Induction of the mammalian stress response gene *GADD153* by oxidative stress: role of AP-1 element

Kathryn Z. GUYTON, Qingbo XU, and Nikki J. HOLBROOK*

Section on Gene Expression and Aging, Gerontology Research Center, National Institute on Aging, 4940 Eastern Ave, Baltimore, MD 21224, U.S.A.

GADD153 is a CCAAT/enhancer-binding-protein-related gene that may function to control cellular growth in response to stress signals. In this study, a variety of oxidant treatments were shown to stimulate endogenous *GADD153* mRNA expression and to transcriptionally activate a *GADD153* promoter–reporter gene construct in transfected HeLa cells. Both commonalities and distinctions in the induction of *GADD153* by H_2O_2 and the thiol-reactive compound arsenite were demonstrated. *GADD153* mRNA induction by both H_2O_2 and arsenite was potentiated by GSH depletion, and completely inhibited by *N*-acetyl-cysteine. *o*-Phenanthroline and mannitol blocked *GADD153* induction by H_2O_2, indicating that iron-generated hydroxyl radical mediates this induction. Concordantly, GSH peroxidase overexpression in WI38 cells attenuated *GADD153* mRNA induction by H_2O_2. However, *GADD153* induction by arsenite was only modestly reduced in the same cells, suggesting a lesser contribution of peroxides to gene activation by arsenite. We also demonstrated that oxidative stress participates in the induction of *GADD153* by UVC (254 nm) irradiation. Finally, both promoter-deletion analysis and point mutation of the AP-1 site in an otherwise intact promoter support a significant role for AP-1 in transcriptional activation of *GADD153* by UVC or oxidant treatment. Indeed, exposure of cells to oxidants or UVC stimulated binding of Fos and Jun to the *GADD153* AP-1 element. Together, these results demonstrate that both free-radical generation and thiol modification can transcriptionally activate *GADD153*, and that AP-1 is critical to oxidative regulation of this gene. This study further supports a role for the *GADD153* gene product in the cellular response to oxidant injury.

INTRODUCTION

Oxidative stress plays a causative role in a variety of human diseases, and is believed to contribute to the degenerative changes that occur with aging [1,2]. Reactive oxygen intermediates (ROI), including molecular oxygen (O_2) superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^\bullet$), are ubiquitously produced in the course of aerobic metabolism and are a constant threat to cellular function. Toxins, carcinogens and environmental agents can both contribute to ROI production and compromise antioxidant defences. Further, exogenous agents can mediate oxidative injury through direct chemical interaction with cellular components, e.g. protein thiol modification by thiol-reactive agents. When the generation of ROI and other oxidizing species exceeds the cellular capacity for detoxication, oxidative stress results and damage to DNA, proteins and lipids ensues.

The mechanisms controlling the cellular response to oxidative stress have been extensively investigated at the molecular level in bacterial systems, where the OxyR and SoxR/S regulons have been shown to co-ordinately regulate the induction of genes by ROI (reviewed in ref. [3]). Similarly, transcription factors have been identified that probably mediate the oxidative stress response in yeast, including yAP-1 and yAP-2 (which have homology with mammalian AP-1), and Mac-1 (reviewed in ref [4]). In mammalian systems, numerous genes have been shown to be responsive to oxidants, although a systematic mechanism for gene regulation by oxidative stress has not been elucidated. Oxidative stress has been shown to alter the expression of mammalian antioxidant enzymes including superoxide dismutase, glutathione peroxidase (GPx), γ-glutamylcysteine synthetase, catalase, glutathione S-transferase and quinone reductase [5-10]. Induction of haem oxygenase by oxidative stress may both function in intracellular signalling [11] and serve to protect cells from further oxidant injury. Oxidants also enhance expression and/or DNA binding of numerous transcription factors, including fos, jun, myc, erg-1, NFκB, HSF and TCF/SCF ([12,13]; reviewed in [4]). That numerous mammalian genes and regulatory proteins involved in transcription are sensitive to oxidative stress speaks for the pivotal role of ROI as likely physiological mediators of gene regulation. As such, oxidants may play a significant role in the molecular response to cellular stress.

Although low in normal proliferating cells, the expression of the mammalian gene *GADD153* (named from the fact that it is inducible by growth arrest and DNA damage) is dramatically increased in response to a variety of stress stimuli, including nutrient depletion, genotoxic agents and Ca^{2+} ionophore [14–16]. A member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, GADD153 may control proliferation in response to cellular stress signals [17,18]. In the present study, the regulation of *GADD153* by oxidative stress was investigated. The roles of $HO^\bullet$ formation, thiol status and cellular GPx levels in mediating *GADD153* induction by H_2O_2 and arsenite were assessed. Further, the contribution of oxidative stress to *GADD153* induction by UVC (254 nm) irradiation was explored. Comparative analysis of the transcriptional activity of

Abbreviations used: ROI, reactive oxygen intermediates; GPx, GSH peroxidase; NAC, *N*-acetyl-cysteine; BSO, buthionine sulphoximine; BCS, bathocuproinedisulphonic acid; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; C/EBP, CCAAT/enhancer-binding protein; SV40, simian virus 40.

* To whom correspondence should be addressed.

serially deleted *GADD153* promoter fragments suggested that an AP-1-binding element contributes significantly to transcriptional activation by UVC or oxidant treatment. This assertion was supported by the marked reduction in transcriptional activity engendered by point mutation of the AP-1 site in an otherwise intact promoter, and by gel mobility-shift studies demonstrating that UVC, arsenite and H_2O_2 treatment of cells stimulates Fos and Jun binding to an oligonucleotide encompassing the *GADD153* AP-1 site.

MATERIALS AND METHODS

Cell culture conditions, transfections and chemical treatments

HeLa (human cervical carcinoma) and simian virus 40 (SV40)-transformed WI38 (human diploid lung) cells were maintained in a humidified atmosphere containing 10% CO_2 in air. Cell lines were grown in Dulbecco's modified Eagle's medium (Gibco/BRL, Bethesda, MD, U.S.A.) supplemented with 10% fetal bovine serum (Hyclone) and 50 μg/ml gentamicin (Gibco). Medium used for WI38 cells also contained sodium selenite (10^{-7} M). Transient transfections were performed with 5 μg of CsCl-purified plasmid DNA by $CaPO_4$ precipitation [19] followed by a 1.5 min exposure to 15% (v/v) glycerol in Dulbecco's modified Eagle's medium 4 h after DNA addition. Relative transfection efficiencies were assessed by normalization of chloramphenicol acetyltransferase (CAT) activity to the activity of a co-transfected actin promoter–luciferase reporter construct. A luciferase assay system kit (Promega, Madison, WI, U.S.A.) was utilized to assess relative actin promoter activity among transfection groups. Transfection efficiencies were not significantly different among deletion and mutation constructs of the *GADD153* promoter linked to the CAT reporter gene.

