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Gene regulation by temperature-sensitive p53 mutants: Identification of p53 response genes

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ABSTRACT **The ability of the p53 protein to act as a sequence-specific transcriptional activator suggests that genes induced by p53 may encode critical mediators of p53 tumor suppression. Using a tetracycline-regulated p53 expression system and cDNA library subtraction procedure, we identified several p53-induced gene transcripts in human Saos-2 osteosarcoma cells that are novel on the basis of their size, regulation, and low abundance. Wild-type p53-dependent induction of these transcripts was observed in cells that are growth arrested by p53, as well as in cells that undergo apoptosis upon expression of an inducible wild-type p53 transgene. These results show that p53 activates the expression of numerous response genes and suggest that multiple effectors may play a role in mediating cellular functions of p53.**

Inactivation or loss of p53 is a common event associated with the development of human cancers. Functional inactivation may occur as a consequence of genetic aberrations within the p53 gene, most commonly missense mutations, or interaction with viral and cellular oncogenes (for reviews see refs. 1–4). Loss of wild-type (wt) p53 functions leads to deregulation of the cell cycle and DNA replication, inefficient DNA repair, selective growth advantage, and, consequently, tumor formation (1–6).

Biochemical studies have suggested several potential mechanisms underlying p53-mediated growth suppression. Of particular interest is the ability of p53 to act as a transcription factor. First, p53 has been shown to suppress a variety of promoters containing TATA elements (e.g., refs. 7–10). This suppression does not appear to involve binding of p53 to promoter sequences and may involve p53 binding to components of the basal transcription machinery, such as the TATA-binding protein (e.g., refs. 11–13). In contrast, transactivation by p53 is sequence dependent and correlates with its binding to specific DNA sequences matching the recently reported consensus binding site 5′-RRRC(A/T)(A/T)GYYY-3′ (14, 15). p53 can efficiently activate transcription from promoters bearing such sites, both *in vivo* and *in vitro* (9, 11, 16–19). Most oncogenic mutants of p53 have lost both the transcription suppression and the sequence-specific transactivation properties displayed by wt p53. However, the role of transcriptional repression in p53-mediated tumor suppression is still unclear. Conversely, recent studies have indicated that the sequence-specific transcriptional activation is essential for growth suppression by p53 (20), suggesting that target genes activated by p53 may play a critical role in mediating the function of p53 as a tumor suppressor. A limited number of endogenous genes have been characterized to be induced by p53 (21–23). Of these, WAF1/CIP1/p21, an inhibitor of cyclin-dependent kinases (24, 25), and GADD45 have been shown to inhibit the growth of tumor cells in culture (23, 26). However, growth suppression appears not as pronounced as that mediated by p53, suggesting that additional potential mediators of p53 may exist.

This study describes an approach we have taken to isolate novel p53 target genes. Using a tetracycline-regulated inducible p53 expression system and cDNA library subtraction procedure (27), we identified p53-induced gene transcripts, at least one of which derives from a direct p53 response gene. Induction of these transcripts was observed in cells that are growth arrested by p53, as well as in cells that undergo apoptosis upon expression of an inducible wt p53 transgene. This demonstration that p53 activates the expression of numerous response genes suggests that multiple effectors may play a role in mediating cellular functions of p53.

MATERIALS AND METHODS

Plasmid Construction. Plasmids pUHD15-1Neo *tet* repressor–herpes simplex virus transactivator protein VP16 (tTA) fusion-protein expression construct, pUHC13-3 [tTA-responsive luciferase construct, contains *tet* operator (tOp) sequences], and pUHG10-3 (tTA-responsive expression vector; ref. 28) were obtained from H. Bujard, University of Heidelberg. The cDNA encoding p53 mutant at codon 143 (V to A substitution) was subcloned from plasmid pC53-SCX3 (29) into the *Bam*HI site of pUHG10-3, giving rise to pTE9.5. pTE3.1 was constructed by subcloning the cDNA encoding p53 mutant at codon 247 (N to I substitution) from plasmid Gal4-53 247 (30) into pUHG10-3. The p53-responsive luciferase reporter construct was generated by replacing the tOp sequences of pUHC13-3 with two copies of a DNA fragment, 5′-TCGAGCTTGCCTGGACTTGCCTGCCAGATCTGTCGACGGAGG-3′, containing the RGC p53 binding site (31), yielding plasmid mRE10. All recombinant constructs were characterized and confirmed by automated DNA sequencing (Applied Biosystems).

