p53-Dependent and independent expression of p21 during cell growth, differentiation, and DNA damage

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Expression of p21 has been shown to be up-regulated by the p53 tumor suppressor gene in vitro in response to DNA-damaging agents. However, p21 expression can be regulated independently of p53, and here we show that expression of p21 in various tissues during development and in the adult mouse occurs in the absence of p53 function. However, most tissues tested did require p53 for p21 induction following exposure of the whole animal to γ irradiation. These results show that normal tissue expression of p21 to high levels is not dependent on p53 and confirm that induction of p21 by DNA-damaging agents does require p53. p21 is expressed upon differentiation of p53-deficient murine erythroleukemia (MEL) cells, and the kinetics of induction of p21 in this system suggest that it may be involved in the growth arrest that precedes terminal differentiation. The gene is up-regulated in mouse fibroblasts in response to serum restimulation but the kinetics and levels of induction differ between wild-type and mutant cells. Expression of p21 message following serum restimulation is superinducible by cycloheximide in wild-type but not in p53-deficient cells. The increases in p21 mRNA are reflected in changes in p21 protein levels. p21 expression also appears to be regulated at the post-transcriptional level because moderate increases in mRNA expression, during differentiation of MEL cells and upon serum restimulation of fibroblasts, are followed by large increases in protein levels. Regulation of the mouse p21 promoter by p53 depends on two critical p53-binding sites located 1.95 and 2.85 kb upstream from the transcriptional initiation site. The sequences mediating serum responsiveness of the promoter map to a region containing the proximal p53 site. p53 appears to play a critical role in p21 induction following DNA damage. Moreover, p21 can be regulated independently of p53 in several situations including during normal tissue development, following serum stimulation, and during cellular differentiation.

[*Key Words*: DNA damage; p53; p21; cellular differentiation]

Cell-cycle progression has been shown to be modulated by a new class of regulators known as cyclin kinase inhibitors. The first of these proteins to be identified and cloned was p21, which is the product of the WAF-1/CIP1 gene and is variously referred to as p21/WAF-1/CIP1 (El-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993a,b). p21 forms a quaternary complex with cyclins, cyclin-dependent kinases (cdks), and proliferating cell nuclear antigen (PCNA) (Xiong et al. 1993a) and acts to regulate cyclin/cdk activity and to directly affect DNA replication through its interaction with PCNA (Waga et al. 1994; Li et al. 1994). p21 can promote cyclin/cdk complex assembly in vitro (Zhang et al. 1994), and because p21 exists in normal cycling cells, it may perform this function in vivo as well. The role of p21 as either a

cyclin/cdk inhibitor or as an assembly factor may depend on the stoichiometry of components within these complexes (Zhang et al. 1994), such that at low concentration p21 may promote complex assembly, whereas at higher concentrations it might be inhibitory.

p21 was cloned independently by virtue of its induction by p53 (El-Deiry et al. 1993), its association with cyclin/cdk complexes (Xiong et al. 1993b; Harper et al. 1993) and its up-regulation in senescent cells (Noda et al 1994). Other cyclin/cdk inhibitors have since been identified and cloned (Serrano et al. 1993; Hannon and Black 1994; Polyak et al. 1994), including p16 (Serrano et al. 1993) and p15 (Hannon and Black 1994), which appear to specifically inhibit cyclin D/cdk4 complexes. p16 is frequently mutated in a variety of tumors (Hussussian et al. 1994; Kamb et al. 1994a,b; Nobori et al. 1994). The p27 cyclin/cdk inhibitor is also involved in mediating

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growth arrest and inhibits cyclin E/cdk2 and cyclin A/cdk2 complexes. p27 is induced in T cells in response to interleukin-2 and in the HL60 cell line in response to retinoic acid as these cells differentiate to form granulocytes (Polyak et al. 1994). However, only p21 has been shown to affect DNA replication (Waga et al. 1994; Li et al. 1994).

p21 is up-regulated by p53 following DNA damage (Xiong et al. 1993a,b; El-Deiry et al. 1993, 1994; Di Leonardo et al. 1994) and is thought to be an integral part of the p53-mediated growth-arrest pathway (El-Deiry et al. 1994). Regulation of p21 by p53 presumably occurs at the level of transcriptional initiation because it is known that the ability of p53 to induce growth arrest requires its trans-activation domain (Pietenpol et al. 1994). Also, loss of p53 results in dramatic reductions in p21 message levels (Xiong et al. 1993a; El-Deiry et al. 1993, 1994). The putative role of p21 in growth arrest mediated by p53 makes it an important gene with respect to the understanding of loss of growth control in transformation processes. Mice that are homozygous for a targeted disruption of the p53 gene are predisposed to tumor formation but develop normally (Donehower et al. 1992; Jacks et al. 1994). Thus, either p21 itself is not essential for normal development or p21 expression is not fully dependent on p53.

To characterize more fully the normal regulation of p21, we have compared its expression in a variety of cells and tissues with or without functional p53. We conclude that p21 expression during development and in the adult mouse can be p53-independent and that the gene can be induced by serum and upon differentiation in the absence of p53 function. However, p53 appears to be necessary for p21 induction following DNA damage, a function that may be critical for its tumor suppressor properties.