The stably transfected cell line *GADD153*–CAT/HeLa has been previously described [20]. The WI38 cell line overexpressing GPx (Renard, P., Zachary, M.-D., Bougelet, C., Mirault, M. E., Haegeman, G., Remacle, J. and Raes, M., unpublished work) is a clonal isolate derived by transfection of SV40-transformed WI38 cells with a plasmid containing the constitutive promoter human cytomegalovirus linked to human GPx. These cells have twice the GPx specific activity of parental cells, correlated with a similar increase in GPx protein measured by Western-blot analysis.

$HgCl_2$, phenylarsine oxide, *t*-butylhydroperoxide and cumene hydroperoxide were from Aldrich (Milwaulkee, WI, U.S.A.). All other chemicals were from Sigma (St. Louis, MO, U.S.A.). Phenylarsine oxide was dissolved in DMSO at 10 μM and stored at −20°C, and arsenite and heavy metals were stored as 100 mM solutions in water. Solutions of H_2O_2, *t*-butyl hydroperoxide, and cumene hydroperoxide in water (1 mM concentrations) were prepared immediately before cell treatment, as were solutions of *N*-acetyl-cysteine (NAC), buthionine sulphoximine (BSO), mannitol and bathocuproinedisulphonic acid (BCS) in medium, and *o*-phenanthroline in ethanol.

To minimize variations in dose effectiveness due to cell number, WI38 cells were seeded at 1×10^6 cells/100 cm² and HeLa cells at 0.5×10^6 cells/100 cm² dish 48 h before all organic peroxide experiments except for transient transfection assays. For transient transfection experiments, cells were seeded to be 50–75% confluent at the time of treatment (approx. 1×10^6 HeLa cells/100 cm² dish seeded 48 h before treatment). To compensate for increased cell number in transient transfection assays, 200 μM rather than 50 μM H_2O_2 was used. Because of the high antioxidant content of serum, all oxidant treatments were performed in serum-free medium or PBS (with Ca^{2+} and Mg^{2+}). A 30 min exposure period was utilized to control for differences in oxidant stability over time among treatments. For UVC treatment, the medium was removed and reserved, and the cells were washed once with PBS. After irradiation at 254 nm, the reserved culture medium was replaced.

RNA isolation and Northern analysis

Total RNA was extracted from treated cells using RNA Stat-60 (Tel-Test 'B', Friendswood, TX, U.S.A.) according to the manufacturer's instructions, using 1 ml of Stat-60 solution per 100 cm² tissue culture dish. RNA (10–20 μg/lane) was size-separated in agarose/formaldehyde gels and transferred to GeneScreen Plus nylon membranes (DuPont/NEN, Boston MA, U.S.A.). *GADD153* cDNA was labelled with [α-^{32}P]dCTP using a random-primer labelling kit (Boehringer-Mannheim, Indianapolis, IN, U.S.A.). Hybridization and washes were performed by the method of Church and Gilbert [21], and the hybridization signal was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). After analysis of the *GADD153* signal, blots were rehybridized to a 24 bp oligonucleotide (5′-ACGGTATCTGATCGTCTCGAACC-3′) complementary to 18 S RNA (synthesized by Midland Certified Reagent Co, Midland, TX, U.S.A.) that had been 3′-end-labelled with [α-^{32}P]dATP by terminal deoxynucleotidyltransferase (Life Technology Laboratories, Gaithersburg, MD, U.S.A.). *GADD153* signals were normalized to 18 S values obtained on the same blot to control for variation in loading and transfer among samples.

CAT assays

CAT assays were performed as previously described [22]. Percentages of chloramphenicol acetylation were obtained over the linear range of the assay (5–45% conversion), and were normalized to protein content.

Gel mobility-shift assays

Control or treated HeLa cells were subjected to Dounce homogenization in 20 mM Hepes, pH 7.5, containing 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.4 M NaCl, 0.2 mM dithiothreitol (DTT), 1 mM Pefablock SC (Boehringer-Mannheim), 20% (v/v) glycerol and 1 μg/ml leupeptin. After centrifugation at 17000 ***g*** for 30 min, 20 μg of protein extract was incubated with 0.5 ng of [γ-^{32}P]ATP-labelled 21 bp oligonucleotides with either the intact *GADD153* AP-1 binding sequence (5′CGATCGCA-**T**GACTCACTCAAT-3′), or a single base change of T to G within this binding element (5′-CGATCGCA-**g**GACTCACTCAAT-3′). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.). Reaction buffer contained 10 mM Tris/HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol and 1 μg of poly (dI-dC) as a non-specific competitor. Supershift assays were performed by the addition of antibodies against Fos or Jun (Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.) following the initial binding reactions between protein extracts and oligonucleotides. Likewise, in competition experiments, 50× unlabelled oligonucleotides were added after binding of protein extracts to [γ-^{32}P]ATP-labelled oligonucleotides had proceeded. Samples were subjected to non-denaturing PAGE in 4% gels, whereupon the gels were dried and protein–DNA complexes visualized by autoradiography.

RESULTS

Oxidant induction of *GADD153*

A variety of oxidants were shown to induce *GADD153* mRNA and activate a *GADD153* promoter–CAT reporter gene construct in stably transfected HeLa Cells (Table 1). Arsenicals and heavy metals caused a 20–50-fold induction, and free-radical-generating compounds consistently induced *GADD153* expression 10–20-fold over control levels. The potency of heavy metals as inducers of *GADD153* corresponded to the reactivity of these agents with GSH [7], suggesting that thiol interaction functions in gene induction by these agents. Oxidant treatment in serum-free medium or PBS showed *GADD153* inductions at significantly lower doses than previously reported for direct addition to growth medium [20], probably because the high serum content of growth medium decreased the effective oxidant dose. Study of *GADD153* induction was thus undertaken within the physiologically relevant range of oxidant dosages. Of the agents tested, arsenite and H_2O_2 were selected for more detailed investigation of oxidative induction of *GADD153*.

Kinetic analysis of the increase in *GADD153* mRNA stimulated by H_2O_2 in HeLa cells indicated that maximal mRNA levels were reached 2 h after treatment (Figure 1A). The induction of *GADD153* mRNA appears to be biphasic with a second increase occurring at 8 h. The induction of *GADD153* mRNA by H_2O_2 was dose-dependent (Figure 1B); 50 μM H_2O_2 consistently mediated a 5–10-fold increase in the mRNA of this gene, with interexperiment variability in fold induction arising from minor changes in the basal level of *GADD153* gene expression. The induction of *GADD153* by H_2O_2 typically declined at doses above 200 μM, presumably because of excessive toxicity.