Development of p53-Expressing Cell Lines. Saos-2 cells were grown as recommended (American Type Culture Collection, Bethesda). Cells were cotransfected by electroporation (GIBCO electroporator) with the tTa expression plasmid pUHD15-1Neo (providing resistance to G418) and either pTE9.5 or pTE3.1 at a 1:10 ratio. Cells were selected in medium containing G418 (250 units/ml, GIBCO) and tetracycline (1 μg/ml, Sigma). G418-resistant colonies were cloned and expanded, yielding Saos-2-A3 and Saos-2-D4. Derivatives of Saos-2-A3 and Saos-2-D4 containing the luciferase reporter construct mRE10 were subsequently established by using pBShygro for positive selection (expresses the bacterial hygromycin-resistance gene; gift of M. Lynch, Bristol-Myers Squibb) of resistant cells in medium containing hygromycin B (150 units/ml), yielding clonal lines Saos-2-A3B and Saos-2-D4H, respectively. EB and clonal EB1 cells were maintained at 37°C and 9% CO_2 in Dulbecco's modified Eagle's medium (GIBCO) as described (32).

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Abbreviations: tOp, *tet* operator; tTA, *tet* repressor–VP16 transactivator; wt, wild type.

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Luciferase Assay. Cell extracts were prepared in 100 μl of 2× luciferase buffer (30 mM glycylglycine, pH 7.8/30 mM $MgSO_4$/1 mM EGTA/2 mM dithiothreitol) containing 1% (vol/vol) Triton X-100. Luciferase activity was determined by reading in a luminometer (Berthold model LB 952 T/16 or Dynatech model ML 1000) the relative luminescence produced by 50 μl of extract (corrected for protein concentration) following injection of 100 μl of assay buffer [1× luciferase buffer with 2 mM ATP (pH 7.0) and 0.5 mM luciferin].

Northern Blot and Western Blot Analyses. Total RNA was prepared by the acid guanidinium thiocyanate single-step isolation method (33). Poly(A)$^+$ RNA was prepared with Oligotex-dT (Qiagen, Chatsworth, CA). Northern blot analysis was as described (34). Quantitation of Northern blots was performed by laser densitometry (Molecular Dynamics) of the autoradiograms or by exposing the blots to phosphorimaging plates followed by analysis on a phosphorimager (Fuji).

Nuclear proteins (80 μg) were resolved by SDS/12% polyacrylamide gel electrophoresis. Proteins were electroblotted onto a poly(vinylidene difluoride) membrane (GIBCO) and immunoblot analysis with p53 monoclonal antibody DO1 (mAB-6, Oncogene Science) was performed as described (35). Western blot reactions were detected by a chemiluminescence-based photoblot system (GIBCO). Quantitation of the autoradiogram was as described above.

cDNA Libraries. The cDNA library subtraction procedure was performed essentially as described (27), except where noted below. cDNA was divided into three aliquots of which one was digested with *Hae* III and one with *Rsa* I. Linker addition, PCR amplification, preparation of biotinylated driver DNA, hybridization, and hybrid removal were as described (27, 34). cDNA cloning and screening began after the third round of subtraction. The cloned cDNA fragment that was identified (W4.5) was then added to the driver to suppress the fragment so that different cDNAs could be enriched in a fourth-round screen.

RESULTS

Development of Cell Lines Carrying Inducible p53 Genes. Saos-2 human osteosarcoma cells were chosen as the parental cell line because (*i*) they are null for p53, (*ii*) overexpression of exogenous wt p53 in these cells inhibits their growth (36), and (*iii*) in transient-expression assays, several temperature-sensitive mutants of human p53, including the naturally occurring mutants p53N247I (30) and p53V143A (29) used in this study, activate at the permissive temperature (30°C) a reporter gene bearing upstream p53-responsive elements (data not shown).

