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WAF1, a Potential Mediator of p53 Tumor Suppression

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Summary

The ability of p53 to activate transcription from specific sequences suggests that genes induced by p53 may mediate its biological role as a tumor suppressor. Using a subtractive hybridization approach, we identified a gene, named *WAF1*, whose induction was associated with wild-type but not mutant p53 gene expression in a human brain tumor cell line. The *WAF1* gene was localized to chromosome 6p21.2, and its sequence, structure, and activation by p53 was conserved in rodents. Introduction of *WAF1* cDNA suppressed the growth of human brain, lung, and colon tumor cells in culture. Using a yeast enhancer trap, a p53-binding site was identified 2.4 kb upstream of *WAF1* coding sequences. The *WAF1* promoter, including this p53-binding site, conferred p53-dependent inducibility upon a heterologous reporter gene. These studies define a gene whose expression is directly induced by p53 and that could be an important mediator of p53-dependent tumor growth suppression.

Introduction

Inactivation of p53 is a common event in the development of human neoplasia (Hollstein et al., 1991). A variety of mechanisms can lead to such functional inactivation, including missense mutation (Baker et al., 1989) and interaction with oncogenic viral or cellular proteins (Mietz et al., 1992; Momand et al., 1992). Wild-type p53 has been shown to be a suppressor of tumor cell growth (for reviews see Mercer, 1992; Oren, 1992; Lane, 1992; Perry and Levine, 1993). Inactivation of p53 by any of the above mechanisms thereby leads to a selective growth advantage, generally observed as tumor progression.

The mechanism underlying p53 growth suppression is still undefined. Several biochemical features of p53 have been elucidated, and at least two of these are currently of much interest. First, p53 has been shown to suppress a variety of promoters containing TATA elements (e.g., Ginsberg et al., 1991; Santhanam et al., 1991; Kley et al., 1992; Mack et al., 1993). This suppression is apparently sequence independent and may involve p53 binding to the TATA-binding protein or to other transcription factors (Seto et al., 1992; Truant et al., 1993; Ragimov et al., 1993; Martin et al., 1993; Liu et al., 1993). Second, p53 can bind to DNA in a sequence-specific manner (Kern et al., 1991). A 20 bp consensus-binding site, consisting of two copies of the 10 bp sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, separated by up to 13 bp, has been identified (El-Deiry et al., 1992; Funk et al., 1992). Both copies of the 10 bp sequence are required for efficient binding by p53. p53 contains a strong transcriptional activation sequence near its amino terminus (Fields and Jang, 1990; Raycroft et al., 1990) and can stimulate the expression of genes downstream of its binding site. Such stimulation has been demonstrated in both mammalian (Kern et al., 1992; Funk et al., 1992; Zambetti et al., 1992) and yeast cells (Scharer and Iggo, 1992; Kern et al., 1992), as well as in an in vitro system (Farmer et al., 1992).

The sequence-specific transcriptional activation by p53 has led to the hypothesis that p53-induced genes may mediate its biological role as a tumor suppressor (Vogelstein and Kinzler, 1992). To date, several genes containing p53-binding sites have been identified. These include muscle creatine kinase (Weintraub et al., 1991; Zambetti et al., 1992), *GADD45* (Kastan et al., 1992), *MDM2* (Barak et al., 1993; Wu et al., 1993), and a *GLN* retroviral element (Zauberman et al., 1993). Each of these genes contains a 20 bp sequence with high homology to the p53 consensus-binding site (Prives and Manfredi, 1993). The p53-binding sites in *GADD45* and *MDM2* are located within introns, the muscle creatine kinase site is 3 kb upstream of the transcription start site, and the *GLN* element is located within a long terminal repeat. The relationship of any of these genes to suppression of cell growth by p53 remains unclear. It has been suggested that *MDM2* may be a feedback regulator of p53 action by being transcriptionally induced (Barak et al., 1993; Wu et al., 1993) and then inhibiting p53 function (Momand et al., 1992; Oliner et al., 1993; Wu et al., 1993). In this regard, *MDM2* functions as an oncogene rather than as a tumor suppressor gene (Fakharzadeh et al., 1991; Finlay, 1993).

In an effort to identify biologically important genes that are transcriptionally regulated by p53, we constructed a cDNA library enriched for the presence of such genes. Using a subtractive hybridization technique, we identified a highly induced gene, named wild-type p53-activated fragment 1 (*WAF1*). We showed that *WAF1* is directly regulated by p53 and can itself suppress tumor cell growth in culture. Thus, *WAF1* may be an important component of the p53 growth suppression pathway.

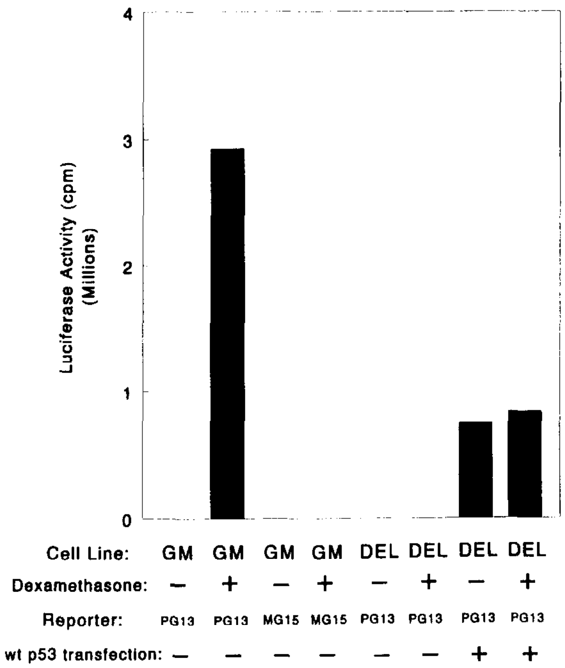

Figure 1. p53-Dependent Transactivation in GM and DEL Cell Lines

GM cells (containing inducible wild-type p53) or DEL cells (containing inducible mutant p53) were transfected with reporter plasmids as indicated, and luciferase activity was measured after 18 hr in the absence (minus) or presence (plus) of dexamethasone. Wild-type (wt) p53 expression plasmid was cotransfected with PG13–Luc into DEL cells in the two lanes at the right.