Arsenite likewise resulted in a time-dependent increase in *GADD153* mRNA in HeLa cells (Figure 2A) with a similar pattern to that occurring with H_2O_2. The first peak of induction occurred at 4 h, and a second increase was evident at 8 h. However, the relative magnitude of the second induction was significantly greater than that seen with H_2O_2. The dose–response relationship for the *GADD153* mRNA increase stimulated by arsenite is shown in Figure 2(B). A dose of 200 μM arsenite consistently mediated at least a 20-fold induction of *GADD153* mRNA, with inter-experiment variability in fold induction arising from minor changes in the basal level of *GADD153* gene expression.

Both arsenite and H_2O_2 enhanced the expression of *GADD153*–CAT in stably transfected HeLa cells (Figure 3). The dose–response relationships for activation of the *GADD153*–CAT reporter gene construct were consistent with those resulting in induction of *GADD153* mRNA. These results indicate a transcriptional mechanism for *GADD153* gene activation by both arsenite and H_2O_2.

Table 1 Oxidant inducers of *GADD153*–CAT and *GADD153* mRNA

HeLa and *GADD153*–CAT/HeLa cells were treated for 30 min with the indicated agents in serum-free medium (A) or PBS (B). CAT activity was measured 24 h later in *GADD153*–CAT/HeLa stable transfectants. RNA was isolated from HeLa cells 4 (A) or 2 (B) h after treatment, and analysed as described in the Materials and methods section. The range of induction of *GADD153*–CAT and *GADD153* mRNA is relative to time-matched untreated controls.

Inducer	Concentration	Fold induction
(A) Thiol-reactive reagents		
Phenylarsine oxide	1–5 nM	20–25
Sodium arsenite	100–400 μM	25–50
Mercury chloride	10–30 μM	10–20
Cadmium chloride	100–400 μM	25–40
Zinc chloride	0.75–1 mM	25–50
(B) Free-radical generators		
H_2O_2	10–100 μM	5–20
t-Butyl hydroperoxide	10–100 μM	2–10
Cumene hydroperoxide	10–100 μM	2–10

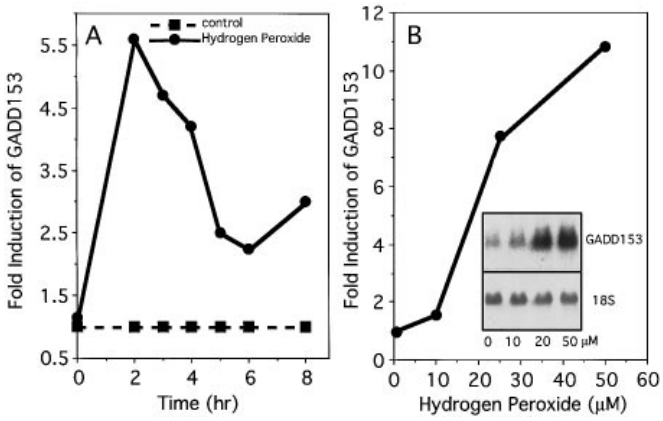

Figure 1 Kinetics and dose–response relationship for induction of *GADD153* by H_2O_2

HeLa cells were treated with H_2O_2 in PBS for 30 min, and harvested for RNA isolation after the indicated incubation times. Northern-blot analysis was performed as described in the Materials and methods section. Values are expressed as fold induction over time-matched controls, and represent the means of at least two independent experiments. (**A**) Time course of *GADD153* induction with 20 μM H_2O_2; (**B**) dose-response analysis at 2 h. Inset, signal for *GADD153* and 18 S on representative RNA blot.

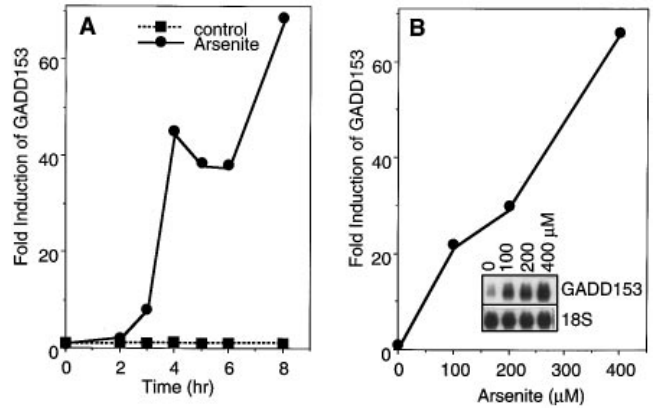

Figure 2 Kinetics and dose–response relationship for induction of *GADD153* by arsenite

HeLa cells were treated with arsenite for 30 min in serum-free medium and harvested for RNA isolation after the indicated incubation times. Northern-blot analysis was performed as described in the Materials and methods section. Values are expressed as fold induction over time-matched controls, and represent the means of at least two independent experiments. (**A**) Time course of *GADD153* induction with 200 μM arsenite; (**B**) dose–response analysis at 4 h. Inset, signal for *GADD153* and 18 S on representative RNA blot.

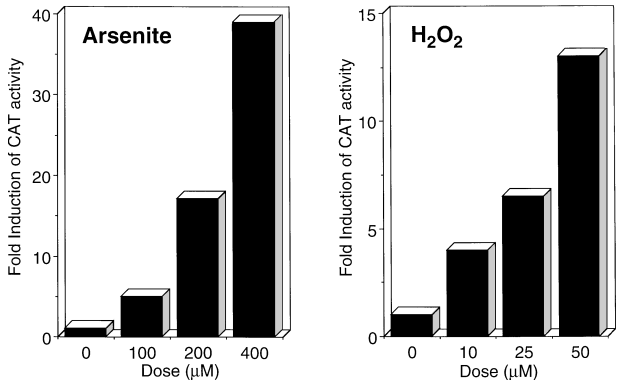

Figure 3 Dose–response relationship for the induction of *GADD153*–CAT activity by arsenite and H_2O_2

HeLa cells stably transfected with *GADD153*–CAT were treated for 30 min with arsenite in serum-free medium (left panel) or H_2O_2 in PBS (right panel). Cells were harvested 24 h later for analysis of CAT activity. Values are expressed as fold induction over control, and represent the means of at least two independent experiments.

