Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9979–9983, November 1991
Biochemistry

Wild-type p53 can down-modulate the activity of various promoters

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Communicated by Robert A. Weinberg, August 16, 1991

ABSTRACT **The wild-type (wt) p53 protein is the product of a tumor suppressor gene that is a frequent target for inactivation in many types of tumors. The nuclear localization of the protein, as well as additional features, suggest that it may be involved in the regulation of gene expression. To explore this possibility, the effects of overproduced wt p53 were investigated in a number of systems. Induction of growth arrest via the antiproliferative effect of wt p53 greatly impaired the ability of cells to exhibit an increase in c-fos mRNA upon serum stimulation. Experiments in which cells were cotransfected with p53 expression plasmids together with a reporter gene linked to various promoters revealed that wt p53 could effectively reduce transcription from a series of promoters derived from serum-inducible genes, but not from a major histocompatibility complex gene. The p53-mediated repression of c-*fos* gene expression occurred even in the presence of cycloheximide. Kinetic studies indicate that the effect of wt p53 is rapid, rather than representing a secondary consequence of growth arrest. These findings support a role for p53 in transcriptional regulation, perhaps by reducing the expression of genes that are needed for ongoing cell proliferation.**

The p53 cellular protein has been implicated in the control of cell proliferation and tumor progression (1). Allelic losses and mutations in the p53 genes are frequently observed in many types of tumors, giving rise to the suggestion that wild-type (wt) p53 is a tumor suppressor (1). This is also supported by the fact that overexpressed wt p53 efficiently inhibits oncogene-mediated transformation (2, 3). The inhibition is mediated through an antiproliferative activity of wt p53, which can lead to a reversible growth arrest (4–7). In addition, wt p53 can completely abolish the tumorigenicity of tumor-derived cells (8). The molecular mechanisms underlying these activities of wt p53 are still unknown. A possible clue is provided by the fact that p53 is predominantly a nuclear protein (9, 10). The functional importance of this nuclear localization is underscored by studies using a temperature-sensitive (ts) mutant of p53, p53val135. When cells overexpressing this mutant are shifted to 32.5°C, a temperature at which the protein assumes a wt-like conformation, their growth is arrested (4). Under these conditions, the protein accumulates preferentially in the nucleus, suggesting that a nuclear activity of p53 is responsible for its inhibitory properties (11–13). One reasonable assumption is that p53 may modulate gene expression. By interfering, directly or indirectly, with the activity of genes whose products are needed for ongoing cell proliferation, p53 may thus restrict growth and prevent tumor progression.

The augmented expression of another tumor suppressor, the retinoblastoma susceptibility gene (RB-1), can reduce the transcriptional activity of the c-*fos* promoter (14). The product of the c-*fos* gene is essential for cell growth (15, 16), and its down-regulation may be growth inhibitory. Since wt p53 also exerts antiproliferative effects, it was of interest to find out whether it could also modulate c-*fos* expression. We report here that wt p53 can inhibit c-*fos* gene expression. This effect of wt p53 is rapid, suggesting that it may precede growth arrest. In addition, cotransfection experiments indicate that wt p53 can down-modulate the activity of a number of promoters. While the effect of wt p53 appeared to be relatively nonspecific, it probably did not reflect a generalized transcriptional shut-off. Our findings suggest that wt p53 may contribute to growth inhibition by down-modulating, directly or indirectly, the expression of genes that are required for ongoing cell proliferation.

MATERIALS AND METHODS

Cells. Primary rat embryo fibroblasts (REFs) were prepared and maintained as described (3). Lines hp53val135-2 and hp53val135-8 were generated by transfecting REFs with p53val135 as described (17). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and 4 mM L-glutamine.

RNA Preparation and Analysis. Cytoplasmic RNA was prepared and analyzed as described (18). Blots were stained with 0.04% methylene blue in 0.5 M sodium acetate to visualize nonradioactive RNA.

Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. Cells (1.5×10^6) growing in a 90-mm dish were transfected and subjected to CAT analysis as described (18).