A tetracycline-regulated expression system established by Gossen and Bujard (28) was used to develop stable cell lines. The p53 cDNAs were cloned downstream of a minimal promoter carrying bacterial tOp sequences. A cytomegalovirus promoter drives constitutive expression of a tTA fusion protein which in the absence of tetracycline binds to tOp and activates transcription of the p53 expression construct. Addition of tetracycline inactivates tTA DNA binding, and thereby transcription. Thus, removal of tetracycline from the cell culture medium should result in accumulation of p53 protein. Distinct clones, Saos-2-A3 and Saos-2-D4, displaying tetracycline-regulated expression of the two mutant p53 proteins (p53N247I and p53V143A, respectively) were selected and expanded. Fig. 1 shows regulated expression of p53 in these cells at 37°C upon removal of tetracycline from the culture medium. Basal expression is essentially undetectable in the Saos-2-D4 cell line. Induction of p53 is >20-fold in the clonal Saos-2-D4 cells and ≈5-fold in Saos-2-A3 cells, with the maximum induced levels of p53 being comparable in both cell lines.

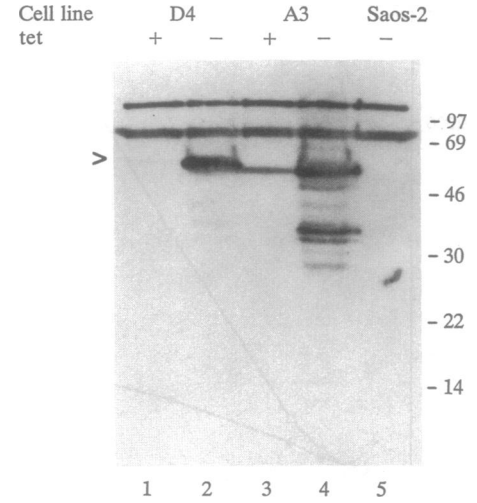

FIG. 1. p53 protein expression in Saos-2-A3 and -D4 cells. Parental Saos-2 cells and the Saos-2-D4 and -A3 derivatives were grown overnight without or with tetracycline (tet, 1 μg/ml) as indicated. Nuclear p53 protein was detected by Western blot analysis. Position of full-length p53 protein is marked by the arrowhead. Location of molecular size standards is indicated at right (scale is in kilodaltons).

Fig. 2 shows that induction of p53 is associated with activation of gene expression. Saos-2-A3B and Saos-2-D4H cells are derivative cell lines containing an integrated luciferase reporter construct with two copies of the RGC p53 binding site (31). Saos-2-A3B and -DH4 cells grown without tetracycline showed strong induction upon incubation at 30°C (Fig. 2*A*). Induction was more pronounced in D4H cells, reaching a 10-fold increase by 4 hr and 200-fold by 24 hr of incubation. No (D4H) or only weak (A3B) induction upon temperature shift was detected in the presence of tetracycline.

Temperature-reactivated p53V143A, besides activating an exogenous promoter carrying a genomic p53-responsive element in an artificial context (Fig. 2*A*), also induced expression of the endogenous hdm-2 gene (Fig. 2*B*), a known p53 target gene (21). In the absence of tetracycline, induction of hdm-2 mRNA in D4H cells was maximal by 8 hr after temperature-shift (10-fold; Fig. 2*B*). The subsequent, rapid decrease in hdm-2 mRNA levels may have resulted, in part, from the rapid decrease in p53 expression occurring during this time period (data not shown).

Identification of p53-Regulated Genes. Experiments described above suggested that the Saos-2-D4H cell line would represent a useful tool for the isolation of p53-regulated genes. To this end, cDNA was generated from RNA prepared 8 hr after temperature shift from cells kept under three different conditions, referred to as: p53 "null" (incubated with tetracycline at 30°C), "wt" (incubated without tetracycline at 30°C), and "mutant" (incubated without tetracycline and maintained at 37°C). The method used to enrich for sequences induced by wt-like p53—i.e., temperature-reactivated p53V143A—was based on a PCR-driven library subtraction procedure (ref. 27; see *Materials and Methods*), the null and mutant PCR fragments serving as driver and the wt fragments as tracer in three successive rounds of hybridization and enrichment.

