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A Mammalian Cell Cycle Checkpoint Pathway Utilizing p53 and *GADD45* Is Defective in Ataxia-Telangiectasia

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Summary

Cell cycle checkpoints can enhance cell survival and limit mutagenic events following DNA damage. Primary murine fibroblasts became deficient in a G1 checkpoint activated by ionizing radiation (IR) when both wild-type p53 alleles were disrupted. In addition, cells from patients with the radiosensitive, cancer-prone disease ataxia-telangiectasia (AT) lacked the IR-induced increase in p53 protein levels seen in normal cells. Finally, IR induction of the human *GADD45* gene, an induction that is also defective in AT cells, was dependent on wild-type p53 function. Wild-type but not mutant p53 bound strongly to a conserved element in the *GADD45* gene, and a p53-containing nuclear factor, which bound this element, was detected in extracts from irradiated cells. Thus, we identified three participants (AT gene(s), p53, and *GADD45*) in a signal transduction pathway that controls cell cycle arrest following DNA damage; abnormalities in this pathway probably contribute to tumor development.

Introduction

Both prokaryotic and eukaryotic cells delay cell division following damage of the DNA. Delays in progression from both G1 into S and from G2 into M occur in most organisms (Tolmach et al., 1977; Painter and Young, 1980; Lau and Pardee, 1982; Weinert and Hartwell, 1988; Kaufmann et al., 1991; O'Connor et al., 1992). These cell cycle checkpoints presumably exist to prevent both replication of a damaged DNA template (the G1 arrest) and segregation of damaged chromosomes (the G2 arrest). It is thought that the transient delays at these checkpoints permit repair of the damaged DNA prior to these critical cellular functions and should thus enhance cell survival and limit propagation of heritable genetic errors (Weinert and Hartwell, 1988). For example, mutations of the *RAD9* gene in yeast result in a defect in the G2 checkpoint; these *RAD9* mutants are radiosensitive compared with wild-type strains and exhibit increased genetic instability following γ-irradiation (Hartwell and Weinert, 1989; Weinert and Hartwell, 1988, 1990).

Though cell cycle delays due to DNA damage have been observed for years in mammalian cells, little is known about the molecular mechanisms that control them. Recently, we found that levels of the tumor suppressor nuclear protein p53 transiently increase in temporal association with the transient decrease in replicative DNA synthesis that follows exposure of human cells to γ-irradiation (Kastan et al., 1991). Since cells with wild-type p53 genes exhibited transient arrests in both G1 and G2 after γ-irradiation, while cells with absent or mutant p53 genes retained only the G2 arrest, it appeared that wild-type p53 played a critical role in the G1 arrest. This concept was further supported by the subsequent observations that transfection of wild-type p53 genes into malignant cells lacking endogenous p53 genes partially restored the G1 arrest after γ-irradiation and that overexpression of a transfected mutant p53 gene in tumor cells with wild-type endogenous p53 genes abrogated the G1 arrest following γ-irradiation (Kuerbitz et al., 1992). Since the tumor cell lines used for the transfection experiments had multiple genetic abnormalities, however, it had still not been formally demonstrated that loss of p53 function alone, with no other genetic changes, was sufficient for loss of the G1 arrest. This is addressed here by evaluating the cell cycle progression following γ-irradiation of normal embryonic fibroblasts obtained from mice in which both p53 genes have been disrupted.

Ataxia-telangiectasia (AT) is a human autosomal recessive disorder with many phenotypic characteristics, including hypersensitivity to ionizing radiation (IR), radioresistant DNA synthesis, a markedly increased incidence of cancer, and progressive cerebellar ataxia with degeneration of Purkinje cells (for reviews see McKinnon, 1987; Gatti et al., 1991). It has been suggested that the inability of AT cells to cease replicating DNA following γ-irradiation contributes to their hypersensitivity (Painter and Young, 1980). Interestingly, the lack of cessation of replicative DNA synthesis in AT cells following γ-ray exposure (Rudolph and Latt, 1989) is virtually identical to the situation observed in cells with abnormal p53 genes (Kastan et al., 1991; Kuerbitz et al., 1992). Therefore, a link between the defect in AT and the signal transduction pathway that utilizes p53 in causing the G1 arrest following γ-irradiation was investigated. If the AT phenotype were due to a defect proximal to p53 in this pathway, AT cells would lack the induction of p53 protein that is seen in normal cells following γ-irradiation. Thus, p53 protein levels were examined in normal and AT cells following γ-irradiation.

Recently, p53 has been shown to be a sequence-specific DNA-binding protein (Bargonetti et al., 1991; Kern et al., 1991), and a genomic consensus sequence has been elucidated that consists of two copies of a symmetric 10 bp motif separated by 0-13 bp (El-Deiry et al., 1992). When this consensus sequence was placed adjacent to a

basal promoter linked to chloramphenicol acetyltransferase (CAT) or luciferase reporter genes, cotransfection of such a construct with a p53 expression vector into mammalian cells resulted in induction of the reporter gene (Kern et al., 1992; Funk et al., 1992). In addition, wild-type p53 can directly activate transcription in vitro (Farmer et al., 1992). Thus, a likely role for p53 in a signaling mechanism that activates a G1 checkpoint after DNA damage is as a transcription factor that activates genes involved in negative growth control. Potential candidates for p53-inducible genes might be those that are differentially regulated after DNA damage and growth arrest.

The five growth arrest and DNA damage–inducible (or gadd) genes were initially isolated on the basis of induction after DNA damage in Chinese hamster ovary cells but have been subsequently found to be induced by DNA-damaging agents or other treatments eliciting growth arrest, such as serum reduction, in a wide variety of mammalian cells (Fornace et al., 1989b). In particular, the *gadd45* and *gadd153* genes have been found to be rapidly and coordinately induced by agents, such as methylmethane sulfonate (MMS), that produce high levels of base damage in DNA in every cell line examined, including human, hamster, murine, and rat cells (Fornace et al., 1989b, 1992). Recently, the human *GADD45* gene was found to be rapidly induced by IR in lymphoblasts and fibroblasts (Papathanasiou et al., 1991). This IR response appeared to be distinct from the gadd response to MMS and other base-damaging agents because only *GADD45* was strongly induced and induction occurred with doses of IR that produce relatively little DNA base damage. Interestingly, this IR response was significantly reduced in four AT compared with four normal lymphoblast lines (Papathanasiou et al., 1991). The function of the mammalian *GADD45* gene is unknown, but it is highly conserved in vertebrate species (unpublished data; Papathanasiou et al., 1991). Based on this information and on the recent finding that the IR induction of *GADD45* is absent in some human tumor cell lines (Fornace et al., 1991), a role for p53 in the IR response of *GADD45* was investigated. After demonstrating that IR induction of *GADD45* is dependent on a wild-type p53 phenotype, a conserved p53-binding site was identified in the human and hamster *GADD45* genes and binding of p53 protein to this sequence was evaluated. Based on the results of these investigations, a model for the steps in this DNA damage–inducible signal transduction pathway resulting in a G1 arrest is presented. Abnormalities at any step in this pathway have the potential to affect cell survival and genomic integrity adversely following certain types of DNA damage, including IR, and thus also to contribute to cellular transformation.