Results

p21 mRNA is expressed in various tissues independently of p53

Given the role of p21 in regulating cell-cycle progression and DNA replication (Harper et al. 1993; Xiong et al. 1993b; Li et al. 1994; Waga et al. 1994), we were interested in determining whether p21 expression was strictly dependent on the presence of functional p53. Our initial studies focused on expression of p21 in tissues from wild-type and p53-deficient mice. As shown in Figure 1A, expression of p21 in wild-type animals is seen in the thymus, lungs, spleen, testes, and brain with very high levels detected in the small intestine. Little or no expression was observed in the kidney, skeletal muscle, liver, or heart. By comparing the pattern of p21 expression in p53-deficient animals, it is clear that the only tissue in which expression is dependent on p53 is the spleen (Fig. 1A). Levels of p21 are comparable between mutant and wild-type animals in the testes, intestine, and brain, although there is slightly reduced expression in mutant thymus and lungs.
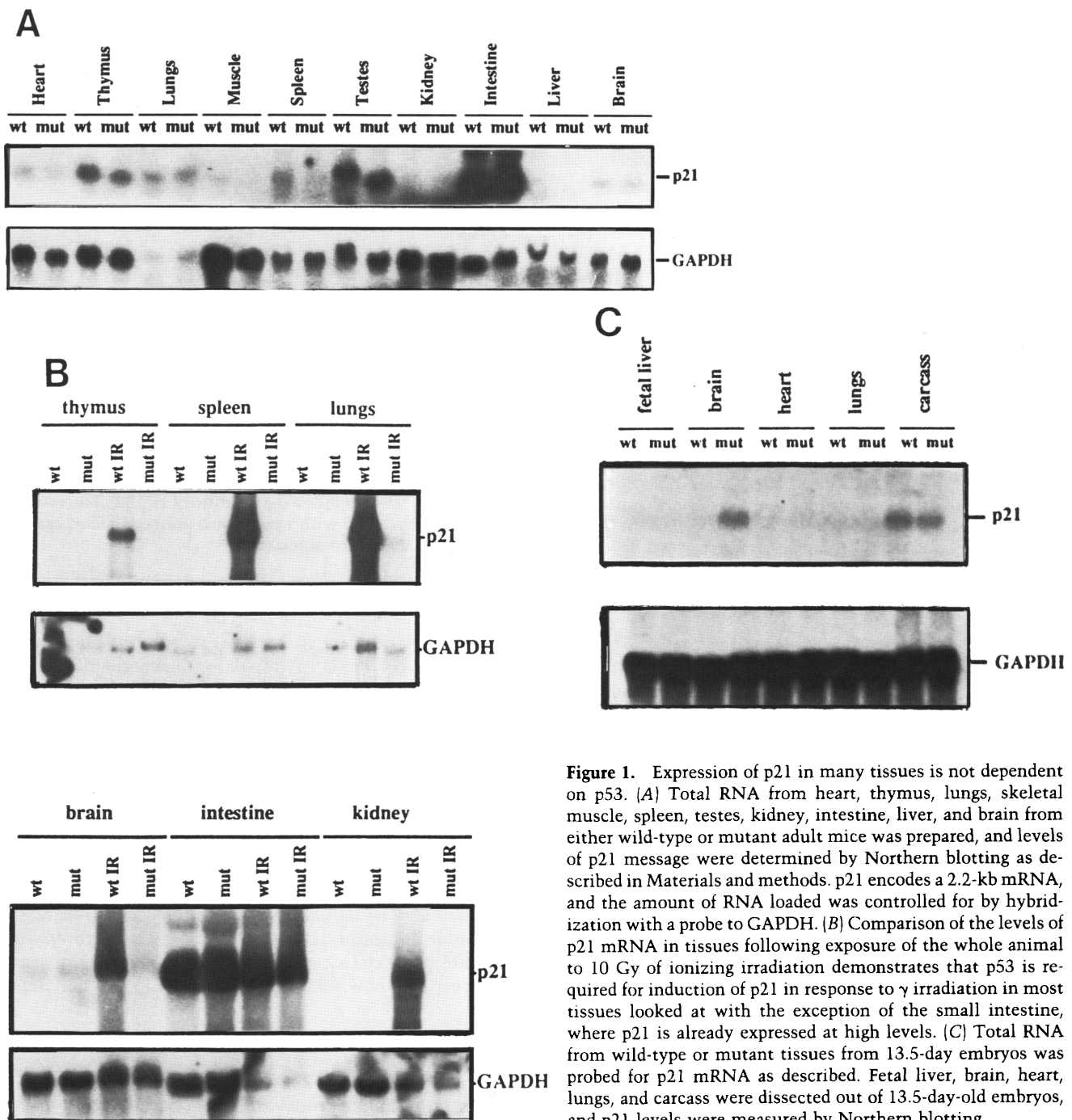
These results suggest that regulation of expression of p21 mRNA in normal mouse tissues occurs by a mechanism not involving p53. However, previous reports have clearly shown the importance of p53 in the induction of p21 following exposure to ionizing radiation and other DNA-damaging agents (Di Leonardo et al. 1994; El-Deiry et al. 1994). Thus, we examined the pattern of p21 expression in wild-type and mutant animals following γ irradiation. As shown in Figure 1B, significant induction of p21 occurs in all wild-type tissues examined, except for the small intestine in which p21 expression is very high prior to irradiation. No induction of p21 mRNA was observed in tissues of irradiated p53-deficient animals (Fig. 1B), confirming the importance of p53 in the irradiation response.