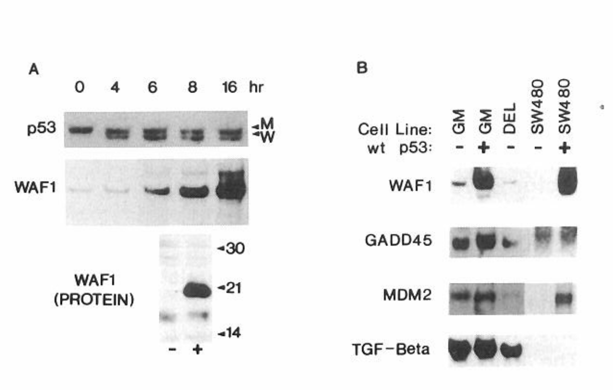

Figure 2. Induction of *WAF1* by p53

(A) A Northern blot was prepared using 10 μg of total RNA isolated from GM cells treated with dexamethasone for 0–16 hr and probed with p53 cDNA or *WAF1* cDNA. The endogenous mutant (m) and induced wild-type (w) p53 mRNA species are indicated. Lysates from GM cells induced with dexamethasone for 22 hr (plus) or uninduced (minus) were used in Western blot analysis with anti-WAF1 antisera.

(B) A Northern blot was prepared from RNA of GM cells in the absence or presence of dexamethasone for 16 hr (lanes 1 and 2, respectively), from DEL cells treated with dexamethasone for 16 hr (lane 3), or from SW480 cells infected with Ad-gal (lane 4) or Ad-p53 (lane 5) for 16 hr. The blot was probed with *WAF1* DNA, *GADD45* DNA, *MDM2* DNA, or TGFβ DNA, as indicated.

Results

Definition of a p53-Responsive System

As a first step toward the isolation of p53-regulated genes, we determined optimal cell culture conditions under which an exogenous wild-type p53 protein could activate transcription through specific DNA binding. A reporter plasmid containing a p53 DNA-binding site upstream of a basal promoter (Kern et al., 1992) linked to a luciferase reporter gene (PG13–Luc) was cloned and cotransfected into SW480 colon cancer cells with either a human wild-type p53 expression plasmid (p53-wt) or a mutant p53 expression plasmid (p53-273). High luciferase activity was observed only when wild-type p53 was present (data not shown). No luciferase activity was detected if the reporter plasmid contained mutant p53-binding sites (MG15–Luc), regardless of whether or not wild-type p53 was present. This validated reporter was then used in a p53-inducible system. The glioblastoma cell line GM contains an endogenous mutant p53 gene (Ullrich et al., 1992) and exogenous wild-type p53 under the control of a steroid responsive promoter. A moderate amount of wild-type p53 is induced in these cells by dexamethasone (Mercer et al., 1990). The related line DEL expresses the same endogenous mutant p53 and an additional dexamethasone-inducible mutant p53 (Lin et al., 1992). Both cell lines were transfected with either PG13–Luc or MG15–Luc and incubated in the presence or absence of dexamethasone. Figure 1 shows that dexamethasone-induced wild-type p53 (GM) but not mutant p53 (DEL) expression activated the luciferase reporter. No luciferase activity was observed when the p53-binding site was mutant (MG15–Luc) or when the p53 protein was mutant (GM without dexamethasone or DEL with or without dexamethasone). Transfection of wild-type p53 into DEL cells activated the PG13–Luc reporter with or without dexamethasone (Figure 1), confirming that the failure of expression of luciferase reporter gene in this cell line was due to the absence of wild-type p53. These experiments demonstrated that reporter gene expression in these two cell lines was dependent on the presence of wild-type p53.

Subtractive Hybridization

Based on the reporter gene experiments, we chose to use subtractive hybridization to identify endogenous genes regulated by p53 in GM cells. To determine the optimal time to isolate RNA enriched for p53-induced genes, Northern blot analysis was performed, using RNA isolated from GM cells at various intervals following dexamethasone induction. Figure 2 shows that under the logarithmic growth conditions used, the exogenous wild-type p53 mRNA was detectable by 4 hr after induction and remained elevated for at least 16 hr in GM cells upon dexamethasone induction. A p53-induced cDNA library was therefore prepared from GM cells treated with dexamethasone for 6 hr (see Experimental Procedures). Of the clones obtained, 80% carried inserts, generally of 1.5–2.0 kb in length. A total of 120,000 clones were screened by hybridization to a subtracted p53-induced cDNA probe. This probe was made from cDNA of dexamethasone-induced GM cells after subtraction with an excess of dexamethasone-induced DEL RNA. Control experiments showed that the subtraction procedure used, involving chemical cross-

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   1   GCC GAA GTC AGT TCC TTG TGG AGC CGG AGC TGG GCG CGG ATT CGC CGA

  49   GGC ACC GAG GCA CTC AGA GGA GGC GCC ATG TCA GAA CCG GCT GGG GAT
   1                                       Met Ser Glu Pro Ala Gly Asp

  97   GTC CGT CAG AAC CCA TGC GGC AGC AAG GCC TGC CGC CGC CTC TTC GGC
   8   Val Arg Gln Asn Pro Cys Gly Ser Lys Ala Cys Arg Arg Leu Phe Gly

 145   CCA GTG GAC AGC GAG CAG CTG AGC CGC GAC TGT GAT GCG CTA ATG GCG
  24   Pro Val Asp Ser Glu Gln Leu Ser Arg Asp Cys Asp Ala Leu Met Ala

 193   GGC TGC ATC CAG GAG GCC CGT GAG CGA TGG AAC TTC GAC TTT GTC ACC
  40   Gly Cys Ile Gln Glu Ala Arg Glu Arg Trp Asn Phe Asp Phe Val Thr

 241   GAG ACA CCA CTG GAG GGT GAC TTC GCC TGG GAG CGT GTG CGG GGC CTT
  56   Glu Thr Pro Leu Glu Gly Asp Phe Ala Trp Glu Arg Val Arg Gly Leu

 289   GGC CTG CCC AAG CTC TAC CTT CCC ACG GGG CCC CGG CGA GGC CGG GAT
  72   Gly Leu Pro Lys Leu Tyr Leu Pro Thr Gly Pro Arg Arg Gly Arg Asp

 337   GAG TTG GGA GGA GGC AGG CGG CCT GGC ACC TCA CCT GCT CTG CTG CAG
  88   Glu Leu Gly Gly Gly Arg Arg Pro Gly Thr Ser Pro Ala Leu Leu Gln

 385   GGG ACA GCA GAG GAA GAC CAT GTG GAC CTG TCA CTG TCT TGT ACC CTT
 104   Gly Thr Ala Glu Glu Asp His Val Asp Leu Ser Leu Ser Cys Thr Leu

 433   GTG CCT CGC TCA GGG GAG CAG GCT GAA GGG TCC CCA GGT GGA CCT GGA
 120   Val Pro Arg Ser Gly Glu Gln Ala Glu Gly Ser Pro Gly Gly Pro Gly