Results

Cell Cycle Perturbations Following IR in Cells from p53 Knockout Mice

To demonstrate that loss of p53 function alone, with no other genetic abnormalities, is sufficient for loss of the G1 arrest following γ-irradiation, cell cycle perturbations of embryonal fibroblasts from mice in which no, one, or two copies of the endogenous p53 alleles had been disrupted by homologous recombination (T. J. and R. Weinberg, unpublished data; see Livingstone et al., 1992) were evaluated following exposure to γ-rays. Cell cycle progression was evaluated at very early passage at a time when the cells were documented to have normal murine karyotypes and morphology (Livingstone et al., 1992). As expected, cells with two wild-type p53 alleles exhibited a marked decrease in the number of cells that continued to progress into S phase from G1 (Figure 1). Cells in which only one p53 allele had been disrupted also exhibited a significant decrease in the percentage of S phase cells following IR. In contrast, cells in which both p53 alleles had been disrupted (two separate isolates were evaluated) lost the ability to arrest in G1 after IR (Figure 1). These results demonstrate that loss of wild-type p53 function alone is sufficient to abrogate the G1 checkpoint following IR.

AT and p53 Following IR

Since AT cells lack the normal delay in DNA synthesis after exposure to IR (e.g., Painter and Young, 1980; Rudolph and Latt, 1989), the relationship between the AT phenotype and p53 function was investigated. As discussed previously, a rapid increase in p53 protein levels appears to be closely linked to the IR-induced G1 arrest. To investigate whether there is a defect in this p53-dependent response pathway in AT, the ability of a number of cell lines from AT patients to increase p53 protein levels following exposure to IR was evaluated. One hour following exposure to 2 Gy, increases in p53 protein levels in Epstein–Barr virus–immortalized lymphoblasts from genetically normal individuals were easily detected by both immunoprecipitation of [^{35}S]methionine-labeled p53 (Figure 2A) and immunoblots detecting p53 from whole-cell extracts (Figure 2B). In contrast, no increase in p53 protein levels following IR was evident in lymphoblasts from patients with AT by immunoprecipitation (Figure 2A; two different cell lines shown) or by immunoblot (Figure 2B; three different cell lines shown). These findings were reproducible in repeated experiments and were also supported by flow cytometric analysis of changes in p53 protein levels following IR (data not shown). (The p53 protein doublet in some cell lines is presumably due to a polymorphism at codon 72 that occurs in some individuals [Buchman et al., 1988] and is independent of the status of the AT or p53 genes in cells.) p53 protein levels following IR did increase in a lymphoblast cell line from an AT heterozygote; whether or not there is a statistically significant, reproducible difference between p53 induction in normal cells and AT heterozygotes, however, will require examination of a larger number of normal and AT heterozygote cell lines.

The induction of p53 protein was also evaluated in diploid fibroblasts from a normal individual and from an AT patient. As was seen in the lymphoblasts, while cells from a normal individual increase p53 protein levels following IR, no increase was evident in fibroblasts from an AT patient (Figure 2C). It is noted that the increase in p53 protein following irradiation of fibroblasts, and possibly nonhematopoietic cells in general (M. B. K., unpublished data), is smaller than that seen in cells of hematopoietic origin.

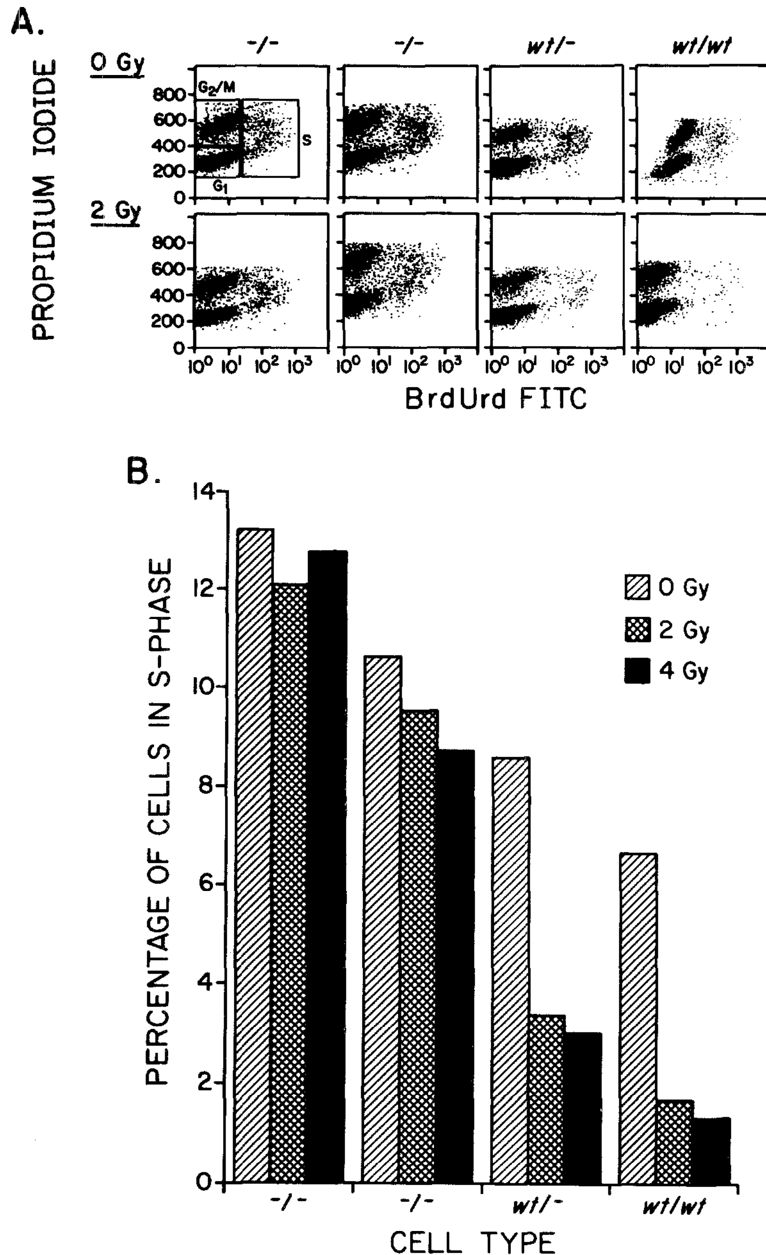

Figure 1. Cell Cycle Changes in Embryonic Fibroblasts after Exposure to IR as a Function of p53 Gene Status

(A) Dot plots of murine embryonic fibroblasts with no intact wild-type p53 alleles (–/–; two separate isolates evaluated), one intact wild-type p53 allele (wt/–), or two intact wild-type p53 alleles (wt/wt) 16 hr after exposure to 0 or 2 Gy IR. The cells were pulsed with BrdUrd for 4 hr, harvested, and stained for replicative DNA synthesis with a fluorescein isothiocyanate–conjugated anti-BrdUrd antibody and for DNA content with propidium iodide. The G1, S, and G2/M phases of the cell cycle defined by this process are illustrated in the top left panel.

