Cell cycle progression is subject to several regulatory controls, of which the p53 protein plays a major role in growth arrest, subsequent to the detection of cellular aberrations. It is well documented that p53 has the ability to inhibit transcription driven by several promoters, possibly via distinct mechanisms. In this report, we show that expression of the cell cycle regulatory transcription factor DP1 is strongly inhibited by p53, at the level of transcription and probably through the basal TATA-less promoter. This inhibitory activity has a relative specificity for the DP1 promoter compared with the functionally related E2F1 promoter or unrelated promoters such as those of the transcription factor ATFa or the thymidine kinase gene. Inhibition of DP1 transcription has implications in one of the several possible mechanisms through which p53 induces cell cycle arrest.

The tumor suppressor protein p53 is generally expressed at low levels in normal cells. Up-regulation of p53 has diverse effects on the expression of a variety of genes and the activity of their encoded proteins, culminating in growth arrest and/or activation of the apoptotic pathway (see Refs. 1–3 for recent reviews). The p53 protein is a genuine transcription factor and can directly activate a subset of genes important for cell cycle arrest and apoptosis, through binding to specific sequences located within the promoter regions or the first introns of these genes (4, 5). However, it has also been reported that p53 can repress the transcription of a large number of genes, which do not have consensus p53 binding sequences within their regulatory regions (6). The exact mechanism underlying this p53-dependent inhibition is presently unclear, but it is thought to be the consequence of p53 inhibiting transcription activators (7, 8) or components of the basal transcription machinery (9–11). This phenomenon, which seemed to be specific for genes with “TATA box” elements, has recently also been demonstrated in promoters without a TATA consensus (12–14).

The E2F/DP family of transcription factors plays a key role in transducing proliferative signals through regulating the expression of genes whose products are involved in DNA synthesis and cell cycle control. E2F/DP significantly contributes to the transcriptional control of cell cycle regulators such as the cyclins A, D, and E, E2F1, E2F2, and c-Myc (15–17). Consequently, E2F/DP functions as a crucial cellular regulator of the G1 to S phase checkpoint, and dysregulated expression of E2F/DP activity is likely to have a drastic effect on the cell and the organism (reviewed in Ref. 18).

E2F/DP is a heterodimeric transcription factor comprising two families of proteins, the E2Fs (E2F1–5) and DPs (DP1–3) (16, 17). The E2F and DP proteins cooperate to bind DNA and activate transcription of target genes. E2F1 and DP1 were the first members of this transcription factor to be cloned and are probably the best characterized (19–21) with respect to their functions and regulation. Several aspects of the DP1 protein have been documented, including its functional domains and its proto-oncogenic potential (16, 22, 23). Its gene locus has also been described recently (24). DP1 is a phosphoprotein, and its activity is believed to be regulated through phosphorylation during the cell cycle (25). One of the kinases involved in phosphorylating DP1 is the cyclin A–cdk2 complex, which phosphorylates DP1 at the end of S phase (26, 27). This results in reduction in DNA binding activity of the E2F1/DP1 heterodimer and down-regulation of target genes at later stages of the cell cycle.

The interaction of the tumor suppressor pocket protein family pRB, p107, and p130 with E2F/DP forms the basis of the mechanism by which E2F/DP activity is regulated, during early phases of the cell cycle (28). Evidence has now emerged that there is cross-talk between the p53 and pRB/E2F pathways and that p53 also has a role in modulating E2F/DP activity. This regulation occurs via direct protein interactions between p53 and components of the E2F1/DP1 heterodimer, resulting in cross-inhibition of p53 and E2F/DP transcription activity (8). Additional level of cross-talk, but in a less direct manner, has been also shown to be mediated by the cyclin-dependent kinase inhibitor, p21 (29, 30). In the present report, we provide evidence for yet another level of control exerted by p53 on E2F/DP activity, through transcriptional repression of the DP1 promoter and to a lesser extent that of the E2F1 promoter.