 481   GAC TCT CAG GGT CGA AAA CGG CGG CAG ACC AGC ATG ACA GAT TTC TAC
 136   Asp Ser Gln Gly Arg Lys Arg Arg Gln Thr Ser Met Thr Asp Phe Tyr

 529   CAC TCC AAA CGC CGG CTG ATC TTC TCC AAG AGG AAG CCC TAA TCC GCC
 152   His Ser Lys Arg Arg Leu Ile Phe Ser Lys Arg Lys Pro ***

 577   CAC AGG AAG CCT GCA GTC CTG GAA GCG CGA GGG CCT CAA AGG CCC GCT
 625   CTA CAT CTT CTG CCT TAG TCT CAG TTT GTG TGT CTT AAT TAT TAT TTG
 673   TGT TTT AAT TTA AAC ACC TCC TCA TGT ACA TAC CCT GGC CGC CCC CTG
 721   CCC CCC AGC CTC TGG CAT TAG AAT TAT TTA AAC AAA AAC TAG GCG GTT
 769   GAA TGA GAG GTT CCT AAG AGT GCT GGG CAT TTT TAT TTT ATG AAA TAC
 817   TAT TTA AAG CCT CCT CAT CCC GTG TTC TCC TTT TCC TCT CTC CCG GAG
 865   GTT GGG TGG GCC GGC TTC ATG CCA GCT ACT TCC TCC TCC CCA CTT GTC
 913   CGC TGG GTG GTA CCC TCT GGA GGG GTG TGG CTC CTT CCC ATC GCT GTC
 961   ACA GGC GGT TAT GAA ATT CAC CCC CTT TCC TGG ACA CTC AGA CCT GAA
1009   TTC TTT TTC ATT TGA GAA GTA AAC AGA TGG CAC TTT GAA GGG GCC TCA
1057   CCG AGT GGG GGC ATC ATC AAA AAC TTT GGA GTC CCC TCA CCT CCT CTA
1105   AGG TTG GGC AGG GTG ACC CTG AAG TGA GCA CAG CCT AGG GCT GAG CTG
1153   GGG ACC TGG TAC CCT CCT GGC TCT TGA TAC CCC CCT CTG TCT TGT GAA
1201   GGC AGG GGG AAG GTG GGG TAC TGG AGC AGA CCA CCC CGC CTG CCC TCA
1249   TGG CCC CTC TGA CCT GCA CTG GGG AGC CCG TCT CAG TGT TGA GCC TTT
1297   TCC CTC TTT GGC TCC CCT GTA CCT TTT GAG GAG CCC CAG CTT ACC CTT
1345   CTT CTC CAG CTG GGC TCT GCA ATT CCC CTC TGC TGC TGT CCC TCC CCC
1393   TTG TCT TTC CCT TCA GTA CCC TCT CAT GCT CCA GGT GGC TCT GAG GTG
1441   CCT GTC CCA CCC CCA CCC CCA GCT CAA TGG ACT GGA AGG GGA AGG GAC
1489   ACA CAA GAA GAA GGG CAC CCT AGT TCT ACC TCA GGC AGC TCA AGC AGC
1537   GAC CGC CCC CTC CTC TAG CTG TGG GGG TGA GGG TCC CAT GTG GTG GCA
1585   CAG GCC CCC TTG AGT GGG GTT ATC TCT GTG TTA GGG GTA TAT GAT GGG
1633   GGA GTA GAT CTT TCT AGG AGG GAG ACA CTG GCC CCT CAA ATC GTC CAG
1681   CGA CCT TCC TCA TCC ACC CCA TCC CTC CCC AGT TCA TTG CAC TTT GAT
1729   TAG CAG CGG AAC AAG GAG TCA GAC ATT TTA AGA TGG TGG CAG TAG AGG
1777   CTA TGG ACA GGG CAT GCC ACG TGG GCT CAT ATG GGG CTG GGA GTA GTT
1825   GTC TTT CCT GGC ACT AAC GTT GAG CCC CTG GAG GCA CTG AAG TGC TTA
1873   GTG TAC TTG GAG TAT TGG GGT CTG ACC CCA AAC ACC TTC CAG CTC CTG
1921   TAA CAT ACT GGC CTG GAC TGT TTT CTC TCG GCT CCC CAT GTG TCC TGG
1969   TTC CCG TTT CTC CAC CTA GAC TGT AAA CCT CTC GAG GGC AGG GAC CAC
2017   ACC CTG TAC TGT TCT GTG TCT TTC ACA GCT CCT CCC ACA ATG CTG AAT
2065   ATA CAG CAG GTG CTC AAT AAA TGA TTC TTA GTG ACT TTA AAA AAA AAA
2113   AAA AAA AAA
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Figure 3. cDNA and Predicted Amino Acid Sequence of Human *WAF1*
The predicted translation begins at nucleotide 76 and ends at nucleotide 567.

linking (Hampson et al., 1992), provided an enrichment of over 100-fold for cDNA sequences not present in the RNA used for subtraction (data not shown). Following hybridization to the subtracted probe, the clones were rehybridized to a probe made from RNA of dexamethasone-induced DEL cells. A total of 99 clones differentially hybridized to the subtracted probe on the initial screen, and 45 of these reproducibly displayed differential hybridization when retested. Hybridization probes were prepared from these clones and used in Northern blots containing RNA isolated from dexamethasone-treated or untreated GM cells. Of the 45 clones, 28 were found to be highly induced upon dexamethasone treatment. The other 17 clones were less robustly induced by dexamethasone and were not studied further. Hybridization, sequencing, and restriction endonuclease analysis indicated that all of the 28 highly induced cDNA clones were derived from a single 2.1 kb mRNA. The gene encoding this message was named *WAF1*. Hybridization of individual *WAF1* clones to the cDNA library revealed that *WAF1* cDNA was present at a frequency of 0.4% following dexamethasone induction.