(B) Quantitative assessment of the percentage of embryonic fibroblast cells remaining in S phase after exposure to 0, 2, or 4 Gy IR evaluated as illustrated in (A).

AT is now known to be a genetically heterogeneous disease with five defined complementation groups (Jaspers et al., 1988). Though the complementation group of every cell line utilized has not been identified, the lymphoblast line, 718 (Figures 2A and 2B), is complementation group A (the most common group). Two lymphoblast lines from AT patients classified as complementation group C (CSA and BMA) exhibited only about 2-fold increases in p53 protein, rather than the 7-fold increase in p53 seen in normal cells (2184; Figure 2D). Thus, group A and group C cells, which make up approximately 83% of all AT families (Jaspers et al., 1988), are both deficient in the ability to induce p53 protein following IR. However, since p53 protein does not increase at all in group A cells (Figures 2A and 2B) and in 1526 cells (complementation group unknown; Figure 2D), it is possible that the molecular defect in group C affects p53 induction after irradiation less than the defect in group A affects p53 induction.

Dependence on p53 Status of *GADD45* Induction by IR

In an effort to identify genes that may be induced by p53 after IR, the response of the *GADD45* gene was examined in human cells where the p53 phenotype is known. As described earlier, this gene has been found to be IR inducible in normal human fibroblasts and lymphoblasts but not in some tumor cell lines. In human lymphoblasts, induction as determined by increased *GADD45* mRNA levels has been seen with doses as low as 0.5 Gy (unpublished data). After 20 Gy there is a strong and prolonged induction for

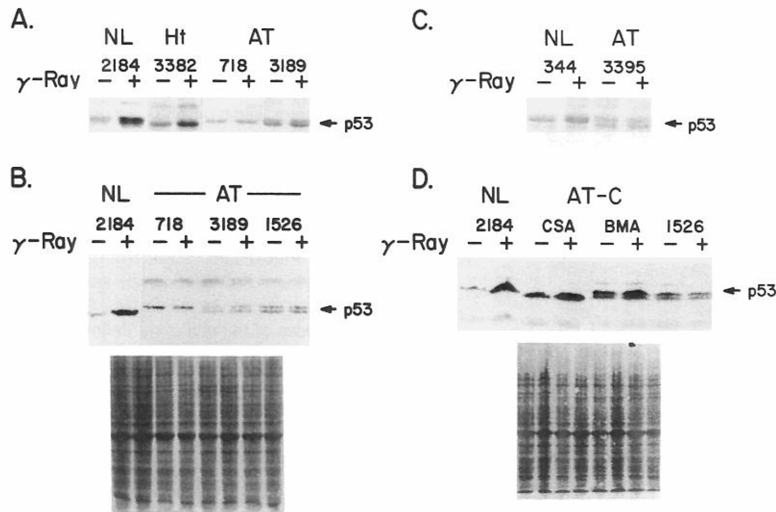

Figure 2. p53 Protein Expression in Normal and AT Cells after IR

(A) Immunoprecipitation of p53 protein metabolically labeled with [^{35}S]methionine for 1 hr after exposure to 0 (minus γ-ray) or 2 (plus γ-ray) Gy in normal (NL; 2184), AT heterozygote (Ht; 3382), or AT homozygote (718 and 3189) lymphoblasts. Equivalent numbers of trichloroacetic acid-precipitable ^{35}S counts per minute were immunoprecipitated in the irradiated and nonirradiated samples from each cell type pair.

(B) Immunoblot for p53 protein in cellular extracts from normal (2184) or AT (718, 3189, and 1526) lymphoblasts made 1 hr after exposure to 0 or 2 Gy. Extracts from 2 × 10^6 cells were loaded in each lane, and total protein loading is illustrated by the fast green staining of the nitrocellulose paper in the lower panel. The exposure time is 2 min for the 2184 lanes and 3 min for the other lanes.

(C) Immunoprecipitation of p53 protein metabolically labeled with [^{35}S]methionine for 1 hr after exposure to 0 or 4 Gy IR in normal (344) or homozygote AT (3395) diploid fibroblasts. Extracts from equivalent cell numbers and containing equivalent ^{35}S counts per minute were immunoprecipitated in the irradiated and nonirradiated samples of both cell type pairs.

(D) Immunoblot for p53 protein in cellular extracts from normal (2184), AT group C (CSA and BMA), and an AT cell line with an undefined complementation group (1526) made 1 hr after exposure to 0 or 2 Gy. Extracts from 2 × 10^6 cells were loaded in each lane, and total protein loading is illustrated by the fast green staining of the nitrocellulose paper in the lower panel.

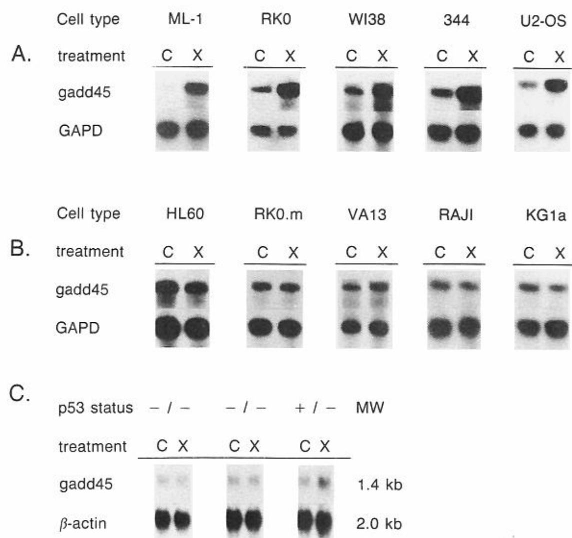

Figure 3. Relationship of p53 Phenotype to the γ-Ray Response of gadd45

(A) RNA from untreated (C) and γ-irradiated (X) human cell lines with a wild-type p53 phenotype were analyzed by RNAase protection assay. Cells were irradiated with 20 Gy 4 hr prior to harvest. For each sample, 10 μg of whole-cell RNA was hybridized with probes complementary to human *GADD45* and *GAPD* mRNA and assayed as described in Experimental Procedures.