MATERIALS AND METHODS

Plasmid Constructs and Transient Transfection Assays—A series of chloramphenicol acetyltransferase (CAT) reporter plasmids were con-
structed by inserting various promoter sequences in front of the cat gene of a promoterless vector (pBlCAT6) (31), generating the following recombinants: mDP1p-CAT contains the wild-type mouse DP1 promoter (mDP1p, between positions −1142 and +170) (Fig. 1 (24)); mDP1pmt1-CAT contains the same mDP1 sequences but with point mutations (CA→AT; TG→CT, at positions −1024 and −1026) that destroy the putative Myc binding site and a deletion of 82 base pairs (between positions −850 to −763) that removes the putative E2F binding site (−842 to −834) and flanking sequences; mDP1pmt2-CAT contains mDP1p sequences identical to the wild type, except for point mutations that destroy the putative Myc binding site (24); and mE2F1p-CAT contains the mouse E2F1 promoter (33); HSV-tkmin contains the minimal promoter of herpes simplex virus (31); and mATFa-CAT contains the minimal promoter of the mouse ATFa gene (32); hATFa-CAT contains the minimal promoter of the human ATFa gene (33). The clon−

### RESULTS

**Exposure of Cells to UV Light Induces Accumulation of p53 and Down-regulates DP1 mRNA Steady-state Levels**—The cloning of the mouse p53 genomic locus and preliminary characterization of the promoter sequences have been reported earlier (24). We presently report the sequence of major upstream regulatory elements (Fig. 1) which, when linked to a luciferase reporter gene and stably expressed in mouse NIH3T3 fibroblasts, mimics the induction kinetics of the endogenous gene. To gain further insight into the mechanisms involved in this regulation, experiments were undertaken to determine the nature of modulatory signals for both the endogenous gene and transfected constructs.

Exponentially growing NIH 3T3 cells were exposed to UV radiation (50 J/m²), and the steady-state levels of DP1 RNA were monitored by Northern blot analysis, over a period of 16 h. The protein level of p53 was also followed by Western analysis over the same time course. An inverse relationship between p53 protein level and DP1 mRNA level was observed, whereas p53 accumulation following UV treatment (Fig. 2A), DP1 expression was progressively reduced, compared with the level of GAPDH transcripts that remained unaffected (Fig. 2B). Because UV radiation is an inducer of p53 expression (1, 42), the measurement of modulatory signals for the involvement of p53 in DP1 regulation.

**Induction of Wild-type p53 in Clone 6 Cells Reduces the Steady-state Levels of DP1 mRNA in Vivo**—To rule out the possibility that the observed modulation of DP1 expression was due to side effects of UV irradiation, unrelated to p53 induction, we examined the pattern of DP1 transcripts under more defined conditions. Rat embryo fibroblasts (clone 6) harboring a temperature-sensitive allele of p53 (9, 40) were shifted to the permissive temperature. Normalization of DP1 mRNA levels as a ratio to GAPDH (Fig. 3B) showed that there was an average 4–5-fold decrease in DP1 mRNA levels, after wild-type p53 induction had occurred. Furthermore, we verified that a similar temperature shift had no effect on DP1

### Footnote

2 B. Bell, E. Sheer, and L. Tora, manuscript in preparation.

3 R. V. Gopalkrishnan, E. W.-F. Lam, and C. Kedinger, unpublished observation.
Northern blotting. DP1 and GAPDH mRNAs were detected by hybridization with 32P-labeled cDNA probes as described (41). Total RNA extracted at times indicated was resolved by formaldehyde-agarose gel electrophoresis, followed by Northern blotting. DP1 and GAPDH mRNAs were detected by hybridization with 32P-labeled cDNA probes as described (41).