Plasmids. pLTRp53cGwt contains a chimera of mouse p53 cDNA and genomic DNA, including introns 2–9, under the transcriptional control of a Harvey sarcoma virus long terminal repeat (4). pLTRp53val135 and pLTRp53phe132 are identical in structure to pLTRp53cGwt but encode mutant proteins with a substitution from alanine to valine at position 135 and from cysteine to phenylalanine at position 132, respectively (4). pLTRp53dl is a deleted derivative of pLTRp53cGval135 missing the bulk of the protein coding region (4). pCMVp53wt and pCMVp53m (3) contain p53 cDNA under the transcriptional control of the immediate-early enhancer/promoter of the human cytomegalovirus (CMV). pCMVp53m encodes a mutant p53 with substitutions at residues 168 and 234. pCMVp53dl is a deleted derivative of pCMVp53wt, encoding only the first 13 amino acids of mouse p53 (3). RSV-c-jun and SVE-c-fos have been described elsewhere (19). All the CAT plasmids described here carry the bacterial CAT gene under the transcriptional control of different promoters contained within the following DNA fragments: pfos-CAT, human c-*fos*, positions −711 to +42 (20); β-actin, a 1.8-kilobase (kb) *Xba* I/*Hin*dIII fragment of the rat β-actin gene (21); hsc70, rat hsc70, positions −2500 to +61 (22); c-jun, a 1.6-kb fragment encompassing the human c-jun promoter (M.Y., unpublished data); p53, a 1.4-kb fragment encompassing the rat p53 gene promoter (D.G., unpublished data); major histocompatibility

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Abbreviations: wt, wild type; ts, temperature sensitive; REF, rat embryo fibroblast; FCS, fetal calf serum; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; MHC, major histocompatibility complex; SRE, serum response element; CHX, cycloheximide; SRF, serum response factor.

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complex (MHC), positions −528 to −38 of the gene coding for PD1, a porcine classical transplantation antigen (23); plasmid −225, human c-*fos*, position −225 to +42 (24); pTF1, human c-*fos*, positions −318 to −284, linked to the fragment extending between nucleotides −225 and +42 of the same promoter (24).

RESULTS

wt p53 Can Inhibit c-*fos* Induction in Response to Serum. Cells of the hp53val135-2 line are derived from primary REFs immortalized by the p53 ts mutant (11). These nontransformed cells grow efficiently at 37.5°C but undergo a complete growth arrest at 32.5°C due to the wt-like activity of the mutant p53 at that temperature (11). When such cells were serum starved at 37.5°C and then induced to resume proliferation upon readdition of serum, c-fos mRNA levels increased very rapidly (Fig. 1). However, if such an experiment was performed at 32.5°C, the induction of c-fos mRNA was clearly less efficient, as judged from a comparison with the levels of these transcripts seen 30 min after serum stimulation of similar cells maintained at 37.5°C. While the inhibition was reproducibly seen at 32.5°C, its precise extent varied among individual experiments (data not shown); in all cases, however, c-fos mRNA levels induced within 30 min of serum stimulation were at least 3 times lower at 32.5°C than at 37.5°C. This was not an effect of the low temperature *per se*, as the parental REFs (Fi) exhibited an efficient serum induction of the c-*fos* gene at 32.5°C (Fig. 1). Thus, excessive wt p53 activity can adversely affect c-*fos* gene expression in this system.

wt p53 Down-Modulates c-*fos* Promoter Activity. We next asked whether wt p53 could mediate a reduction in the transcriptional activity of the c-*fos* promoter. To that end, expression vectors for various forms of murine p53 were transfected into REFs together with pfos-CAT, a plasmid in which the human c-*fos* promoter (nucleotide positions −711 to +42; ref. 20) had been linked to the CAT gene. Cotransfection of a plasmid encoding wt p53 caused a severalfold reduction in CAT activity (Fig. 2 *A* and *B*) relative to the activity seen in the presence of an internally deleted derivative of the same plasmid, missing the bulk of p53 protein-coding sequences. Reduction was achieved by using two different plasmids, in