Identification of Intermediate-Abundance p53-Regulated Transcripts. All colonies that hybridized strongly with the third-round-enriched probe were composed of one cDNA fragment, called W4.5, which hybridized in Northern blot analysis to an ≈2.2-kb mRNA that was strongly induced

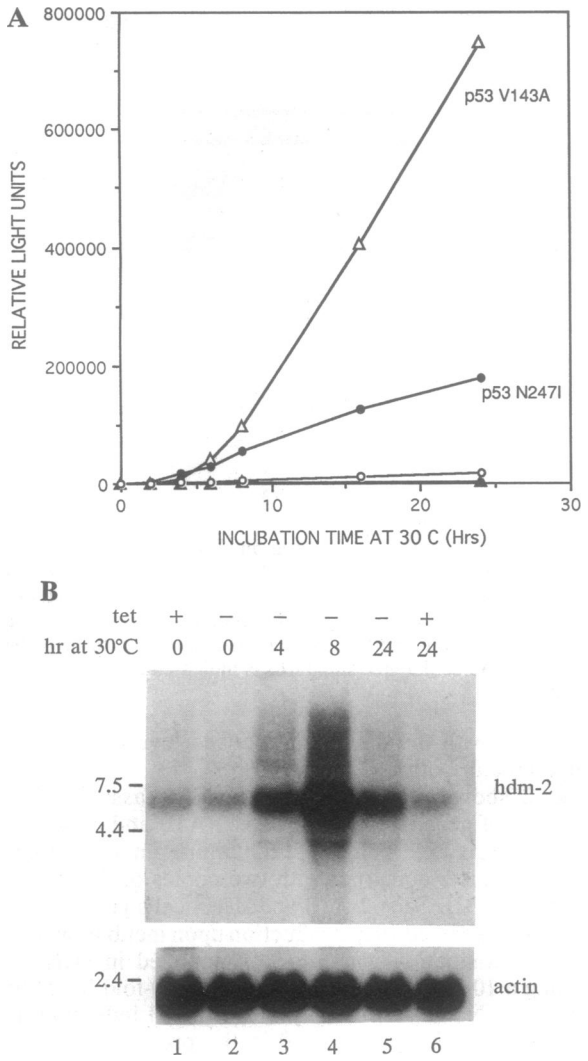

FIG. 2. Induction of gene expression by temperature-sensitive p53 mutants. (*A*) Activation of a p53-responsive luciferase reporter gene. Saos-2-A3B and -D4H cells were grown in medium without (● and △, respectively) or with (○ and ▲, respectively) tetracycline (1 μg/ml). After 16 hr of incubation at 37°C, cells were switched to 30°C (0 time) for the indicated times. (*B*) Induction of hdm-2 gene expression in Saos-2-D4H cells. Saos-2-D4H cells were grown overnight without or with tetracycline (tet, 1 μg/ml) and subsequently incubated at 30°C for the time indicated. Total RNA (10 μg) was analyzed by Northern blot analysis.

upon shifting cells to the permissive temperature of 30°C (wt status). Fifteen-fold induction was achieved by 8 hr after temperature shift (Fig. 3). No DNA sequence homologies to W4.5 were initially detected by a search of the GenBank data base. However, while we were characterizing this gene in more detail, a report by El-Deiry *et al.* (23) appeared,

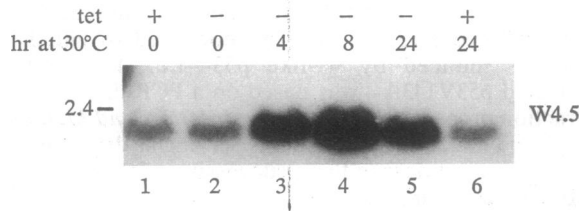

FIG. 3. Enriched cDNA fragment W4.5 identifies a p53-regulated transcript. Northern blot was prepared using total RNA (10 μg per lane) isolated from Saos-2-D4H cells incubated in the absence or presence of tetracycline (tet, 1 μg/ml) at 30°C as described for Fig. 2*B*.