(B) Human tumor cell lines with a mutant or null p53 phenotype were analyzed as in (A).

(C) Primary fibroblasts from mice with the designated p53 genotype were irradiated as above and analyzed by Northern blot. Samples of whole-cell RNA (10 μg) were size separated and hybridized with a hamster gadd45 probe; the blot was stripped and then hybridized with a hamster β-actin probe. Only the hybridizing bands are shown with the estimated sizes (kilobases) to the right.

more than 4 hr; the increase in *GADD45* mRNA at 4 hr has been found to be representative of this induction (Papathanasiou et al., 1991; unpublished data). When mRNA levels were determined by RNAase protection, a clear increase for *GADD45* was observed in cells with a wild-type p53 phenotype, while the control transcript GAPD was unchanged (Figure 3A). In contrast, appreciable induction was not evident in cells lacking a wild-type p53 phenotype (Figure 3B).

The relationship of p53 status to *GADD45* induction was examined in more detail by estimating the magnitude of the induction in various human cell lines, as measured by RNAase protection (Table 1). The accuracy of this approach was confirmed by comparing induction in representative poly(A) RNA samples measured by either RNAase protection or quantitative dot-blot hybridization (Hollander and Fornace, 1990); the results with both approaches were similar and typically varied by <25% (data not shown). It should be noted that this analysis (Table 1) was done in a blinded fashion with the identity of the cell types withheld until all quantitations were completed. The relative abundance of *GADD45* mRNA in irradiated cells was estimated by first normalizing to the value for *GAPD* in each sample and then dividing this value for irradiated cells by that of its control. Normal lymphoblasts and fibroblasts and tumor cells with a wild-type p53 status all exhibited greater than 2-fold increases in *GADD45* mRNA after irradiation, with a range up to 10-fold. Induction of *GADD45* mRNA also correlated with the activation by IR of the G1 checkpoint in these cells, which have normal p53 function (Kastan et al., 1991; Kuerbitz et al., 1992). In contrast, cells with mutant (SW480, Raji, and KG1a) or absent (HL60)

p53 genes failed to show appreciable induction of *GADD45* mRNA after IR (Table 1). Loss of *GADD45* responsiveness after IR also correlated with loss of the G1 checkpoint (Table 1) and loss of induction of p53 protein (Kastan et al., 1991; Kuerbitz et al., 1992). It is further noted that AT cells, which fail to increase p53 protein levels in response to IR (see above), similarly have reduced *GADD45* induction following IR (Papathanasiou et al., 1991).

The basal levels of *GADD45* mRNA did not correlate with p53 status and were low in all cells. It was estimated that the abundance of *GADD45* mRNA was >100-fold lower than that of *GAPD* mRNA in these cell lines. The relative levels of *GAPD* mRNA could be accurately estimated by quantiative dot–blot hybridization using whole-cell RNA and normalized to the poly(A) content of the cells. When this was done (data not shown), this value was used to compute the relative level of *GADD45* mRNA in different cell types (also employing the values in Table 1) and to confirm that the level of *GAPD* mRNA remained constant after IR.

Cells with wild-type p53 genes, but expressing viral products that interfere with p53 function, similarly lacked normal IR-mediated *GADD45* induction. VA13 is a derivative of WI38 that was obtained by transformation with SV40 (Girardi et al., 1966). The T antigen of this virus is known to bind to p53 protein (Lane and Crawford, 1979), and VA13 cells were deficient in induction of *GADD45* mRNA (Table 1). HeLa cells have been infected with HPV-18 that contains an E6 protein that inhibits normal p53 function (Werness et al., 1990; Scheffner et al., 1990, 1991; Crook et al., 1991). Activation of the G1 checkpoint and induction of *GADD45* was substantially less in HeLa cells than in the cell lines with normal p53 function (Table 1).

To demonstrate that it was the status of the p53 gene and not some other difference between these cell lines that was responsible for the differences in *gadd45* induction, *gadd45* induction was evaluated in cells in which the p53 gene had been manipulated. RKO colorectal carcinoma cells stably overexpressing a mutant (codon 143) p53 gene have previously been shown to lose the G1 arrest following IR (Kuerbitz et al., 1992). In contrast with parental RKO cells (Figure 3A) and RKO cells transfected with a control vector lacking the p53 gene insert, RKO cells overexpressing the mutant p53 allele did not significantly increase *GADD45* mRNA levels following IR (Table 1; Figure 3B). Similarly, the murine embryonic fibroblasts discussed above in which the p53 genes had been disrupted by homologous recombination failed to increase *gadd45* mRNA levels following IR, while the heterozygous cells with only one intact wild-type p53 allele remaining still induced *gadd45* mRNA following IR (Figure 3C; +/+ cells were not available in sufficient quantities for this experiment). (Since these cells are murine and the only rodent probe available for the *gadd45* gene is hamster, these evaluations were done by Northern analysis rather than by RNAase protection, which requires a homologous probe).

Binding of p53 Protein to a Conserved Element in the *GADD45* Gene

Based on the relationship of p53 to *GADD45* mRNA expression after IR, a search was undertaken to identify elements in the *GADD45* gene that may interact with p53.

Table 1. Fold Increase of *GADD45* mRNA after γ-Irradiation

Cell Line	Cell Type	p53 Status	γ-Ray G1 Arrest[a]	Relative Abundance of mRNA[b]
Normal p53 function				
ML-1	Myeloid leukemia	wt/wt	+	9.9
U2-OS	Osteosarcoma	wt/wt	+	3.0
AG1522	Skin fibroblast	wt/wt	ND	4.2
344	Skin fibroblast	wt/wt	+	3.3
WI38	Lung fibroblast	wt/wt	ND	2.0
RKO	Colorectal carcinoma	wt/wt	+	3.1
RKO.cp[c]	Colorectal carcinoma	wt/wt	+	3.2
RKO.c[d]	Colorectal carcinoma	wt/wt	+	4.1
Abnormal p53 function				
RKO.m[e]	Colorectal carcinoma	wt/wt, mut	−	1.4
Raji	Lymphoid leukemia	wt/mut	−	0.7
SW480	Colorectal carcinoma	mut/−	−	1.4
KG1a	Myeloid leukemia	mut/−	−	0.8
HL60	Myeloid leukemia	−/−	−	1.0
VA13	Lung fibroblast	?, SV40 transformed	ND	1.0
HeLa	Cervical carcinoma	wt/wt, HPV-18 infected	±[f]	1.6

[a] Activation of arrest in the G1 phase of the cell cycle following γ-irradiation as published previously (Kastan et al., 1991; Kuerbitz et al., 1992) ND, not determined.
[b] Relative values for samples harvested 4 hr after 20 Gy compared with untreated controls as determined by RNAase protection assay (see Experimental Procedures).
[c] Polyclonal population, transfected with control vector lacking p53 gene insert (Kuerbitz et al., 1992).
[d] Clonal population, transfected with control vector lacking p53 gene insert (Kuerbitz et al., 1992).
[e] Clonal population, transfected with mp53 vector (RKO.p53.13; Kuerbitz et al., 1992).
[f] Measurable decrease in S phase, but markedly less than cells with normal p53 function.
wt, wild type; mut, mutant.