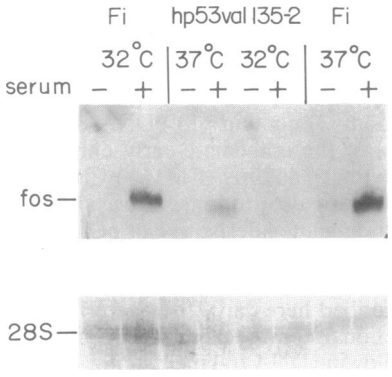

FIG. 1. Inhibition of the serum-induction of c-fos mRNA in cells overexpressing a ts p53 mutant. Subconfluent cultures of Fischer REFs (Fi) and cells of line hp53val135-2 immortalized by a ts mutant of p53 (11) were starved for 24 hr in medium containing 0.5% FCS and then either stimulated by the addition of 20% FCS (lanes +) or left in 0.5% FCS (lanes −). Cytoplasmic RNA was extracted from the cells 0.5 hr later. The experiment was performed in parallel with cells maintained at either 37.5°C or at 32.5°C; in the latter case, cultures were transferred to 32.5°C 16 hr before the onset of serum starvation. Thirty micrograms of each RNA sample was subjected to Northern blot analysis, using a mouse c-*fos* cDNA probe (*Upper*). (*Lower*) Bands of 28S rRNA obtained upon staining the blot with methylene blue.

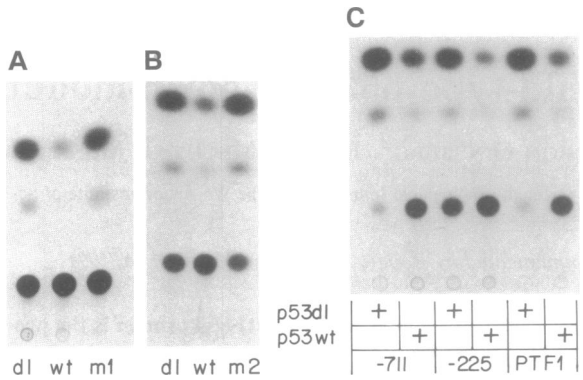

FIG. 2. (*A* and *B*) Inhibition of c-*fos* promoter activity by wt p53. Exponentially growing REFs maintained in 90-mm dishes were transfected with 3 μg of pfos-CAT and 10 μg of the respective p53 expression plasmid indicated below, driven either by the Moloney leukemia virus long terminal repeat (LTR) (*A*) or by the CMV enhancer/promoter (*B*). (*A*) Lanes: wt, pLTRp53cGwt encoding wt mouse p53 (4); m1, pLTRp53cGphe132 encoding mutant p53 with a substitution at residue 132 (4); dl, pLTRp53dl, a derivative of pLTRp53cGwt, missing the bulk of the coding region (4). (*B*) Lanes: wt, pCMVp53wt (3); m2, pCMVp53m encoding mutant p53 with substitutions at residues 168 and 234 (3); dl, pCMVp53dl, a deleted derivative of pCMVp53wt (3). Transfections and CAT analysis are described in *Materials and Methods*. Extracts were prepared 40 hr posttransfection. (*C*) Effect of wt p53 on deleted derivatives of the c-*fos* promoter. REFs were transfected with 10 μg of either pLTRp53cGwt or pLTRp53dl, along with 3 μg of either pfos-CAT (lanes −711) or a CAT plasmid carrying the first 225 upstream nucleotides of the human c-*fos* gene (lanes −225) or pTF1, containing these 225 nucleotides preceded by a segment carrying the SRE (nucleotides −318 to −284) (24). Experimental details are the same as in *A* and *B*.

which the expression of wt p53 was driven either by a retroviral long terminal repeat or by the CMV immediate-early enhancer/promoter (Fig. 2 *A* and *B*). No such reduction was effected by either of two plasmids encoding different tumor-derived p53 mutants, despite the fact that such plasmids cause the production of higher levels of p53 than the wt p53 plasmids (3, 25). It is noteworthy that both these p53 mutants are completely devoid of any antiproliferative activity (2–4, 26). Similar results were also obtained with a third mutant, p53val135 (data not shown). Hence, the ability of p53 to exhibit growth-inhibitory effects is tightly linked with its ability to down-regulate c-*fos* promoter activity.