describing the identification of a p53-induced gene encoding a transcript of ≈2.1 kb, called WAF1. Sequence comparison revealed that the DNA sequence of W4.5 is identical to that of a 466-bp *Rsa* I restriction fragment present in the 3′ untranslated region of the published WAF1 cDNA sequence (data not shown). By comparison with the abundance of β-actin mRNA, the W4.5 transcript belongs to a class of intermediate-abundance transcripts (0.1–0.05% of total mRNA).

The PCR-based library subtraction procedure tends to efficiently enrich for the most abundant and differentially expressed transcripts. To enrich for less abundant but regulated cDNA sequences, W4.5 fragments were driven out of the enriched cDNA library. This led to the identification of two nonoverlapping cDNA fragments, W5.5 and B26, both of which turned out to represent sequences derived from the hdm-2 gene. Similar to the results obtained with the W4.5 probe, the hdm-2 transcript belongs to the intermediate-abundance transcripts in these cells.

Identification of Low-Abundance p53-Regulated Transcripts. After repeated library screening, single clones were identified for two nonoverlapping cDNA fragments, A26 and A28. Upon hybridization to the starting material both fragments appeared to be encoded by low-abundance but regulated transcripts. This was confirmed by Northern blot analysis. Due to the low abundance of these transcripts (<0.005% of total mRNA, based on comparison with β-actin), poly(A)$^+$ RNA was prepared from DH4 cells grown in the absence of tetracycline and maintained at 37°C or 30°C for 7 hr.

Northern blot analysis using probe A28 revealed an ≈2.5-kb hybridizing mRNA that was induced upon temperature shift in a p53-dependent manner (Fig. 4, lane 1 vs. lane 2). No induction was observed in the presence of tetracycline (data not shown). The size of the mRNA is distinct from that of other characterized p53-activated transcripts and appears to be encoded by a novel regulated gene. No sequence homologies were detected upon searching the GenBank data base (data not shown). The gene encoding the A28 sequence appears to be a direct p53 response gene. This was examined in cells that were treated as before, except that the protein synthesis inhibitor cycloheximide (10 μg/ml) was added 30 min before temperature shift (cycloheximide treatment resulted in >95% inhibition of protein synthesis as judged by [^{35}S]methionine labeling of cells; data not shown). Cycloheximide treatment increased the basal (noninduced) levels of the A28 transcript, but did not prevent p53-dependent induc-

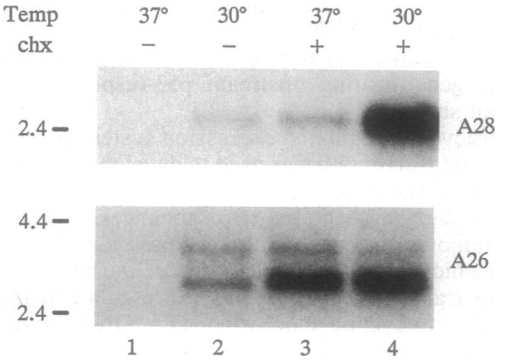

FIG. 4. Clones A28 and A26 identify transcripts regulated by the temperature-sensitive mutant p53V143A in Saos-2-D4H cells. Saos-2-D4H cells were grown in the absence of tetracycline at 37°C. Cells were treated with vehicle (water) or cycloheximide (chx, 10 μg/ml) for 30 min and subsequently incubated for 7 hr at 37°C or 30°C as indicated. Northern blots were prepared with equal amounts of poly(A)$^+$ RNA and hybridized sequentially with ^{32}P-labeled cDNA probes for A28 and A26. Autoradiograms are shown with the position of RNA size markers (kb) indicated on the left.

tion of this transcript (12-fold induction; Fig. 4, lane 3 vs. lane 4). Cycloheximide-mediated RNA stabilization, referred to as superinduction, has been observed for a number of RNA species and has been well characterized for the immediate-early serum response genes (for review, see ref. 37). As previously proposed, the lack of feedback inhibition or the turnover of labile inhibitors could account for this stabilization.