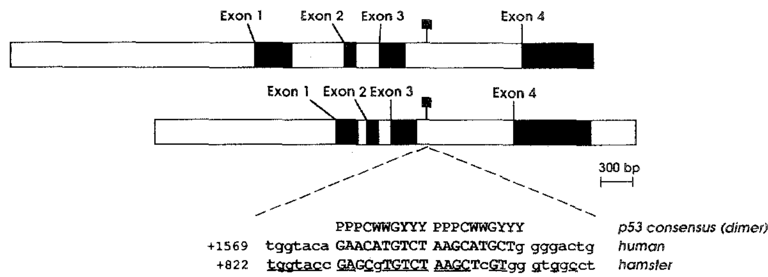

Figure 4. Organization of the Human and Hamster *gadd45* Genes

The determined sequences of the two genes are shown diagrammatically; the only regions with convincing homology to the p53 consensus sequence (El-Deiry et al., 1992) are designated with closed squares. The nucleotide sequences of this region from the human and hamster genes are shown below with the consensus sequence. Nucleotides matching the consensus sequence are shown in uppercase letters, and homologies with the hamster sequence are underlined.

Recently, the human and hamster *gadd45* genes have been isolated and sequenced (M. C. Hollander et al., unpublished data). Both genes consist of four exons that are highly conserved between human and hamster (Papathanasiou et al., 1991); the first two introns show no appreciable homology, while the larger third intron is 61% homologous between the two species (M. C. Hollander et al., unpublished data). Both genes were found to contain only one site with convincing homology to the published p53 consensus sequence (Figure 4). Interestingly, this site is not in the 5′ promoter region of these genes but instead is in the third intron at the same corresponding position in both genes. It should be noted that this intron is slightly larger in the human gene because of several small insertions elsewhere in the intron. This putative p53-binding site in the human *GADD45* gene matched the consensus sequence (El-Deiry et al., 1992) in 19 of 20 bp and in the hamster in 16 of 20 bp (Figure 4). The invariant C at positions 4 and 14 and G at position 7 and 17 of the consensus sequence are present in both the human and hamster genes.

To demonstrate that p53 can bind to this particular sequence, binding studies were undertaken with baculovirus-produced p53 protein. By immunoprecipitation with an anti-p53 antibody, wild-type p53 protein was found to bind a 30 bp oligomer containing the putative binding site from the human gene (Figure 5A). In contrast, a mutant p53 protein did not bind to the oligomer (Figure 5A). To demonstrate binding of wild-type p53 to the *GADD45* gene itself, a genomic plasmid subclone containing 6 kb of human

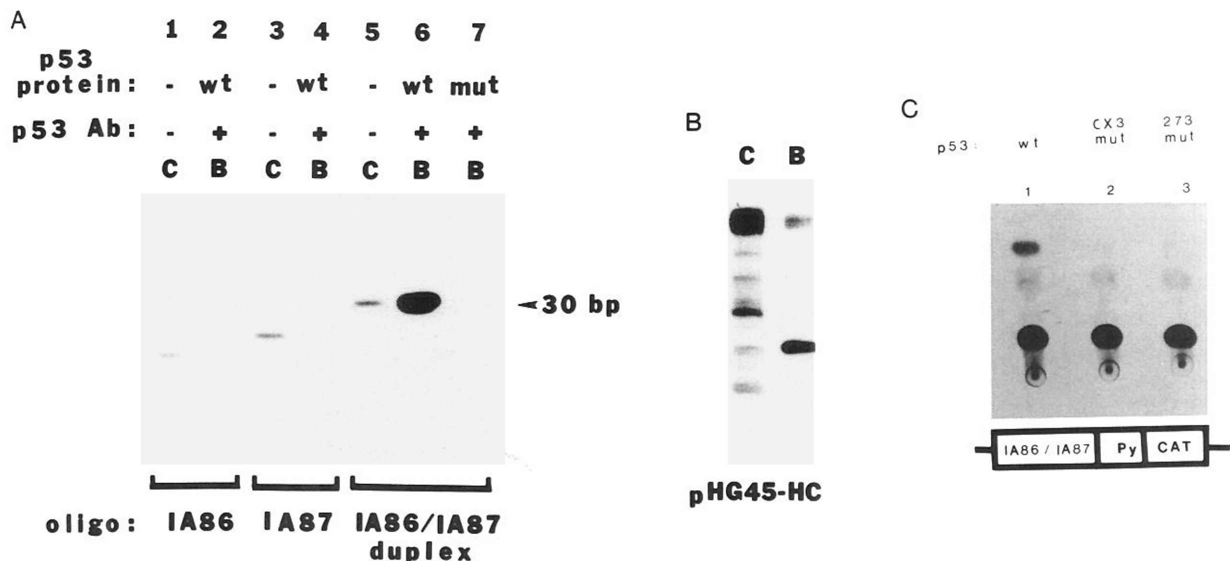

Figure 5. Binding of p53 Protein to *GADD45* Gene Sequences

(A) Immunoprecipitation of ^{32}P-labeled 30 bp oligomers from the *GADD45* intronic sequence with wild-type and mutant p53 protein. Single-strand oligomers IA86 and IA87 were allowed to autoanneal (lanes 2 and 4, respectively) or anneal with each other (lanes 6 and 7) prior to p53 protein binding and immunoprecipitation. Control (C) lanes (1, 3, and 5) contain 2% of the total end-labeled DNA used in each oligomer binding assay. The oligomers were immunoprecipitated with wild-type (lanes 2, 4, and 6) or mutant (Arg to His, codon 273; lane 7) p53 protein prior to electrophoresis on a 15% polyacrylamide gel.

(B) Binding of wild-type p53 protein to a restriction fragment from a GADD45 genomic clone. The pHG45-HC clone was digested with SauIIIAI, end labeled with [α-^{32}P]dCTP, and either directly electrophoresed (2% of total labeled fragments; lane C) or immunoprecipitated in the presence of wild-type p53 protein (lane B) prior to electrophoresis on a 7.5% polyacrylamide gel. A fragment of ~150 bp, the expected size for the intronic fragment containing the p53 consensus binding site following SauIIIAI restriction, is immunoprecipitated by wild-type p53.

(C) A CAT construct containing the *GADD45* oligomer sequence was cotransfected into SW480 cells with wild-type (lane 1) or mutant (lanes 2 and 3) p53 expression vectors, and CAT activity was assessed.

