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Distinct Mechanisms Are Utilized to Induce Stress Sensor *gadd45b* by Different Stress Stimuli

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ABSTRACT

The GADD45 family of proteins consists of three small proteins, GADD45A, GADD45B, and GADD45G, implicated in modulating the cellular response to genotoxic/physiological stressors. Despite similarities in sequence, structure and function, each gadd45 gene is induced differentially by different stress stimuli. Studies on stress-mediated induction of the gadd45 genes have predominantly focused on gadd45a, with knowledge of gadd45b and gadd45g regulation lacking. To generate a more complete understanding of the regulation of gadd45 genes, a comprehensive analysis of stress-mediated induction of human gadd45b has been carried out using human RKO colorectal carcinoma cells as a model system. Novel data indicate that gadd45b induction in RKO cells is regulated by distinct mechanisms in a stress-specific manner. Methylmethane sulfonate (MMS), a DNA alkylating agent, induces gadd45b transcription through a cohort of both constitutive and inducible bound factors, including NFY, Sp1 and Egr1. In contrast, in a hyperosmotic environment generated with sorbitol, gadd45b mRNA is induced exclusively by mRNA stabilization. These findings indicate that the stress-mediated induction of gadd45b is largely distinct from gadd45a. Furthermore, data obtained provide a novel paradigm for stress–response gene induction, indicating that gadd45b induction by distinct stressors, in the same cell type and under the same experimental settings, is differentially regulated at the level of mRNA transcription or mRNA stability. Importantly, this study also provides the groundwork to further examine the regulation of gadd45b expression in in vivo settings using animal models and tissues obtained from normal individuals and cancer patients prior to and after chemotherapeutic intervention. J. Cell. Biochem. 108: 1220–1231, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: GADD45B; EXPRESSION; TRANSCRIPTION; RNA STABILITY

gadd45b (*gadd45β/MyD118*) is a member of a family of structurally and functionally related genes, that includes *gadd45a* (gadd45α/gadd45) and *gadd45g* (gadd45γ/CR6), which encode small (18 kDa), evolutionarily conserved proteins, sharing high homology (55–57%), high acidity (pI 4.0–4.2), and which are primarily localized to the nucleus [Liebermann and Hoffman, 2002]. *gadd45b* was first identified as a primary response gene in the myeloid differentiation program, when leukemic myeloblasts (M1) were induced to differentiate with IL-6 [Abdollahi et al., 1991]. Shortly thereafter, it was observed that *gadd45b* and other gadd45 gene members are also induced by a wide variety of genotoxic stress stimuli [Zhan et al., 1999] such as MMS and UV [Wang et al., 1999; Vairapandi et al., 2002] in addition to being expressed in essentially all tissues [Takekawa and Saito, 1998].

Gadd45 genes have been implicated in stress signaling in response to physiological and genotoxic stressors, including MMS, UV, and sorbitol [Liebermann and Hoffman, 2008], which results in either cell cycle arrest, DNA repair, cell survival and senescence, or apoptosis. On the other hand, aberrant expression of GADD45 has also been implicated in the development of cancer [Cretu et al., 2009].

Evidence accumulated implies that Gadd45 stress sensor functions are mediated by a complex interplay of physical interactions with other cellular proteins that are implicated in cell cycle regulation and the response of cells to stress. These include PCNA, p21, cdc2/cyclinB1, and the p38 and JNK stress–response kinases [Smith et al., 1994, 2000; Vairapandi et al., 1996, 2000, 2002; Takekawa and Saito, 1998; Azam et al., 2000; Bulavin et al.,

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2003; Liebermann and Hoffman, 2008]. Recently, the tertiary structure of GADD45B and GADD45G has been elucidated [Papa et al., 2007; Schrag et al., 2008; Tornatore et al., 2008]. These data indicate that GADD45B and GADD45G consist of a central four-stranded β-sheet surrounded by five α-helices with two acidic loops that extend outward from the structure proper. It was further determined that GADD45B exists in solution as a dimer, but is also capable of hetero-dimerizing with either GADD45A or GADD45G [Kovalski et al., 2001; Tornatore et al., 2008]. It is clear that this homo-dimerization is essential for GADD45B to functionally interact with and modulate the activity of other proteins. In contrast, GADD45A exists primarily as a tetramer in solution [Kovalski et al., 2001].

Despite these structural and functional similarities, each gadd45 gene is induced differentially, depending on the type of stress stimuli. For example, the alkylating agent methylmethane sulfonate (MMS) rapidly induces all three genes, whereas hydrogen peroxide and sorbitol preferentially induce gadd45a and gadd45b, respectively [Takekawa and Saito, 1998].

Little is known about the mechanism by which diverse stress stimuli induce *gadd45b*. Clearly, given the pleiotropic function of Gdda45b as a cellular stress–response gene that modulates the response of cells to physiological and environmental stressors, and that aberrant expression has been implicated in tumorigenesis, an important task is to gain a better understanding of mechanisms that modulate Gadd45b expression in response to distinct stressors.

Thus, in order to generate a more complete understanding of the collective regulation of the gadd45 genes, a comprehensive analysis of the stress-mediated induction of gadd45b has been carried out. Towards this end, a gadd45b promoter–reporter construct was generated. Employing human colorectal carcinoma cells (RKO), in which gadd45b mRNA levels were observed to be profoundly induced by various stress stimuli, including MMS, UVC, or sorbitol, we provide novel data that gadd45b induction by distinct stress agents, such as MMS and sorbitol, is regulated differentially at the level of either mRNA transcription or mRNA stability, respectively.

