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Presetting of chromatin structure and transcription factor binding poise the human *GADD45* gene for rapid transcriptional up-regulation

Dawn M. Graunke, Albert J. Fornace Jr[1] and Russell O. Pieper*

DNA Damage and Repair, Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Room C2058-7, Baton Rouge, LA 70808, USA and [1]Division of Basic Science, National Cancer Institute, Bethesda, MD 20982, USA

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ABSTRACT

***GADD45* has been suggested to coordinate cell cycle regulation with the repair of DNA damage following ionizing radiation (IR). Although the *GADD45* gene is transcriptionally up-regulated in response to IR, alterations in *in vivo* transcription factor (TF) binding or chromatin structure associated with up-regulation have not been defined. To understand how chromatin structure might influence TF binding and *GADD45* up-regulation, key regulatory regions of the gene were identified by *in vivo* DNase I hypersensitivity (HS) analysis. Chromatin structure and *in vivo* TF binding in these regions were subsequently monitored in both non-irradiated and irradiated human ML-1 cells. In non-irradiated cells expressing basal levels of *GADD45*, the gene exhibited a highly organized chromatin structure with distinctly positioned nucleosomes. Also identified in non-irradiated cells were DNA–protein interactions at octamer binding motifs and a CCAAT box in the promoter and at consensus binding sites for AP-1 and p53 within intron 3. Upon irradiation and a subsequent 15-fold increase in *GADD45* mRNA levels, neither the chromatin structure nor the pattern of TF binding in key regulatory regions was altered. These results suggest that the *GADD45* gene is poised for up-regulation and can be rapidly induced independent of gross changes in chromatin structure or TF binding.**

INTRODUCTION

The *GADD* gene family consists of genes cloned on the basis of their up-regulation following DNA damage and/or growth arrest (1,2). The most widely characterized of these genes is *GADD45*. In human cells, *GADD45* is up-regulated in response to growth arrest conditions or after exposure to ultraviolet radiation (UV), hydrogen peroxide, methyl methanesulfonate (MMS) or ionizing radiation (IR) (1,3). The direct action of increased levels of *GADD45* protein remains uncertain although increases in *GADD45* protein levels are correlated with a block in S phase progression as well as a stimulation of excision repair (4–6). *GADD45* has, therefore, been suggested to link the processes of cell cycle arrest with DNA repair. As such, an understanding of the mechanism by which *GADD45* is up-regulated is important in understanding the cellular response to DNA damage.

Up-regulation of *GADD45* occurs primarily at the transcriptional level and appears to follow one of two pathways. Under conditions of growth arrest or following exposure of cells to UV or MMS, *GADD45* expression is up-regulated 5- to 10-fold (3). Because similar up-regulation was noted in transient gene expression systems in which the *GADD45* promoter was linked to a reporter gene, *GADD45* up-regulation in response to UV has been suggested to be promoter-dependent (3). Although it remains unclear which sequences in the *GADD45* promoter control *GADD45* up-regulation, a variety of consensus binding sites for transcription factors have been identified, including those for WT-1 and POU family members (3). Transcriptional up-regulation mediated by the *GADD45* promoter therefore potentially involves the action of a number of transcription factors. While *GADD45* up-regulation following UV exposure or stress is promoter-dependent, a second regulatory pathway is activated following exposure of cells to IR. This pathway is more complex than that noted following UV exposure in that it appears, at least in transient transfection systems, to require intron 3 sequence of the *GADD45* gene in addition to promoter sequence (7,8). The intron 3 region of the *GADD45* gene contains a consensus p53 binding site and may involve DNA–protein interactions in both the promoter and intron 3 regions and potentially interactions between complexes in both regions (8).

Although the identification of transcription factor binding sites in the *GADD45* gene has proven useful in beginning to understand *GADD45* regulation, an important and as yet uninvestigated factor in *GADD45* gene regulation is chromatin structure. It is becoming increasingly appreciated that while information can be derived by studying TF/DNA interactions *in vitro* or in transient transfection systems, many interactions *in vivo* require transcription factors to gain access to and bind to recognition sequences. This accessibility is in turn dependent upon the chromatin organization of regulatory

*To whom correspondence should be addressed at: UCSF Cancer Center, 2340 Sutter Street, Room N261, Box 0128, San Francisco, CA 94115, USA. Tel: +1 415 502 7132; Fax: +1 415 502 3179; Email: rpieper@cc.ucsf.edu

sequences. For many inducible genes (e.g. steroid receptor targets and the yeast PHO5 gene) up-regulation first requires the movement or 'remodeling' of nucleosomes (9,10). The changes in chromatin structure allow TF binding which in turn drives gene expression. For other inducible genes, regulatory regions are maintained in a conformation in which nucleosomes are arranged around transcription factor binding sites with TF free to bind at all times (11–13). In these so-called 'preset' genes, up-regulation is independent of chromatin remodeling and nucleosomal movement but rather is dependent upon the binding of newly synthesized TF or on the modification of prebound TF (11–13). With regard to the *GADD45* gene, it is not known how chromatin structure relates to TF binding in the presumed regulatory regions of the gene nor is it known whether induction of expression involves changes in this chromatin structure. As the chromatin structure of regulatory regions shapes the series of events necessary for the induction of gene expression, we examined the chromatin structure of the *GADD45* gene *in vivo* both prior to and following IR and correlated the chromatin structure with TF binding. The results of this study show that prior to up-regulation, the *GADD45* gene is highly organized, with nucleosomes positioned around but not within the promoter and intron 3 regions. These accessible regulatory regions of DNA are in turn occupied in non-irradiated cells by a variety of TFs. Surprisingly, upon irradiation and *GADD45* up-regulation, neither the chromatin structure of the *GADD45* gene nor the pattern of TF binding in the key regulatory regions was altered. These results suggest that the presetting of both chromatin structure and TF binding poise the *GADD45* gene for rapid transcriptional up-regulation following DNA damage.

MATERIALS AND METHODS

Cells and cell treatment

A human myeloid leukemia cell line (ML-1) was grown in RPMI-1640 medium (2 mM L-glutamine, penicillin/streptomycin and 10% bovine calf serum). For the irradiation studies, log phase ML-1 cells (1×10^6 cells, 50 ml) were exposed to 20 Gy of γ-irradiation using a ^{137}Cs source (5.7 Gy/min). Following irradiation, cells were collected by centrifugation and either harvested immediately or resuspended in fresh growth medium and incubated at 37°C for 4 h prior to analysis. ML-1 cells exposed to chemical agents were incubated in fresh medium containing the agent prior to exposure to 20 Gy of IR and then either harvested immediately or grown for an additional 4 h prior to harvesting.