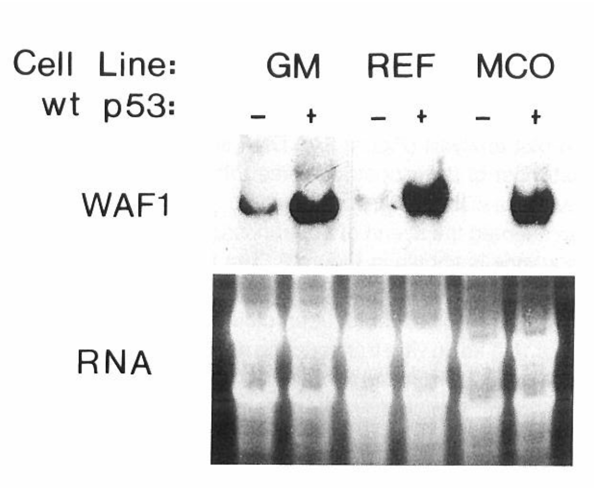
Structural Analysis of *WAF1*

Of the 28 *WAF1* clones, 18 appeared to contain near full-length cDNA, predicted to be 2.1 kb on the basis of Northern blot analysis (Figure 2A). DNA sequencing revealed that most of the clones contained the same 5′ end. Because the cDNA library used was not amplified, this likely represented the 5′ end of the transcript. The *WAF1* cDNA sequence is shown in Figure 3. The first ATG codon occurred at nucleotide 76, and an in-frame termination codon occurred at nucleotide 570, predicting a translation product of 18.1 kd. In vitro transcription and translation of *WAF1* cDNA clones produced a protein of the expected size (data not shown). Additionally, GM cells induced with dexamethasone produced a protein of 21 kd reactive with anti-WAF1 antibodies (see Figure 2A). These antibodies localized WAF1 protein to the nucleus of dexamethasone-induced GM cells (W. S. E.-D., B. V., M. Burrell, and D. Hill, unpublished data). Analysis of the amino acid sequence of WAF1 protein revealed a cysteine-rich region $C(X)_4C(X)_{15}C(X)_6C$ between amino acids 13 and 41 with the potential for zinc binding (Berg, 1986) as well as a basic region between amino acids 140 and 163 containing two potential bipartite nuclear localization signals (Robbins et al., 1991) near the carboxyl terminus. No significant homologies at the amino acid level were found to known proteins (National Biomedical Research Foundation PIR release #35.0). Southern blot analysis showed that *WAF1* was probably a single copy gene, with no close relatives in the human genome (data not shown).

To identify the chromosomal location of the *WAF1* gene, a human genomic *P1* clone (*P1-WAF1*) containing *WAF1* sequences was obtained (see Experimental Procedures). The clone contained about 85 kb of DNA, and partial sequencing revealed that the *WAF1* gene consisted of three exons of 68, 450, and 1600 bp (exons 1, 2, and 3, respectively). The translation initiation signal was contained in exon 2, a relatively long coding exon (Sterner and Berget, 1993). The *P1-WAF1* clone was labeled with biotin and hybridized to metaphase chromosomes as previously described (Meltzer et al., 1992). A total of 18 metaphase cells were examined, and each had at least one double fluorescent signal (i.e., signals on each of two chromatids) on the middle of the short arm of chromosome 6. In 15 of 18 cells, double signals were observed on both chromosome 6 homologs. Only chromosomes in which both chromatids displayed a signal were included for analysis, making the background hybridization close to zero. The same cells subjected to fluorescence in situ hybridization had been previously G-banded using trypsin–Giemsa and photographed to allow direct comparison of the results. The results demonstrated that sequences hybridizing to *WAF1* DNA fragment were localized to 6p21.2.

Induction of *WAF1*

If *WAF1* is important for p53 function, one might expect that it would be induced in more than one human cell type following wild-type p53 expression; that it would be highly conserved among species, because p53 is conserved both functionally and structurally; and that its induction by p53



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  1                                             Met Ser Asn Pro Gly Asp Val
       CT TCT TGT GTT TCA GCC ACA GGC ACC ATG TCC AAT CCT GGT GAT GTC

  8   Arg Pro Val Pro His Arg Ser Lys Val Cys Arg Cys Leu Phe Gly Pro
      CGA CCT GTT CCG CAC AGG AGC AAA GTG TGC CGT TGT CTC TTC GGT CCC

 24   Val Asp Ser Glu Gln Leu Ser Arg Asp Cys Asp Ala Leu Met Ala Gly
      GTG GAC AGT GAG CAG TTG AGC CGT GAT TGC GAT GCG CTC ATG GCG GGC

 40   Cys Leu Gln Glu Ala Arg Glu Arg Trp Asn Phe Asp Phe Val Thr Glu
      TGT CTC CAG GAG GCC CGA GAA CGG TGG AAC TTT GAC TTC GTC ACG GAG

 56   Arg Gln Leu Glu Gly Asn Phe Val Trp Glu Arg Val Arg Ser Leu Gly
      AGG CAG CTG GAG GGC AAC TTC GTC TGG GAG CGC GTT CGG AGC CTA GGG

 72   Leu Pro Lys Val Tyr Leu Ser Pro Gly Ser Arg Ser Arg Asp Asp Leu
      CTG CCC AAG GTC TAC CTG AGC CCT GGG TCC CGC AGC CGT GAC GAC CTG

 88   Gly Gly Asp Lys Arg Pro Ser Thr Ser Ser Ala Leu Leu Gln Gly Pro
      GGA GGG GAC AAG AGG CCC AGT ACT TCC TCT GCC CTG CTG CAG GGG CCA

104   Ala Pro Glu Asp His Val Ala Leu Ser Leu Ser Cys Thr Leu Val Ser
      GCT CCG GAG GAC CAC GTG GCC TTG TCG CTG TCT TGC ACT CTG GTG TCT

120   Glu Arg Pro Glu Asp Ser Pro Gly Gly Pro Gly Thr Ser Gln Gly Arg
      GAG CGG CCT GAA GAT TCC CCG GGT GGG CCC GGA ACA TCT CAG GGC CGA

136   Lys Arg Arg Gln Thr Ser Leu Thr
      AAA CGG AGG CAG ACC AGC CTG ACA GGT AAG GAC AGG AGC AGA GAA GGA
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Figure 4. *WAF1* Induction by p53 Is Conserved in Rat and Mouse

A Northern blot was prepared using RNA from GM cells, untreated (lane 1) or treated for 6 hr with dexamethasone (lane 2), from REF-112 cells grown at 37°C (uninduced; lane 3) or 31°C (lane 4), or from MCO1 cells infected with Ad-gal (lane 5) or Ad-p53 (lane 6). The RNA was hybridized with a human (lanes 1 and 2) or mouse (lanes 3–6) *WAF1* probe. An ethidium bromide stain of the gel prior to transfer is also shown. The nucleotide and predicted amino acid sequence of the mouse *WAF1* second exon is shown at the bottom.

would also extend across species. These predictions were tested in the following series of experiments.

Figure 2B illustrates the expression of *WAF1* in GM cells following dexamethasone treatment for 16 hr (lane 2), compared with either uninduced GM cells (lane 1) or dexamethasone-treated DEL cells containing induced mutant p53 (lane 3). Controls for the experiment included two other genes known to be induced by p53, *MDM2* and *GADD45*, as well as an unrelated gene, transforming growth factor β (TGFβ). Both *MDM2* and *GADD45* were induced in GM cells when wild-type p53 was present, but less so than *WAF1* (see Figure 2B).