Probe A26 detected two RNA species of about 3 and 4 kb upon Northern blot analysis. Both RNA species were specifically induced in a p53-dependent manner upon temperature shift of D4H cells (5-fold; Fig. 4, lane 1 vs. lane 2). No induction was observed in the presence of tetracycline (data not shown). However, although A26 is regulated, it is unclear to date as to whether it represents a direct p53 response gene. In cycloheximide-treated cells, the basal A26 transcript was also increased; however, in contrast to the A28 transcript, no induction was observed upon subsequent temperature shift (Fig. 4, lane 3 vs. lane 4).

Induction of p53-Regulated Transcripts Correlates with p53-Induced Apoptosis. To complement our studies on the regulation of the A28 and A26 transcripts in Saos-2 cells, whose growth is reduced, but not abolished, upon activation of p53V143A as a consequence of temperature shift (data not shown), we studied the regulation of these transcripts in a colorectal carcinoma cell line (EB) carrying an inducible wt p53 gene. In these EB cells, expression of wt p53 is placed under the control of a metallothionein promoter that is activated by metal ions such as zinc or cadmium (32). Expression of p53 in these cells, both in culture and as an established tumor in animals, leads to apoptosis (32). Poly(A)$^+$ RNA was prepared from cultured parental EB and clonal EB1 cells (carrying the wt p53 transgene) treated without or with cadmium (6 μM) for 10 hr. Northern blot analysis showed that the A28 transcript was strongly and specifically induced (>20-fold; Fig. 5) in cadmium-treated, p53-positive clonal EB1 cells. Similarly, transcripts hybridizing to probe A26 were induced (5-fold; Fig. 5). Interestingly, the major RNA species detected by the A26 probe were 2 and 3 kb, compared with 3 and 4 kb for Saos-2-D4H cells.

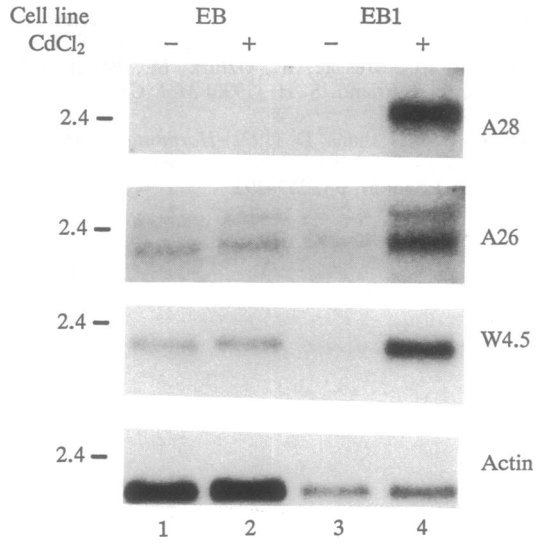

FIG. 5. A28 and A26 transcripts are induced by wt p53 in EB1 colon carcinoma cells. Parental EB and clonal EB1 cells (containing the wt p53 gene under control of the metallothionein promoter) were treated with vehicle (water) or 6 μM $CdCl_2$ for 10 hr as indicated. Poly(A)$^+$ RNA was isolated and Northern blots were prepared in quadruplicate. Blots were hybridized with probes for clones A28, A26, W4.5, or β-actin. Autoradiograms are shown with the position of molecular size markers (kb) indicated on the left.

Upon longer exposure a 4-kb RNA species was also detected (data not shown), suggesting that A26 transcripts may be alternatively spliced in different tissues. WAF1/CIP1/p21 mRNA was induced 8-fold under these conditions (Fig. 5), whereas β-actin mRNA levels remained unchanged.