MATERIALS AND METHODS

PLASMIDS

A wild-type 8.1 kb gaddd45b gene/promoter fragment was isolated from a human genomic lambda library (Clontech). A 8,172 bp *gadd45b* promoter DNA fragment isolated from the lambda library was cloned into pBluescript II SK+ as a blunted *Fsp*I (*gadd45b*)-*Eco*RV (pBluescript), and *Bam*HI (pBluescript)-BglII (*gadd45b*) fusion. A ~8.5 kb *gadd45b* promoter–luciferase construct was generated by a *Bgl*II–*Bgl*II and *Nco*I–*Nco*I fusion between *gadd45b*-pBluescript and pGl3-basic (Promega). This construct contains 3,877 bp of *gadd45b* promoter sequence upstream from the transcription start site and 219 bp of sequence downstream from the transcription start site, up to the native *gadd45b* AUG translation start site codon. To generate 5′gross deletions, restriction sites were used within the *gadd45b* promoter to generate successively shorter *gadd45b* promoter fragments, from the 5′ end, in the pgl3-basic backbone. A total of nine, 5′-gross deletion *gadd45b* promoter–luciferase constructs were generated using the following restriction sites: 1. *Eco*R1, *gadd45b*-2194luc; 2. *Eco*R1, *gadd45b*-1656luc; 3. *Sbf*I, *gadd45b*-1514luc; 4. *gadd45b*-1118luc; 5. *Pvu*II, *gadd45b*-638luc; 6. *Rsr*II, *gadd45b*-387luc; 7. *Bst*EII, *gadd45b*-227luc; 8. *Bsg*I, *gadd45b*-133luc; 9. *Eco*RI, *gadd45b*-81luc. Linker-scanning mutant constructs were produced on the *gadd45b* promoter in the pGL3-basic luciferase backbone. A 15-bp DNA linker (5′-*ggtaccgagctctta*-3′) replaced wild-type gadd45b sequence in two parts using the Stratagene Quikchange II XL mutagenesis system. First, a specific 15-bp wild-type sequence was deleted from the Gadd45b-1656LUC construct. Then, the 15-bp linker cassette was inserted into the deleted sequence location. Linker-scanning mutant constructs were screened for successful insertion by restriction digest analysis, and then confirmed by DNA sequence analysis using primers to the pGL3-basic backbone, GL3 and RV2. Site-directed mutagenesis was carried out using the Stratagene Quikchange II XL system to mutate individual *cis*-elements. The base change mutations were made in order to eliminate the binding sites for Egr1/Sp1/or NFY. The specific sequence changes required to eliminate binding were determined from the literature and then tested by EMSA. All mutant constructs were sequence verified for accuracy.

TISSUE CULTURE

A human colorectal carcinoma cell line (RKO) was maintained in Dulbeco's modified Eagle's Media (DMEM) containing 10% fetal bovine serum (FBS) (without antibiotics) at 37°C and 10% CO_2. Cells were maintained and split every 3 days to a density of 30–40%.

NORTHERN BLOT

RKO cells (2.0×10^6) were plated in 10 ml DMEM containing 10% FBS on 10-cm gas plasma-treated tissue culture plates (Falcon), and grown for 24 h to ~70% confluency before treatment. For treatment, plates were washed once with room temperature (RT) 1X PBS, then DMEM media containing a stress agent was added back to the plate (For UVB/C, cells were exposed to radiation prior to adding DMEM with FBS). At the desired time point, the plate was washed with PBS and 3 ml Trizol (Invitrogen) was added at RT for 5 min. The Trizol-cell slurry was collected in 15-ml round bottom polypropylene tubes (Falcon) and total RNA extraction carried out according to the Trizol product insert. The final RNA pellet was resuspended in 30 μl Rnase/Dnase-free water (Invitrogen) and quantified spectrophotometrically. Ten micrograms total RNA (in 9.5 μl) in 36.5 μl loading buffer (8.7% (v/v) 10X MOPS, 43% (v/v) de-ionized formamide, 14% (v/v) 37% formaldehyde, 52 ng/μl ethidium bromide, 10% 10X RNA loading buffer) from each sample was run for 3 h at 5 V/cm on a denaturing 1% agarose gel (10% (v/v) 10X MOPS, 16.5% (v/v) 37% formaldehyde) in denaturing running buffer (10% (v/v) 10X MOPS, 7.5% (v/v) 37% formaldehyde). RNA quality in the gel was assessed by UV imaging of the 18S and 28S ribosomal bands for RNA integrity. RNA was transferred to a Duralon UV (Stratagene Stratalinker) membrane by conventional capillary transfer for 16 h at RT. RNA was fixed to the membrane by UV irradiation at 1,200 J/m^2 (Stratagene Stratalinker). Twenty-five nanograms of a 1.3 kb *gadd45b* cDNA fragment was radiolabeled with the DNA radprime labeling system (Invitrogen) according to the product insert. The membrane was prehybridized in 12 ml buffer (50% (v/v) formamide, 1 M NaCl, 1% (v/v) SDS, 1% (v/v) H_2O, 10% (v/v) dextran

sulfate, 100 μg/ml denatured sheared salmon sperm DNA) at 42°C for 2.5 h, and hybridized overnight (16 h) at 42°C in fresh buffer with hot probe. The membrane was washed twice at RT in 2X SSC, followed by two washes at 65°C (0.1X SSC, 0.1% SDS), before being exposed to film wrapped in saran wrap.

NUCLEAR RUN-ON ASSAY

To prepare nuclei for the nuclear run-on transcription reaction, 1.4×10^7 RKO cells were seeded into 15-cm tissue culture dishes (30 ml) and treated 24 h later. At the desired time-point, nuclei were isolated according to *Current Protocols in Molecular Biology* [Greenberg and Bender, 2007]. Nonidet P-40 (NP-40) lysis buffer A (10 mM Tris–Cl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) NP-40) was used and the protocol was followed verbatim, with the final nuclei pellet stored in 200 μl chilled glycerol storage buffer (50 mM Tris–Cl, pH 8.3, 40% (w/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) in liquid nitrogen until ready for the run-on transcription reaction. The nuclear run-on transcription reaction was carried out in 15-ml polypropylene tubes (Falcon) according to *Current Protocols in Molecular Biology* [Greenberg and Bender, 2007]. It was imperative that fresh [a-^{32}P]UTP (10 mCi/ml) be used in the reaction to obtain enough signal in the hybridization. Following the run-on reaction at 30°C for 30 min, the labeled RNA transcripts were extracted and purified according to a modified protocol by Schubeler et al. [1996] as follows. Forty-one microliters Dnase I (Promega, 1 U/μl) was added to each tube followed by an additional 15 min incubation at 30°C. Trizol LS (1,350 μl; Invitrogen) was added to each tube and the reaction was homogenized by passing through a pipet tip several times, followed by a 5 min RT incubation. Chloroform (360 μl) was added and the tube was vortexed vigorously for 15 s within a closed 50-ml conical tube (Falcon), followed by a 10 min RT incubation. The 15-ml reaction tube was centrifuged 12,000*g*, 15 min, 4°C, and the top layer was transferred to a fresh 15-ml tube containing 900 μl isopropanol, which was mixed and incubated at RT for 10 min. The 15-ml reaction tube was centrifuged a second time at 12,000*g*, 10 min, 4°C, the supernatant was removed, and 1.8 ml of 75% ethanol was added and mixed by vortexing briefly. The tube was again centrifuged at 7,500*g*, 5 min, 4°C to pellet the RNA, the supernatant was removed and the pellet air-dried 5–10 min at RT. The RNA pellet was resuspended in 100 μl Rnase-free H_2O (Invitrogen), and incubated at 60°C for 10 min to dissolve the pellet. radiolabeled transcript (3×10^6 CPM) was hybridized to gene screen plus nylon membranes (NEN) containing 10 μg linearized target cDNAs prepared in a Schleicher & Schuell vacuum slot–blot apparatus. A previously prepared DNA slot–blot membrane (described below) was placed, DNA side in, into a 20 ml glass vial to which 2 ml hybridization buffer (1% SDS, 10% dextran sulfate, 1.4 M NaCl, 325 μg/ml sheared salmon sperm DNA, 325 μg/ml *E. coli* tRNA) was added. The membrane was prehybridized at 60°C for 2 h with rotation. To the tube, 13 μl Rnasin (Promega, 40 U/μl), plus 80 μl 1 M DTT was added, and incubated at 60°C for 10 min. CPM radiolabeled transcript (3×10^6) was then added and allowed to hybridize at 60°C for 24 h. The washing protocol was derived from Schubeler et al. [1996] as follows. The membrane was transferred to a series of nine 50 ml conical tubes containing the following wash solutions and incubated at the following temperatures, for the following times: 1. 25 ml 2X SSC, 5 min RT; 2. 25 ml 2X SSC, 5 min RT; 3. 25 ml 2X SSC, 1% SDS, 15 min 65°C; 4. 25 ml 2X SSC, 5 min RT; 5. 8 ml 2X SSC, 10 μg/ml RNase A, 10 min 37°C; 6. 25 ml 2X SSC, 5 min RT; 7. 25 ml 0.1X SSC, 5 min RT; 8. 25 ml 0.1X SSC, 5 min RT; 9. 25 ml 0.1X SSC, 5 min RT. The membrane was blotted dry and taped to Whatman filter paper and then exposed to film.