To map more precisely the region of the c-*fos* promoter that is responsive to the inhibitory effect of wt p53, deleted versions of pfos-CAT (24) were used. As shown in Fig. 2*C*, the sequences necessary for down-regulation reside within a segment encompassing the proximal 225 nucleotides of the promoter; the presence of the serum response element (SRE), located further upstream, does not affect the response (compare plasmids −225 and PTF1).

wt p53 Down-Modulates the Activity of Various Promoters. To determine whether the observed down-regulation was specific to the c-*fos* promoter, the effect of wt p53 on a series of other promoters was assessed. As shown in Fig. 3, overexpression of wt p53 in REFs reduced the activity of a variety of promoters, including those of the β-actin, p53, hsc70, and c-*jun* genes. Thus, a very broad specificity was apparently displayed. Yet, this effect did not result from a general block of RNA polymerase II-dependent transcription, since the activity of a class I MHC gene promoter was not affected at all (Fig. 3).

Kinetics of Transcriptional Repression by wt p53. These effects of wt p53 could either reflect an ability of wt p53 to affect transcription, or else they may represent a secondary consequence of growth arrest. To distinguish between these

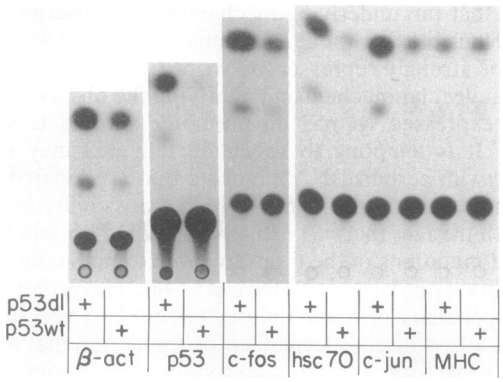

FIG. 3. Effects of wt p53 on the activity of various promoters. REFs were transfected with 10 μg of either pLTRp53cGwt or pLTRp53dl, along with 3 μg of a plasmid in which the CAT gene was linked to one of the following promoters: β-actin (β-act), p53, c-*fos*, hsc70, c-*jun*, a porcine class I MHC gene (MHC). See *Materials and Methods* for details of the plasmids. Experimental details are the same as in Fig. 2. Due to marked differences in the relative activities of some promoters, the data shown represent nonidentical exposure times.

possibilities, a kinetic analysis was performed. Cells of an additional cell line, hp53val135-8, possessing the same phenotype as hp53val135-2 (data not shown) were used. As in the case of hp53val135-2 (Fig. 1), prolonged exposure to 32.5°C resulted in a marked reduction in the ability of the c-*fos* gene to respond to serum (Fig. 4*A*). Most remarkably, a prominent inhibitory effect (75% of the maximum inhibition) was already evident after as early as 3 hr at 32.5°C. At this time, while the vast majority of p53 is already in the nucleus, the translocation is still incomplete (data not shown). This nuclear translocation is correlated with, and probably conducive to, the growth arrest (11–13). Furthermore, p53 appears to arrest cells primarily at a point near the G_1/S boundary (4–6, 13), and it seems most unlikely that 75% of the cells could have reached this point and entered a growth arrest within 3 hr. It thus appears that inhibition of c-*fos* promoter activity is an early effect of overexpressed wt p53, which is exerted well before most cells have become growth arrested. Additional support for this conclusion was provided by a cotransfection experiment (Fig. 4*B*). When cells were harvested 8 hr after transfection, very low CAT activity levels were present, indicating that products of the transfected genes were just beginning to accumulate. Yet, even at this early stage, the extent of c-*fos* promoter inhibition was already as prominent as after 24 hr. Both these experiments support the notion that the inhibition of promoter activity by wt p53 is not a secondary consequence of growth arrest.