DNA probes used in northern blot analysis and in DNase I/MNase hypersensitivity assays

A 1.4 kb *GADD45* cDNA and a 900 bp glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) cDNA were used as probes for northern blot analysis. The 1.4 kb *GADD45* cDNA probe was isolated from the pHUL145B2 plasmid by sequential digestions using the restriction enzymes *Kpn*I and *Not*I. The final digestion products were size fractionated through a 0.8% low melting temperature agarose gel. The *Kpn*I–*Not*I fragment of ~1.4 kb was excised from the gel and purified by centrifugation through glass wool (13 000 *g*, 30 s). The 900 bp *GAPDH* cDNA was isolated from a plasmid (provided by Mark R. Kelley, Indiana University) by *Pst*I digestion and agarose gel purified as described above.

Two probes were used in the hypersensitivity assays. The 5′D probe (nt –816 to nt –232) recognizes the 5′-end of a 3.3 kb *Bam*HI fragment of the *GADD45* gene (nt –816 to nt +2455) (Fig. 1C) and was isolated by *Bam*HI and *Bss*HII digestion of a plasmid (pHg45) containing the entire *GADD45* gene. The 584 bp *Bam*HI–*Bss*HII DNA fragment was gel purified as described above. The 3′D probe (nt +1721 to +2499) recognizes the 3′-end of the same 3.3 kb *Bam*HI fragment of the *GADD45* gene (Fig. 1C) and was isolated by restriction enzyme digestion of the pHg45 plasmid using *Bam*HI and *Eco*RI. The resulting 778 bp fragment was gel purified as described above.

DNA probes were uniformly radiolabeled using the random primer method (Prime-A-Gene; Promega) incorporating [α-^{32}P]dCTP (sp. act. $>1 \times 10^9$ c.p.m./μg). For use in DNase I and MNase hypersensitivity assays, either a 1 kb DNA ladder or a 100 bp DNA ladder were radiolabeled using [γ-^{32}P]ATP by the phosphate exchange reaction (Gibco Life Technologies). Following labeling and removal of unincorporated nucleotides by spin column chromatography, incorporation of ^{32}P was quantified using a scintillation counter.

Analysis of *GADD45* mRNA up-regulation in response to IR

RNA from 1×10^7 ML-1 cells (irradiated and non-irradiated) was isolated using a standard guanidinium isothiocyanate lysis procedure (14). RNA (15 μg) was fractionated through a 0.8% denaturing agarose gel and transferred to a nylon membrane by capillary action. The membrane was prehybridized in a solution of 50% formamide, 5× SSPE (1 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.7), 10% dextran sulfate, 1% SDS, 1× Denhardt's solution, with 0.25 mg/ml sheared salmon sperm DNA for 3 h, after which a ^{32}P-end-labeled 1.4 kb *GADD45* cDNA probe was added to a final count of 2×10^6 c.p.m./ml. After hybridization (36 h, 42°C), the membrane was washed as previously described (15). The amount of probe hybridized to the membrane was quantified using a Betascope 603 analyzer (BetaGen). Following removal of the *GADD45* probe, the membrane was rehybridized using the radiolabeled *GAPDH* cDNA probe (15 h, 42°C). Unbound probe was removed from the membrane by washing (6 min, 60°C) in a solution of 0.1% SSPE, 0.1% SDS in a modified Disk-Wisk washing system (Schleicher & Schuell). The amount of probe hybridized to membranes was quantified as described above. Following quantification, membranes were exposed to X-ray film (X-Omat AR-5; Kodak) for 16–20 h to generate autoradiographic data.

The effect of actinomycin D and cycloheximide on up-regulation of *GADD45* in response to IR

Control ML-1 cells were incubated with the RNA synthesis inhibitor actinomycin D (ActD, 5 μg/ml, 37°C, 250 min) as previously described (16) prior to RNA isolation. ML-1 cells treated with ActD and exposed to IR were incubated with ActD (10 min, 37°C), irradiated as described above, supplied with fresh medium containing ActD and incubated (4 h, 37°C) prior to isolation of RNA for northern blot analysis. ML-1 cells were treated with cycloheximide (CHX, 5 μg/ml) in a manner consistent with previously published work (17). Control ML-1 cells were incubated with CHX (16 h, 37°C) prior to RNA

isolation. Cells treated with both CHX and IR were incubated overnight (12 h) in CHX, irradiated as described above, then supplied with fresh medium containing CHX and incubated (4 h, 37°C) prior to isolation of RNA for northern blot analysis. Northern blot analysis was performed as described above.

Isolation of nuclei for DNase I hypersensitivity assays

ML-1 cells (5×10^7 non-irradiated or irradiated) were collected by centrifugation, washed in ice-cold PBS and lysed in 600 µl lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 2 min, 4°C). Nuclei were collected by centrifugation at 10 000 *g* for 30 s, washed in ice-cold RSB (10 mM Tris, pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$), resuspended in cold RSB and divided equally among six reactions.

Analysis of DNase I hypersensitivity within the *GADD45* gene

Nuclei from ML-1 cells, the equivalent of 25 µg of DNA/sample, were incubated with DNase I (0, 2.5, 5, 10, 25 or 50 U, 10 min, 37°C) and then lysed (20 mM Tris, pH 8, 20 mM NaCl, 20 mM EDTA, 1% SDS, 600 µg/ml proteinase K) (18). Following protein digestion, RNase A (50 µg/ml) was added to the samples and incubated for a minimum of 1 h at 37°C. The DNase I-cleaved DNA was phenol:chloroform extracted, ethanol precipitated, resuspended and digested overnight with *Bam*HI. For preparation of the 'naked' DNA samples used in these studies, protein-free DNA from ML-1 cells was incubated with DNase I (0.005, 0.01, 0.02 and 0.05 U, 5 min, 37°C). DNase I was inactivated by phenol:chloroform extraction and the DNA was ethanol precipitated, resuspended and cleaved with *Bam*HI. For Southern blot analysis, DNA (20 µg) was size-fractionated through a 1.1% agarose gel (25 V, 14 h) and transferred to a nylon membrane by capillary action. Either the radiolabeled 5′D or 3′D probe (see Fig. 1C) was hybridized to the membrane-bound DNA as described above (>48 h, 42°C). Unbound probe was removed from the membranes as described above for the *GAPDH* probe. Autoradiographs were generated by exposing hybridized membranes to X-ray film for 16–20 h.

Ligation-mediated PCR (LMPCR) analysis of DNA–protein interactions

Non-irradiated and irradiated ML-1 cells were analyzed for *in vivo* DNA–protein interactions within the *GADD45* gene by LMPCR. *In vivo* DMS-treated DNA and LMPCR-suitable DNA devoid of DNA–protein interactions was generated as previously described (19). LMPCR was performed using a modification of the method of Garrity *et al*. (20). Following primer extension and 18 rounds of PCR amplification using a gene-specific primer and the linker-specific primer, two additional rounds of amplification were performed using a third, nested, ^{32}P-end-labeled primer so that amplification products could be analyzed by autoradiography following polyacrylamide gel electrophoresis. LMPCR primers used in the analysis of the *GADD45* promoter and intron 3 regions were: linker primer LLP, 5′-GCGGTGACCCGGGAGATCTGAATTC-3′; *GADD45* primers GAD4, 5′-GATCTGTGGTAGGTGGGGGTC-3′; GAD5, 5′-GGAGGGTGGCTGCCTTTGTCCGACT-3′; GAD6, 5′-GGAGGGTGGCTGCCTTTGTCCGACTAGAG-3′; GAD10, 5′-CGCCTCCCGCGTGGCTCCT-3′; GAD11, 5′-CCCTTTT-CCGCTCCTCTCAACCTGA-3′; GAD12, 5′-CCCTTTTCCG-CTCCTCTCAACCTGACTCC-3′.