DNA slot–blot membranes were prepared using Gene Screen Plus II Nylon membranes (NEN). Membrane was soaked for 5 min in 0.4 M Tris, pH 7.5 and assembled in the slot–blot apparatus (Schleicher & Schell vacuum slot–blot apparatus) according to the manufacturer's recommendations. Ten micrograms of linearized cDNAs in 198 μl H_2O was denatured for 10 min at RT by adding to each tube: 6.25 μl 10 N NaOH, 25 μl 5 M NaCl, and 20.75 μl H_2O. Each sample was diluted to 787.5 μl with 0.1X SSC, 0.125 N NaOH. One hundred seventy-five microliters of each sample was loaded per slot and allowed to incubate for 30 min at RT without suction. Light suction was then applied until the solution was drawn through. The membrane was then neutralized in a solution of 0.5 N NaCl, 0.5 M Tris–Cl, pH 7.5 and cross-linked by UV (Stratagene Stratalinker) at 1,200 J/m^2 while still moist. The membrane was allowed to dry at RT, then cut up intro strips, rolled up and placed in 20 ml glass vials and stored under vacuum in a desiccator until ready for hybridization.

DNA SEQUENCING AND ANALYSIS

All constructs generated and cloned were screened and/or sequence verified using the DNA sequencing facility at the University of Pennsylvania, Philadelphia, Pennsylvania.

LUCIFERASE PROMOTER–REPORTER ASSAY

To test the activity of the *gadd45b* promoter–luciferase constructs generated, the Dual Luciferase Reporter Assay (Promega) was performed using the single tube Berthold Luminometer. RKO cells (1.0×10^5) were seeded into ten 3.5 cm Falcon tissue culture treated dishes for each promoter–luciferase construct to be tested. Twenty-four hours after seeding, each construct was transfected (0.5 μg/plate) and co-transfected with pRL-null (Renilla luciferase, 50 ng/plate) using lipofectin (5 μl/plate, Invitrogen), according to the product insert procedure. Following the protocol, 1 ml of lipofectin/DNA mix was added in the absence of FBS at 1 ml per plate and incubated overnight (16 h) before media was replaced with 2 ml DMEM + 10% FBS per plate. Thirty-three to 35 h after the initial transfection, plates were treated or left untreated and harvested at the desired time-point (usually 8–10 h). To harvest, the protocol from the Dual Luciferase Assay kit was followed. Five hundred microliters of 1X Passive Lysis buffer was used per plate to harvest according to protocol. Lysate was diluted 1:10 in 1X Passive Lysis buffer and stored at −80° until ready to assay. To assay, lysate was thawed at RT until samples were RT. Samples were randomly assayed at RT by using 10 μl lysate with 100 μl Luciferase Assay Reagent II (LARII) and 100 μl Stop and Glo Reagent. Relative Luciferase Activity for each promoter construct, for a given treatment, was determined as follows: the absolute activity value for any given sample was attained by averaging the ratio of the Firefly luciferase light units to the Renilla luciferase light units.

The Relative light unit values were then attained by taking the ratio of treated to untreated light units calculated above. Values were normalized to the control construct, *gadd45b*-1656luc, to which a value of 100 was assigned. All luciferase experiments were repeated at least three times.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

To identify and/or confirm the binding of specific proteins or protein complexes to the *gadd45b* promoter in vitro, the EMSA was carried out. RKO cells (2×10^6) were seeded in 10 cm tissue culture plates and treated after 24 h. At the appropriate time, the nuclear extracts were isolated using the NE-PER reagent (Pierce). Protein concentration was determined prior to freezing and storing the nuclear extracts at −80°C. Three PAGE purified duplex oligonucleotides (IDTDNA) were radiolabeled with gamma-P32 (3,000 Ci/mmol) with T4 polynucleotide kinase (NEB), purified with oligo spin column (Roche), and 70 fmol used per binding reaction. Twenty microliters binding reactions included 10 mM Tris, 50 mM KCl, 1 mM DTT pH 7.5, 1 μg poly(dI–dC), 70 fmol radiolabeled probe and 5 μg nuclear protein extract. Reactions were incubated at RT for 20 min before adding 1X loading buffer and loading onto precast 6% DNA retardation gels (Invitrogen) in 0.5X TBE at 100 V for 1 h. Gel was dried at 80°C for 25 min and exposed to film.