Effect of Cycloheximide (CHX) on Transcriptional Repression by wt p53. It has previously been suggested that p53 may be a positive regulator of gene expression (27–29). It is thus possible that the observed inhibition of promoter activity by wt p53 may reflect not an ability to repress transcription directly, but rather a capacity to induce the synthesis of transcriptional repressors. Even though the data shown in Fig. 4 argue that the effect of p53 is rapid, they do not necessarily imply that it is direct. Some insight into the underlying mechanism may be gained by assessing the influence of protein synthesis inhibitors on p53-mediated transcriptional repression. Therefore, cells immortalized by p53val135 were tested for their ability to restrict c-*fos* gene induction at 32.5°C in the presence of CHX. Cells were maintained for 24 hr in 0.5% FCS at either 37.5°C or 32.5°C. CHX (15 μg/ml) was then added to some of the dishes, and 2.5 hr later, part of the dishes were serum stimulated, whereas the remainder were left in low serum. In all cases, c-fos mRNA levels were determined after an additional 30

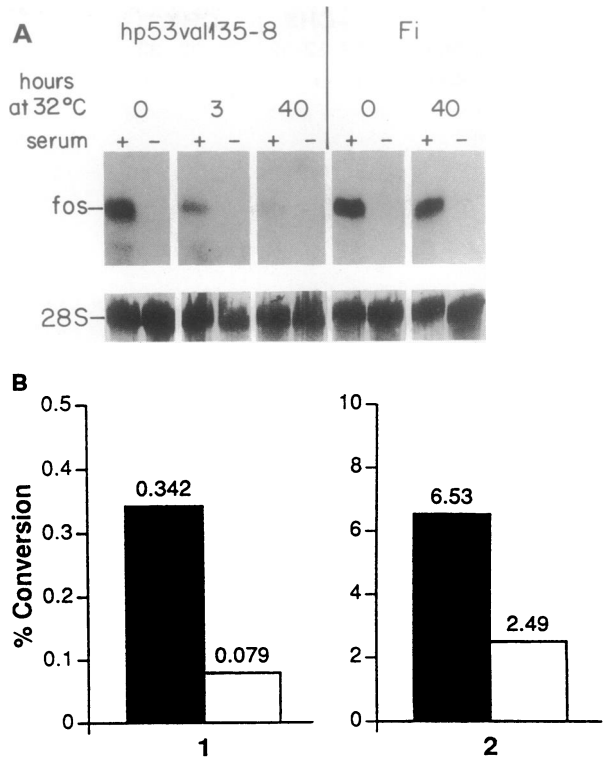

FIG. 4. (*A*) Rapid inhibition of c-fos mRNA serum induction by a ts p53 mutant. Subconfluent cultures of Fischer REFs (Fi) or cells of line hp53val135-8, immortalized by a ts mutant of p53, were starved for 24 hr in medium containing 0.5% FCS and then either stimulated by the addition of 20% FCS (lanes +) or left in 0.5% FCS (lanes −). Cytoplasmic RNA was extracted from the cells 0.5 hr later. The experiment was performed in parallel with cells maintained throughout at 37.5°C (time 0), cells maintained at 37.5°C and transferred to 32.5°C for the last 3 hr of the starvation period, and cells maintained at 32.5°C starting 16 hr prior to the onset of serum starvation (a total of 40 hr at 32.5°C). Thirty micrograms of each RNA sample was subjected to Northern blot analysis, using a mouse c-*fos* cDNA probe (*Upper*). (*Lower*) Stained bands of 28S rRNA are shown. (*B*) Kinetics of inhibition of c-*fos* promoter activity by wt p53. Exponentially growing REFs were transfected with 3 μg of pfos-CAT and 10 μg of either of the following p53 expression plasmids: pLTRp53cGwt (open bars) and pLTRp53dl (solid bars) (see Fig. 2). Cells were glycerol shocked 4 hr after transfection and collected 4 (bars 1) or 20 (bars 2) hr later. CAT analysis was as described.