To examine the induction of *WAF1* by p53 in a different human cell line, a wild-type p53 construct in an adenoviral vector (Ad-p53) was used to infect SW480 colon cancer cells. That Ad-p53 produced transcriptionally active p53 was demonstrated by assaying an SW480 cell line carrying a stably integrated reporter responsive to wild-type but not mutant p53 (see Experimental Procedures). SW480 cells were infected with either Ad-p53 or Ad-gal (a control adenoviral vector producing β-galactosidase instead of p53) for 16 hr. *WAF1* mRNA was highly induced in SW480 cells infected with Ad-p53 (see Figure 2B, lane 5), but not those infected with Ad-gal (lane 4).

We next assessed the evolutionary conservation of *WAF1*. So-called zoo blots revealed that single copy sequences from mouse and rat cells hybridized to the human *WAF1* clone, and we obtained a clone containing the *WAF1* gene by screening a mouse genomic library. The nucleotide and predicted amino acid sequence of the mouse *WAF1* second exon is shown in Figure 4. The mouse and human *WAF1* second exon sequences were 75% identical and 79% similar at the amino acid level. A stretch of 26 amino acids (human amino acids 21–56) was almost perfectly conserved, as was the zinc finger–like motif between amino acids 13 and 41 in human WAF1 ($H(X)_4C(X)_{15}C(X)_6C$ in the mouse). The positions of introns surrounding exon 2 in the *WAF1* gene were identical in human and mouse.

To determine whether rodent *WAF1* gene expression was induced by wild-type p53, two experimental systems were used. The first consisted of rat embryo fibroblasts containing a stably integrated murine temperature-sensitive mutant p53 (REF-112 cells; Michalovitz et al., 1990). These cells were transfected with the PG13–Luc reporter and incubated either at 37°C (mutant p53 conformation) or 31°C (wild-type p53 conformation) for 24 hr. No measurable increase in luciferase activity was observed at 37°C, but luciferase activity increased 1000-fold at 31°C, confirming the presence of transcriptionally active murine wild-type p53 at the latter temperature. RNA was then prepared from REF-112 cells incubated for 14 hr either at 37°C or 31°C. Figure 4 shows that expression of *WAF1* mRNA was detected at 31°C but not at 37°C, demonstrating that the *WAF1* gene is conserved in rat and that the gene is inducible by the murine p53 at the wild-type permissive temperature.

Second, the murine fibrosarcoma cell line MCO1 (Halevy et al., 1991), which lacks p53 owing to a splice site mutation and a deletion, was infected with either Ad-p53 or Ad-gal. At 22 hr following adenoviral infection, RNA was prepared and used in Northern blot analysis. Figure 4 shows that mouse *WAF1* was highly induced in MCO1 cells infected with Ad-p53, but not in cells infected with Ad-gal. Thus, *WAF1* induction by p53 was conserved in both rat and mouse cells.

WAF1 Suppresses Tumor Cell Growth

If *WAF1* plays a role in mediating the tumor growth inhibition of p53, one might expect it to have a growth suppressive role of its own. To address this possibility, mammalian expression vectors containing p53 cDNA or *WAF1* cDNA in either the sense (pC-WAF1-S) or antisense (pC-WAF1-AS) orientation were constructed. The vectors each contained a gene conferring hygromycin resistance in addition to the cDNA. The vectors were transfected into SW480 cells previously shown to be inhibited by wild-type p53 expression (Baker et al., 1990). Following transfection, cells were grown in the presence of hygromycin, and the number of colonies was scored after 2–3 weeks. The

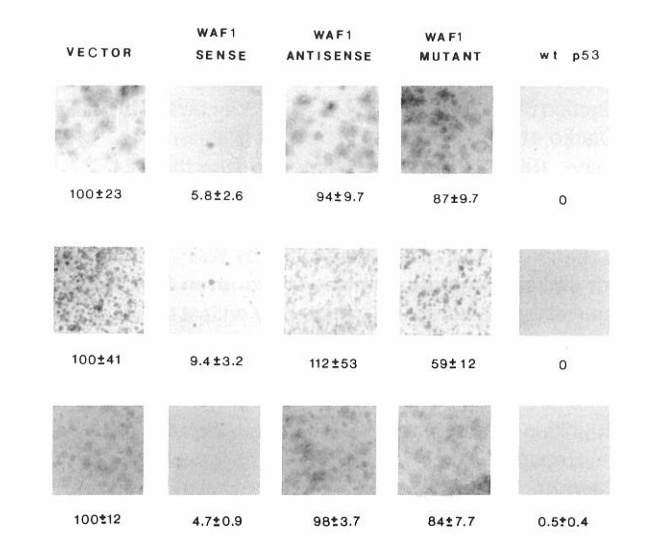

Figure 5. *WAF1* Suppresses the Growth of Human Tumor Cells

The human brain tumor line DEL (top), the human colon tumor line SW480 (middle), or the lung adenocarcinoma line H1299 (bottom) were transfected with the pCEP4 vector or with vectors encoding sense *WAF1*, antisense *WAF1*, mutant *WAF1*, or wild-type (wt) p53. The photographs show low power views of the transfected flasks following 17 days of hygromycin selection. Below each photograph, the fraction of colonies (in percent) in each flask compared with the vector transfected cells is indicated (mean of three flasks ± SD). The vector transfectants contained an average of 310, 850, and 427 colonies, respectively.

data in Figure 5 show that introduction of *WAF1* sense cDNA expression vectors resulted in substantial growth suppression, as seen by a 10- to 20-fold decrease in the number of hygromycin-resistant colonies. This growth suppression was similar to, but not as complete as, that observed with p53 (Figure 5). Introduction of the *WAF1* antisense cDNA expression vector or of the vector devoid of *WAF1* sequences resulted in a similar number of clones. The few clones that did appear after transfection of the *WAF1* sense cDNA expression vector generally grew at a slow rate and were not easily passaged. Similar results were obtained in four separate experiments, each with triplicate transfections, using different preparations of plasmid DNA. Additionally, we used the brain tumor cell lines GM and DEL and the lung adenocarcinoma line H1299 in similar experiments and found that their growth was also suppressed by the introduction of wild-type *WAF1* (Figure 5; data not shown). As an additional control, we constructed a *WAF1* mutant (pC-WAF1-ES) with a stop codon at nucleotide 222. Introduction of pC-WAF1-ES into either SW480, H1299, or DEL cells did not result in significant growth suppression (Figure 5).