Role of cellular thiol status in *GADD153* gene activation by arsenite and H_2O_2

Depletion of cellular GSH by BSO pretreatment markedly potentiated *GADD153* induction by arsenite and H_2O_2 (Figure 4). At identical doses, BSO-pretreated HeLa cells showed an approx. 2-fold greater induction of *GADD153* mRNA by both arsenite and H_2O_2 relative to that seen in the absence of BSO. In contrast, NAC pretreatment, which elevates cellular GSH levels, completely prevented *GADD153* mRNA induction by either arsenite or H_2O_2 (Figure 5). NAC completely blocked induction by 200 μM arsenite, and reduced the 60-fold induction by 400 μM arsenite by 96% (left panel). Likewise, NAC diminished the 6-fold induction mediated by 50 μM H_2O_2 by 85% (right panel). In the absence of NAC, 100 μM H_2O_2 induced significant toxicity,

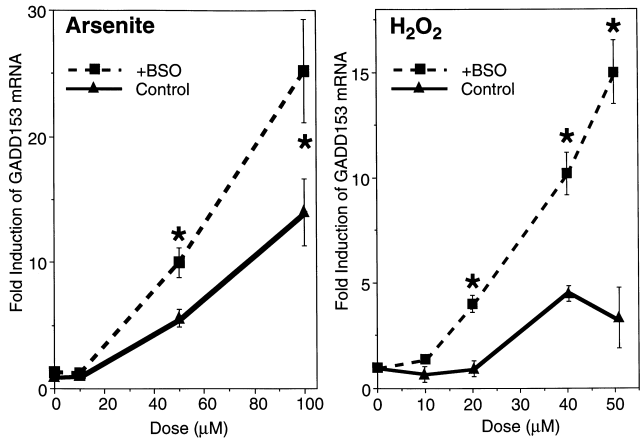

Figure 4 BSO potentiates *GADD153* mRNA induction by arsenite and H_2O_2

GSH depletion (> 90%) was achieved by pretreatment of HeLa cells with 1 mM BSO for 18 h. Arsenite treatment was for 30 min in serum-free medium, and cells were harvested 4 h later (left panel). Cells were treated with H_2O_2 for 30 min in PBS, and harvested 2 h later (right panel). Northern-blot analysis was performed as described in the Materials and methods section, and values are expressed as fold induction over BSO alone (– – – –) or untreated (——) controls. Data represent the means ± S.D. of four experiments. *BSO values significantly different from control cells ($P < 0.01$; Student's *t*-test.)

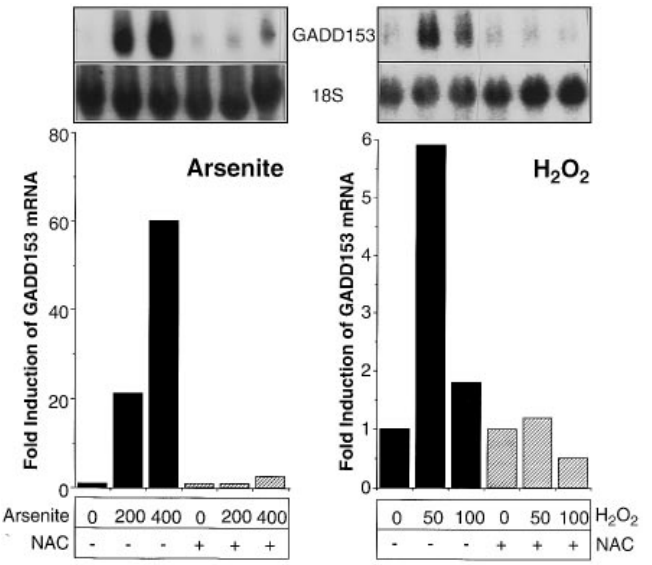

Figure 5 NAC inhibits *GADD153* mRNA induction by arsenite and H_2O_2

HeLa cells were pretreated for 1 h with 20 mM NAC. Arsenite treatment was for 30 min in serum-free medium, and cells were harvested 4 h later for RNA isolation (left). Cells were treated with H_2O_2 for 30 min in PBS, and harvested 2 h later for RNA isolation (right panel). Northern-blot analysis was performed as described in the Materials and methods section. The signal for *GADD153* and 18 S on RNA blots is shown above the quantification of these data, with values expressed as fold induction over untreated (■) or NAC alone (▨) controls.

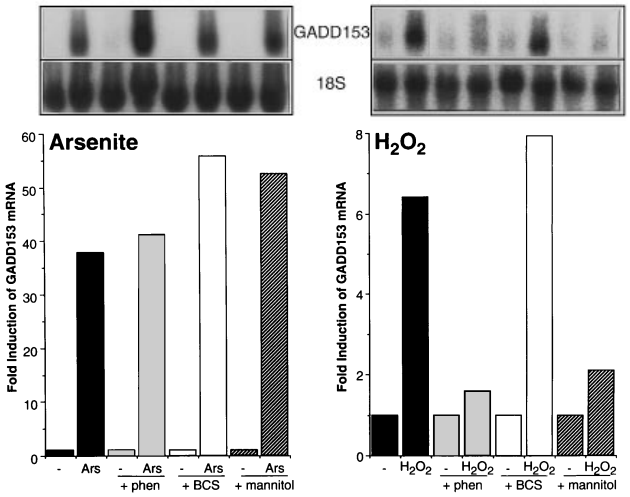

Figure 6 HO$^{\cdot}$ mediates *GADD153* induction by H_2O_2

HeLa cells were pretreated for 1 h with 0.1 mM *o*-phenanthroline (phen), 1 mM BCS or 100 mM mannitol. Cells were treated with arsenite for 30 min in serum-free medium, and harvested 4 h later for RNA isolation (left panel). H_2O_2 was added for 30 min in PBS, and cells were harvested 2 h later for RNA isolation (right panel). Northern-blot analysis was performed as described in the Materials and methods section. The signal for *GADD153* and 18 S on RNA blots is shown above the quantification of these data, with values expressed as fold induction over untreated (■) or inhibitor alone controls.

and was thus beyond the dose of maximal *GADD153* induction; however, NAC treatment resulted in significant protection against the toxicity induced by 100 μM H_2O_2 and prevented *GADD153* induction (right panel).

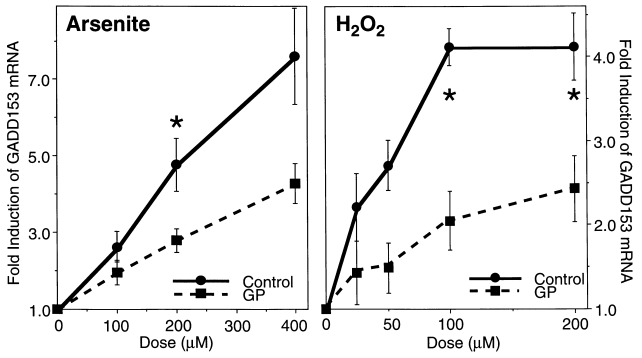

Figure 7 GPx overexpression attenuates *GADD153* mRNA expression by both arsenite and H_2O_2

Parental (Control) or GPx-overexpressing (GP) WI38 cells were treated with arsenite in serum-free medium for 30 min and harvested 4 h later (left panel) or with H_2O_2 in PBS for 30 min and harvested 5 h later (right panel). Northern-blot analysis was performed as described in the Materials and methods section, and values are expressed as fold induction over time-matched untreated controls. Data represent means ± S.D. of four experiments. *Control WI38 cells that are significantly different from GPx transfectants ($P < 0.01$; Student's t test).