min. In agreement with earlier findings (30), exposure of serum-starved cells to CHX caused a mild induction of c-fos mRNA steady-state levels (Fig. 5). The addition of serum resulted in a pronounced increase in c-fos mRNA, which was slightly augmented by the presence of CHX [under our experimental conditions, only minimal "superinduction" (30) could be observed]. As before, the increase in c-fos mRNA was interfered with by the wt-like activity of p53val135 at 32.5°C; most importantly, a significant reduction of c-fos mRNA was seen in the presence of CHX (compare lanes 5 and 6). Thus, p53-mediated inhibition of c-fos induction does not appear to require the ongoing synthesis of a short-lived protein, which could act directly as a transcriptional repressor. It should be noted, however, that the experimental conditions used in Fig. 5 do not rule out the possibility that p53 turns on the synthesis of a stable transcriptional repressor.

DISCUSSION

The results presented above demonstrate that overexpressed wt p53 can lead to a reduction in the transcriptional activity of a variety of promoters. Most of the experiments focused on the c-*fos* gene. In nontransformed cells overexpressing a

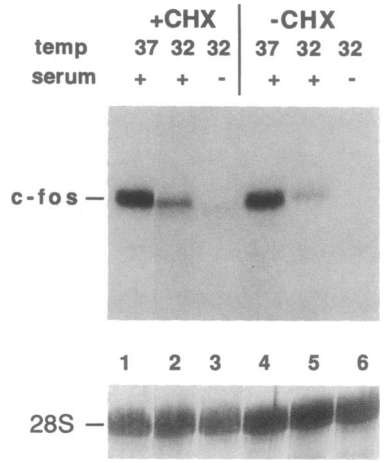

FIG. 5. Effect of CHX on the ability of wt p53 to repress serum-induced c-*fos* gene expression. Cells of line hcGp53val135-2 were subjected to serum stimulation at either 32.5°C or 37.5°C essentially as described for Fig. 1, except that CHX (15 μg/ml) was added to some of the dishes 3 hr before harvesting. Under these conditions, protein synthesis was inhibited by >98% (data not shown). Analysis of the RNA was as described in Fig. 1.

ts p53 mutant, the levels of c-fos mRNA seen after serum stimulation were reduced in the presence of wt p53 activity. Serum induction of c-fos transcription is mediated by the serum response factor (SRF) (31). In principle, one could thus propose that overexpressed wt p53 interferes with SRF-dependent signal transduction. The transient cotransfection experiments, though, do not support this notion. Thus, the inhibitory effect of wt p53 was exerted in the absence of the SRE, which is the site of action of SRF (31). In fact, no further increase in the extent of p53-mediated repression was seen when the SRE was included in the c-*fos* promoter (Fig. 2*C*). Hence, all our findings could effectively be accounted for by an ability of wt p53 to interfere with c-*fos* basal promoter activity, which would also prevent a full activation of the promoter under conditions of serum stimulation. One should bear in mind, though, that the experimental conditions used for analysis of the stable p53 overexpressors (Fig. 1) were rather different from those pertaining to the transient assays. Consequently, an SRF-mediated effect in the former case cannot be rigorously excluded at the moment.

The responsive promoters described in this study share the property of being derived from genes whose expression can be affected, to varying degrees, by the presence of serum growth factors as well as by transformation-related processes (18, 32–37). Interestingly, wt p53 also reduced the activity of the p53 gene promoter (Fig. 3), suggesting the existence of an autoregulatory mechanism.

The same cellular genes whose promoters were found to be down-regulated by p53 can often be activated by various oncogene products. For instance, the c-*fos* promoter is activated by a mutated ras protein (38). Similarly, p53 gene transcription can be enhanced by adenovirus E1A (39, 40). Both c-*myc* and E1A gene products can stimulate the hsp70 promoter (41, 42). It is therefore conceivable that products of tumor suppressor genes may be operating, at least partially, through repression of the same promoters. The ensuing reduction in the expression of a battery of growth-related genes could then provide the molecular basis for the antiproliferative effect of wt p53. This notion is in agreement with the results of Mercer *et al.* (43), reported while this manuscript was in preparation, who have demonstrated a down-regulation of proliferating cell nuclear antigen (PCNA) mRNA and protein by wt p53; although it still remains to be proven that the underlying mechanism is transcriptional, it seems plausible that the PCNA gene promoter will also turn out to be strongly repressed by wt p53.