**FIG. 2. Opposite effects of UV irradiation on p53 and DP1 expression.** A, Western blot of p53 protein in mouse NIH3T3 fibroblasts following UV irradiation. Whole cell extract (50 μg) collected at times indicated was separated by SDS-polyacrylamide gels. Following Western blotting, p53 protein was detected by the CM5 antibody. B, Northern blot analysis of DP1 mRNA levels in mouse NIH3T3 fibroblasts following UV irradiation. Total RNA extracted at times indicated was resolved by formaldehyde-agarose gel electrophoresis, followed by Northern blotting. DP1 and GAPDH mRNAs were detected by hybridization with 32P-labeled cDNA probes as described (41).

**TABLE I**

*p53 inhibits the mDP1 promoter activity*

| mDP1p-CAT reporter | Relative CAT activity | Fold inhibition by p53 |
|---------------------|-----------------------|------------------------|
| −p53                | +p53                  |                        |
| 0.5 μg CAT          | 19                    | 2                      |
| 1.0 μg CAT          | 39                    | 8                      |
| 2.0 μg CAT          | 56                    | 16                     |
| 4.0 μg CAT          | 88                    | 22                     |

Saos-2 cells were transfected using a range (0.5 to 4.0 μg) of mDP1p-CAT reporter constructs in the absence (1 μg of empty vector; −p53) or presence of p53 expression constructs (1 μg of p53WT, +p53). This amount of expression vector has previously been determined to show detectable inhibitory activity without causing nonspecific squelching effects. CAT activities were determined, and the extent of reporter inhibition was expressed as a ratio of activity observed in the absence to that in the presence of p53.

**FIG. 3. Induction of a temperature-sensitive p53 results in decreased mDP1 expression.** A, Northern blot analysis of DP1 and GAPDH mRNAs in rat embryo fibroblasts expressing a temperature-sensitive (ts) p53. Total RNA extracted at times indicated after temperature shift from 37 to 32.5 °C was subjected to Northern blot analysis as described (41). B, expression of DP1 mRNA following induction of the temperature-sensitive p53. The relative DP1 mRNA expression levels were calculated from the ratio of the DP1 to GAPDH mRNA expression levels that were quantitated using a Hoefer GS300 scanning densitometer.

expression in wild-type rat embryo fibroblasts (not shown). It therefore appeared that accumulation of active wild-type p53 was capable of down-regulating the steady-state levels of endogenous DP1 mRNA in vivo.

**Expression of p53 Inhibits the Activity of mDP1 Reporter Constructs**—To examine the effect of p53 on DP1 promoter activity in a more direct way, transient transfection experiments were performed in p53-negative Saos-2 cells, using a DP1 reporter construct (mDP1p-CAT) and assaying its activity in the absence or presence of a p53 expression vector (Table I). In accordance with the observations above, expression of p53 resulted in a marked inhibition of the DP1 promoter, as revealed by the decrease in reporter activity. A maximal level of about 10-fold inhibition was observed with 0.5 μg of reporter construct. Reduction in levels of inhibition was observed with increasing amounts of DP1 reporter DNA and constant amount of p53 expression construct, suggesting that the assay system was responsive to the amount of p53 in the cell; excess of DP1 promoter sequences partially overcame inhibition, probably when p53 levels became limiting.

**Wild-type but Not Mutant p53 Inhibits mDP1 Promoter Activity**—To determine whether inhibition of the mDP1 promoter was specifically achieved by the wild-type but not mutant p53 protein, as suggested in the temperature-sensitive mutant experiments (Fig. 3), transient transfection experiments were performed in Saos-2 cells with identical amounts of expression construct encoding either the wild-type or distinct mutant forms of p53 (see “Materials and Methods”). Only wild-type p53 had a significant ability to inhibit the activity of the mDP1 promoter (Fig. 4A). The extent of inhibition was, on an average, 7-fold compared with the activity of the mDP1 promoter co-transfected with an equivalent amount of unloaded vector or mutant constructs, under identical assay conditions.