Given the results with adult tissues, we next examined p21 mRNA levels in tissues from wild-type and p53 − / − embryos. Most embryonic cell types are still proliferating and the role of p21 in modulating cell-cycle progression may result in a different pattern of p21 expression in embryonic tissues compared with adult tissues. As shown in Figure 1C, p21 is expressed in the carcass of wild-type embryos at 13.5 days of gestation but not in fetal liver, brain, lungs, or heart. As was observed in adult tissues, expression in embryonic carcass is not dependent on p53 because high levels of expression are also seen in p53 − / − embryos (Fig. 1C). Quite unexpectedly, absence of p53 actually resulted in increased expression of p21 in embryonic brain.

p21 mRNA and protein are induced during MEL cell differentiation

Murine erythroleukemia (MEL) cells are derived from erythoid tumors that develop in mice infected by the Friend virus complex. These leukemic cells are growth-factor independent, carry activated forms of Ets family oncogenes, and are mutant for p53 (Ben-David and Bernstein 1991). The cell line is effectively blocked in erythroid differentiation and proliferates in culture until induced to terminally differentiate by various agents such as dimethylsulfoxide (DMSO) or N, N'-hexamethylene-bis-acetamide (HMBA). Alterations in the cell cycle of MEL cells following their induction to differentiate are well characterized. Induction of differentiation in MEL cells results in a prolonged G_1 phase, up-regulated expression of the retinoblastoma protein (Rb) tumor suppressor gene, and increased levels of hypophosphorylated Rb. Induced MEL cells proceed through two to four cell cycles before arresting in the following G_1 phase of the cell cycle (Richon et al. 1992) and expressing markers of terminal erythroid differentiation such as β^{major} globin. We were interested in determining whether p21 might be involved in the growth arrest and terminal differentiation of MEL cells, particularly because they lack p53 function.

We examined both p21 mRNA and protein levels following addition of the inducing agent, HMBA. p21 message levels showed no overall change when measured at 1, 2, 3, 4, or 5 days following induction to differentiate

Figure 1. Expression of p21 in many tissues is not dependent on p53. (A) Total RNA from heart, thymus, lungs, skeletal muscle, spleen, testes, kidney, intestine, liver, and brain from either wild-type or mutant adult mice was prepared, and levels of p21 message were determined by Northern blotting as described in Materials and methods. p21 encodes a 2.2-kb mRNA, and the amount of RNA loaded was controlled for by hybridization with a probe to GAPDH. (B) Comparison of the levels of p21 mRNA in tissues following exposure of the whole animal to 10 Gy of ionizing irradiation demonstrates that p53 is required for induction of p21 in response to γ irradiation in most tissues looked at with the exception of the small intestine, where p21 is already expressed at high levels. (C) Total RNA from wild-type or mutant tissues from 13.5-day embryos was probed for p21 mRNA as described. Fetal liver, brain, heart, lungs, and carcass were dissected out of 13.5-day-old embryos, and p21 levels were measured by Northern blotting.

(data not shown). These cells were clearly differentiating, because ~95% expressed globin at 3 days following addition of HMBA. However, examination of p21 mRNA levels in the first several hours following induction to differentiate revealed a three- to fivefold increase in p21 message levels after ~2 hr (Fig. 2A). The levels seen at 2 hr decreased to uninduced levels by 4 hr following HMBA addition. Thus, although p21 may have a role in initiating growth arrest prior to differentiation, it seems not to be involved in maintaining the growth-arrested state. Only 15–20% of MEL cells in the population be-

come committed to differentiation during the first cell cycle following addition of inducer, and it may require another two to four cycles for 100% of cells to become committed (Kiyokawa et al. 1993).

The levels of p21 protein also increased dramatically within 2 hr following the induction of differentiation (Fig. 2B). We observe that the increase in p21 protein levels in differentiating MEL cells is significantly more substantial than the increase in mRNA levels. There is an ~10-fold increase in protein levels compared with a 2- to 3-fold increase in mRNA levels, suggesting that p21
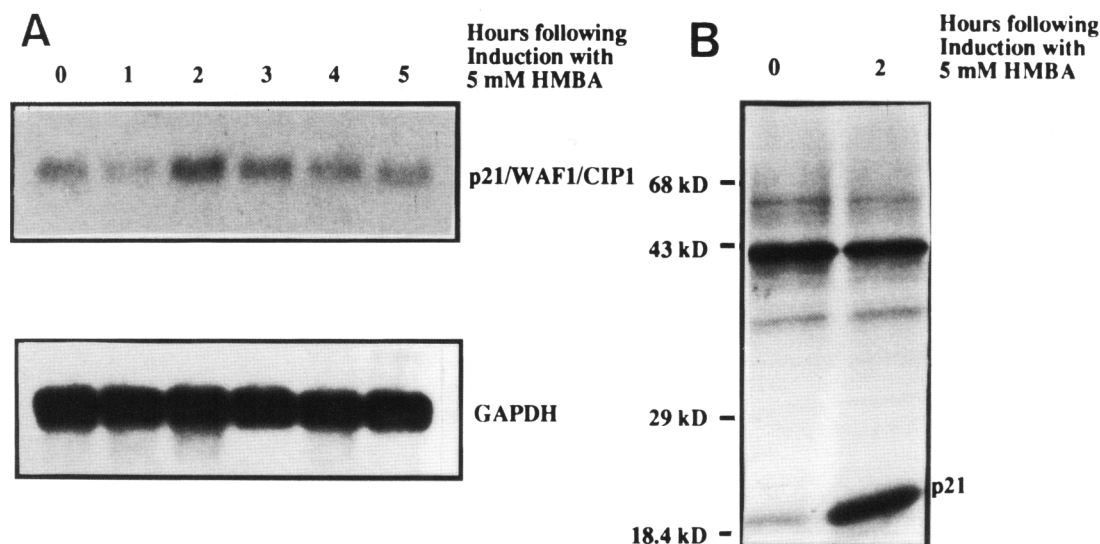
Figure 2. p21 is up-regulated in differentiating MEL cells. Exponentially growing MEL cells were induced to differentiate by addition of 5 mM HMBA. (*A*) Northern blot analysis shows that p21 mRNA levels increase at ~2 hr following addition of HMBA. The loading of RNA is controlled for by hybridization to a probe for GAPDH. (*B*) Immunoprecipitation with a polyclonal antibody raised against mouse p21 showed that p21 protein also increases 2 hr following induction of MEL cells to differentiate.