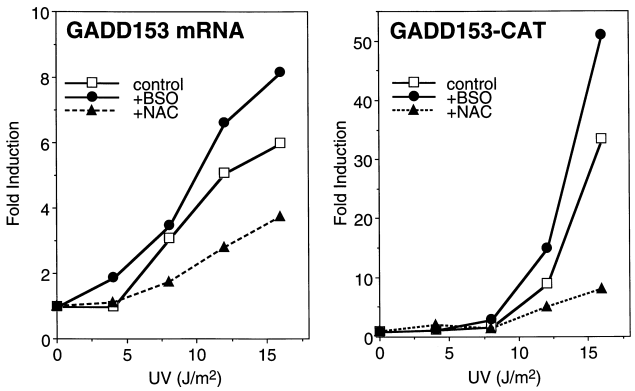

Figure 8 Induction of *GADD153* by UVC is partially modulated by thiol status

Left panel, HeLa cells pretreated with BSO (1 mM; 18 h) or NAC (20 mM; 1 h) were exposed to UVC (254 nm) and harvested for RNA isolation 2 h later. Northern-blot analysis was performed as described in the Materials and methods section. Right panel, HeLa cells stably expressing *GADD153*–CAT were pretreated with BSO (1 mM; 18 h) or NAC (20 mM; 1 h), exposed to UVC (254 nm) and harvested for analysis of CAT activity 24 h later. RNA and CAT values are expressed as fold induction over untreated (□), BSO alone (●) or NAC alone (▲) controls, and represent the means of at least two independent experiments.

Role of intracellular ROI in *GADD153* gene activation by arsenite and H_2O_2

Induction of *GADD153* by H_2O_2 was blocked by the iron chelator *o*-phenanthroline, whereas the copper chelator BCS was without effect (Figure 6, right panel). H_2O_2-stimulated *GADD153* induction was also diminished in the presence of mannitol, an $HO^{\bullet}$ scavenger. Taken together, these results suggest that iron-dependent $HO^{\bullet}$ formation mediates *GADD153* gene activation by H_2O_2. In contrast, neither metal chelators nor mannitol inhibited *GADD153* mRNA induction by arsenite (Figure 6, left panel). However, more direct assessment of the role of ROI in *GADD153* induction by constitutive over-expression of GPx suggested a partial contribution of peroxide moieties to *GADD153* induction by arsenite. In cells over-expressing GPx, *GADD153* activation by arsenite was reduced approx. 35% (Figure 7, left panel). *GADD153* induction by H_2O_2 was 2-fold lower in GPx-overexpressing cells (right panel), indicating a greater degree of ROI involvement in gene activation by H_2O_2 than arsenite.

Involvement of oxidative stress in *GADD153* induction by UVC

Modulation of cellular thiol status also moderately influenced the ability of UVC to induce *GADD153* (Figure 8). The induction of *GADD153* mRNA by UVC in HeLa cells was somewhat enhanced by depletion of intracellular thiols with BSO, and partially blocked by NAC (left). The effects of BSO and NAC were confirmed in HeLa/*GADD153*–CAT stable transfectants (right). Although pronounced, the sensitivity of UVC-induced *GADD153* expression to cellular thiol status was not as dramatic as that for H_2O_2- or arsenite-induced expression. NAC had completely abolished both H_2O_2 and arsenite induction of *GADD153* (Figure 5); in contrast, NAC decreased UVC induction of *GADD153*–CAT by 45% and 75% for UVC doses of 12 and 16 J/m² respectively (Figure 8). Potentiation of the UVC response by BSO (50–60% increase) was also less than that seen with arsenite and H_2O_2 (compare Figures 4 and 8). Further, UVC-induced *GADD153* mRNA expression was shown to be reduced 50% by *o*-phenanthroline pretreatment and 40% by mannitol pretreatment (results not shown). These results indicate that, although oxidative stress contributes to the induction of *GADD153* by UVC, $HO^{\bullet}$ is not the sole mediator of the response, as appears to be the case with H_2O_2 (Figure 6). Because UVC does not result in a significant induction of *GADD153* mRNA in WI38 cells, a comparison of the ability of GPx overexpression to modulate induction of *GADD153* mRNA by UVC *vis à vis* arsenite and H_2O_2 could not be undertaken.

Common promoter elements function in the induction of *GADD153* by arsenite and H_2O_2

The *GADD153* promoter contains numerous regulatory elements that are likely to function in controlling the expression of this gene in response to cellular stress [23] (Figure 9A). Serial deletion of the *GADD153* promoter was undertaken to highlight regions responsive to oxidative stimuli (Figure 9B). The magnitude of promoter activation in response to arsenite, H_2O_2 or UVC was diminished by decreasing the promoter size from −778/+21 to −483/+21, indicating removal of positive regulatory elements; however, basal activity increased nearly 2-fold with loss of DNA sequences in this region. Interestingly, the magnitude and pattern of expression that results on further deletion from −483 to −36 was nearly identical for arsenite and H_2O_2. Deletion of the sequences from −483 to −250 resulted in an increased responsiveness to arsenite and H_2O_2, suggesting that this region contains negative regulatory elements for these agents. In contrast, deletion of these same sequences resulted in a diminished response to UVC. These findings support the view that distinct regulatory elements are involved in mediating *GADD153* gene activation in response to UVC and the oxidative agents arsenite and H_2O_2. Nonetheless, further deletion of promoter from −250 to −225, a 25 bp region that contains an AP-1-binding element, resulted in a similar 2-fold reduction in the remaining activity for all three agents, indicating commonalities among cellular signals leading to gene activation by UVC, arsenite and H_2O_2.