CHROMATIN IMMUNOPRECIPITATION ASSAY (ChIP)

To identify and/or confirm the binding of specific proteins or protein complexes to the *gadd45b* promoter in vivo, the ChIP was carried out. RKO cells (2.5×10^6) were seeded into 10 cm plates. Twenty-four hours after seeding, cells were either treated or left untreated for 8 h prior to in vivo cross-linking and lysis using the EZ ChIP system (Upstate). Sonication was carried out on 8.2×10^6 cells in 410 μl SDS lysis buffer on a setting of 3 for 30 pulses per tube. Cell equivalents (2×10^6) in 100 μl SDS lysis buffer was used for each immunoprecipitation in the protocol. All steps were followed verbatim for the cross-linking, DNA shearing, DNA isolation, immunoprecipitation, and PCR according to the EZ ChIP protocol (Upstate). The one exception was in the preclearing step prior to the immunoprecipitation, in which an additional overnight incubation with sepharose beads was carried out. For immunoprecipitation, 5 μg of the following antibodies were used: Egr1 (Santa Cruz SC189X, C-19), NFY-A (Santa Cruz SC-10779X, H-20), MZF1 (Santa Cruz SC-764X, N-262), Sp1 (Santa Cruz SC-420X, 1C6). PCR primers to *gadd45b* were designed as suggested in the protocol. The following primers were used to generate a 183-bp amplicon from the gadd45b promoter: upstream, 5′-*ggcattcgcggtcacctaccc*-3′, downstream, 5′-*ccactgaggccacgcccaat*-3′. PCR reaction, Roche Faststart PCR mastermix was used in 20 μl total reaction volume, with the following cycling conditions: 95C 3 min; 95C 20s; 68C 30s; 72C 30s; 72C 2 min, for a total of 32 cycles.

siRNA

RKO cells (1.0×10^6) were seeded per 10 cm plate. After 24 h siRNA oligonucleotides (50 nM Egr1, Ambion Silencer predesigned siRNA AM16704, ID: 146223; 20 μM scrambled oligo) were transfected using lipofectin (Invitrogen) at 30 μl per plate according to product insert. Six milliliters of the siRNA/lipofectin mixes were transferred to each plate for an overnight incubation in 10% CO_2 incubator. Media was removed and replaced with 10%FBS/DMEM for 8 h before treating the plates with MMS (100 μg/ml). After 8 h, cells were harvested and total RNA was isolated with Trizol (Invitrogen). To determine the levels of *gadd45b* and Egr1 mRNA, Northern blot analysis was carriedout.

DNA ISOLATION, QUANTIFICATION, AND QUALITY ANALYSIS

Sequence-verified *gadd45b* promoter–luciferase constructs were produced and isolated by High Speed Maxi-prep (Qiagen). All luciferase constructs used in the luciferase assay were stored at −20°C in a frost-less freezer, subjected to only one freeze–thaw. DNA quantity and quality were assessed spectrophotometrically and by gel electrophoresis.

SEQUENCE ANALYSIS

To identify candidate stress-mediated *cis*-element in the *gadd45b* promoter, the gadd45B promoter sequence was computationally analyzed and compared with two independent transcription factor analysis programs. First, the sequence was analyzed against the TRANSFAC transcription factor database in the program TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). These data were confirmed by analysis of the same sequence with the Transcription Factor Element Search System from the University of Pennsylvania (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

RESULTS

gadd45b IS ROBUSTLY INDUCED BY DIVERSE STRESS STIMULI IN RKO CELLS

In order to determine the levels of *gadd45b* mRNA in RKO cells following various types of stress treatment, Northern blot analysis was carried out on total RNA isolated from stress-treated RKO cells over an 8-h time course (Fig. 1). As a reference point, *gadd45a* mRNA levels were determined as well, by re-probing the same blot. To verify equal sample loading, the 28S and 18S ribosomal RNA bands are included. It can be seen that basal levels of *gadd45b* and *gadd45a* mRNA in untreated RKO cells is almost undetectable (Fig. 1, lane 1). Both MMS (100 μg/ml) and UVC (500 J/M^2) profoundly induced *gadd45b* mRNA, beginning at <1-h post-treatment and achieving maximal levels by 8 h (Fig. 1, lane 4 and data not shown); maximum increases of 17- and 15-fold at 8 h post-MMS and -UVC treatment were observed, respectively. In contrast, *gadd45a* MMS- or UVC-mediated induction is different. For *gadd45a*, we observed a marginal increase following UVC treatment, that is maintained over the entire 8-h time course, while for MMS treatment, we observed a more abrupt increase at 4 h that is maintained up to 8 h (Fig. 1, lanes 2–4).

For sorbitol and anisomycin treatments, we observe similar kinetic profiles for *gadd45b* induction, in which an abrupt and robust increase in *gadd45b* mRNA is seen at 4 h, and is sustained

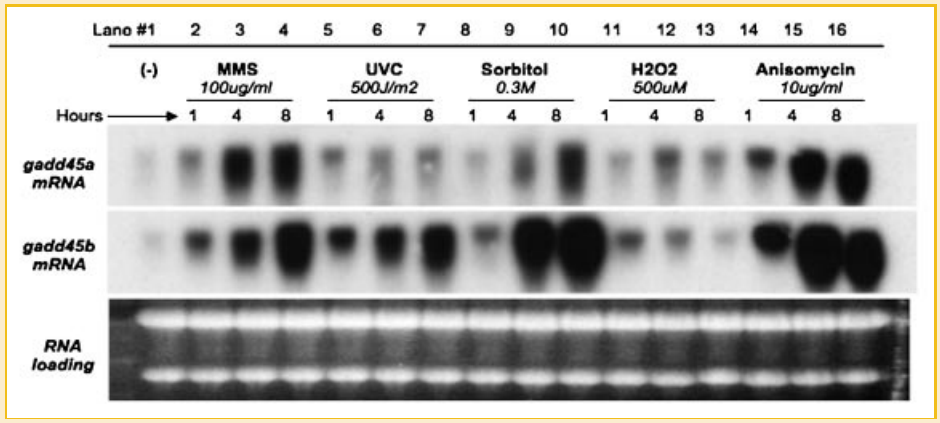

Fig. 1. *gadd45b* mRNA levels increase in mammalian cells following treatment with various stress stimuli. Northern blot analysis using a human 1.3 kb *gadd45b*- or 0.7 kb *gadd45a*-cDNA probe on 10 μg total RNA from human RKO cells following treatment with different stress agents. Total RNA was extracted with Trizol (Invitrogen) at 1, 4, and 8 h following treatment of RKO cells. Treatments were applied 24 h after seeding 2.5×10^6 cells per 10-cm tissue culture dish.

through 8 h (Fig. 1, lanes 8–10, 14–16). The maximum levels of induction for *gadd45b* mRNA is 27-fold for sorbitol and 27-fold for anisomycin. Less robust induction is observed for sorbitol-mediated gadd45a induction, with a gradual increase of mRNA over 8 h (Fig. 1, lanes 8–10); however, induction of *gadd45a* mRNA by anisomycin is similar to *gadd45b* (Fig. 1, lanes 12–16). Finally, hydrogen peroxide (500 μM) treatment results in a maximum increase of fivefold for *gadd45b* at 1 h and slowly declines thereafter. Gadd45a mRNA peaks fivefold at 4 h to and then declines.

In summary gadd45b induction is stress specific and is distinct from gadd45a induction.