may also be regulated post-transcriptionally. The increased level of p21 protein was associated with decreased H1 kinase activity present in both p21 and cdk2 immunoprecipitates (data not shown), again consistent with the notion that p21 participates in growth arrest in this differentiation program. Because MEL cells are mutant in both alleles of p53 (Mowat et al. 1985; Munroe et al. 1990), the increase in p21 expression in this differentiation system may constitute another example of p53-independent regulation.

p53 is required for normal serum induction of p21

We examined the regulation of p21 in primary fibroblasts isolated from wild-type and p53-deficient embryos. As shown in Figure 3 (A,B), the steady-state level of p21 in wild-type cells growing in 10% serum is considerably higher than that seen in p53 − / − cells. Following serum starvation and restimulation of wild-type cells, there is an approximately twofold increase in p21 mRNA at 1 hr following serum addition (Fig. 3A). This increase in message is short-lived, however, and by 2 hr following serum stimulation, p21 mRNA levels return to unstimulated levels.
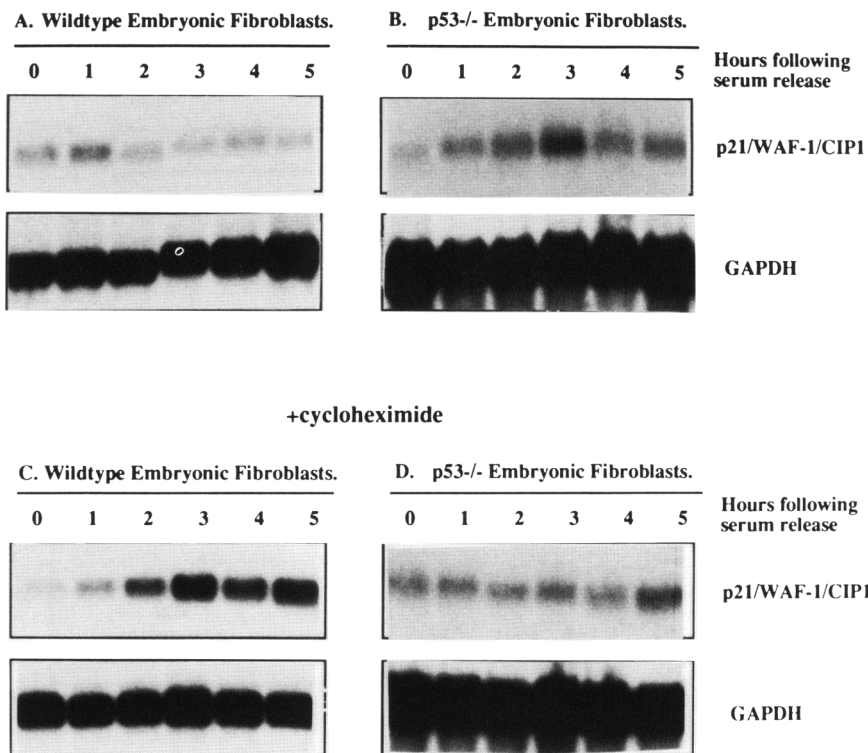
In contrast to the situation in wild-type cells, there is a dramatic induction of p21 mRNA in serum-stimulated p53 − / − cells. Serum-starved p53 − / − cells have barely detectable levels of p21 mRNA. Within 3 hr of serum restimulation, we observe a 35-fold induction of the p21 message (Fig. 3B). As in wild-type cells, the levels of p21 subsequently decline in the p53 − / − cells. Thus, p21 is serum inducible in both wild-type and p53 mutant cells although with different kinetics and to differing levels.

This serum inducibility is transient and thus may imply that p21 levels are tightly regulated during the cell cycle.

Addition of cycloheximide during serum stimulation of wild-type cells resulted in a 40-fold superinduction of p21 expression (Fig. 3C), suggesting that serum induction of p21 in wild-type cells is inhibited by a labile protein factor. When cycloheximide was added during serum restimulation of p53-deficient cells, p21 induction was delayed or blocked (Fig. 3D). Thus, the factor(s) responsible for serum induction of p21 in the absence of p53 may also be labile. It will be interesting to identify the labile inhibitor acting in wild-type cells to down-regulate p21 expression following serum induction and to investigate how this factor might act in p53-deficient cells.

Elements within the p21 promoter-mediating serum induction overlap with a functional p53 DNA-binding site

To begin to address the mechanism of p53-independent induction of p21 mRNA, we examined the activity of a series of CAT reporter constructs controlled by different 5′ deletions of the p21 promoter (Fig. 4A). The transcriptional activity of these plasmids was assayed in wild-type and p53-deficient fibroblasts following transient transfection. CAT activity was determined in both serum-starved cells and cells starved of serum and then restimulated with 10% serum. As shown in Figure 4B, the activity of the p21 promoter in wild-type cells is substantially reduced upon sequential deletion of two p53 DNA-binding sites, located between − 2853 and − 2834 bp and between − 1961 and − 1941 bp relative to