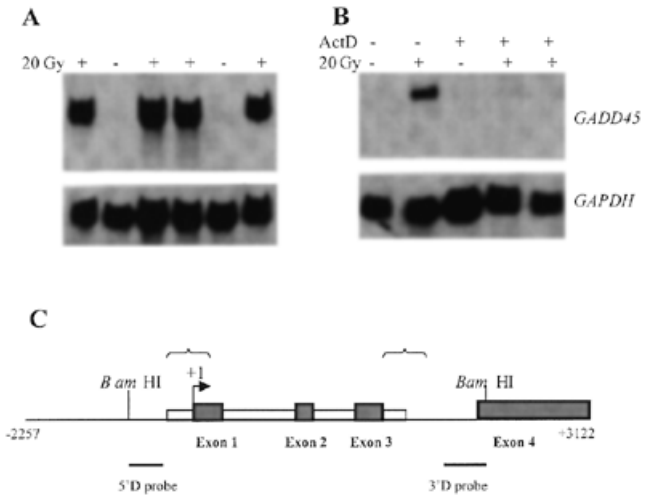

Figure 1. The *GADD45* gene is transcriptionally up-regulated in response to IR. (**A**) Northern blot analysis of *GADD45* expression in ML-1 cells following 20 Gy of IR. (**B**) Northern blot analysis of the effect of ActD on *GADD45* expression in non-irradiated and irradiated cells. (**C**) A schematic representation of the 3.3 kb *Bam*HI fragment of the *GADD45* gene. The white box represents the CpG island sequence, shaded boxes represent exons of the *GADD45* gene and the arrow at +1 marks the transcription start site. Braces denote the regions of the *GADD45* gene in which DNA–protein interactions were investigated. The location of DNA probes used in DHS and MNase HS analysis are noted.

Isolation of nuclei for MNase analysis

Non-irradiated ML-1 cells (5×10^7) were collected, washed once in ice-cold PBS and lysed in 600 µl ice-cold MNase digestion buffer (50 mM Tris, pH 7.4, 60 mM KCl, 3 mM $CaCl_2$, 0.34 M sucrose) plus 0.5% Nonidet P-40 (2 min, 4°C). Nuclei were collected by centrifugation (10 000 *g*, 30 s), washed and resuspended in ice-cold MNase digestion buffer and divided equally among six reactions.

Analysis of MNase hypersensitivity within the *GADD45* gene

Nuclei from non-irradiated ML-1 cells (the equivalent of 25 µg of DNA/sample) were incubated with MNase (0, 0.01, 0.02, 0.04, 0.05 or 0.1 U, 10 min, 37°C), lysed and incubated with RNase A (1 h, 37°C). Following RNase A incubation, the DNA was phenol:chloroform extracted, ethanol precipitated, resuspended and digested using *Bam*HI (10 U/µg DNA, 12 h, 37°C). DNA purified from ML-1 cells was cleaved with MNase (0.01 or 0.02 U, 5 min, 37°C), phenol:chloroform extracted, ethanol precipitated, resuspended and cleaved using *Bam*HI. Southern blot analysis was performed as previously described, using the 5′D probe.

RESULTS

IR-induced up-regulation of *GADD45*

To verify that *GADD45* mRNA levels in ML-1 cells increase following exposure to IR, up-regulation of *GADD45* mRNA

levels in ML-1 cells was measured by northern blot analysis 4 h following a 20 Gy exposure to IR. In ML-1 cells, *GADD45* mRNA levels increased 15.7 ± 1.5-fold over basal levels (n = 7) (representative results shown in Fig. 1A) following exposure to IR, consistent with previously published results (2,6,15). Previous studies have also demonstrated increased transcription of the *GADD45* gene following exposure to IR (2). To verify this response, ML-1 cells were incubated with the RNA synthesis inhibitor ActD prior to irradiation. Control ML-1 cells displayed a 15-fold induction of *GADD45* mRNA levels 4 h following irradiation, while cells pretreated with a concentration of ActD previously shown to block RNA synthesis in ML-1 cells (16) showed no IR-induced increase in *GADD45* mRNA levels (representative results shown in Fig. 1B). To determine the role of protein synthesis in *GADD45* up-regulation, ML-1 cells were incubated with the protein translation inhibitor CHX prior to irradiation. While control cells displayed a 15-fold induction of *GADD45* mRNA levels following irradiation, cells preincubated with a concentration of CHX that blocked protein synthesis by 98% demonstrated a 6.9 ± 2.7-fold induction of *GADD45* mRNA levels following irradiation (data not shown), consistent with previously published results (17). As a whole, these studies verify that *GADD45* induction following IR occurs at least in part at the transcriptional level and that this IR-induced *GADD45* up-regulation can occur in the absence of protein synthesis.

Localization of sites of DNase I hypersensitivity (DHS) within the *GADD45* gene

TF interactions often occur at nucleosome-free, DNase I hypersensitive regions of DNA. In order to localize potential transcriptional control sites within the *GADD45* gene *in vivo* and to determine whether or not these sites changed in number or location following IR, regions of DHS were identified within the *GADD45* gene. DHS analysis was performed by isolating nuclei from either non-irradiated or irradiated ML-1 cells and exposing the nuclei to varying concentrations of DNase I. DNA was purified, cleaved with *Bam*HI and subjected to Southern blot analysis using probes complementary to either the 5′- or the 3′-end of a 3.3 kb *Bam*HI fragment of the *GADD45* gene (Fig. 1B). As shown in Figure 2A, exposure of nuclei to increasing concentrations of DNase I increased the cleavage of genomic DNA. The results of Southern blot analysis using this DNA and a probe complementary to the 5′-end of the *GADD45* gene are shown in Figure 2B. In DNA from non-irradiated nuclei not exposed to DNase I (lane 1), the probe hybridized to a 3.3 kb DNA fragment. In DNA from non-irradiated nuclei exposed to increasing concentrations of DNase I *in vivo* (lanes 2–5), DNase I cleavage occurred within the 3.3 kb *GADD45 Bam*HI fragment, resulting in gradual disappearance of the fragment. Rather than being random, however, this DNA cleavage preferentially occurred in two regions of the 3.3 kb *GADD45 Bam*HI fragment (upper and lower arrows, Fig. 2B). The locations of these DHS regions were confirmed by rehybridization of the membrane-bound DNA to a probe (3′D) complementary to the 3′-end of the *GADD45 Bam*HI fragment. The 3′D probe hybridized to the 3.3 kb *GADD45 Bam*HI DNA fragment in DNA from non-irradiated cells not exposed to DNase I (lane 1) and to lower molecular weight fragments generated by DNase I cleavage (upper and lower arrows, Fig. 2C). Given the resolution of the technique used, DHS could be localized to ~700–1000 bp and 2.3–2.5 kb from the 3′-end of the 3.3 kb fragment or ~800–1000 bp and 2.3–2.6 kb from the 5′-end of the *GADD45 Bam*HI fragment, corresponding to two regions of DHS located 1400 bp apart (see Fig. 1C). Alignment of the two DHS sites with the *GADD45* gene sequence revealed that the identified regions of preferential DNase I cleavage