IDENTIFICATION OF MMS-STRESS-RESPONSIVE REGIONS IN gadd45b PROMOTER

To determine the stress-responsiveness of the gadd45b promoter, a 3.9 kb promoter fragment was isolated from a human genomic library and cloned into the luciferase reporter vector pGL3-basic (Fig. 2A). The luciferase activity of nuclear extracts from treated cells was determined at 10 h post-treatment using the dual luciferase assay (Promega). Several 5′-gross deletions of the gadd45b promoter were generated from this construct to identify specific regions of stress-mediated promoter activity. As shown in Figure 2B, the 1.656 kb *gadd45b* promoter–luciferase reporter construct was observed to be activated by treatment with MMS or UVC treatment, similar to the 3.9 kb fragment (Fig. 2C), but not following sorbitol treatment.

Two regions within the *gadd45b* proximal promoter, located within the first 227 bp upstream from the transcription start site, were found to be required for maximal MMS-mediated *gadd45b* promoter induction (Fig. 2C). It also can be seen that induction by MMS was partially lost (60% of max) with a construct containing 133 bp upstream from the transcription start site, and complete loss of inducibility was observed with a −81 construct. These data establish the importance of two regions within the gadd45b promoter for MMS-mediated transcriptional activity, the −227 and −133 bp region, and the region between −133 and −81 upstream from the transcription start site. In contrast, none of these constructs appeared to mediate sorbitol induction.

IDENTIFICATION OF MMS-RESPONSIVE *cis*-ELEMENTS IN gadd45b PROMOTER

To localize MMS-responsive *cis*-elements in the two regions of the gadd45b promoter that were identified as mediating MMS inducibility, a series of *gadd45b* linker-scanning mutant promoter–luciferase constructs were generated (on a backbone of the 1.656 kb construct), by replacing 15-bp wild-type sequence with 15-bp "inert" linker DNA sequence spanning the entire −227 to −81 region of the promoter (Fig. 3A,B). Linker-scanning mutants were generated, as opposed to deletion constructs, in order to alter the wild-type sequence while preserving the spatial and topological landscape of the DNA. In so doing, specific transcription factor binding is disrupted while the overall tertiary structure of the promoter-transcription apparatus interface is maintained. From four independent experiments, we observed a loss of MMS-mediated *gadd45b* promoter activity in six of ten linker scanning mutants shown in Figure 3B. (1) Loss of ∼25% activity in LS17 (−180/−166 bp), LS18 (−167/−153 bp), and LS22 (−115/−101 bp); (2) loss of ∼35% activity in LS19 (−154/−140 bp); and (3) loss of ∼40% activity in LS20 (−141/−127 bp) and in LS23 (−102/−88 bp). The losses of activity indicated above are in reference to the wild-type, *gadd45b*-1656 luc construct. In three linker-scanning mutant constructs, LS15 (−206/−192 bp), LS16 (−193/−179), and LS21 (−128/−114), we observe slight, but insignificant, increases in activity relative to wild-type control.

Furthermore, it is shown that gadd45b induction by MMS was further impaired in double linker scanning constructs LS19/23 and LS19/20 LS20/23 compared to single linker scan mutants, and that induction was almost completely abolished in the triple linker scan mutant LS19/20/23 (Fig. 3B).

Collectively, these findings indicate that MMS-mediated inducibility of gadd45b is combinatorally regulated by three MMS responsive elements located at the −154/−140 bp, −141/−127 bp, and −102/−88 sites of the *gadd45b* promoter (Fig. 3A arrows).