A. Wildtype Embryonic Fibroblasts.   **B.   p53-/- Embryonic Fibroblasts.**

+cycloheximide

C. Wildtype Embryonic Fibroblasts.   **D.   p53-/- Embryonic Fibroblasts.**

Figure 3. p21 is serum inducible in a p53-independent manner. Primary mouse embryonic fibroblasts (MEFs) were serum starved for 48 hr in 0.1% fetal bovine serum and then restimulated with 10% serum. Total RNA was collected from wild-type (A) and p53 mutant fibroblasts (B) at different time points after restimulation with serum and p21 levels measured by Northern blotting. Northern blots carrying RNAs from wild-type cells were exposed for 16 hr, whereas those carrying RNAs from p53-deficient cells were exposed for 40 hr. The effect of addition of 10 µg/ml of cycloheximide on the induction of p21 in wild-type (C) and p53 mutant (D) fibroblasts was also assessed.

the start site of transcription (T. Tokino, K. Kinzler, and B. Vogelstein, unpubl.). The large deletion that removes the distal p53-binding site reduced promoter activity in p53-deficient cells as well, indicating that important regulatory factors other than p53, may also bind in this region. However, deletion of the proximal p53 DNA-binding site resulted in a slight increase in CAT activity in serum-starved p53-deficient cells (Fig. 4B, cf. pCAT6 and pCAT7), in contrast to the result obtained in wild-type cells.

In both wild-type and p53-deficient cells, serum inducibility appears to map to a region located between positions -2581 and -1871 because the pCAT6 plasmid showed increased activity following serum addition in both cell types, whereas the pCAT7 plasmid was unresponsive in both (Fig. 4B). Given that one of the putative p53-binding sites maps to this region and the serum inducibility of the native p21 promoter is stronger in p53-deficient cells, it is tempting to speculate that the factor responsible for serum induction might bind to a site that overlaps the p53-binding site (Fig. 4C). We note that the p53-binding site in this region does overlap with a consensus Ets-binding site (Fig. 4C). Members of the Ets family of transcription factors have been associated with serum induction of other promoters (Prosser et al. 1992; Hill et al. 1993; Marais et al. 1993).

Serum induction of p21 protein

p21 protein has been proposed to act as both an assembly factor for cyclin/cdk complex formation and, at higher

concentrations, as a cyclin/cdk inhibitor (Zhang et al. 1994). To determine whether the observed induction of p21 mRNA in wild-type and mutant cells was reflected in increased p21 protein levels, we immunoprecipitated p21 from asynchronously cycling as well as serum-starved and restimulated cells. As shown in Figure 5, in both the presence and absence of p53, levels of p21 protein are higher in the serum-restimulated cells compared with asynchronously growing cells. Increased amounts of p21 protein in G_1-enriched populations of cells further support its role in G_1 regulation and are fully consistent with the mRNA analysis (Fig. 3). However, relative levels of p21 mRNA 2–4 hr after serum stimulation in wild-type cells are ~10-fold greater than in p53 $-/-$ cells (Fig. 3), yet the amount of p21 protein in these cells at these times is comparable (Fig. 5). Thus, as observed in differentiating MEL cells, p21 regulation in embryonic fibroblasts may also occur at the level of translation and/or protein stability.

p21 levels correlate with cdk activity

Finally, we assessed the effects of p21 induction on cyclin/cdk activity by performing in vitro H1 kinase assays with anti-p21 immunoprecipitates. Wild-type and p53 $-/-$ fibroblasts were serum starved and restimulated with 10% serum. At hourly time points following restimulation, cell lysates were prepared and immunoprecipitated with polyclonal anti-p21 antibodies. Immunocomplexes were then incubated with [γ-^{32}P]ATP and histone H1 as a kinase substrate. Consistent with the