The molecular mechanism underlying the observed effects of overexpressed wt p53 on promoter activity is still unknown. It is tempting to speculate that p53 may interact directly with certain DNA elements that are located within transcriptional control regions. Such a possibility is consistent with the fact that p53 is a DNA-binding protein (44, 45) and that mutations of the types found in tumors compromise the ability of p53 to bind DNA (45, 46). Nevertheless, the broad specificity of the inhibitory effect of wt p53 seems to support a less direct mechanism of transcriptional modulation. In its wt form, p53 interacts avidly with a variety of viral proteins including simian virus 40 large tumor antigen, the adenovirus E1B 55-kDa protein, and the human papilloma virus 16 E6 protein (reviewed in ref. 47). The best studied interaction, which involves the simian virus 40 large tumor antigen, is mediated through highly conserved regions of the p53 protein (48). Such interaction has been shown to perturb the biochemical activity of the large tumor antigen, at least as assayed in a simian virus 40 DNA replication system (49, 50). It is therefore tempting to speculate that p53 may engage in similar interactions with transcription factors. These interactions may then sequester the activity of such factors and eventually repress the expression of a variety of genes that are positively regulated by these factors.

Furthermore, the data presented here do not necessarily imply that p53 can interact directly with the transcriptional machinery. For instance, p53 could modulate the activity of a nuclear protein kinase that targets a particular transcription factor. Alternatively, p53 could somehow modify the intranuclear environment in a way that represses transcription rather broadly and nonspecifically. Yet, the fact that at least one promoter does not appear to be affected, along with the recent demonstration that another promoter can actually be strongly stimulated by wt p53 (51), argues against a general shut-off of the transcription machinery.

The rather low degree of specificity of the inhibitory effect of p53 may be a consequence of overexpression. It is possible that some or all of the promoters studied by us represent genes that are not physiological targets of p53. In fact, it is very unlikely that the effect of p53 on c-fos is responsible for p53-mediated growth arrest. While a reduction of c-fos induction by p53val135 at 32.5°C is seen in all nontransformed cell lines studied by us thus far (this paper and data not shown), such an effect could not be observed in transformed lines that overexpress p53val135 along with mutant ras (data not shown); this may represent the consequence of deregulated ras activity. Yet, an efficient growth arrest is induced by p53val135 in both types of cell lines (4, 11–13). Thus, other genes are probably involved in this process. It is possible that, when expressed at low physiological levels, wt p53 down-modulates only a small subset of promoters; it will be of great interest to identify the corresponding genes.

Note Added in Proof. Transcriptional repression of several promoters by wild-type p53 has also recently been reported by Santhanam *et al.* (52).

We wish to thank Jill Rodel-Traub for excellent technical assistance; D. Michael for providing cell lines hcGp53val135-2 and hcGp53val135-8; Dr. R. Prywes for the gift of plasmids −225 and pTF1; M. A. Dieckmann and P. Berg for plasmid pSV2hph; and Drs. P. Sassone-Corsi, U. Nudel, R. Ehrlich, and H. R. B. Pelham for plasmids pfos-CAT, β-actin-CAT, pN(−38), and hsc70-CAT, respectively. We are indebted to P. Angel and P. Herrlich for the gift of the human c-*jun* promoter, and to J. M. Gauthier and B. Bourachot for communicating unpublished results. This work was supported in part by Grant RO1 CA 40099 from the National Cancer Institute, by a grant from the Ministry of Science and Technology,

Israel, and the French Ministry of Research and Technology, and by grants from the Wolfson Research Awards administered by the Israel Academy of Science and Humanities, and the Israel Cancer Research Fund. M.O. is a scholar of the Leukemia Society of America, Inc.

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