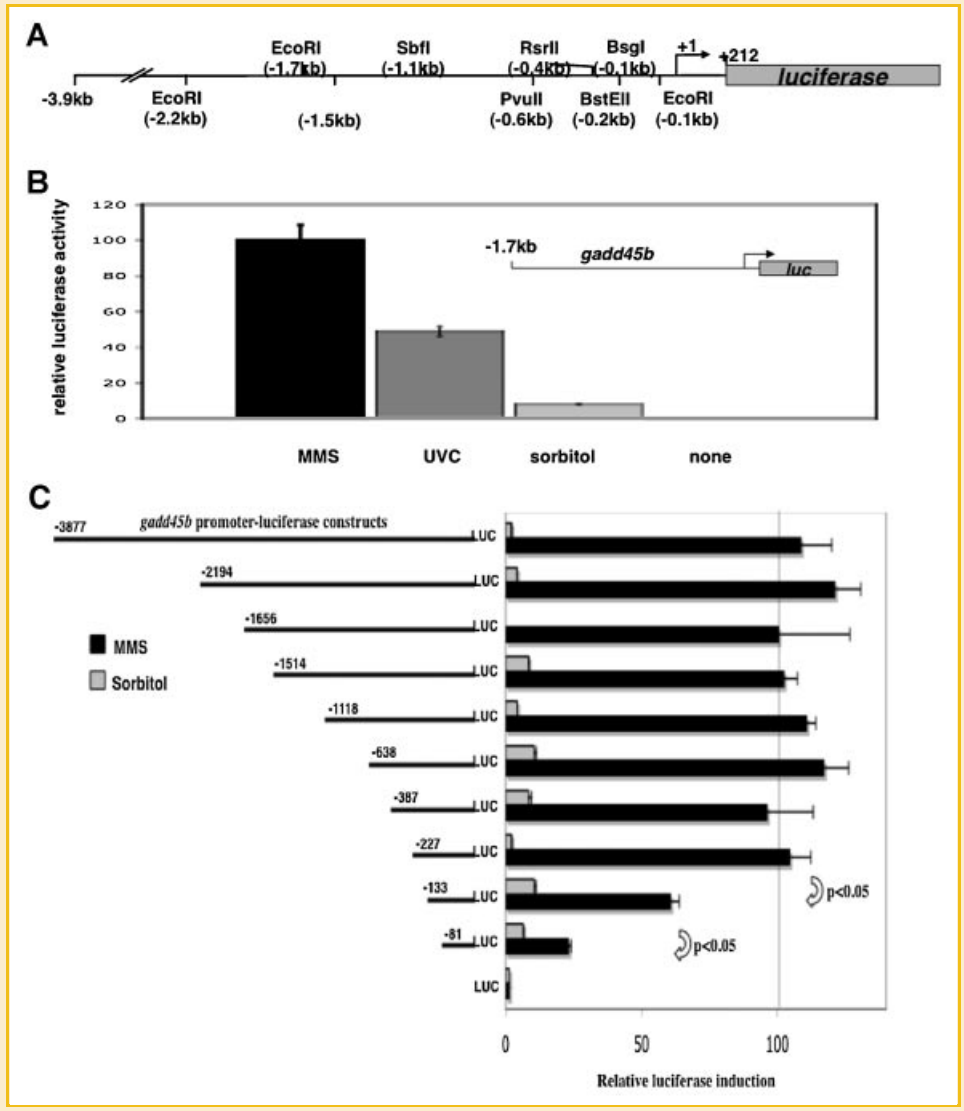

Fig. 2. Identification of stress-responsive regions in the gadd45b promoter-two regions within 227 bp upstream of the transcription start site of the *gadd45b* promoter are important for MMS-mediated promoter activity. A: Sequence of the −284 to +54 region of the gadd45b gene and schematic diagram of the gadd45b promoter fragment cloned into a luciferase reporter vector. B: Comparative analysis of gadd45b promoter by MMS (100 μg/ml), UVC (500 J/M^2) and sorbitol (0.3 M). C: Identification of MMS-stress-responsive regions in gadd45b promoter. The Dual Luciferase Assay (Promega) was carried out on extracts from treated or untreated RKO cells transfected with a series of 5′ gross deletion *gadd45b*-promoter constructs. Extracts were harvested 10 h after treatment with either MMS (100 μg/ml), sorbitol (0.3 M), or untreated control. N = 5 for each sample and the experiment was repeated five times. Relative luciferase activity represents values normalized to the gadd45b–1656 (1.7 kb) luc construct maximally induced (100) by MMS (B), or to the gadd45b-2194 luc construct (C).

IDENTIFICATION OF Egr-1, Sp1, AND NFY AS TRANSCRIPTION FACTORS THAT BIND TO MMS-RESPONSIVE *cis*-ELEMENTS IN gadd45b PROMOTER AND CO-OPERATE IN gadd45b MMS INDUCTION

In order to identify putative transcription factors that bind to *cis*-elements that mediate MMS inducibilty of the *gadd45b* promoter, we carried out EMSAs. We first sought to determine which proteins bind to the most distal of the two response regions, located at −154/−127 bp upstream of the transcription start site. To this end, a radiolabeled double stranded oligonucleotide, "EMSA oligo −174/−124" (Fig. 4A), which spans this MMS-responsive region, was used in EMSA binding reactions with nuclear extracts from RKO cells untreated- or MMS treated (Fig. 4B). We observed at least three distinct bands in binding reactions using either untreated or MMS-treated extracts. Interestingly, a fourth band is observed in the EMSA only when MMS-treated extracts are used; this band is not present using the untreated extracts. Thus, we can conclude that at least three proteins or protein complexes bind constitutively, and one protein binds specifically following MMS treatment, to the most distal MMS-response region.

In order to identify the proteins binding to this region of the *gadd45b* promoter, we carried out a number of EMSA competition and/or supershift experiments, in which specific bound proteins could be identified in binding competition assays using either cold

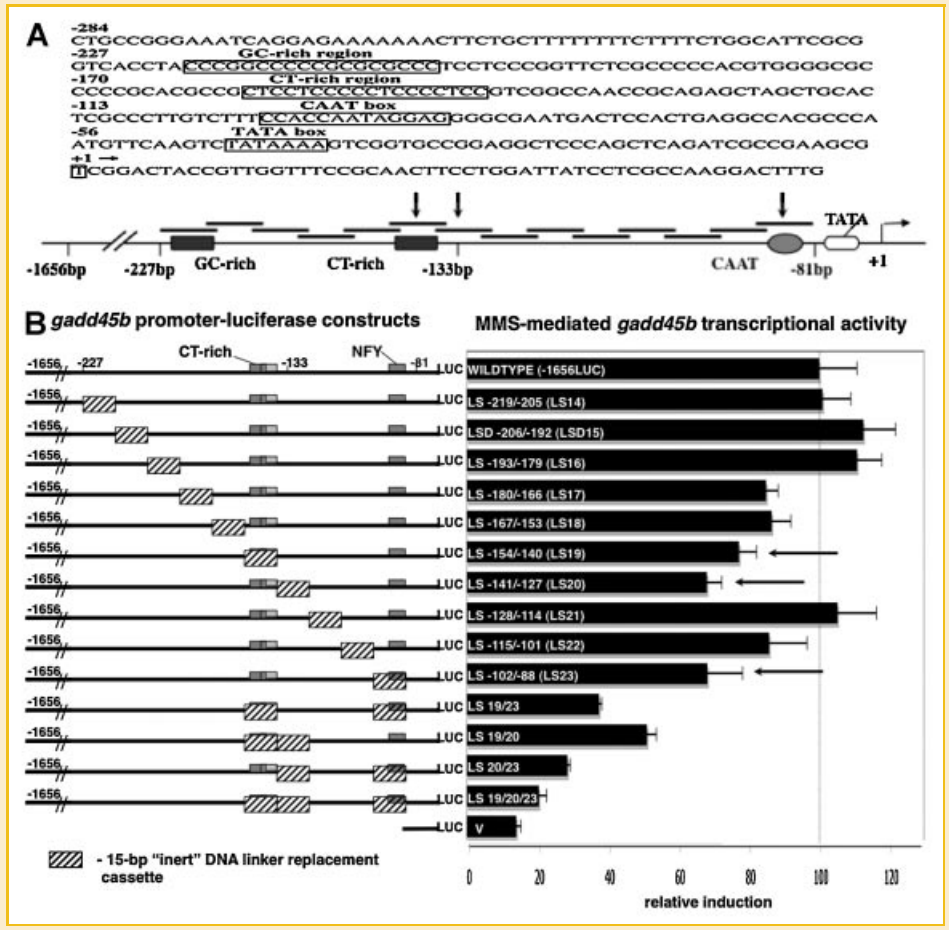

Fig. 3. Identification of MMS-responsive *cis*-elements in gadd45b promoter located between −154/−140, −141/−127, and −102/−88 bp of the gadd45b promoter. A: Sequence of the minimal MMS responsive region of the gadd45b promoter and schematic diagram of positioning of linker scans. Bars represent linker scans. Arrows identify linker scans LS19, LS20, and LS23. B: Schematic diagram of linker-scan mutants generated on the backbone of the 1.656 kb gadd45b promoter region (left), and results of luciferase assays (right). Dual luciferase assay was carried out as previously described on RKO cell extracts. N = 5 per sample and four independent experiments were carried out with similar results.

oligonucleotide competitors or antibodies to a putative bound protein. As a starting point in these experiments, we focused on the transcription factors most likely to bind to the MMS-response distal region based on observed homology to the consensus binding sequences within this region, notably an Egr-1/Sp1 site (Fig. 4C).