A

B
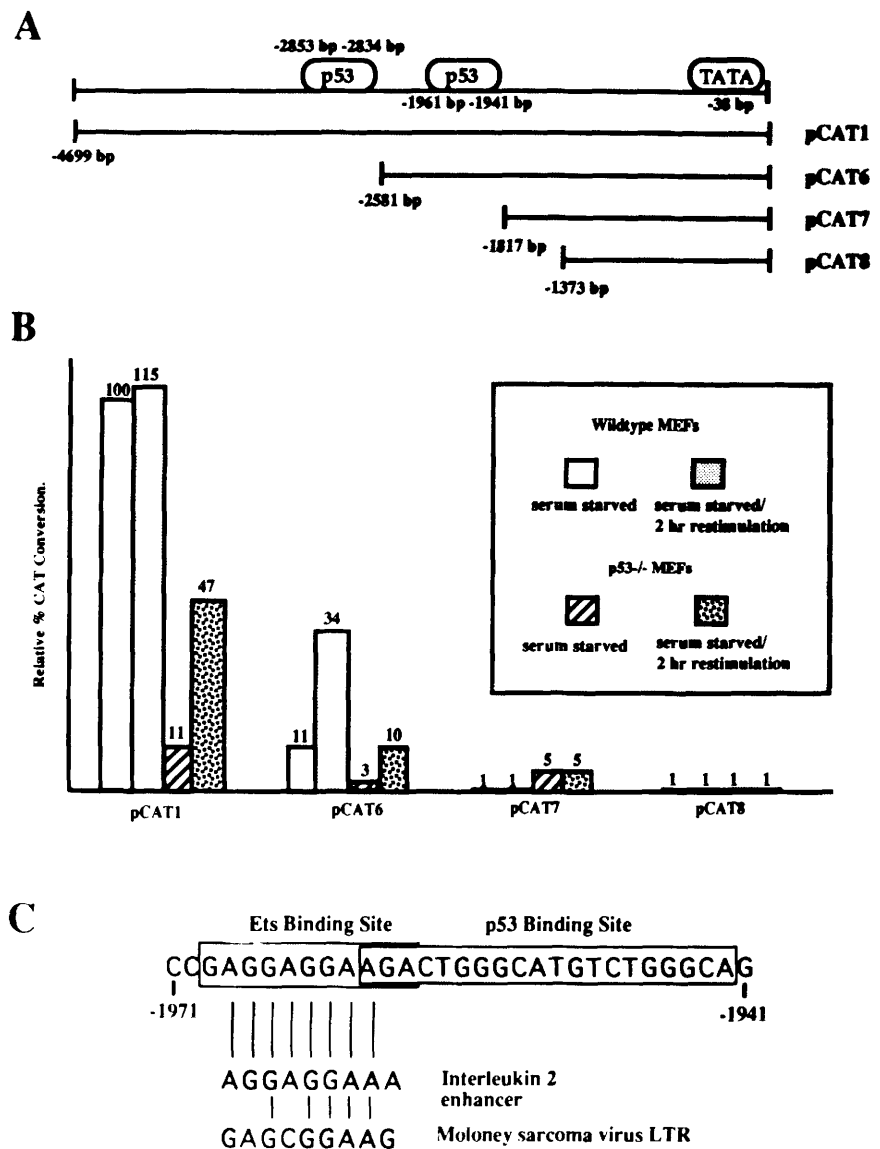
Figure 4. The serum responsiveness of the p21 promoter maps to a region containing a functional p53 site. 5′ Deletion mutants of the p21 promoter were constructed and are diagramed in *A*. The effect on promoter activity in wild-type and p53 mutant fibroblasts following serum starvation and restimulation is shown in *B*. Percent CAT conversion was normalized for transfection efficiency and then expressed relative to the activity of the full-length promoter construct in serum-starved cells. The results are the average of two series of experiments. All available substrate had been exhausted by the end of the reaction with equal amounts of extracts from 2-hr serum-restimulated wild-type cells transfected with pCAT1, explaining the poor induction relative to unstimulated cells. The sequence of the p21 promoter region containing the proximal p53-binding site and the extent of homology of the overlapping, putative Ets-binding site to characterized Ets-binding sites is shown in *C*.

C

proposed role of p21 in promoting cyclin/cdk complex assembly, we observe maximal H1 kinase activity at time points known to correspond to the peak of p21 mRNA levels in wild-type and mutant cells (Fig. 6). H1 kinase activity was highest at ~2 hr following serum addition in wild-type cells and after ~4 hr in p53-deficient cells. Once again, despite considerable differences in the p21 mRNA levels in the two cell types at these time points, the level of p21-associated kinase activity was comparable, further supporting the existence of some form of post-transcriptional control of p21.

Discussion

The recent discovery of a class of low-molecular-weight cyclin/cdk inhibitors coupled with the observation that the p21 inhibitor can be up-regulated by the p53 tumor suppressor protein underscores the close connection between cell-cycle regulation and tumorigenesis. We have studied the expression of p21 in a variety of settings in which p53 is absent to determine what other signals could induce this important cell-cycle regulator. Our results indicate that the pattern and extent of p21 expression are comparable in wild-type and p53-deficient animals. These data suggest that transcriptional regulation of p21 in adult mouse tissues can be accomplished by other transcriptional regulators besides p53. The spleen is the only tissue tested in which p53 was required for normal p21 expression levels. Although p21 is expressed at equal levels in wild-type and mutant tissues, the possibility remains that steady-state levels of p21 expression are dependent on p53 in wild-type mice and that a compensatory mechanism activates p21 expression in p53-deficient animals. Following whole-body irradiation, there was a marked increase in the levels of p21 mRNA in most wild-type mouse tissues but not in p53-
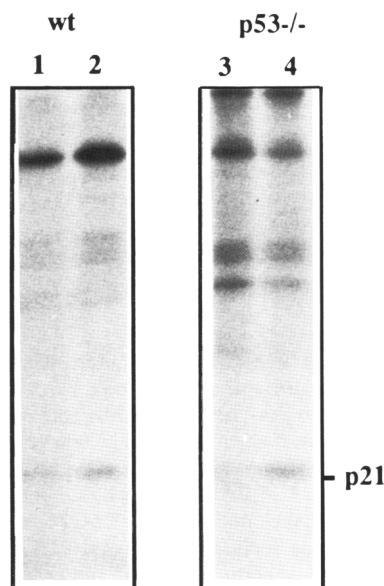
Figure 5. p21 protein levels are serum inducible in a p53-independent manner. Changes in p21 protein levels following serum restimulation were measured by immunoprecipitation. Serum-starved cells were restimulated with 10% dialyzed serum in the presence of [^{35}S]methionine as described in Materials and methods. Cells were radiolabeled for 4 hr following addition of serum, and cell lysates were prepared as described. p21 was immunoprecipitated with the rabbit polyclonal serum used previously. p21 immunoprecipitates were also prepared from asynchronously cycling radiolabeled cells. Asynchronously cycling MEFs (lanes 1,3); serum-starved and 4-hr serum released (lanes 2,4); wild-type (lanes 1,2), and p53 mutant (lanes 3,4) MEFs.

deficient tissues. Thus, the regulation of p21 by p53 appears to be critical to this stress response. The intestine was the one wild-type tissue tested that failed to induce higher levels of p21 mRNA following irradiation. It has been shown that p53 is required for radiation-induced apoptosis in the intestine (Merrit et al. 1994), but the lack of p21 induction in this tissue under these conditions suggests that p21 will not be a critical effector of the cell death process in these circumstances.