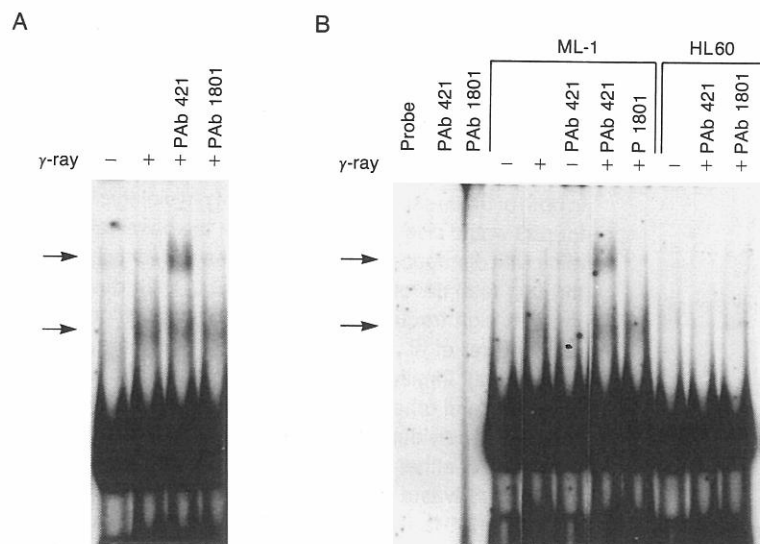

Figure 6. Mobility Shift Assays with the *GADD-45* p53-Binding Site and Extracts from Irradiated Cells

(A) Nuclear extracts from ML-1 cells isolated 3 hr after 20 Gy or nonirradiated cells were incubated with labeled DNA corresponding to the human *GADD45* p53-binding site and then were electrophoresed in a neutral acrylamide gel as described in Experimental Procedures. In the designated lanes, the p53 antibodies PAb421 or PAb1801 were included.

(B) In a separate experiment, a similar analysis was carried out using extracts from ML-1 or HL60 cells. The first three lanes consist of controls where probe alone or probe with antibody were used in the absence of nuclear extracts. The bottom arrow indicates the position of the IR-induced band, and the top arrow indicates the position of the supershifted band seen with PAb421.

DNA including this gene was digested with a restriction enzyme and immunoprecipitated in the presence of p53 protein (Figure 5B). As predicted by the known sequence of the *GADD45* gene, only one restriction fragment, which corresponded to the third intron site, was immunoprecipitated in the presence of wild-type but not mutant p53. No specific binding of wild-type p53 was seen with other DNA fragments, including a promoter fragment tested separately (data not shown for promoter fragment). These in vitro studies suggest that the human *GADD45* gene contains only one p53-binding site, which is in the third intron. This oligomer sequence also activated CAT transcription when a vector containing one copy of this putative p53-binding site upstream of a basal promotor adjacent to the CAT gene was cotransfected with wild-type, but not two different mutant, p53 expression vectors (Figure 5C).

Gel mobility shift assays were carried out to determine whether increased GADD45 binding activity could be detected in nuclear extracts from γ-irradiated cells. In extracts from nonirradiated ML-1 cells, several bands are evident, some of which may represent constitutive DNA-binding proteins; however, a distinct band (Figure 6A, bottom arrow) that is clearly visible in the IR extract is not detected in the extract from untreated cells. With the inclusion of the PAb421 antibody to p53, a higher supershifted band is observed (Figure 6A, top arrow). Interestingly, as was found previously (Funk et al., 1992) with other p53-binding oligomers and with p53 protein produced by an expression vector, the p53 antibody PAb1801, which binds to the amino terminus of p53 protein (in contrast with the carboxy-terminal binding of PAb421 [Wade-Evans and Jenkins, 1985]), did not produce a supershifted band. The reason for this difference in the antibodies is uncertain. In a second similar experiment (Figure 6B), results without nuclear extracts and with extracts from HL60 cells were compared with those from ML-1 cells. In contrast with ML-1 cells, neither the induced band nor the supershifted band is apparent in extracts from irradiated HL60 cells, which have a null p53 genotype. In addition to using the antibodies and the HL60 cells, the specificity of p53 binding in these experiments was further demonstrated by blocking both shifted bands with addition of excess unlabeled identical oligomer while no inhibition of binding was seen with addition of oligomer in which the invariant C and G positions had been replaced (data not shown). These results indicate that an IR-inducible nuclear factor, which binds to the *GADD45* p53 site, is present in ML-1 cells and that this factor contains p53. This demonstrates binding of an endogenous p53 gene product to a specific DNA sequence.

Discussion

Exposure to DNA-damaging agents probably contributes to the development of a significant percentage of human cancers (Doll and Peto, 1981). Cell cycle checkpoints appear to be an important mechanism for limiting the heritable genetic changes that lead to tumor formation following DNA damage. We had previously demonstrated that exposure of cells to IR leads to a transient increase in p53 protein levels by a posttranscriptional mechanism that temporally correlates with a cessation of replicative DNA synthesis (Kastan et al., 1991); tumor cells with mutated or absent p53 genes lost the ability to arrest in G1 following IR (Kastan et al., 1991; Kuerbitz et al., 1992). In the present study, this observation has been extended by demonstrating that the loss of both p53 alleles in otherwise normal murine fibroblasts similarly led to loss of this G1 arrest after IR. Our studies then identified two additional gene products that are involved in this pathway: one or more of the genes that is defective in the syndrome AT is required for the increase in p53 protein levels following IR, and the induction of *GADD45* following IR is dependent on a functional p53 gene product. The additional observations that AT cells are also defective in IR-induced enhancement of *GADD45* expression and that wild-type p53 binds

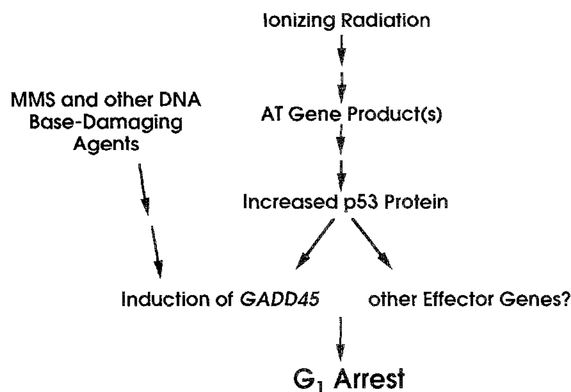

Figure 7. Schematic Representation of IR-Induced Cell Cycle Checkpoint Pathway in Mammalian Cells

Exposure to IR results in an increase in p53 protein levels; this increase is dependent on normal function of the gene(s) defective in AT. The increase in p53 protein levels results in enhancement of *GADD45* transcription and in an inhibition of progression of cells from the G1 into the S phase of the cell cycle. Whether the *GADD45* gene product plays a critical role in causing the G1 arrest is not yet clear. Induction of *GADD45* expression by other DNA-damaging agents does not appear to be as strictly dependent on normal p53 function, and the effects of these agents on cell cycle progression have not been characterized here.

to a conserved intronic sequence of the *GADD45* gene further support a role for p53 in the regulation of *GADD45* expression. A scenario can thus be proposed for this pathway (Figure 7): following IR-type damage, the cell "recognizes" this damage (probably damage to DNA [Holbrook and Fornace, 1991; unpublished data]) and increases p53 protein levels via a mechanism involving the AT gene product. Subsequently, p53 functions as a transcription factor to up-regulate the expression of *GADD45* and possibly other effector genes, which triggers the G1 checkpoint.