Role of AP-1 in oxidative induction of *GADD153*

Several experimental approaches were utilized to explore the role of the AP-1 site in regulating the transcriptional activity of

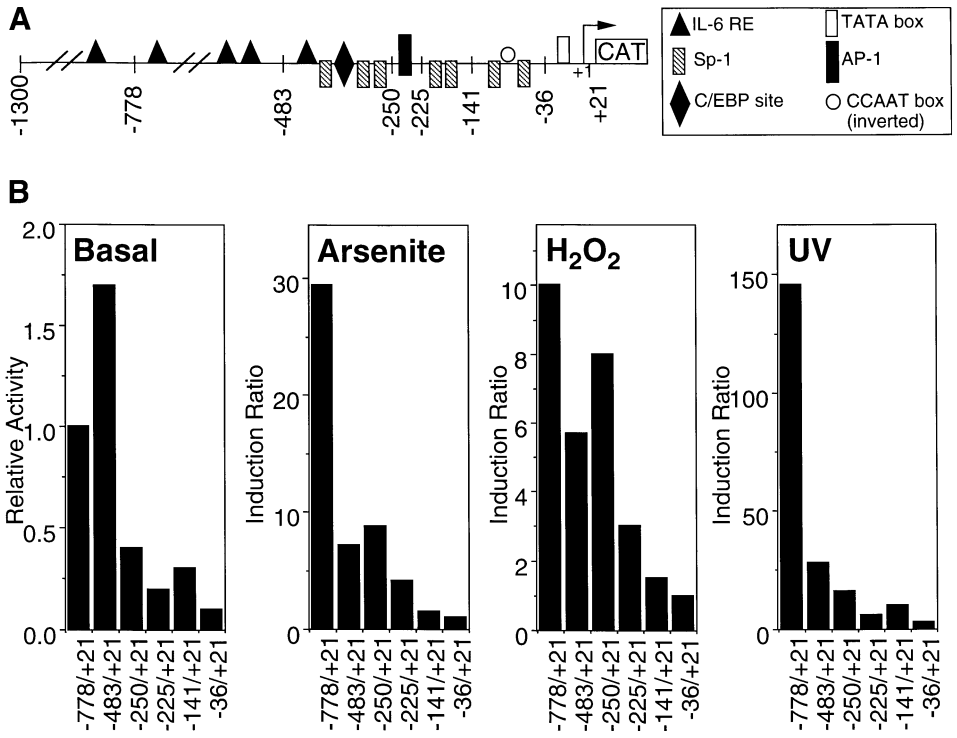

Figure 9 Deletion analysis of *GADD153* promoter induction by arsenite, H_2O_2 and UVC

(**A**) Schematic of the *GADD153* promoter region. IL-6 RE, interleukin 6 response element. (**B**) Induction of CAT activity in untreated cells or arsenite-, H_2O_2 or UVC-treated cells. HeLa cells were transiently transfected with the indicated *GADD153* promoter–CAT deletion constructs as described in the Materials and methods section. The following day, cells were treated with arsenite (400 μM) in serum-free medium, H_2O_2 (200 μM) in PBS for 30 min or UVC (20 J/m^2). Cells were harvested for determination of CAT activity 24 h after treatment (48 h after transfection). Limited evaluation of transfection efficiency as described in the Materials and methods section revealed no differences among *GADD153* promoter-deletion constructs. Basal activities of the deletion constructs are expressed relative to $-778/+21$, and induction ratios are expressed relative to untreated controls. Values are means of two independent experiments.

GADD153 in response to oxidative stress. Gel mobility-shift assays were employed to determine whether oxidative stress stimulates binding of cellular factors to an oligonucleotide containing the *GADD153* AP-1 sequence. H_2O_2 and arsenite treatment resulted in a time-dependent increase in binding activity to the oligonucleotide containing the *GADD153* AP-1 site (Figure 10A). UVC treatment similarly stimulated *GADD153* AP-1 DNA-binding activity. By contrast, *GADD153*mutAP-1, a 21 bp oligonucleotide containing a single base pair mutation within the AP-1 consensus sequence (TGACTCA changed to gGACTCA), did not bind proteins from arsenite-, H_2O_2- or UVC-treated cells (Figure 10B). Further, whereas unlabelled *GADD153* AP-1 oligonucleotide was an effective competitor for radiolabelled DNA in the DNA–protein complex, the *GADD153*mutAP-1 oligonucleotide was not (Figure 10C). Finally, addition of Fos or Jun antibody after binding of *GADD153* AP-1 DNA to protein from treated cells caused a shift in the mobility of the resultant complexes to a more slowly migrating species (Figure 10C). This supershift indicates the presence of Fos and Jun in complexes of the *GADD153* AP-1 oligonucleotide and proteins from oxidant-treated cells.

Two additional experiments directly demonstrated the functional activity of the AP-1-binding element in regulating *GADD153* transcription. First, a full-length promoter construct was generated which contains the same T to G mutated *GADD153* AP-1 site shown to lack protein-binding activity in gel mobility-shift assays above ($-778/+21$mutAP-1). In the absence of treatment, the point mutation did not affect promoter activity. However, the induction ratio of CAT activity for arsenite, H_2O_2 or UVC was reduced by approx. 50% when the T to G point mutation was introduced in the AP-1 site of an otherwise intact *GADD153* promoter fragment (Figure 11). Secondly, the functional capacity of the AP-1 sequence was directly demonstrated using a construct containing a trimer of this site linked to a minimal *GADD153* promoter construct ($-36/+21$ fragment). The enhanced expression of the 3× AP-1 construct displayed in transiently transfected cells treated with arsenite, H_2O_2 or UVC confirmed the transcriptional activity of this particular promoter element (Figure 11).

DISCUSSION

GADD153 gene expression is known to be ubiquitously enhanced in response to a diversity of stressful stimuli. This report provides a comprehensive examination of the regulation of *GADD153* by oxidative stress, with particular focus on the cellular factors and promoter elements that contribute to transcriptional activation after oxidant injury. Although both thiol-reactive agents and free-radical-generating compounds transcriptionally activate *GADD153*, more detailed analysis revealed some differences in the gene inductive signal for the two classes of oxidants. The magnitude of *GADD153* induction by the thiol-reactive reagent arsenite was 2–5-fold greater than that seen with the free-radical-generating compound H_2O_2 (Figures 1 and 2). Similar kinetics of induction were found for both H_2O_2 and arsenite, characterized by a biphasic pattern with the second peak evident 8 h after treatment. The first peak of induction, however, occurred at 2 h after treatment with H_2O_2, and 4 h after exposure to arsenite.