Because p21 expression is induced as MEL cells differentiate, we have suggested that this protein may be involved in growth arrest as part of the differentiation process. p21 mRNA has also been shown to be induced in the p53-deficient myeloid cell line HL-60 induced to differentiate with a variety of agents (Jiang et al. 1994; Steinman et al. 1994). As shown here, many adult tissues show high-level expression of p21 including the thymus, brain, and intestine. Other tissues do not express detectable levels of p21 in either the wild-type or mutant animals (e.g., liver, muscle, and kidney), and it is possible that other cyclin/cdk inhibitors, such as p27^{KIP1}, are functioning in these tissue types to induce growth arrest prior to differentiation. Alternatively, these tissues may have reached a state of stable maintenance of growth arrest such that the activity of any of these inhibitors is no longer required.

In comparison with adult tissues, the cells of embryonic tissues are less well differentiated and are more actively proliferating. Thus, it is perhaps not surprising that p21 expression is higher in the adult than in the embryo. Embryonic expression of p21 was limited to the carcass, a region of extensive muscle differentiation. Halevny and co-workers (1995) have recently shown that myoblast differentiation is accompanied by p21 induction, also independently of p53. Interestingly, despite comparable p21 expression in the carcass of wild-type and mutant embryos, significantly higher p21 mRNA levels are observed in wild-type compared with mutant fibroblasts grown in culture. Thus, some aspect of tissue culture may represent a form of environmental stress that induces a p53-dependent response. Curiously, we observe expression of p21 in the brain of p53-deficient but not wild-type embryos. It appears that loss of p53 function induces p21 expression in this tissue. Induction of p21 in p53-deficient embryonic brain is one of the few expression differences observed between wild-type and p53 mutant animals in vivo and may be related to our recent observation of a defect in neural tube formation in a proportion of p53-deficient embryos (V.P. Sah, L.D. Attardi, G.J. Mulligan, B.O. Williams, R.T. Bronson, and T. Jacks, unpubl.).

We and others (Michieli et al. 1994) have shown that p21 is induced in serum-starved wild-type and p53-deficient embryonic fibroblasts when these cells are restimulated with 10% fetal calf serum (FCS). The kinetics of induction differ between wild-type and mutant fibroblasts. p21 mRNA is induced in wild-type fibroblasts within 1 hr of serum restimulation, whereas peak p21 induction in p53 − / − cells occurs at 3 hr. The levels of induction in the two cell types also differ, with higher induction in mutant than wild-type cells. Elevated expression of p21 in the first 1–3 hr following serum release may jump-start the cell into cycle by facilitating complex formation between cyclins and cdks.

Increased p21 mRNA levels in wild-type cells reach a peak by 1 hr following serum induction but decrease again by 2 hr, implying that there is a homeostatic control mechanism operating to regulate p21 mRNA levels. This mechanism is clearly sensitive to agents that block protein synthesis because cycloheximide is shown here to superinduce p21 message. Other genes have been shown previously to be superinducible by cycloheximide following serum release, notably the immediate early response genes like c-*myc* and c-*fos* (Sassone-Corsi et al. 1988; Penn et al. 1990). Interestingly, superinduction of p21 in wild-type cells appears to depend on p53. Thus, cycloheximide may activate the transcriptional properties of p53.

We have identified a region mapping from −1817 to −2581 bp relative to the transcriptional initiation site of the p21 promoter that mediates serum induction in both wild-type and mutant cells. This region contains one of two critical p53 DNA-binding sites (T. Tokino, K. Kinzler, and B. Vogelstein, unpubl.). There is also a putative Ets-binding site in this region, which actually overlaps the p53-binding site. Members of the Ets family of tran-

Figure 6. p21-associated H1 kinase activity increases as cells are serum stimulated. The H1 kinase activity of p21 immunoprecipitates from serum-starved cells was measured at different time points following restimulation with 10% serum. H1 kinase activity was measured at 0, 1, 2, 3, 4, and 5 hr following addition of serum to MEFs that had been serum starved for 48 hr.
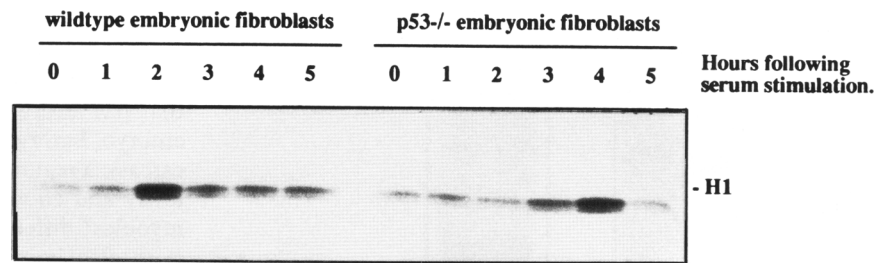
scription factors have been linked to serum responsiveness of promoters, frequently by interactions with other factors (Prosser et al. 1992; Hill et al. 1993; Marais et al. 1993).

We have described two examples of apparent post-transcriptional control of p21. During the differentiation of MEL cells, p21 mRNA levels rise by three- to fivefold by 2 hr following addition of HMBA, whereas protein levels increase by 10-fold. Also, despite the differences in absolute levels of p21 mRNA in wild-type and p53-deficient fibroblasts following serum release, there appear to be equivalent levels of p21 protein and p21-associated H1 kinase activity, suggesting that p21 is post-transcriptionally regulated. Given the proposed role of p21 as a cyclin/cdk assembly factor, it is not surprising that it is subjected to many levels of regulation.