p53 Activation of the *WAF1* Promoter

Having demonstrated that *WAF1* expression is induced by wild-type p53, we attempted to determine whether this resulted from a direct interaction of p53 with regulatory elements in *WAF1*. To search for sequences transcriptionally responsive to p53, we used the genomic clone *P1-WAF1* in a yeast enhancer trap system. In this system, yeast cells auxotrophic for histidine were transformed with a plasmid library constructed by insertion of random fragments of *P1-WAF1* upstream of a truncated *GAL1* promoter regulating *HIS3* reporter gene expression. Clones were selected for histidine prototrophy in the presence of human p53 expression. Three libraries were constructed, using AluI, HaeIII, or Sau3AI fragments of *P1-WAF1*. Through the screening of 1.6 × 10^5 transformants, 22 wild-type p53-dependent histidine prototrophs were obtained. No histidine prototrophy was observed if yeast expressed mutant instead of wild-type p53. All but 1 of the 22 clones were found to contain either of two sequence elements, both matching the previously defined p53-binding site consensus. Mapping revealed that one of them was located 2.4 kb upstream of *WAF1* coding sequences (Figure 6); the other was more than 8 kb upstream (T. Waldman and W. S. E.-D., unpublished data) and was not studied further.

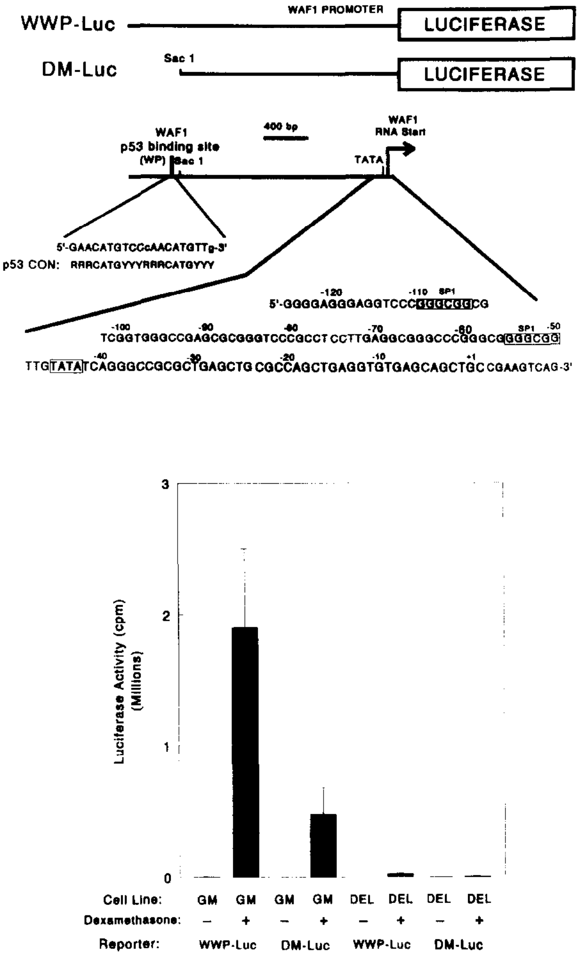

Figure 6. *WAF1* Transcriptional Regulatory Region

The diagram shows the promoter–reporter constructs and partial DNA sequence of the *WAF1* promoter. The consensus p53-binding site is compared with the p53-binding site (WP) 2.4 kb upstream of *WAF1*. The TATA element and Sp1 recognition sequences within the *WAF1* promoter are indicated by boxes. GM or DEL cells were transfected with the WWP–Luc or DM–Luc reporters (bottom), and luciferase activity was measured after incubation with or without dexamethasone for 14 hr.

The yeast experiments showed that at least one p53-binding site was present near *WAF1*, and this element, when placed in an artificial system with a foreign promoter, could stimulate expression of a reporter gene in the presence of wild-type p53. To determine whether the natural promoter elements of *WAF1* could mediate p53-dependent transcriptional activation, a 2.4 kb genomic fragment, with its 3′ end at nucleotide 11 of *WAF1* cDNA, was cloned upstream of a promoterless luciferase reporter gene. A partial sequence of the *WAF1* promoter and a map of this clone are shown in Figure 6. This promoter was G:C rich and contained a TATA element 43 nt upstream of the putative transcription start site. Two Sp1-binding sites were located at nucleotides −50 and −104, and there was a sequence weakly matching the p53-binding site consensus at nucleotide −75. The p53 responsive element identified in the yeast experiments was 2.4 kb upstream of *WAF1*.

Figure 6 shows that the *WAF1* promoter construct WWP–Luc activated expression of luciferase only in the presence of wild-type p53. In the absence of wild-type p53 (GM cells without dexamethasone or DEL with or without dexamethasone), expression of this reporter was less than 3% of levels observed in the presence of wild-type p53. When the 2.4 kb upstream p53-binding site was deleted (DM–Luc), the majority of the luciferase activity was abolished, though the residual activity was still wild-type p53 dependent. This observation suggests the presence of a second (weaker) p53 response element within the *WAF1* promoter, perhaps at nucleotide −75 (Figure 6).

Discussion

One of the goals of tumor biology is to unravel the pathways leading to growth suppression. For the tumor suppressor p53, a clue to the pathway was provided when it was found that p53 can bind to DNA in a sequence-specific manner and activate transcription from adjacent genes (see Introduction). This suggests that genes whose expression is activated by p53 might be mediators of p53 action (Vogelstein and Kinzler, 1992). The data described here show that *WAF1* may represent such a gene. *WAF1* expression was induced by p53, and this induction was observed in cell lines from human, mouse, and rat. Not only are the coding sequences and exon structure of *WAF1* conserved, but also its regulation by p53. This is consistent with the fact that p53 tumor suppressive function is also conserved between rodents and humans and the expectation that the mechanism of this suppression would be similarly conserved. The activation of a gene following wild-type p53 expression could be indirect, a result of induction by a second gene primarily controlled by p53. In the case of *WAF1*, the p53 induction was likely to be direct, as at least one strong functionally active binding site existed within its transcription regulatory region. The binding site functioned in yeast as well as in mammalian cells. Finally, *WAF1* could itself mimic the growth suppression of p53 when introduced into four different tumor cell lines.