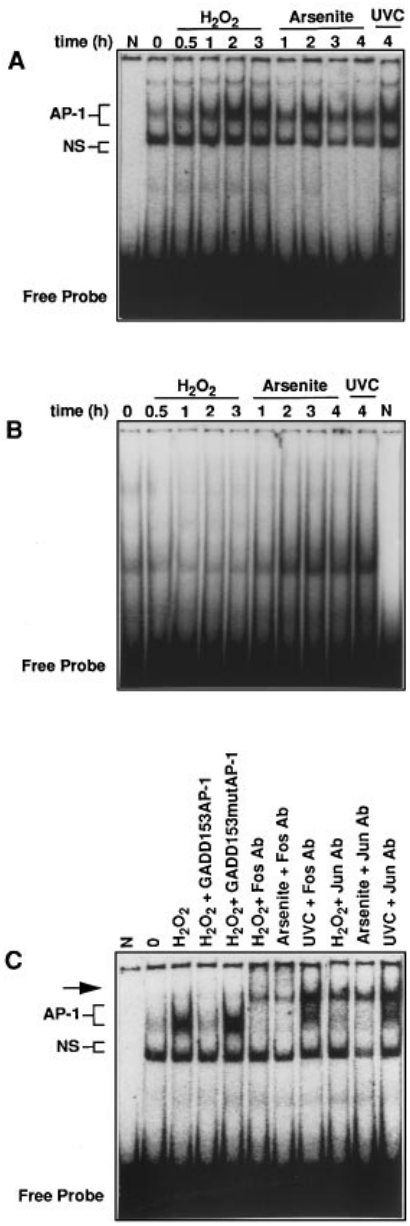

Figure 10 Oxidant and UVC treatments stimulate *GADD153* AP-1 binding activity

Gel mobility-shift analysis using 21 bp oligonucleotides having either (**A**) the intact *GADD153* AP-1-binding sequence (5′-CGATCGCA**B**GACTCACTCAAT-3′) or (**B**) a single base change of T to G within this binding element (5′-CGATCGCA**g**GACTCACTCAAT-3′) (*GADD153*mutAP-1). (**C**) Binding to the intact *GADD153* AP-1 site is not subject to competition with GADD153mutAP-1 (50:1); the complexes of *GADD153* AP-1 oligonucleitide and proteins from H_2O_2- (2 h), arsenite- (2 h) or UVC- (4 h) treated cells supershift in the presence of Fos and Jun antibodies (Ab). HeLa cells were treated as indicated, and gel mobility-shift analyses using the resultant protein extracts were performed as described in the Materials and methods section. NS, Non-specific binding; AP-1, *GADD153* AP-1 binding; arrow, supershifted DNA-binding complexes indicating the presence of Fos and Jun proteins.

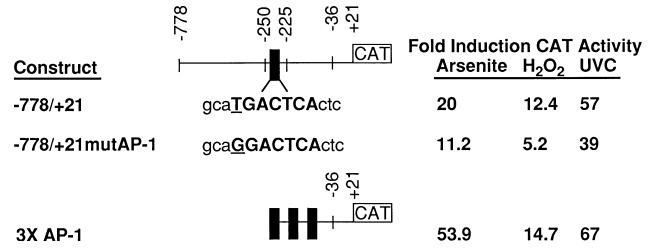

Figure 11 Transcriptional activity of *GADD153* AP-1 site

Oxidant-induced CAT activity from *GADD153* constructs containing wild-type −778/+21, −778/+21mutAP-1 (in which a single T to G mutation is introduced in the AP-1-binding site) or 3 × AP-1 (three copies of the *GADD153* AP-1 site linked to the minimal *GADD153* promoter fragment). HeLa cells were transiently transfected with the indicated CAT reporter gene constructs as described in the Materials and methods section. The following day, cells were treated with arsenite (400 μM) in serum-free medium, H_2O_2 (200 μM) in PBS for 30 min or UVC (20 J/m^2). Cells were harvested for determination of CAT activity 24 h after treatment (48 h after transfection). Limited assessment of transfection efficiencies as described in the Materials and methods section revealed no difference between wild type −778/+21 and −778/+21mutAP-1 *GADD153* constructs. Activation is expressed relative to untreated controls, and values are means of two independent experiments.

Depletion of cellular GSH by BSO pretreatment significantly potentiated *GADD153* induction by both H_2O_2 and arsenite (Figure 4). Conversely, NAC pretreatment, which is known to elevate GSH levels [24], completely abolished *GADD153* induction by these oxidants (Figure 5). That either BSO or NAC treatment alone can mildly stimulate *GADD153* expression indicates that oxidation or reduction of critical thiol moieties may mediate *GADD153* induction. The ability of the reducing agent DTT to stimulate *GADD153* induction further supports this assertion [25]. However, the relatively small magnitude of *GADD153* induction stimulated by BSO alone indicates that H_2O_2 and arsenite do not function solely through GSH depletion. Instead, BSO and NAC are likely to influence opposingly the accessibility of intracellular targets for oxidative modification of H_2O_2 and arsenite.

The cellular effects of H_2O_2 are thought to be largely mediated through the metal-catalysed production of HO$^\bullet$, which will react indiscriminately with the entire spectrum of biomolecules, including proteins, lipids and DNA (reviewed in ref [26]). The inhibition of H_2O_2-induced *GADD153* expression by *o*-phenanthroline and mannitol indicates that iron-mediated conversion of H_2O_2 to HO$^\bullet$ plays a significant role in gene activation by H_2O_2 (Figure 6). That GPx overexpression also diminishes the induction of *GADD153* by H_2O_2 strengthens this conclusion. Whereas GPx acts to detoxify H_2O_2 directly, peroxide moieties generated in the course of HO$^\bullet$ reactions with cellular components will also serve as a substrate for this enzyme. Thus GPx may not only serve to diminish the effective dose of H_2O_2, but it may also act on oxidatively damaged lipids or proteins.

The role of ROI in the induction of *GADD153* by arsenite was less pronounced. Neither metal iron chelators nor the HO$^\bullet$ scavenger, mannitol, inhibited *GADD153* induction by arsenite (Figure 6), and GPx overexpression only modestly influenced *GADD153* mRNA induction by arsenite (Figure 7). These data are in keeping with the assertion that arsenite can stimulate the intracellular production of oxidative stress, and that peroxide-moiety generation arising as a consequence of the ensuing reactions of ROI with lipids may function in gene activation by arsenite [27]. However, because arsenite has considerable preferential reactivity with vicinal dithiol moieties, its primary mechanism of action is likely to be through the direct oxidation of critical cellular thiols [28–31].

Oxidative stress has also been proposed to contribute, in part, to the cellular effects of UVC [32,33]. Indeed, induction of *GADD153* by UVC displayed moderate sensitivity to intracellular thiol status modification by BSO and NAC (Figure 8). Similarly, induction of *GADD153* could be partially inhibited by

mannitol and *o*-phenanthroline, lending support to the notion that the generation of ROI, particularly $HO^{\bullet}$, contributes to the transcriptional activation of *GADD153* by UVC.