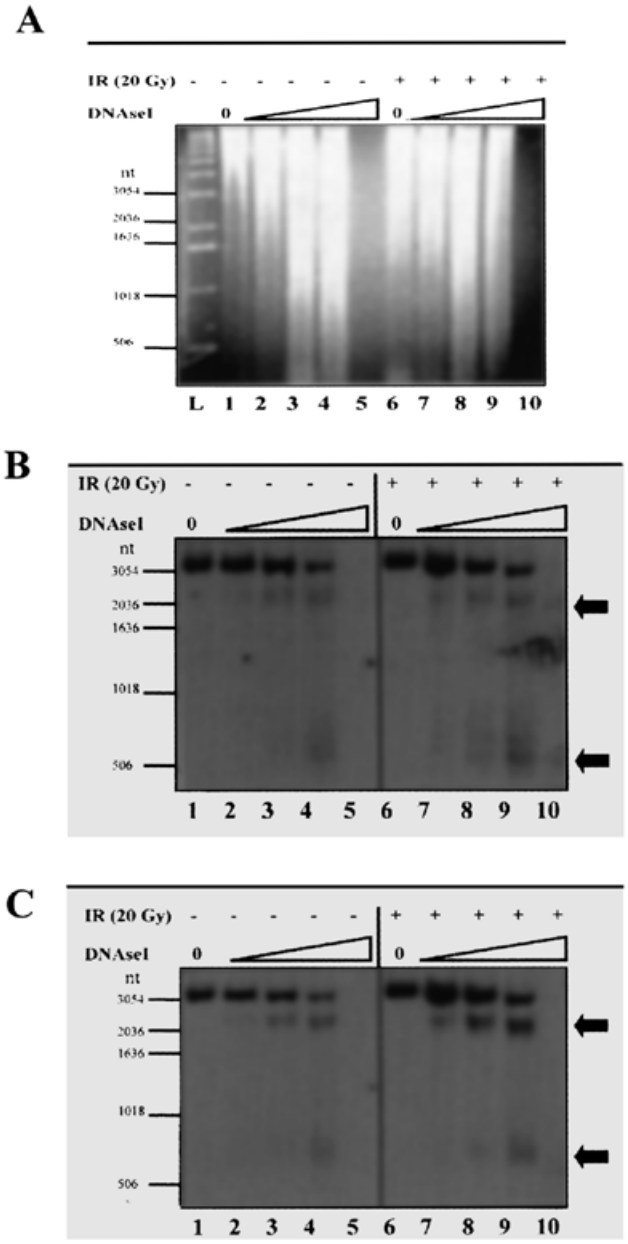

Figure 2. DHS exists within the *GADD45* gene in both non-irradiated and irradiated ML-1 cells. Nuclei from non-irradiated cells were incubated with increasing concentrations of DNase I. (**A**) A photograph of an ethidium bromide (EtBr) stained agarose gel following fractionation of DNA. Lanes 1–5, nuclei isolated from non-irradiated ML-1 cells; lanes 6–10, nuclei isolated from ML-1 cells 4 h after exposure to 20 Gy of IR. Lane L, 1 kb DNA ladder. (**B**) Autoradiograph of Southern blot analysis performed using the 5′D probe. Arrows to the right of the figure denote DNase I cleavage fragments. (**C**) Autoradiograph generated by stripping the membrane used in (B) and rehybridization using the 3′D probe. Results shown are representative of four independent experiments.

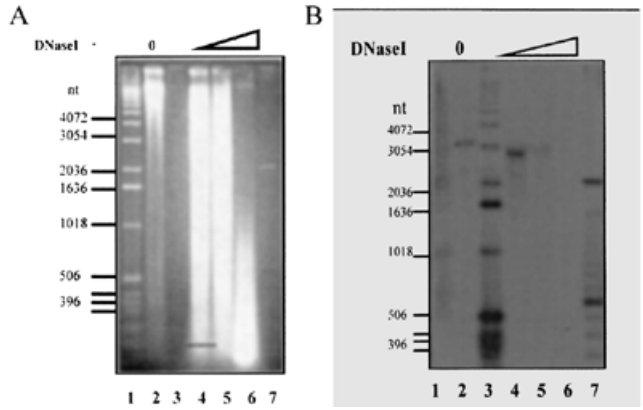

Figure 3. DHS present in ML-1 nuclei is not present in naked ML-1 DNA. Purified DNA from ML-1 cells was incubated with increasing concentrations of DNase I. The following DNA samples were used: 1 kb DNA ladder (lane 1); *Bam*HI-digested ML-1 DNA (lane 2); ^{32}P-radiolabeled 1 kb DNA ladder (lane 3); DNase I-digested ML-1 DNA (lanes 4–6); ^{32}P-radiolabeled 100 bp DNA ladder (lane 7). (**A**) Photograph of the EtBr stained gel prior to capillary transfer. (**B**) Autoradiograph of Southern blot analysis using the 5′D probe. The results shown are representative of three independent experiments.

coincided with the promoter and intron 3 regions of the gene. These data suggest that even in the uninduced state, two regions of DHS exist within the *GADD45* gene, one in the 5′ promoter, the other in intron 3.

Having identified regions of DHS within the *GADD45* gene prior to IR, it was next addressed whether these hypersensitive regions were altered following induction of gene expression by IR. Southern blot analyses using nuclei from irradiated ML-1 cells were performed in parallel with studies using nuclei from non-irradiated cells. In DNA samples that had not been exposed to DNase I, the 5′ probe recognized the expected 3.3 kb DNA fragment (Fig. 2B, lane 6). In DNA samples exposed to increasing concentrations of DNase I *in vivo*, the 3.3 kb DNA fragment disappeared as DNase I concentrations increased (Fig. 2B, lanes 7–10). As in the studies using the nuclei of non-irradiated cells, two regions of DHS were identified in the *GADD45 Bam*HI fragment (arrows, Fig. 2B) from irradiated ML-1 cells. The location of these sites was confirmed by rehybridization of the membrane-bound DNA to the 3′D probe, which revealed DNA fragments in the same size range as those identified in non-irradiated cells (arrows, Fig. 2C). The location and size of regions of DHS in irradiated ML-1 cells, as analyzed using either the 5′D or 3′D probe, were identical to the DNase I hypersensitive sites in non-irradiated cells (compare lanes 1–5 to lanes 6–10 in Fig. 2B and C). These data suggest that the regions of DHS in the promoter and intron 3 regions of the *GADD45* gene present in non-irradiated cells do not change following up-regulation of *GADD45* expression.