Considering that activation of this pathway was defective in more than one AT complementation group, one or more early steps in this pathway probably involve multiple gene products. One possibility is that a step in this pathway consists of a complex involving different proteins (representing the different AT complementation groups), all of which must be normal for it to be functional. The radiosensitivity and radioresistant DNA synthesis of AT cells from different complementation groups (Jaspers et al., 1988) are remarkably similar and would be consistent with such a complex. The defect in AT cannot be in p53 itself since the AT genes have been localized to the q arm of chromosome 11 (Gatti et al., 1988), while p53 is located on the p arm of chromosome 17 (van Tuinen et al., 1988). It should also be recognized that many experiments with AT cell lines in the past have utilized SV40-infected cells that should have dysfunctional p53 protein; therefore, interpretation of physiologic variables from these experiments should be made with caution and with this pathway in mind.

The fact that lymphoid malignancies are the most common tumor seen both in AT patients (Morrell et al., 1986; Hecht and Hecht, 1990) and in p53-deficient mice (Donehower et al., 1992; T. J. and R. Weinberg, unpublished data) is also consistent with a shared physiologic pathway between p53 and AT gene products. It is of interest that lymphoid cells normally experience DNA strand breaks during gene rearrangements. Thus, abnormalities in the response to strand breaks may be the link between the response to IR and the high incidence of lymphoid tumors in both of these situations. This type of physiologic function for p53 would also be consistent with the observation that mice with disruption of both p53 alleles appear to progress through fetal development normally but then develop tumors at high frequencies at young postgestational ages (Donehower et al., 1992; T. J. and R. Weinberg, unpublished data). Finally, it is also intriguing that the incidence of breast (and other nonlymphoid) cancer is markedly increased in individuals heterozygous for inherited germline mutations of either p53 (Li–Fraumeni syndrome; Malkin et al., 1990; Srivasta et al., 1990) or the AT gene(s) (Swift et al., 1987, 1991). Interestingly, the increased incidence of breast cancer in AT heterozygotes has been suggested to be related to exposure to low doses of IR (Swift et al., 1991).

The dependence of *GADD45* induction following IR on the status of p53 function in the cell is a novel insight into mechanisms of p53 action. The finding that wild-type but not mutant p53 protein binds to this gene suggests that p53 protein is directly responsible for activation of *GADD45* transcription. The location of the p53-binding site in an intronic region, rather than in a 5′ promoter region, suggests a role for p53 as an enhancer of *GADD45* transcription. Such a role is consistent with the observation that *GADD45* mRNA is expressed at low levels in many cells and increases in abundance following IR in cells with wild-type p53 function. The conservation of this intronic sequence between human and rodent species suggests an important function for this region of the *GADD45* gene. The regulation of the *GADD45* gene is complex, and the p53-dependent pathway is only one of multiple mechanisms that can affect its expression. In addition to IR, it is inducible by agents, such as MMS or ultraviolet radiation, that produce much higher levels of base damage in DNA than IR at similar toxicity (Holbrook and Fornace, 1991). The MMS response has been seen in many mammalian cells, including tumor cells defective in p53 function, such as HeLa and HL60 (Fornace et al., 1989b). When the 5′ promoter regions of either the *GADD45* or *GADD153* genes have been linked to CAT reporter genes (Holbrook and Fornace, 1991; unpublished data), both have been found to be MMS, but not IR, inducible. A significant role for other transcription factors in regulating *GADD45* expression thus also seems likely. This specificity of the IR/*GADD45* response for cells containing wild-type p53 also has the potential to be a useful assay for wild-type p53 function in laboratory and clinical studies.

One predicted physiologic consequence of abnormalities in this p53-dependent pathway is that a cell will have an increased chance of developing heritable genetic abnormalities following DNA damage, such as those caused by IR and similar agents. Such an increase in genetic instability following IR has been observed in yeast with *RAD9* mutations, which lack a G2 checkpoint (Hartwell and Wein-

ert, 1989; Weinert and Hartwell, 1990). Since AT cells have increased genetic instability following IR (e.g., Zampetti-Bosseler and Scott, 1981; Nagasawa et al., 1985), abnormalities in other steps in this p53-dependent pathway probably also contribute to increase genetic changes following IR. It is possible that the high frequency with which tumor cells exhibit both p53 gene abnormalities (Hollstein et al., 1991) and gross chromosomal changes (e.g., Yunis, 1990) is causally related through this response pathway to DNA damage. A potential link between p53 gene abnormalities and one type of genetic instability, gene amplification frequency, has been suggested recently (Livingstone et al., 1992). Interference with normal p53 function by viral proteins or even by abnormal binding of p53 protein to endogenous cellular proteins (Momand et al., 1992; Oliner et al., 1992) could also lead to the same abnormal response to DNA damage and contribute to tumor cell development. Implications for cancer treatment are also apparent: loss of this pathway in tumors with abnormal p53 function could contribute to their responsiveness to IR and other cytotoxic agents and could be exploited in future therapeutic strategies.

Experimental Procedures

Cells, Cell Treatment, and Cell Cycle Analysis

AT lymphoblastoid cell lines 718, 3189, and 1526, AT heterozygote lymphoblastoid cell line 3382, and normal lymphoblastoid cell line 2184 were all obtained from the National Institute of General Medical Science Human Genetic Mutant Cell Repository (Camden, New Jersey). AT cell line 718 had been previously characterized as being complementation group A group (718 = AT012; Gatti et al., 1988). AT lymphoblastoid cell lines CSA and BMA, previously characterized as complementation group C (BMA = AT4LA and CSA = AT13LA; Jaspers et al., 1988), were provided by Dr. Richard Gatti (University of California at Los Angeles). AT fibroblast cell line 3395 was provided by Dr. Thea Tlsty (University of North Carolina). Embryonic fibroblasts from mice with manipulated p53 genes were obtained and characterized as previously described (T. J. and R. Weinberg, unpublished data; see Livingstone et al., 1992). Other cell types utilized were previously described (Kastan et al., 1991; Kuerbitz et al., 1992). Cells were maintained in culture and exposed to IR as previously described (Kastan et al., 1991; Kuerbitz et al., 1992), except in the gel mobility shift assays where cells were irradiated with a ^{137}Cs source at 5.5 Gy/min. Cell cycle analysis was assessed by pulsing cells with 10 μM BrdUrd for 4 hr at the selected time after irradiation and subsequently staining cells for replicative DNA synthesis with a fluorescein isothiocyanate-conjugated anti-BrdUrd antibody and for DNA content with propidium iodide as described (Kastan et al., 1991).