The role of p21 up-regulation following treatment of cells with ionizing radiation is clearly important to our understanding of p53-mediated growth arrest. However, the regulation of this gene by other factors acting independently of p53 suggests that it is a more universal cell cycle regulator. p21 is clearly regulated in the absence of p53 function: under conditions of serum release, induction to differentiate, and in adult and embryonic tissues. The mechanisms by which this regulation operates are both transcriptional and post-transcriptional and are subject to homeostatic control. Identifying the other factors able to induce p21 should improve the understanding of how different extracellular signals induce growth arrest and differentiation, and how these signaling pathways are affected in tumorigenesis.

Materials and methods

Adult and embryonic mouse tissues

Tissues were dissected from wild-type and homozygous p53 mutant adult mice and homogenized in PBS. Tissues were prepared from irradiated adult mice in the same manner. Wild-type and p53 − / − mice were exposed to 10 Gy of γ irradiation from a [137]Cesium source at a constant dose rate 52 Gy/hr. The mice were sacrificed 3 hr following irradiation and tissues prepared as described. Tissues were also obtained from 13.5-day wild-type and p53 − / − embryos and homogenized by successive passage through a 23-gauge and then a 26-gauge syringe needle.

Cell culture conditions

Mouse embryonic fibroblasts (MEFs) were obtained from the carcasses of wild-type and p53 − / − 12.5-day-old embryos as described previously (Robertson 1987), and cultured in 10% FCS (Intergen)/5 mM L-glutamine/Dublecco's modified Eagle medium (DMEM) up to passage 6. Given the role of p21 in senescence and the potential effect of loss of p53 on genome stability, cells cultured beyond passage 6 were not used in the experiments described here. MEFs were plated at a density of 10^5/ml in 10 ml in 9-cm dishes in 10% FCS/DMEM, and 24 hr later, growth medium was replaced with 0.1% FCS/DMEM. Cells were incubated in low serum for 48 hr and then released from serum deprivation with 10% FCS/DMEM. Cycloheximide was added at this point, at a final concentration of 10 μg/ml, where indicated. Cells were harvested at different time intervals following serum release, depending on the assay. The murine erythroleukemic cell line MEL-J was grown in 10% FCS and induced to differentiate with 5 mM HMBA, as reviewed previously (Marks and Rifkind 1978).

RNA extraction and Northern blot analysis

Total RNA was extracted by use of the RNAzol B method from homogenized mouse tissues, MEFs, thymocytes, or MEL cells, and ~20 μg of RNA was electrophoretically separated on a 1% agarose/1× MOPS/16% formaldehyde gel. The RNAs were then transferred to nylon membranes in 20× SSC, UV cross-linked to the membrane, and prehybridized, hybridized, and washed as described (Shackleford et al. 1987). The p21 mRNA was detected by probing of the membranes with 50 ng of a 720-bp fragment obtained from the mouse p21 cDNA by BamHI–XhoI digestion and labeled to high specific activity by random oligonucleotide labeling with a Quickprime kit (Stratagene).

Immunoprecipitation and H1 kinase assays

Serum-starved or asynchronously cycling MEFs (70–75% confluent in a 9-cm plate) or differentiating MEL cells were metabolically labeled for 2–4 hr in 2 ml of 10% dialyzed FCS/methionine- and cysteine-free DMEM/50 μCi ^{35}S-labeled methionine + cysteine (EXPRE[35]S[35]S protein labeling mix from NEN Dupont). Lysis of labeled cells and immunoprecipitation of p21-containing complexes was carried out as described previously (Xiong et al. 1993a,b).

To measure the kinase activity of p21-containing complexes, anti-p21 immunoprecipitates were prepared as described above, except that cell lysates were not metabolically labeled. After the immunoprecitate was washed in lysis buffer, it was then washed in kinase buffer (50 mM HEPES at pH 7.0, 10 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT) and resuspended in 50 μl of kinase buffer + 10 μM ATP. Kinase reactions were then carried out by addition of 20 μCi of [γ-^{32}P]ATP and 2 μg histone H1 and incubation at 37°C for 30 min. The reaction was stopped by addition of 50 μl of 2× protein loading buffer and subjected to 15% SDS-PAGE as described above.

p21 promoter analysis by transient transfection

Deletion constructs of the p21 promoter cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene were used to determine the regions of the p21 promoter responsible for serum inducibility in p53−/− MEFs or in differentiating MEL cells. Reporter plasmid (5 μg) was transfected with 5 μg of the pTKGH standardization plasmid by lipofection (DOTAP, Boehringer Mannheim) into wild-type or p53−/− MEFs growing at 60–70% confluence in Opti-MEM (GIBCO-BRL) in 9-cm dishes. Six hours after lipofection, Opti-MEM was removed and replaced with 0.1% FCS/DMEM. Cells were incubated for 48 hr in low serum and then released from serum deprivation by addition of 10% FCS/DMEM. Growth medium (1 ml) was recovered prior to the addition of serum, and hGH levels were measured by radioimmunoassay (Nicholls Allegro hGH TGES). Cell lysates were prepared from serum-starved cells and serum-released cells by the scraping of cells into 1 ml of PBS and resuspension of the cell pellet in 200 μl of 0.25 M Tris-HCl (pH 7.8). CAT assays were carried out, as described previously (Gorman et al. 1982), on quantities of lysate that had been adjusted for transfection efficiency on the basis of levels of expression of hGH.

Acknowledgments

Note added in proof

Following submission of this paper, S.B. Parker et al. [*Science* 267: 1024–1027 (1995)] reported that p21 is expressed in a p53-independent manner in several tissues undergoing terminal differentiation. These results are consistent with work described here.

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