In order to confirm that regions of DHS identified by the studies represented in Figure 2 were a result of *in vivo* chromatin structure and were not merely due to sequence-specific DNase I cleavage, Southern blot analyses similar to those described above were performed with purified, non-chromatin-associated DNA isolated from ML-1 cells (referred to as naked DNA). Incubation of naked DNA *in vitro* with increasing concentrations of DNase I yielded DNA fragments digested to the same degree as the *in vivo* DNase I-treated samples (compare Fig. 3A to 2A). In Southern blot analyses of this DNA, the 5′D probe hybridized to a 3.3 kb *GADD45 Bam*HI fragment in naked DNA not exposed to DNase I (Fig. 3B, lane 2). At higher concentrations, DNase I cleaved within the 3.3 kb fragment, resulting in its gradual disappearance (Fig. 3B, lanes 4–6). Unlike the results of *in vivo* DNase I cleavage, however, there were no sites of preferential DNase I cleavage within the 3.3 kb *GADD45 Bam*HI fragment in naked DNA. These results suggest that preferential DNase I cleavage within the *GADD45* gene in nuclei is not due to a sequence preference of DNase I, but rather is due to *in vivo* chromatin structure.

LMPCR analysis of DNA–protein interactions within the *GADD45* promoter

As regions of DHS are typically nucleosome free and contain DNA–protein interactions *in vivo*, the DHS regions of the *GADD45* gene were analyzed for DNA–protein interactions in ML-1 cells. Specific DNA–protein interactions in the *GADD45* promoter were examined by LMPCR amplification of DNA from ML-1 cells exposed to dimethyl sulfate (DMS). DMS methylates the N7 position of guanine and, to a lesser degree, the N3 position of adenine (21). This modification of DNA is blocked by sequence-specific DNA-binding proteins (e.g. TFs) but is not blocked by nucleosomes (22). DMS-treated DNA can be cleaved at sites of adduction by incubation with piperidine. For LMPCR, the 3′-ends of piperidine-cleaved DNA are ligated to a linker molecule. Following PCR amplification using a gene-specific primer and a linker-specific primer, PCR products are analyzed by polyacrylamide gel electrophoresis and autoradiography. This process results in the generation of a ladder of fragments, the 3′-end of each corresponding to a site of DMS adduction and subsequent cleavage. Using this method, sites of *in vivo* DNA–protein interactions appear as holes or ‘footprints’ in the LMPCR-generated guanine ladder. Analysis of DNA–protein interactions in the *GADD45* gene were carried out in non-irradiated ML-1 cells, immediately following IR (identifying rapidly induced DNA–protein interactions) and 4 h following IR (persistent DNA–protein interactions would be noted). An LMPCR analysis of *in vivo* DNA–protein interactions in the transcribed strand of the *GADD45* promoter is shown in Figure 4. LMPCR analysis using the plasmid pHg45, which contains the entire *GADD45* gene sequence, yielded a sequence ladder consistent with the published *GADD45* sequence (lanes 1 and 9). LMPCR analysis using ML-1 DNA which was purified prior to DMS exposure also yielded the expected sequence ladder (lane 2) and confirmed that the more complex genomic setting of the *GADD45* promoter had little effect on DMS reactivity. Results from LMPCR analysis using as template DNA from cells prior to irradiation (B), from cells immediately following exposure to IR (IR-0) and from cells 4 h following irradiation (IR-4) are shown in lanes 3–8. The sequence ladder in lanes 3 and 4 differed slightly from that in lanes 1 and 2 in the disappearance of DNA fragments corresponding to cleavage at guanines –83 and –76 (open circles, Fig. 4). The guanine at nt –83 (lower circle), although not located within one of the consensus TF binding sites in the *GADD45*

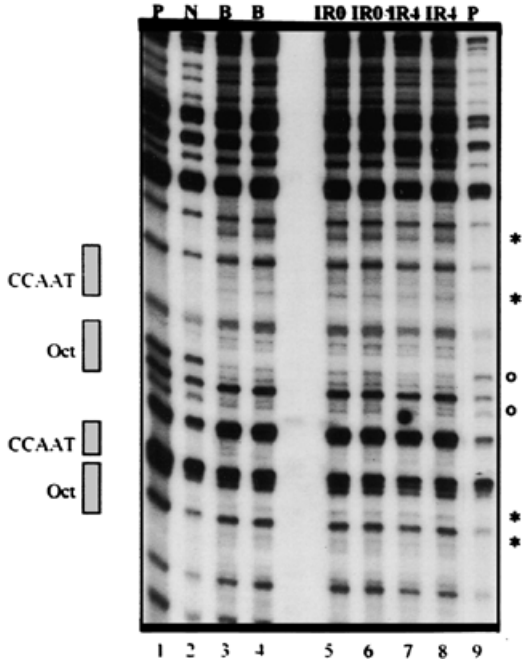

Figure 4. Identification of DNA–protein interactions within the transcribed strand of the DNase I HS *GADD45* promoter. LMPCR was performed using primers GAD10–12 and plasmid DNA (P, lanes 1 and 9), naked ML-1 DNA (N, lane 2), DNA from non-irradiated cells (B, lanes 3 and 4) and DNA from cells exposed to IR, either immediately following irradiation (IR-0, lanes 5 and 6) or 4 h after irradiation (IR-4, lanes 7 and 8). Putative TF binding sites are represented by bars to the left of the panel while guanines and adenines involved in DNA–protein interactions are marked with a circle or an asterisk, respectively. Results shown are representative of three independent experiments.