Although all these experiments suggest that *WAF1* plays an important role in the p53 pathway, the results should be interpreted cautiously. First, we do not know whether WAF1-mediated growth inhibition results from the induction of apoptosis (Shaw et al., 1992; Lowe et al., 1993; Clarke et al., 1993) or of G1 arrest (Kastan et al., 1991; Lane, 1992). Second, we cannot be sure that *WAF1* is a critical target for p53. It is conceivable that the p53 DNA-binding site near the *WAF1* promoter is coincidental and that the growth inhibition mediated by *WAF1* results from an entirely separate pathway. Third, even if *WAF1* is a critical target, it may not be the only critical target. It may be part of a genetic program of growth arrest mediated by wild-type p53, and p53 may induce several downstream effectors, each with the potential to play a role in growth inhibition in some cells under certain circumstances. In this regard, we have noted in preliminary experiments that DNA damage induced by ultraviolet radiation (known to induce p53 expression; Maltzman and Czyzyk, 1984; Zhan et al., 1993) induces *WAF1* expression, but at lower levels than those observed in GM cells induced to express wild-type p53 with dexamethasone. Perhaps several growth arrest pathways exist, depending on the type of cell and its environment, as suggested by other experiments (Livingstone et al., 1992; Yin et al., 1992; Lowe et al., 1993; Clarke et al., 1993; Sherley, 1991; Zhan et al., 1993). We also note that G1 arrest induced in GM cells by mimosine or serum starvation, in the absence of wild-type p53, did not induce *WAF1* gene expression (W. S. E.-D. and B. V., unpublished data).

In the future, some of the above issues can be tested in *WAF1* mutagenesis and "knock out" experiments. A subset of the effects of p53 would be predicted to be *WAF1* dependent, assuming *WAF1* function was not redundant with other downstream genes. A detailed analysis of the cell cycle in stable cell lines carrying an inducible *WAF1* transgene may similarly provide clues as to its function in p53-mediated growth inhibition. Additionally, some tumors without p53 mutation might contain mutations of *WAF1*. Several tumors have been noted to have losses of the chromosomal region (6p21) containing *WAF1* (Solomon et al., 1991; Sato et al., 1991; Cliby et al., 1993; Lukeis et al., 1990; Morita et al., 1991; Vogelstein et al., 1989), consistent with the idea that a tumor suppressor gene resides in this area. Finally, identification of *WAF1* and its regulatory region potentially provides a novel drug discovery approach: compounds that activate expression of *WAF1* might bypass the p53 defect in tumors with endogenous p53 mutation.

After acceptance of this manuscript for publication, we learned that Harper et al. (1993 [this issue of *Cell*]) have identified a gene called *CIP1* whose product binds to cyclin complexes and inhibits the function of cyclin-dependent kinases. The sequence of *CIP1* is identical to that of *WAF1*. These results provide a dramatic example of the interplay between tumor suppressor genes and the cell cycle. In particular, the combined data suggest the following model for p53 function: p53 is not required for normal development, but in certain cellular environments (DNA damage, cellular stress), p53 expression is stimulated. In turn, p53 binds to *WAF1* regulatory elements and transcriptionally activates its expression. The WAF1 protein subsequently binds to and inhibits cyclin-dependent kinase activity, preventing phosphorylation of critical cyclin-dependent kinase substrates and blocking cell cycle progression. In tumor cells with inactive p53, this pathway would thereby be defective, permitting unregulated growth.

Experimental Procedures

Cell Culture and Transfection

The SW480-IAB3 cell line was obtained following cotransfection of SW480 cells with plasmids PG13-Gal (see below) and pCMV-Neo-Bam (Baker et al., 1990) and selection with genetecin. Individual clones were isolated by limiting dilution and tested for the presence of stably integrated intact reporter by transfection with either plasmid p53-wt or p53-143 (Kern et al., 1992) and by in situ X-Gal staining. REF-112 and MCO1 cells were obtained from M. Oren and H1299 cells were obtained from A. J. Fornace. The GM4723 (GM cells) and del4A (DEL cells) lines were passaged in Earle's minimal essential media, and log phase cells were induced with dexamethasone as previously described (Mercer et al., 1990). For transfection experiments, 1.5 × 10^6 cells were plated in 25 cm^2 tissue culture flasks 24 hr before transfection. A total of 5 μg of DNA and 25 μg of lipofectin (Bethesda Research Laboratories, Gaithersburg, Maryland) were used for transfections. For growth inhibition experiments (Figure 5), hygromycin (0.25 mg/ml) selection began 24 hr after transfection.

Plasmid Constructs

PG13–Luc and MG15–Luc plasmids were cloned by inserting the HindIII–EcoRI fragments containing wild-type or mutant p53-binding elements (PG13–CAT and MG15–CAT; Kern et al., 1992) into the HindIII–EcoRI sites of pBluescript II SK(+) (Stratagene, La Jolla, California). PG13 contains 13 copies of a p53-binding site, while MG15 contains 15 copies of a subtly mutated p53-binding site. The 200 bp EcoRI–BamHI fragment containing the polyoma promoter (from pBE-L.Py; Munholland et al., 1992) was cloned into pBluescript II SK(+) constructs containing either PG13 or MG15. A 2.6 kb SacI luciferase cassette or a 3 kb β-galactosidase cassette without promoter elements was then cloned downstream to create either PG13–Luc, MG15–Luc, or PG13–Gal, respectively. pC-WAF1-S (sense) and pC-WAF1-AS (antisense) expression plasmids were prepared by cloning the full-length *WAF1* cDNA as a NotI fragment from a cDNA library clone (pZL-WAF1) into the NotI site of pCEP4 (Invitrogen). The pC-WAF1-ES mutant vector was similarly obtained from a polymerase chain reaction (PCR)-generated cDNA insert, containing a G to A transition at nucleotide 222, resulting in a stop codon instead of tryptophan at amino acid 49. The 2.4 kb *WAF1* promoter region was obtained by PCR amplification using a *P1-WAF1* subclone as template and the primers 5′-CCACAAGCTTCTGACTTCGGCAG-3′ and 5′-CCCAGGAACAAGCTTGGGCAGCAG-3′. This PCR fragment was cloned into the HindIII site of pBluescript KS(+) (Stratagene) to yield plasmid pWWP. The plasmid pDM, which lacks the p53-binding element 2.4 kb upstream of *WAF1*, was obtained by digesting pWWP with SacI and recircularization. WWP–Luc and DM–Luc plasmids were cloned by inserting the 2.6 kb BamHI luciferase cassette from PG13–Luc into the XhoI sites of pWWP and pDM.

RNA Isolation and Northern Blot Analysis

Total RNA was prepared by CsCl gradient ultracentrifugation of guanidine isothiocyanate–lysed cells as described (Davis et al., 1986). Northern blot analysis was performed as previously described (El-Deiry et al., 1991) except that Quickhyb (Stratagene, La Jolla, California) was used for hybridization. The *MDM2* probe was made from a 1.6 kb cDNA fragment (Oliner et al., 1993), and the *GADD45* probe was made from a 5.0 kb genomic fragment (provided by A. J. Fornace; Kastan et al., 1992) through random priming (Feinberg and Vogelstein, 1983).