DISCUSSION

Recent studies have indicated that the sequence-specific transcriptional activation is essential for p53-mediated growth suppression. Most, if not all, mutant forms of p53 fail to activate transcription from binding sites found within genomic DNA fragments (2, 20). Alternatively, the N- and C-terminal ends of p53 appear to be functionally replaceable with foreign transactivation and dimerization domains, respectively, indicating that conservation of the central region of p53, which mediates sequence-specific DNA binding, is sufficient and necessary to confer growth-suppressive properties on such hybrid proteins (20). Taken together, these studies imply that target genes activated by p53 play a critical role in the p53 tumor-suppression pathway. Two growth-inhibitory p53 response genes, WAF1/CIP1/p21 (23), coding for an inhibitor of cyclin-dependent kinases, and GADD45 (22, 26), a growth-arrest- and DNA-damage-induced gene, may encode such potential mediators of p53 tumor suppression. In this study we identified two additional gene transcripts induced by wt p53, but not mutant forms of p53, that may encode mediators of p53 functions.

Certain naturally occurring temperature-sensitive mutants of p53 retain their ability to bind to DNA and activate transcription from genomic p53 binding sites when expressed at the permissive temperature (usually 30–34°C). Taking advantage of this phenomenon, we established Saos-2 human osteosarcoma cells (deficient in endogenous p53 expression) conditionally expressing two naturally occurring mutants of p53, p53N247I, previously recognized as a temperature-sensitive mutant (30), and p53V143A, characterized as a very active temperature-sensitive mutant during the course of this study. Both mutant proteins activated transcription from an integrated reporter gene containing a genomic RGC p53 binding site and from the endogenous hdm-2 gene, demonstrating that these temperature-reactivated mutant proteins can mimic the sequence-specific transactivation function of wt p53. Given its sensitivity to p53-dependent transactivation, the p53V143A-expressing cell line, Saos-2-D4H, was used to identify p53-induced gene transcripts. The more abundant cDNAs, isolated first, represented sequences encoded by the recently cloned WAF1/CIP1/p21 and hdm-2 genes (see Introduction), thus confirming that the temperature-reactivated p53V143A protein efficiently activates endogenous p53 target genes. Less abundant cDNAs derived from novel p53-regulated transcripts of low abundance (expression is at least 1 order of magnitude less than that for WAF1/CIP1/p21 and hdm-2 transcripts). Of these, at least one, A28, is encoded by a direct response gene, as ongoing protein synthesis is not required for induction. In contrast, it is unclear whether the gene encoding A26 is a direct response gene, as induction by p53 is blocked by cycloheximide. This may be interpreted in several ways: (*i*) the A26 encoding gene is not a direct response gene; (*ii*) due to the stabilization of the A26 RNA and possible activation of alternative signaling pathways upon treatment with cycloheximide, direct induction by p53 may be obscured; or (*iii*) the kinetics of induction of the A26 transcripts by p53 is comparable to that of direct response genes such as WAF1/CIP1/p21 and hdm-2, suggesting that the A26 gene may be a direct response gene. In such an event, direct activation by p53 would require the presence of a short-lived coactivator protein and possibly be mediated through a DNA response element distinct from the p53 20-bp consensus binding site (31), as dictated by the

putative coactivator. Cloning and characterization of the promoter should address this issue.

Induction of these transcripts is not restricted to Saos-2 osteosarcoma cells, whose growth is arrested by p53 (36). A pronounced induction was also observed in EB1 colorectal carcinoma cells (32), which undergo apoptosis upon expression of wt p53. Furthermore, preliminary studies indicate that these transcripts are induced not only by overexpression of exogenous p53 proteins but also by DNA-damaging stimuli (e.g., UV radiation and treatment with doxorubicin), which lead to an accumulation of endogenous p53 protein in a variety of cultured normal and tumor cells (S. Velasco-Miguel and N.K., unpublished observations). Induction correlates with the wt p53 status in these cells, suggesting that induction is p53 dependent.

In summary, we have shown that wt p53 activates multiple signaling pathways by inducing transcripts derived from at least several genes; these may encode potential mediators of p53 tumor suppression. Whether these proteins play a role in p53-mediated growth arrest or apoptosis remains to be determined.

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