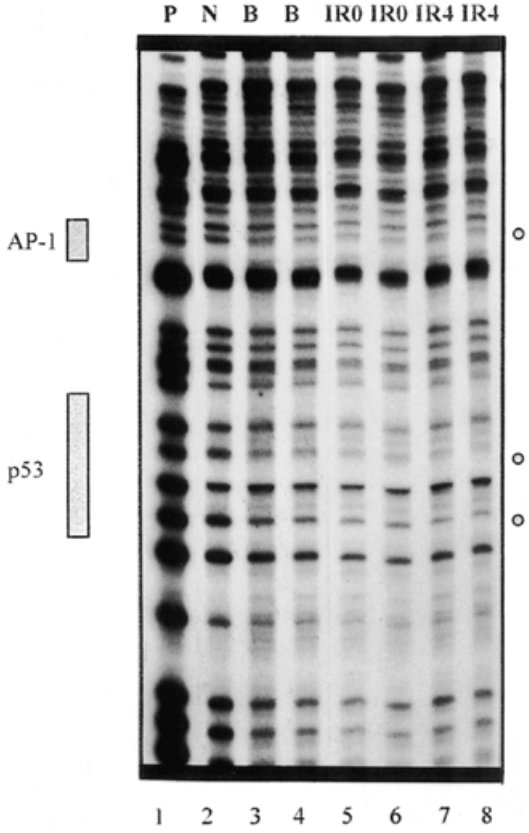

Figure 5. Analysis of DNA–protein interactions within the transcribed strand of the DNase I HS intron 3 of the *GADD45* gene. LMPCR was performed using primers GAD4–6 with DNA from the following sources: plasmid (P, lane 1); naked ML-1 DNA (N, lane 2); non-irradiated ML-1 cells (B, lanes 3 and 4); cells exposed to IR immediately following irradiation (IR-0, lanes 5 and 6) or 4 h after irradiation (IR-4, lanes 7 and 8). Putative TF binding sites are represented by bars to the left of the panel. Guanines involved in DNA–protein interactions are marked with a circle. Results are representative of three independent experiments.

promoter (bars to the left of Fig. 4), was most likely protected by the same DNA–protein interaction as the guanine at nt –76, an interaction at a consensus octamer binding motif. The sequence ladder in lanes 3 and 4 also differed from that in lanes 1 and 2 in the appearance of adenines (nt –96, –92, –53 and –39, marked with asterisks in Fig. 4) hypersensitive to DMS-induced damage and subsequent cleavage. Hyper-reactive adenines at nt –92 and –96 (lower asterisks) were located within an additional octamer binding motif. Adenines at nt –53 and –39 (upper asterisks), while not located within a consensus TF binding site, appeared hypersensitive to DMS-induced damage, most likely due to a DNA–protein interaction within the CCAAT box they flank. These data suggest the presence of three DNA–protein interactions in the *GADD45* promoter of ML-1 cells prior to irradiation, two involving the POU family of transcription factors (possibly the ubiquitously expressed Oct-1 protein or the lymphoid cell-specific Oct-2 protein), the other most likely involving the CCAAT box-binding C/EBP protein. The LMPCR-generated sequence ladder from reactions using template DNA from irradiated cells (Fig. 4, lanes 5–8) was identical to the results from amplification of DNA from non-irradiated cells (lanes 3 and 4), suggesting that in the transcribed strand of the *GADD45* promoter the DNA–protein interactions present during basal transcription do not change upon *GADD45* up-regulation. Similar analyses of DNA from non-irradiated and irradiated ML-1 cells performed using primers specific for the non-transcribed strand of the *GADD45* promoter revealed no DNA–protein interactions (data not shown), suggesting that the interactions noted in the *GADD45* promoter were single-stranded in nature.

LMPCR analysis of DNA–protein interactions within intron 3 of the *GADD45* gene

The results of LMPCR analysis of the transcribed strand of intron 3 are shown in Figure 5. LMPCR analysis using as template either a plasmid containing the entire *GADD45* gene or purified ML-1 DNA yielded the expected sequence ladder (Fig. 5, lanes 1 and 2). The results of LMPCR analysis using DNA from non-irradiated ML-1 cells immediately post-irradiation (IR-0) and cells 4 h post-irradiation (IR-4) are shown in lanes 3–8. The sequence ladder generated using DNA from non-irradiated cells (lanes 3 and 4) differed slightly from that generated from plasmid DNA and purified DNA (lanes 1 and 2). Specifically, in DNA from non-irradiated cells the guanine at nt +1629 (top open circle, Fig. 5) was protected from DMS adduction and cleavage. This guanine occurs in a consensus AP-1 binding site (bar to the left of Fig. 5),

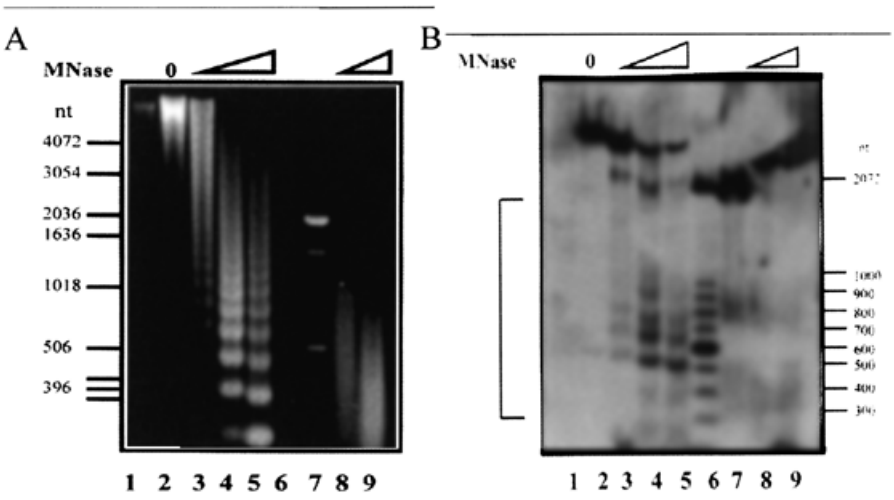

Figure 6. Analysis of local chromatin structure of the *GADD45* gene. Nuclei from non-irradiated ML-1 cells were incubated with increasing amounts of MNase. (**A**) Photograph of the EtBr stained agarose gel containing the following DNA samples: non-radiolabeled 1 kb DNA ladder (lane 1); DNA isolated from nuclei exposed to increasing concentrations of MNase (lanes 2–5); ^{32}P-radiolabeled 100 bp DNA ladder (lane 6); non-radiolabeled 100 bp DNA ladder (lane 7); ML-1 DNA incubated with MNase *in vitro* (lanes 8 and 9). (**B**) Southern blot analysis using the 5′D probe. The bracket denotes the region in which MNase HS sites were identified. The results shown are representative of two independent experiments.

suggesting a DNA–protein interaction at this site during basal expression of the *GADD45* gene. Other guanines within intron 3 of the *GADD45* gene were also involved in DNA–protein interactions. Two guanines located within a p53 consensus binding site (nt +1588 and +1578) were protected in DNA from non-irradiated cells (lanes 3 and 4) when compared to plasmid or 'naked' DNA (lanes 1 and 2, respectively). The p53 consensus binding site within the *GADD45* intron 3 has been shown *in vitro* to be bound by p53 post-IR (23) and with regard to the present data suggests an interaction of the *GADD45* intron 3 DNA with p53 prior to irradiation of ML-1 cells. No DNA–protein interactions at the consensus p53 binding site were identified on the non-transcribed strand of intron 3 (data not shown), which is consistent with the single-stranded nature of the p53 interaction (23). The sequence ladder generated using DNA from irradiated cells (lanes 5–8) was very similar to that derived from amplification of DNA from non-irradiated cells (lanes 3 and 4), i.e. there were DNA–protein interactions at both the AP-1 and p53 consensus binding sequences. These results suggest that DNA–protein interactions exist within the promoter and intron 3 of the *GADD45* gene during basal transcription and that no new DNA–protein interactions are created following induction of *GADD45* expression.