Library Screening

Poly(A)$^+$ RNA (3.5 μg) obtained from GM cells induced with dexamethasone for 6 hr was used to make an oligo(dT)-primed cDNA library with the SuperScript Choice System (GIBCO BRL Research Products Life Technologies, Grand Island, New York). A total of 100 ng of cDNA, comprising the 1.5–5 kb fraction, was ligated to λ Ziplox EcoRI arms (GIBCO BRL Life Technologies, Incorporated, Gaithersburg, Maryland), and phage clones were obtained following infection of Escherichia coli strain Y1090ZL. Phage clones were screened by hybridization of colony lifts to either subtracted or unsubtracted cDNA probes prepared as described below. Excision of pZL1 plasmid clones was carried out by phage infection of the excision strain DH10B-Zip (Elledge et al., 1991).

Unsubtracted cDNA probes were prepared from 2 μg of poly(A)$^+$ RNA "driver" using oligo(dT) as primer and Moloney murine leukemia virus SuperScript II as described (Hampson et al., 1992), except that following alkaline hydrolysis with NaOH and neutralization with HCl, the cDNA was isopropanol precipitated in the presence of 0.17 M sodium perchlorate, washed with 70% ethanol, vacuum dried, and resuspended in 10 μl of water (Kinzler and Vogelstein, 1989). Unsubtracted cDNA (20 ng) was then labeled with random primers using Sequenase as described (Hampson et al., 1992). Subtracted cDNA probes were prepared by hybridizing for 22 hr of 500 ng of target cDNA to 10 μg of poly(A)$^+$ driver RNA, chemical cross-linking with 2,5-diaziridinyl-1,4-benzoquinone (provided by J. Butler), and labeling as described (Hampson et al., 1992).

A mouse *WAF1* genomic clone was isolated by screening 1 × 10^6 clones of a mouse genomic DNA library in λFix II (Stratagene), using the human *WAF1* cDNA as a probe. One hybridizing clone was obtained. An 11 kb HindIII fragment containing the second exon of mouse *WAF1* was subcloned into the HindIII site of pBluescript II SK(+). An 0.3 kb PstI fragment from this clone (containing part of mouse *WAF1* exon 2) was used to probe the Northern blot in Figure 4.

Wild-Type p53-Producing Defective Adenovirus

The cDNA for p53 was obtained as a BamHI fragment from the p53-wt vector (Baker et al., 1990; Kern et al., 1992) and cloned into the BamHI site of pMV10 (Wilkinson and Akrigg, 1992). The HindIII fragment of pMV10-p53-wt was then subcloned into the HindIII site of the pMV60 vector (Wilkinson and Akrigg, 1992) to make the vector pMV60-p53-wt. The plasmids pMV60-p53-wt and pJM17 (Wilkinson and Akrigg, 1992) were cotransfected into 293 cells. Recombinants were plaque purified and tested for production of transcriptionally active p53 by infection of the SW480-IAB3 cell line. A plaque-purified recombinant (Ad-p53) induced β-galactosidase activity in infected SW480-IAB3 cells. The β-galactosidase-producing defective adenovirus (Ad-gal) was obtained from plaque-purified recombinants following cotransfection of 293 cells with pMV35 and pJM17. Both Ad-p53 and Ad-gal were further purified by CsCl banding.

Isolation of a p53-Responsive Element Using a Yeast Enhancer Trap

The *P1-WAF1* clone was digested to completion with HaeIII, AluI, or Sau3AI, subcloned into the plasmid pBM947, and used to identify p53-binding sites by genetic selection in yeast (Wilson et al., 1991; T. T. et al., unpublished data). A total of 530,000 clones were obtained in E. coli, and the DNA from these clones was used to transfect Saccharomyces cerevisiae cells containing a p53 expression vector and a *HIS3* gene under the control of p53 binding sequences (Nigro et al., 1992; Kern et al., 1992; T. T. and S. Thiagalingam, unpublished data). A total of 160,000 yeast clones were assayed for histidine prototrophy. Selection in the absence of histidine allowed the isolation of clones containing a p53 binding sequence; transcriptional activation by p53 resulted in *HIS3* production and subsequent survival of the yeast transformants. DNA was isolated from such clones and tested for induction of histidine prototrophy in yeast strains with or without human p53 expression vectors. The sequence of one of the sites is shown in Figure 6, and the sequence of the second site (greater than 8 kb upstream) was 5′-GGCCTTGCCCGGGCTTGTCT-3′.

Chromosomal Localization

A screen of human genomic *P1* clones for *WAF1* was performed using the primers 5′-CTTTCTAGGAGGGAGACAC-3′ and 5′-GTTCCGCTGCTAATCAAAG-3′ from *WAF1* exon 3 for PCR (Genome Systems, Incorporated, St. Louis, Missouri). The PCR was performed using the Bind-Aid kit (U. S. Biochemical, Cleveland, Ohio) in a 25 μl reaction containing 2.5 μl of 10× PCR buffer (U. S. Biochemical), 2 μl of 2.5 mM each dNTP (dATP, dCTP, dGTP, and TTP), 0.5 μl of Bind-Aid

(0.5 µg/µl SSB), 0.5 µl of each primer (350 ng/µl), 10 ng of DNA template, and 2 U of AmpliTaq (Perkin-Elmer Cetus, Norwalk, Connecticut). Amplification was carried out for 35 cycles (following the profile of 95°C for 30 s, 57.5°C for 1 min, and 70°C for 1 min), yielding a 99 bp PCR product. The *P1* clone obtained (*P1-WAF1*) contained approximately 85 kb, including at least 21 kb upstream of exon 1 and 7 kb downstream of exon 3 of *WAF1*. *P1-WAF1* DNA was labeled with biotin and hybridized to metaphase chromosomes as previously described (Meltzer et al., 1992). Eighteen metaphase nuclei were examined for *WAF1* localization.

Luciferase Assays

Transfected cells were washed twice with 4 ml of Dulbecco's phosphate-buffered saline per T-25 flask. The cells were lysed with 0.3 ml (per T-25) of 1 × CCLR buffer (Promega) for 10 min at room temperature. After a 5 s spin to pellet large debris, 10 µl of supernatant was added to 90 µl of reconstituted luciferase assay reagent (Promega). Light emission was detected by scintillation counting.

Isolation of WAF1 Antisera and Western Blot Analysis

A WAF1–glutathione S-transferase fusion construct was prepared in pGEX-2T (Pharmacia). Electroeluted WAF1–glutathione S-transferase fusion protein was used to immunize mice as described previously (Smith et al., 1993). Western blots using 1:500 dilution of mouse polyclonal sera were performed and analyzed as previously described (Smith et al., 1993).

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