To examine whether the different p53 expression constructs used in transfection assays expressed equivalent levels of proteins, extracts were made from appropriately transfected cells and analyzed by Western blotting, using two monoclonal antibodies recognizing distinct epitopes on the p53 molecule (Fig. 4B, top). Except for the negative control (Saos-2 cells transfected with empty vector), all four p53 constructs expressed the corresponding proteins to approximately equivalent levels under the conditions used. Furthermore, the different transfected p53 proteins displayed their expected capacity for transcriptional activation; as previously reported (Ref. 1 and references therein), only the wild type and to a much lesser extent the 22,23 mutant (35) showed biological activity based on the ability to drive expression of a cat reporter gene placed downstream of a synthetic reporter containing multiple p53 binding sites (Fig. 4B, bottom).

Under our experimental conditions, no significant levels of cell death were observed between cells that expressed wild-type p53 and those expressing mutant constructs (data not shown). Also, cells expressing wild-type p53 protein were not compromised in general transcriptional functions, since other promoters transfected in parallel exhibited comparable activity either in the presence or absence of p53 (see below).

Altogether, these observations strongly supported the conclusion that inhibition of the DP1 promoter was specifically...
exhibited only by the wild-type, functional p53 protein and not by the mutant forms. These results were also indicative of the fact that nonspecific inhibition or "squelching" of transcription by titration effects due to excess, exogenously introduced DNA or p53 protein expression was not the likely cause of this repression.

Comparative Inhibition by p53 of the mDP1p and Other Promoter Constructs—The p53 protein has been previously reported to inhibit a number of promoters (7–11) that do not contain specific p53 binding sites. To determine the specificity of inhibition, a series of transfection assays were performed using mE2F1p-, hATFap-, and the HSV-tkminp-CAT constructs (see "Materials and Methods"), in parallel with the mDP1p-CAT reporter. All four promoter constructs had identical pBLCAT6 reporter backbones. Although the basal activities of these constructs were intrinsically different, their relative levels of inhibition by p53 (Fig. 5A) were quite distinct; it appeared that the mDP1 promoter was most susceptible to inhibition by p53, followed by the mE2F1 promoter; the other two promoters were repressed to a much lesser extent. It is probably more than coincidental that the DP1 and E2F1 promoters were most sensitive to inhibition by p53, since both promoters exhibit temporally overlapping activities during the cell cycle and because both DP1 and E2F products participate in cell cycle regulation. Additionally, both genes most likely share regulatory elements that are not in common to the other two promoters.

p53 Inhibits Both Wild-type and Mutated mDP1 Promoters—To determine the mechanism by which p53 exerted its inhibitory effects on the mDP1 promoter, transfection experiments were performed using the wild-type mDP1p-CAT and two mutated mDP1 constructs as follows: mDP1mut1-CAT, mutated in the putative Myc binding site and lacking a putative enhancer element, centered around an E2F binding site and mDP1mut2-CAT point-mutated in only the putative Myc binding site (see "Materials and Methods"). Although mDP1mut2 had activity comparable to that of the wild-type promoter, the mDP1mut1 construct had approximately 3–5-fold reduced basal activity (data not shown). Despite this difference in basal activities, both mutated promoters exhibited similar fold inhibition by p53 (Fig. 5B).

This indicated that, although the mDP1mut1 construct was lacking in a putative enhancer region that encompassed sequences important for mDP1 promoter activity, it was still inhibited by p53. Thus p53 does not apparently mediate its major inhibitory effects at the level of this putative enhancer element, since deletion of those sequences, while reducing overall promoter activity, actually resulted in a greater inhibitory response to p53 (Fig. 5B).

To approach this question from another angle, E2F1, DP1,
and Sp1 proteins, all of which possess putative binding sites in the mDP1 promoter (24) and have the reported capacity to interact with p53 (7, 8, 43, 44), were unable to reverse inhibition (data not shown). Such experiments were technically difficult to perform, in terms of obtaining an optimal balance of several different overexpressed factors under the regulation of different promoters. In general, however, in every case where activation of the reporter was obtained by overexpression of an exogenous factor, the extent of p53 inhibition was still in the range of 5–7-fold, similar to when these factors were not exogenously expressed. It seems likely, therefore, that p53-mediated inhibition of the DP1 promoter does not occur at the level of enhancer sequences nor mediated through interactions with enhancer-binding proteins.