Nucleosome-like positioning in the *GADD45* gene

The results of DHS and LMPCR analyses suggest that the *GADD45* gene assumes a chromatin structure that favors TF binding in two regulatory regions and that neither this chromatin structure nor TF binding in these regions changes following irradiation. To further define this chromatin structure, nucleosome positioning in the *GADD45* gene was investigated using MNase. MNase is an endonuclease that preferentially cleaves DNA between nucleosomes *in vivo*. Regions of DNA in which nucleosomes are randomly positioned will be randomly digested *in vivo* with MNase while regions in which nucleosomes are positioned in the same location in all or most cells will display periodic cleavage.

Nuclei from non-irradiated ML-1 cells were isolated and incubated with varying concentrations of MNase. The DNA was purified, digested with *Bam*HI and subjected to Southern blot analysis using the same 5′D probe as used in the DHS analysis. As shown in Figure 6A, increasing the *in vivo* exposure of nuclei from ML-1 cells to MNase resulted in increased cleavage of ML-1 DNA. Note that because MNase cleaves between nucleosomes *in vivo*, a nucleosomal ladder results from incubation of nuclei with increased concentrations of MNase (Fig. 6A, lanes 2–5). As MNase cleavage can display sequence specificity, naked ML-1 DNA exposed to varying concentrations of MNase *in vitro* and cleaved to the same degree as experimental groups was also included in this study (compare lanes 8 and 9 to lanes 4 and 5 in Fig. 6A). Figure 6B shows the results of Southern blot analysis using the 5′D probe. In DNA from nuclei not exposed to MNase, the 5′D probe hybridized to a 3.3 kb DNA fragment (lane 1). DNA isolated from nuclei exposed to increasing amounts of MNase was cleaved within the 3.3 kb *GADD45 Bam*HI fragment. Rather than being random, however, this MNase cleavage of the 3.3 kb fragment preferentially occurred ~400, 560, 720, 820, 950, 2300 and 2500 bp from the 5′-end of the *GADD45 Bam*HI fragment (range noted by the left bracket, Fig. 6B). Periodic cleavage was not noted in ML-1 DNA digested to an equal extent by *in vitro* MNase exposure (lanes 8 and 9). Alignment of the sites of MNase hypersensitivity with the *GADD45* gene sequence suggested that MNase cleaved at the transcription start site, the boundaries of regions of DHS and at periodic intervals around regions of TF binding (Fig. 7A). The appearance of these periodically spaced cleavage sites in DNA from nuclei exposed to MNase suggests the positioning of nucleosome-like structures upstream of the TF-binding *GADD45* promoter region and between the promoter and the

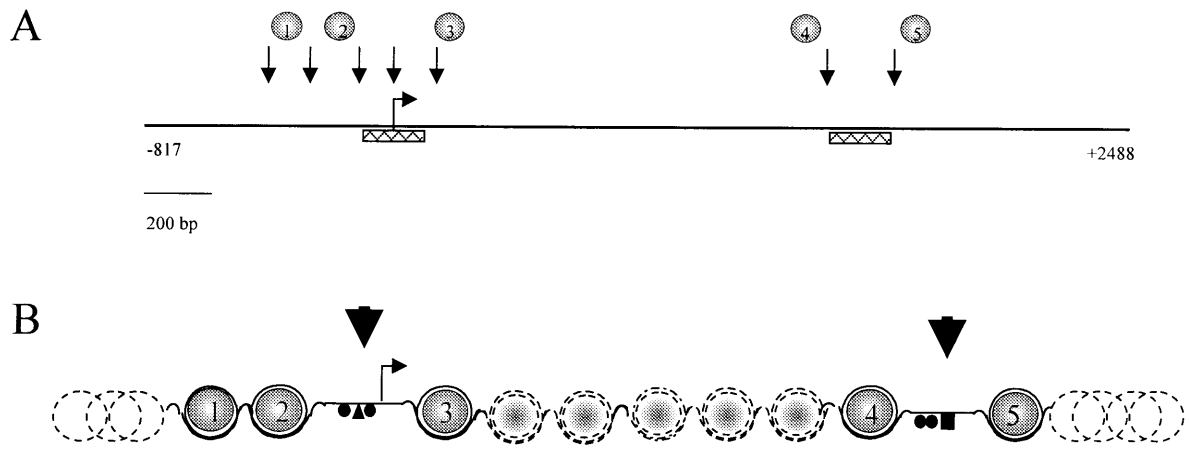

Figure 7. (**A**) Map of the MNase HS sites and proposed nucleosomes within the *GADD45* gene. The position of MNase HS sites relative to the transcription start site and the regions of DNase I HS (stippled boxes) are denoted by downward arrows. Proposed nucleosome-like structures are represented by filled, numbered circles. (**B**) Model for the organization of the *GADD45* gene *in vivo*. Basal expression of *GADD45* is associated with two nucleosome-free regions of TF binding that are surrounded by at least five positioned nucleosome-like structures. No detectable changes in TF binding or nuclease HS are associated with induction following IR. Large arrowheads denote regions of DNase I HS, filled, numbered circles represent positioned nucleosome-like structures while the reverse shaded, non-numbered circles represent hypothetical positioned nucleosome-like structures. *In vivo* DNA–protein interactions are represented by the geometric shapes located within the regions of DNase I HS.

TF-binding intron 3 region (Fig. 7A). The higher order chromatin structure of the *GADD45* gene in cells expressing basal levels of *GADD45*, in combination with the lack of changes in TF binding and chromatin structure following irradiation, suggests that the *GADD45* gene is organized as depicted in Figure 7B. Based on the data presented, the *GADD45* gene appears to be poised for rapid up-regulation in the absence of gross changes in TF binding or chromatin structure.

