpressed (50, 52), and its synthesis is down-regulated by p53 (Ref. 12 and this study). Nonetheless, our data suggest that down-regulation of survivin is not required for the induction of apoptosis. Apparently, p53 triggers apoptosis primarily through the induction of pro-apoptotic

**Fig. 9. Influence of c-Myc on the mRNA levels of p21/CDKN1A, survivin, and stathmin.** A, c-Myc as an antagonist of p53-mediated repression. H1299 cells were transduced with adenovirus vectors to express c-Myc and/or p53 in the indicated combinations (multiplicity of infection = 20). The mRNA levels of the indicated genes were determined by RT-PCR as described in the legend to Fig. 1B. B, failure of c-Myc to block p21-mediated repression. H1299 cells were transduced to express p21 and/or c-Myc, followed by RT-PCR analysis as in A.

**A**

| Gene       | Expr. c-Myc | Expr. p53 | Total RNA | UbC | PIG3 | Survivin | STMN1 |
|------------|-------------|-----------|-----------|-----|------|----------|-------|
| Ad c-myc   | -           | -         | 285/185   | 322 | 340  | 308      | 319   |
| Ad p53     | +           | -         | 285/185   | 322 | 340  | 308      | 319   |

**B**

| Gene       | Expr. c-Myc | Expr. p21 | Total RNA | UbC | Survivin | STMN1 |
|------------|-------------|-----------|-----------|-----|----------|-------|
| Ad-Myc     | -           | +         | 285/185   | 322 | 319      |       |
| Ad-p21     | +           | -         | 285/185   | 322 | 319      |       |

**DISCUSSION**

Our results demonstrate that the induction of p21/CDKN1A is essential for p53-mediated negative regulation of transcription, whereas the onset of apoptosis is not. This strongly argues that p53 reduces the expression of certain genes indirectly by enhancing the expression of p21/CDKN1A. c-Myc negatively regulates the expression of p21/CDKN1A and also prevents p53-mediated repression.
genes rather than the repression of anti-apoptotic genes.
p21 induces cell cycle arrest in the phases G1 and G2. p53-mediated G2 arrest does not occur in the absence of p21 (20).Consistently, the survivin gene was not only reported to inhibit apoptosis but also to promote mitosis (53). We conclude that indirect negative regulation of genes, including survivin, by p53, may be required for the G2 arrest, rather than for the induction of apoptosis. Further, the fact that overexpression of cyclin B1 or a dominant active form of cdc2 can override p53-mediated G2 arrest (54) argues that repression of cdc2 and cyclin B1 by p53 through p21 may be necessary for p53-triggered arrest in G2.

If p53 can down-regulate genes indirectly through the induction of an effector gene, similar mechanisms might apply to the regulation of certain p53-activated genes. A subset of p53-inducible genes may not be induced directly through binding of p53 to their promoters but rather indirectly through the enhanced expression of a transcriptional regulator. An example may be represented by the induction of the insulin-like growth factor-binding protein 3 gene by p53, because the product of this gene interferes with insulin-like growth factor signaling and the subsequent transcriptional regulation (55). After its discovery, a plethora of activities exerted by p53 was described in vitro and in vivo, leading to an extremely complex picture and some confusion about which of these activities are essential for tumor suppression (56). Recent knock-in studies revealed that a germline mutation corresponding to the amino-terminal domain of p53 in mice yields a phenotype that apparently cannot be distinguished from p53−/− animals (57, 58). This would imply that functions of the amino-terminal domain of p53 in mice yield a phenotype that apparently cannot be distinguished from p53−/− animals (57, 58). This would imply that functions of the amino-terminal domain of p53 in mice yield a phenotype that apparently cannot be distinguished from p53−/− animals (57, 58).

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