**p53 Inhibition at the Level of the mDP1 Basal Promoter—**A possible mechanism of p53-mediated inhibition, reported for several TATA box containing promoters, has been attributed to its ability to interact with diverse components of the basal transcription machinery. A majority of such studies has demonstrated the ability of components of the basal machinery such as TBP (9, 45–48), TFIIH (10, 49), and the TAFs (50–52) to at least partially counteract the inhibitory activity of p53 through overexpression of that component. Similar studies have been performed by overexpressing transcription factors such as Sp1 (7) or Mdm2 (53) to relieve inhibition.

We therefore attempted to reverse p53-mediated inhibition in cotransfection experiments by overexpressing potential competing factors, like Mdm2, TBP, and TAFII70. These experiments were performed with the HSV-tkmin- and mDP1p-CAT reporters in parallel, in the presence and absence of p53; representative data are shown for some factors (Fig. 6A). Whereas p53 alone had little inhibitory effect on the HSV-tkmin promoter activity, TBP and TAFII70β expression resulted in the inhibition of basal levels of promoter activity. This was actually reversed by p53 overexpression (compare −p53 and +p53 lanes in TBP and TAFII70β columns, respectively). Expression of Mdm2 had no effect in the presence or absence of p53 on the HSV-tkmin promoter (data not shown). When an identical series of experiments was performed with the mDP1 promoter, in the absence of p53, neither TBP (data not shown), TAFII70β, or Mdm2 had any effect on promoter activity. The ability of these proteins to overcome the inhibitory effects of p53 was, however, also very limited (Fig. 6B). As revealed by the overlapping standard deviations, there were significant fluctuations, from one experiment to the other, between experiments (a total of three independent experiments like that shown in Fig. 6A were carried out and averaged as indicated in Fig. 6B). However, within each individual experiment, the relative levels of reporter activity always varied in the same direction, depending on the cotransfected vector. The observed variations between the mean values calculated for the mDP1 promoter are very likely therefore to reflect a partial reversal of the p53-induced inhibition.

Thus, expression of basal factors seemed to perturb the activity of the HSV-tkmin promoter, an effect that was actually reversed by p53 expression, indicative of functional interactions occurring between the overexpressed proteins. However, expression of the same set of proteins in the mDP1 promoter context had significantly different effects, most likely reflecting intrinsic differences in respective promoter compositions. Neither TBP, TAFII70β, nor Mdm2 affected basal mDP1 promoter activity in the absence of p53. In its presence, small but reproducible levels of rescue from inhibition were obtained. Therefore, it seems likely that p53 exerted a major part of its inhibitory effect on the mDP1 promoter via interactions with the basal transcription machinery. It was not possible to perform a similar series of experiments using mDP1 promoter mutants deleted in the basal promoter or with more extensive deletions than mDP1mut1, since their intrinsic activity was too low to determine transcriptional inhibition or rescue.

**DISCUSSION**

In response to DNA damage, the cellular level of p53 protein increases, causing either G1 cell cycle arrest and/or apoptosis. However, the molecular mechanisms and the cellular target genes involved in this process are not well characterized. The growth-suppression property of p53 appears to be primarily related to its ability to regulate the expression of genes whose products are important for cell cycle progression and/or DNA replication. For example, the p53 protein has been shown to activate directly the transcription of negative regulators of cell growth and DNA synthesis, including p21 (54) and GADD45 (55), through binding to consensus sequences (54) within their promoters. Besides its function as a sequence-specific transactivator, p53 can also induce cell cycle arrest by repressing transcription from a variety of growth promoting genes, including those of the c-Fos (6), c-Myc (9), MDR1 (56), interleukin-6 (57), proliferating cell nuclear antigen (58, 59), O6-methylguanine-DNA methyltransferase (60), which lack consensus p53 binding se-
sequences. Transcription from this group of p53 targeted genes is directed from TATA box containing promoters, and their transcriptional repression by p53 is believed to be mediated through the TATA box binding protein (TBP).