DISCUSSION

The results presented in this study suggest that the rapid up-regulation of *GADD45* expression following IR does not require synthesis of new transcription factors, changes in chromatin structure or novel IR-induced DNA–protein interactions. As such, the *GADD45* gene appears to fall into the category of 'preset' genes whose up-regulation is independent of changes in chromatin structure. The data supporting these conclusions are internally consistent as well as being consistent with previous studies concerning *GADD45* expression and inducible gene expression. Prior to irradiation, DNA–protein interactions in the *GADD45* promoter exist at two consensus octamer binding motifs and at a CCAAT box sequence, consistent with previously published *in vitro* data (3). An interaction at the consensus AP-1 site was noted in the intron 3 region of the *GADD45* gene during basal expression, consistent with both the presence of a consensus AP-1 site in this region, as well as with the presence of AP-1 interactions in regulatory regions of other IR-inducible genes prior to irradiation (23). A DNA–protein interaction at a consensus p53 binding site in the intron 3 region of the gene is also consistent with previously published *in vitro* data (6,7,23) and with the observation that non-irradiated ML-1 cells express wild-type p53 (7; data not shown). The results of the present study differ slightly from those of Chin *et al*. who, using DNase I as an *in vivo* 'footprinting' agent, suggested that the p53 interaction with the *GADD45* intron 3 binding site only occurs 30 min following irradiation (23). The interaction identified in the present study, and in the aforementioned study, is relatively weak, making an assessment of when the interaction occurs and how it changes over time difficult. DMS and DNase I also have differing sensitivities to DNA–protein interactions and perhaps the constitutive p53 binding demonstrated using DMS was not detectable in the previous study using DNase I and the interaction detected in the previous study using DNase I was perhaps a conformational or other qualitative change in the p53 interaction. Nonetheless, both studies suggest an early and relatively constant interaction between p53 and intron 3 of the *GADD45* gene. The regions of the *GADD45* gene shown in the current study to interact with proteins *in vivo* coincided both with those regions previously suggested to be involved in *GADD45* gene regulation (3,6,7) and with regions identified to be nucleosome free and DNase I HS. During basal expression, MNase cleaved the *GADD45* gene at the outer boundaries of TF-binding regions, at the transcription start site or at nucleosome sized intervals. The pattern of MNase hypersensitivity could be a result of interaction of the *GADD45* gene with non-nucleosomal proteins. This is unlikely, however, due to the periodicity of cleavage, the lack of detectable DNA–protein interactions (nucleosomes, as opposed to sequence-specific DNA-binding proteins, do not protect DNA from DMS-induced damage) and the lack of DHS within the region of proposed nucleosome-like structures. Rather, the periodic cleavage of the *GADD45* gene at regular nucleosome sized intervals by MNase is consistent with the positioning of nucleosomes. Such nucleosome-like positioning is in turn consistently found in CpG islands (24,25), one of which spans the TF-binding regions of the *GADD45* gene. The lack of change in DNA–protein interactions in the *GADD45* gene

following IR-induced transcriptional up-regulation is consistent with the observation that *GADD45* up-regulation can occur in the absence of protein synthesis, as well as with the lack of chromatin reorganization of the gene following IR.

One issue that complicates interpretation of the present study is the fact that DNA damage, including damage caused by IR, induces *GADD45* expression. Therefore, the DNA-damaging techniques used to identify regions of *in vivo* DNA–protein interactions and regions of DNase I/MNase HS may themselves induce *GADD45* expression. As such, the DNA–protein interactions and hypersensitive sites seen in non-irradiated cells may be artifacts of the processes used for their identification and may obscure IR-induced alterations. If this were true, however, the DNA damage-induced changes in DNA–protein interactions and chromatin structure would be immediate as exposures of cells to DNase I/MNase or DMS were for 10 and 2 min, respectively. Additionally, with regard to DMS, it should be noted that the large concentration of DMS (10 mM) used for *in vivo* footprinting analysis reduced, rather than induced, expression of *GADD45* and completely blocked IR-induced *GADD45* up-regulation in ML-1 cells (data not shown). As such, it appears that the large concentrations of DMS used for *in vivo* footprinting extensively methylated cellular components (DNA and proteins), 'froze' cells in their pre-exposure state and precluded cellular responses to DMS-induced DNA damage. Finally, and most importantly, it should be noted that the *GADD45* gene contains a CpG island beginning upstream of the promoter region and ending at the third intron (26). DNA damage-based analysis of DNA–protein interactions and chromatin structure have demonstrated that all CpG islands examined to date contain TF-binding, nucleosome-free regions which coincide with the regulatory regions of genes (24,25). The identification of nucleosome-free, TF-binding regions in the CpG islands of genes not inducible by DNA damage as well as in the damage inducible *GADD45* gene suggests that the TF-binding, nucleosome-free regions noted in the *GADD45* gene are not likely an experimental artifact, but rather are present in non-irradiated as well as irradiated cells.

The present study has significant impact on how *GADD45* up-regulation can be viewed. As is the case for most genes whose expression is transcriptionally up-regulated, many control mechanisms can be envisioned. IR may trigger changes in chromatin structure which could subsequently allow TF binding to regulatory regions previously not available for interaction. Alternatively, exposure to IR could increase the levels of, or modify, a number of different TF, any one of which could interact with the *GADD45* regulatory regions and increase transcription. Finally IR could alter *GADD45* expression in the absence of changes in TF binding and chromatin structure simply by altering prebound TF interactions. With regard to the first scenario, while changes in gene expression are commonly associated with changes in chromatin structure (9,27,28), the present studies provide little evidence for IR-induced changes in chromatin structure in the *GADD45* gene. With regard to the second possibility, because *GADD45* mRNA levels can increase following IR even in the absence of protein synthesis, it seems unlikely that new transcription factors relevant for *GADD45* expression are synthesized in response to IR. IR could, however, induce post-translational modification of TF, which could result in increased TF binding and subsequent *GADD45* up-regulation. IR-induced post-translational modification of p53 is a particularly appealing possibility as p53 is known to play a role in IR-induced *GADD45* up-regulation and is known to be stabilized following IR by post-translational modification. The issue of increased levels of p53 binding to intron 3 of the *GADD45* gene following IR is only partially addressed in the present study. Non-irradiated, logarithmically growing ML-1 cells express p53 and data from the present study show that p53 binds to the *GADD45* intron 3 region even prior to irradiation. Additionally, p53 binding did not quantitatively change following IR, suggesting that *GADD45* up-regulation was not likely a result of increased p53 binding. Rather, the presence of TF interactions in the *GADD45* regulatory regions prior to IR is most consistent with the possibility that IR alters the interactions between prebound TF. In this regard it is worth noting that p53 has recently been shown to activate UV-induced *GADD45* expression not by direct DNA interaction, but rather by interaction with bound TF and/or the transcriptional machinery itself (29). If p53 functioned similarly following IR, *GADD45* up-regulation could be accomplished by IR-induced stabilization of p53 followed not by increased p53 DNA binding in the *GADD45* exon 3 region, but rather by increased p53 interaction with prebound TF or the transcriptional machinery. Such up-regulation could be accomplished in the absence of protein synthesis and would not require changes in chromatin structure or TF binding, all of which would be consistent with the data presented in this study.

In summary, the present studies, by linking chromatin structure of the *GADD45* gene with the DNA–protein interactions that contribute to *GADD45* transcriptional up-regulation, suggest that the presetting of both chromatin structure and TF binding poise the *GADD45* gene for rapid transcriptional up-regulation following DNA damage. Further understanding of *GADD45* up-regulation will likely require a better understanding of how IR triggers changes in TF activation and/or coupling to the transcriptional machinery.

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