Thus, EMSA was carried out with MMS-treated nuclear extracts using the radiolabeled EMSA oligo −163/−138 probe in the presence a number of competitors (Fig. 4A,C). In lanes 1 and 2, are untreated and MMS-treated nuclear extracts respectively, in which no binding competitors were included. These results show the band pattern observed in Figure 4B—three constitutively bound bands and an additional band in the MMS-treated lane 2. The top band was observed to be significantly diminished when 1 μg of Sp1 antibody was included in the binding reaction (lane 3), or excess competitor Sp1 consensus sequence oligonucleotide (lane 10); the band is restored in the presence of a mutant Sp1 sequence oligonucleotide (lane 11), thereby identifying Sp1 as the bound protein.

Next, EMSA was carried out using a cold competitor oligonucleotide that contains the Egr1 consensus binding sequence (Fig. 4C). As shown in lane 8, a wild-type cold oligonucleotide containing the consensus Egr1 binding site was able to compete away the MMS-inducible band. Furthermore, when a similar binding reaction was carried out with MMS treated nuclear extracts in the presence of two different Egr1 antibodies (lanes 4 and 5), a specific supershift of the inducible band was observed. These data indicate that following MMS treatment Egr-1 is bound to a MMS responsive *cis*-element within the *gadd45b* promoter.

Finally, using an EMSA probe that spans the proximal MMS-response element, −124/−80, we observed a constitutively bound protein using both untreated and MMS treated RKO cell extracts (Fig. 4D). A cold competitor probe in which the two adenine residues of the CCAAT box were changed to guanine failed to compete for binding as was observed with the wild-type cold probe. Furthermore, incubation of the nuclear extract with NFY antibody resulted in a supershift of the bound protein. These data identify the NFY transcription factor complex as the protein bound to this *cis*-element, which is required for full MMS induction of the *gadd45b* promoter.

In conclusion, using the EMSA, we have shown that Sp1, and NFY bind constitutively and that Egr1 binds inducibly to the MMS-response regions of the *gadd45b* promoter. The EMSA is useful for identifying specifically interacting proteins in vitro. However, due to the artificial nature of in vitro binding assays, we sought to verify that these proteins bind to the *gadd45b* promoter in vivo. To accomplish this, we carried out the ChIP to assess in vivo binding of Egr1, Sp1, and NFY to the *gadd45b* promoter (Fig. 4E). It can be seen that all three transcription factors are indeed bound to the gadd45b promoter following MMS treatment. Unexpectedly, in this in vivo setting, in addition to increased binding of EGR-1 increased binding also was observed for Sp1; this most likely reflects the different status of endogenous DNA. Additionally, using siRNA interference, we observed that that Egr1 knockdown by siRNA reduces the MMS-mediated induction of gadd45b significantly (Fig. 4F).

Collectively these data identify Egr-1, Sp-1, and NFY as transcription factors that cooperate in the regulation of MMS-inducible expression of gadd45b. The identity of the third constitutive protein (protein complex) that was observed to be bound to the gadd45b promoter (Fig. 4B) remains elusive, since it