Deletion analysis of the *GADD153* promoter region suggested that multiple elements within an 800 bp region are likely to contribute to regulation of *GADD153* activity in response to oxidant stress. Activation by arsenite and H_2O_2 relied on certain elements common to those involved in mediating the response to UVC, but also others that are distinct. In particular, the region spanning nucleotides −250 and −225 was equally important for *GADD153* induction by UVC, H_2O_2 and arsenite. Additional experiments identified an AP-1-binding element within this region as an important regulator of *GADD153* expression. A single base-pair change in the AP-1-binding sequence abolished the formation of complexes of Fos and Jun with the *GADD153* AP-1 site after oxidant treatment, and significantly diminished the transcriptional activity of an otherwise intact *GADD153* promoter (Figures 10 and 11). Further, a construct containing multimers of the *GADD153* AP-1 site alone demonstrated enhanced transcriptional activity in response to oxidative treatments (Figure 11). Similar sequences have been shown to play a role in the induction of other genes by UVC and oxidative stress [7,8,33–35]. Interestingly, AP-1 activity is also responsive to both hypoxic and antioxidant stimuli [36–38]; likewise, induction of *GADD153* can occur in response to both thiol-oxidizing and -reducing compounds (Table 1; [25]).

In summary, we have clearly demonstrated that oxidative stress results in the rapid induction of *GADD153* gene expression, and that this induction is mediated, at least in part, by an AP-1 site in the *GADD153* promoter. These data suggest that *GADD153* may participate in the mammalian response to oxidant injury. Although the precise function served by *GADD153* has not been clarified, as a transcription factor it may take part in the regulation of other genes expression after oxidative stress. In addition, *GADD153* has been implicated in mediating growth arrest in response to cellular stress, allowing critical repair processes to proceed before cell cycling [17,18]. As such, this gene may play a pivotal role in survival subsequent to oxidant injury.

We thank Dr. Jose Remacle for providing WI38 cells stably overexpressing GPx, and Dr. Myriam Gorospe for critically reading the manuscript.

REFERENCES

1 Harman, D. (1956) J. Gerontol. **11**, 298–300
2 Edgington, S. M. (1994) Biotechnology **12**, 37–40
3 Demple, B. and Amabile-Cuevas, C. F. (1991) Cell **67**, 837–839
4 Pahl, H. L. and Baeuerle, P. A. (1994) BioEssays **16**, 497–502
5 Shi, M. M., Kugelman, A., Iwamoto, T., Tian, L. and Forman, H. J. (1994) J. Biol. Chem. **269**, 26512–26517
6 Shull, S. Heintz, N. H., Periasamy, M., Manohar, M., Janssen, Y. M. W., Marsh, J. P. and Mossman, B. T. (1991) J. Biol. Chem. **266**, 24398–24403
7 Prestera, T., Holtzclaw, W. D., Zhang, Y. and Talalay, P. (1993). Proc. Natl. Acad. Sci. U.S.A. **90**, 2965–2969
8 Li, Y. and Jaiswal, A. K. (1992) J. Biol. Chem. **267**, 15097–15104
9 Cowan, D. B., Weisel, R. D., Williams, W. G. and Mickle, D. A. G. (1993) J. Biol. Chem. **268**, 26904–26910
10 Kayanoki, Y., Fujii, J., Suzuki, K., Kawata, S., Matsuzawa, Y. and Taniguchi, N. (1994) J. Biol. Chem. **269**, 15488–15492
11 Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V. and Snyder, S. H. (1993) Science **259**, 381–384
12 Ohba, M., Shibanuma, M., Kuroki, T. and Nose, K. (1994) J. Cell Biol. **126**, 1079–1087
13 Shibanuma, M., Kuroki, T. and Nose, K. (1988) Oncogene **3**, 17–21
14 Fornace, A. J., Nebert, D. W., Hollander, M. C., Luethy, J. D., Papathanasiou, M., Fargnoli, J. and Holbrook, N. J. (1989) Mol. Cell. Biol. **9**, 4196–4203
15 Bartlett, J. D., Luethy, J. D., Carlson, S. G., Sollott, S. J. and Holbrook, N. J. (1992) J. Biol. Chem. **267**, 20465–20470
16 Carlson, S. G., Fawcett, T. W., Bartlett, J. D., Bernier, M. and Holbrook, N. J. (1993) Mol. Cell. Biol. **13**, 4736–4744
17 Zhan, Q., Lord, K. A., Alamo, I. Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A. and Fornace, A. J., Jr. (1994) Mol. Cell. Biol. **14**, 2361–2371
18 Barone, M. V., Crozat, A., Tabaee, A., Philipson, L. and Ron, D. (1994) Genes Dev. **8**, 453–464
19 Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) Mol. Cell. Biol. **2**, 1044–1051
20 Luethy, J. D. and Holbrook, N. J. (1992) Cancer Res. **52**, 5–10
21 Church, G. M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 1991–1995
22 Holbrook, N. J., Luethy, J. D. and Kin, J. (1987) Mol. Cell. Biol. **6**, 2610–2613
23 Park, J. S., Luethy, J. D., Wang, M. G., Fargnoli, J., Fornace, A. J., Jr., McBride, O. W. and Holbrook, N. J. (1992) Gene **116**, 259–267
24 Meister, A. (1991) Pharmacol. Ther. **51**, 155–194
25 Chen, Q., Yu, K., Holbrook, N. J. and Stevens, J. L. (1992) J. Biol. Chem. **267**, 8207–8212
26 Fischer, A. B. (1988) in Oxygen Radicals and Tissue Injury (Halliwell, B., ed.), pp. 34–42, Upjohn Co., Bethesda, MD
27 Yamanaka, K., Hasegawa, A., Sawamura, R. and Okada, S. (1991) Toxicol. Appl. Pharmacol. **198**, 205–213
28 Delnomdedieu, M., Basti, M. M., Otvos, J. D. and Thomas, D. J. (1993) Chem. Res. Toxicol. **6**, 598–602
29 Chakraborti, P. K., Garabedian, M. J., Yamamoto, K. R. and Simons, S. S., Jr. (1992) J. Biol. Chem. **267**, 11366–11373
30 Jha, A. N., Noditi, M., Nilsson, R. and Natarajan, A. T. (1992) Mutat. Res. **284**, 215–221
31 Petronilli, V., Costantini, P., Scorrano, L., Colanna, R., Passamonti, S. and Bernardi, P. (1994) J. Biol. Chem. **269**, 16638–16642
32 Black, H. S. (1987) Photochem. Photobiol. **46**, 213–221
33 Devary, Y., Gottlieb, R. A., Smeal, T. and Karin, M. (1992) Cell **71**, 1081–1091
34 Alam, J. and Zhining, D. (1992) J. Biol. Chem. **267**, 21894–21900
35 Friling, R. S., Bergelson, A. and Daniel, V. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 668–672
36 Yao, K.-S., Xanthoudakis, S., Curran, T. and O'Dwyer, P. J. (1994) Mol. Cell. Biol. **14**, 5997–6003
37 Meyer, M., Schreck, R. and Baeuerle, P. A. (1993) EMBO J. **12**, 2005–2015
38 Schenk, H., Klein, M., Erdbrugger, W., Droge, W. and Schulze-Oxthoff, K. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 1672–1676

Received 29 August 1995/30 October 1995; accepted 7 November 1995