Plasmid Clones

The following cDNA clones were used: pXR45m, a nearly full-length Chinese hamster gadd45 clone (Papathanasiou et al., 1991), and pA2, a 1.2 kb Chinese hamster β-actin clone (Fornace et al., 1989a). The plasmid pGAPD4, which was provided by Dr. F. G. Kern (Georgetown University), contained an insert spanning positions 256–359 of the human glyceraldehyde-3-dehydorgenase cDNA that was subcloned between the HindIII and EcoRI sites of pGEM-7zf. The plasmid pRibo-Hg45 consisted of a 269 bp fragment spanning positions 296–565 of the human *gadd45* cDNA (Papathanasiou et al., 1991) that was subcloned between the EcoRI and SmaI sites of pBluescript II SK.

p53 Protein Assays

Immunoprecipitation of [^{35}S]methionine-labeled p53 protein was done as previously described (Kuerbitz et al., 1992) with equivalent loading of ^{35}S counts per minute in each pair of lanes (−/+ γRT). For immunoblots, cells were washed with phosphate-buffered saline and counted, and the cell pellet was resuspended in Laemmli sample buffer (62 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue) at 0.1 × 10^6 cells per microliter. After boiling, cellular proteins were separated by 10% SDS–PAGE (20 μl = 2 × 10^6 cell equivalents loaded per lane) and transferred to nitrocellulose paper. The nitrocellulose paper was stained with fast green and photographed to document equivalent protein loading in the lanes. Following blocking with 5% milk, 10 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20, the blot was incubated for 2 hr at room temperature with the anti-p53 antibodies Ab-1 and Ab-2 (Oncogene Science, Manhassett, New York) diluted 1:100 in the blocking solution. The blot was then washed, incubated for 1 hr at room temperature with horseradish peroxidase–conjugated goat anti-mouse immunoglobulin G (Pierce Chemical Company, Rockford, Illinois), washed again, and autoradiographed utilizing enhanced chemiluminescence according to the instructions of the manufacturer (Amersham, Arlington Heights, Illinois).

RNA Isolation and Analysis

Cells were lysed in 4 M guanidine thiocyanate, and RNA was isolated by the acid phenol method (Chomczynski and Sacchi, 1987). Northern (RNA) blot analysis (Fornace et al., 1989b) or quantitative RNA dot-blot hybridization (Hollander and Fornace, 1990) was carried out as described previously.

For RNAase protection assays, reagents were obtained from Ambion, Incorporated, and the procedure was similar to that of the manufacturer with only minor modifications. The plasmids pRibo-Hg45 and pGAPD4 were linearized with HindIII or BamHI, respectively, and in vitro transcription was carried out at 4°C for 1 hr with T3 or T7 RNA polymerase, respectively. GADD45 and GAPD riboprobes were labeled with [α-^{32}P]UTP at 1400 or 15 Ci/mmol, respectively. Whole-cell RNA (10 μg) was hybridized with both riboprobes simultaneously (in the same test tube) at 53°C for 15 hr and then digested with RNAase A and RNAase T1. Following proteinase K digestion and phenol–chloroform extraction, the samples were analyzed on an 8 M urea, 5% acrylamide gel. Protected bands were visualized by autoradiography and were quantitated with a Betascope (Betagen, Incorporated). The relative level of *GADD45* mRNA was determined by normalizing the Betascope counts (less background) for *GADD45* to that of *GAPD* for each sample.

Gel Mobility Shift Assay

Nuclear extracts were prepared as described previously (Dignam et al., 1983; Carrier et al., 1992). DNA binding reactions were carried out for 20 min at room temperature in a buffer containing 20 mM HEPES (pH 7.8), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 μg of sonicated salmon sperm DNA, 10^4 disintegrations per minute of labeled probe, 10% glycerol, and 10 μg of nuclear protein extract. The probe used was a 30-mer double-stranded synthetic oligonucleotide containing the sequence from positions 1569–1598 of the human *GADD45* gene (Figure 4). Each strand was labeled separately with T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP, 5000 Ci/mmol, and the strands were then allowed to reanneal. Where indicated, 0.2 μg of monoclonal anti-p53 antibodies (PAb421 or PAb1801) was added prior to the addition of nuclear extract. PAb421 immunoglobulin G was purified from ascites fluid on protein A–agarose (ImmunoPure Plus, Pierce Chemical Company, Rockford, Illinois); PAb1801 immunoglobulin G (Oncogene Science) was used directly. The samples were then analyzed on a 4% nondenaturing acrylamide gel (Carrier et al., 1992).

Oligonucleotide/p53 Immunoprecipitations and CAT Assays

The synthetic oligonucleotides IA86 (containing the sequence from positions 1569 to 1598 of the human *GADD45* gene [Figure 4]) and IA87 (containing the complementary sequence) were radioactively labeled with [γ-^{32}P]ATP and T4 kinase. The labeled oligomers were ethanol precipitated, washed with 70% ethanol, and either allowed to autoanneal or to anneal with each other by incubation at 65°C for 5 min in 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 5% (w/v) polyethylene glycol 8000, followed by slow cooling over 30 min to room temperature. The annealed DNA was extracted with phenol–chloroform, ethanol precipitated, washed with 70% ethanol, and resuspended in 3 mM Tris (pH 7.5), 0.2 M EDTA. The *GADD45* human genomic clone pHG45-HC was digested with SauIIIAI, and the resulting fragments were end labeled with [α-^{32}P]dCTP, extracted with

phenol–chloroform, ethanol precipitated, washed with 70% ethanol, and resuspended in 3 mM Tris (pH 7.5), 0.2 M EDTA. Labeled DNA fragments were allowed to bind to baculovirus-produced p53 protein, immunoprecipitated, and analyzed as described (El-Deiry et al., 1992). For CAT assays, the IA86/IA87 duplex oligomer was cloned into the EcoRV site of the pBCPy CAT vector, which was constructed by cloning the BglII–BamHI PyCAT insert of pPyOICAT (Murakami et al., 1990) into pBC KS(+) (Stratagene, La Jolla, California). CAT assays were performed as previously described (Kern et al., 1992; the GADD45 oligomer was merely substituted for the PG sequence) with cotransfection of 2 μg of IA86/IA87–PyCAT with 3 μg of p53 expression plasmids.

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