In the present study, we demonstrate that p53 represses transcription from the mouse DP1 promoter, which contains no consensus TATA box element in its core promoter. This repression of DP1 expression by p53 is physiologically significant since induction of functional p53 by UV radiation (NIH3T3 cells) or temperature shift (rat embryonic clone 6 fibroblasts) leads to down-regulation of endogenous DP1 mRNA. More importantly, in both cases, the drop in DP1 expression is followed by cell cycle arrest in G1, as determined by fluorescence-activated cell sorter analysis (data not shown). Our finding corroborates recent reports showing that p53 also represses other promoters lacking TATA box elements, like those of the topoisomerase IIa (12, 14), ETS-1 and ETS-2 (13) genes.

It is rather unlikely that the p53-mediated repression involves direct binding of p53 to regulatory elements on the DP1 promoter, as no homology to consensus p53 binding sites could be identified within this promoter. In addition, sequence-specific binding by p53 is usually associated with transcriptional activation. Apparently, the p53-mediated repression most probably acts through protein-protein interactions with factors bound to the DP1 core promoter. Indicative of this is the fact that DP1 promoter constructs deleted or point-mutated in regions affecting overall promoter activity by a factor of 3–5-fold (data not shown), in regions located outside the basal promoter region, failed to uncouple repression by p53.

Several studies have documented the involvement of TBP (9), hTAFII70, and hTAFII31 (50–52) via direct protein contacts in mediating p53 transcriptional functions. Similarly, transcription factors such as Sp1 (7) or regulators such as Mdm2 (61) have been shown to reverse p53 inhibitory activity. Gene disruption studies in mice (62, 63) have unequivocally demonstrated an in vivo role for Mdm2 in reversing and/or potentiating the growth inhibitory and transcriptional effects of p53, in agreement with compelling in vitro data on the functional interactions between these two proteins (53). We were able to only partially reverse p53-induced transcriptional repression of the mDP1 promoter; coexpression of Mdm2 resulted in a maximum 2-fold reversal, whereas overexpression of TAFII70 and TBP had even lower or undetectable effects.

It has been suggested that the sensitivity of a particular promoter to p53 depends on the composition of the transcription initiation and/or upstream activator complex (11). The partial reversal effects we observe may actually reflect the affinity of p53 for the specific (basal) transcription factor configuration present on the mDP1 promoter. Comparative promoter strength studies have indicated that the mDP1 reporter construct exhibits higher activity than the virally derived SV40 consensus TATA box element in its core promoter. This repression of p53 represses DP1 expression at the transcriptional level, which is at least partly mediated through modulation of promoter activity. Our studies have demonstrated an alternative pathway by which p53 could turn down E2F-DP1 activity and block cell cycle progression. Our studies have indicated that the E2F1 promoter is also susceptible to p53 inhibition (Fig. 5). Although the extent of this inhibition is lower than that of the mDP1 promoter, it may clearly have relevance to cell cycle arrest phenomena.

In summary, this work provides evidence that DP1 is a physiologically important cellular target of p53. Overexpression of p53 represses DP1 expression at the transcriptional level, which is at least partly mediated through modulation of promoter activity. Our results suggest that p53 very likely represses DP1 expression by targeting the basal core promoter through protein contact-mediated mechanisms. At present, it is unclear if common features or inhibitory mechanisms exist between the various TATA-less containing promoters repressible by p53. The inhibition of DP1 transcription by p53 could represent an alternative mechanism whereby p53 accumulation can induce cell cycle arrest. The exact mechanism of p53 repression of DP1 transcription and its specific functional significance in cell cycle regulation remain to be determined and are the subject of our present investigations.

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