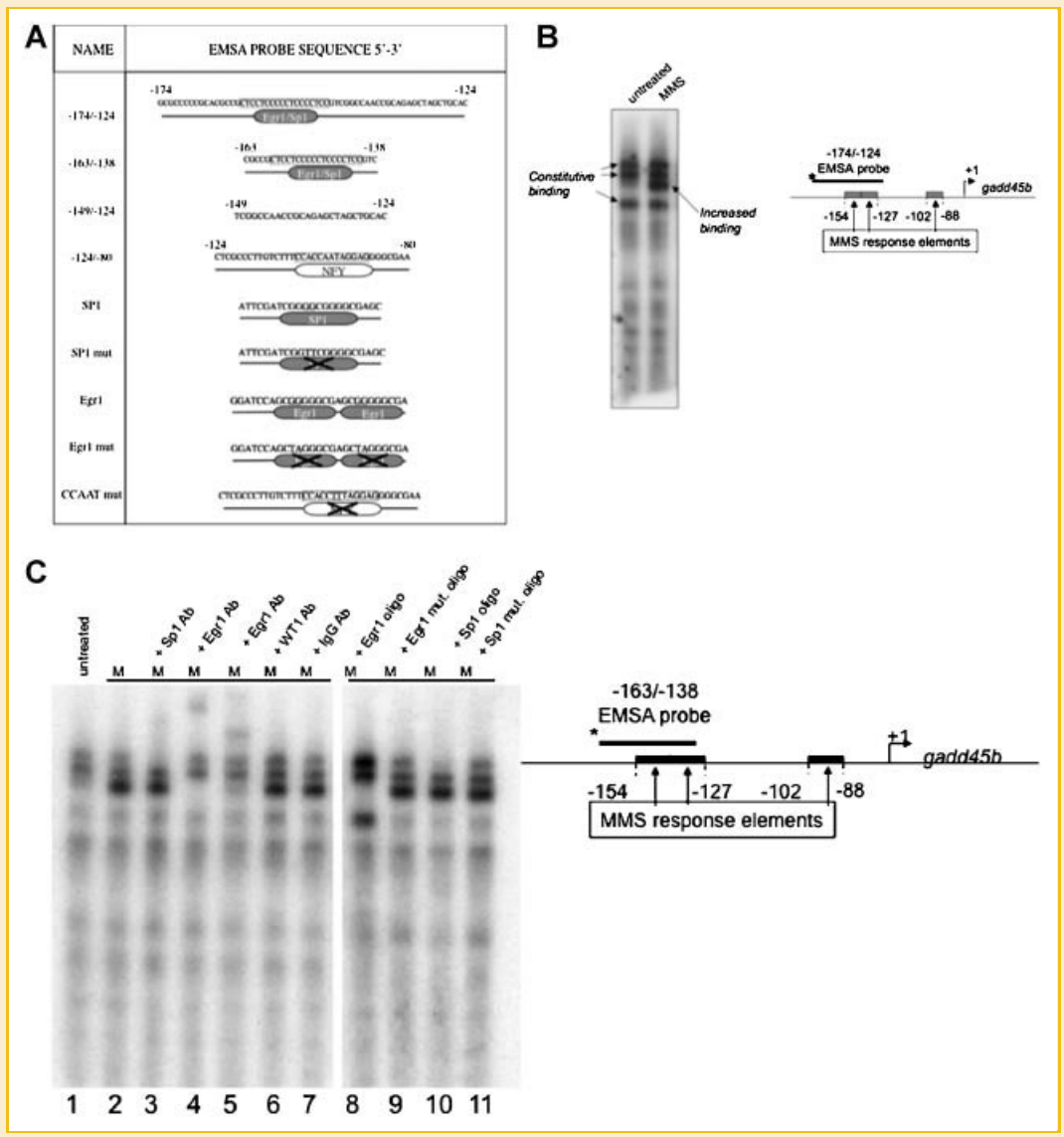

Fig. 4. Identification of Egr-1, Sp1, and NFY as transcription factors that bind to MMS-responsive *cis*-elements in gadd45b promoter. A: Schematic diagrams and sequences of EMSA oilgos used. B: EMSA binding reactions of the −174/124 EMSA probe with nuclear extracts from RKO cells untreated- or MMS treated, identify three protein complexes that are constitutively bound and one MMS-inducible complex. C: EMSA competition and/or supershift experiments identify constitutive bound Sp1 and increased binding of Egr-1 to the distal −172/−124 region. D. EMSA binding reactions of the −124/−80 EMSA probe with nuclear extracts from RKO cells untreated- or MMS-treated and EMSA competition/supershift experiments identify constitutive bound NFY to the proximal regulatory region. E: Binding of Egr1, Sp1, and NFY-A to the endogenous gadd45b promoter as determined by Chromatin Immunoprecipitation (ChIP). F: RNAi knockdown of Egr1 by siRNA in RKO cells reduces MMS-mediated gadd45b induction. Experiments were performed as detailed in the Materials and Methods Section. Data shown are representative of at least three independent experiments with reproducible results.

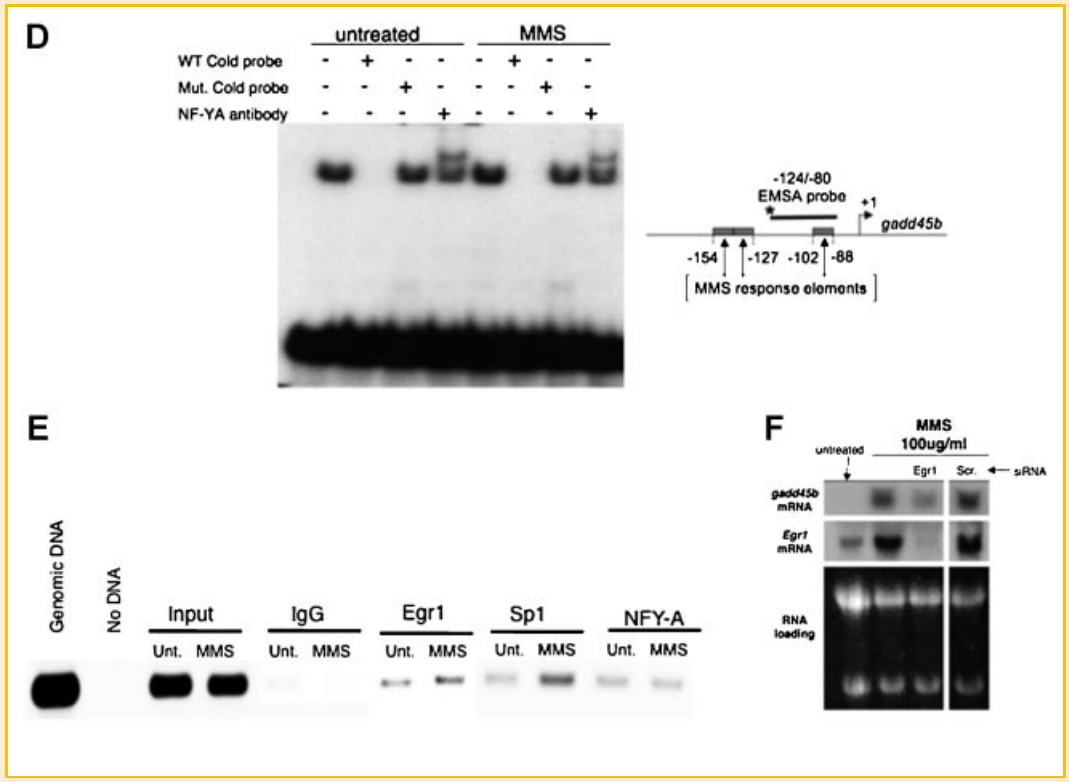

Fig. 4. (*Continued*)

could not be competed/super shifted by consensus oligos/antibodies to putative transcription factors suggested to bind to this region using two independent sequence analysis programs, TFSEARCH and TESS (University of Pennsylvania) (data not shown).

gadd45b INDUCTION BY MMS IS REGULATED AT THE TRANSCRIPTION LEVEL WHEREAS SORBITOL INDUCES gadd45b BY A NON-TRANSCRIPTIONAL MECHANISM INVOLVING mRNA STABILIZATION

Treatment of RKO cells with MMS, but not with sorbitol, was observed to result in increased promoter activity using the luciferase assay (Fig. 2). This observation raised the possibility that in contrast to transcriptionally regulated MMS inducibility of the gadd45b gene, sorbitol induced expression may be regulated at the post-transcriptional level. Alternatively, lack of inducibility of gadd45b by sorbitol in luciferase assays, may reflect the lack of essential sorbitol-*cis* acting elements in the 3.9 kb promoter region (Fig. 2A) that was used in this study.

To distinguish between these possibilities a nuclear run on assay was performed to measure endogenous transcriptional activity of the gadd45b gene following treatment of RKO cells with MMS as opposed to sorbitol. As shown in Figure 5A, in contrast to elevated transcription of gadd45b following treatment of cells with MMS (normalized to β-actin as loading control), gadd45b transcription was not enhanced following treatment with sorbitol. It is notable, however, that MMS induced increase in gadd45b transcription, using the run on assay, was less than what was observed using the luciferase assay; this may reflect regulation at both the level of transcription initiation and elongation. Nevertheless, these data indicate that gadd45b-mRNA induction by sorbitol is regulated at a post-transcriptional level, perhaps by increasing gadd45b-mRNA stability.

To test this conjecture we inhibited transcription with Actinomycin D, to assess gadd45b-mRNA half-life following treatment with sorbitol as opposed to MMS (Fig. 5B); data obtained were quantified by band densitometry, and normalized to mRNA loading. Following treatment cells with sorbitol gadd45b mRNA stability was significantly increased compared to following treatment with MMS. Similar results were observed when RKO cells are pretreated with Actinomycin D, followed by either MMS, sorbitol, or DMEM treatment (Fig. 6B–D). Clearly a rapid decay was observed in *gadd45b* mRNA in both untreated and MMS-treated RKO cells, whereas sorbitol treated cells maintained higher levels of *gadd45b* mRNA over time.

Collectively these data indicate that stress mediated induction of gadd45b by two different stressors is distinctly regulated. MMS mediated induction of gadd45b is regulated at the transcription level, whereas induction by sorbitol is regulated by mRNA stabilization.

DISCUSSION

In this study, we provide novel data showing that the stress sensor gadd45b is regulated by distinct mechanisms in a stress-specific

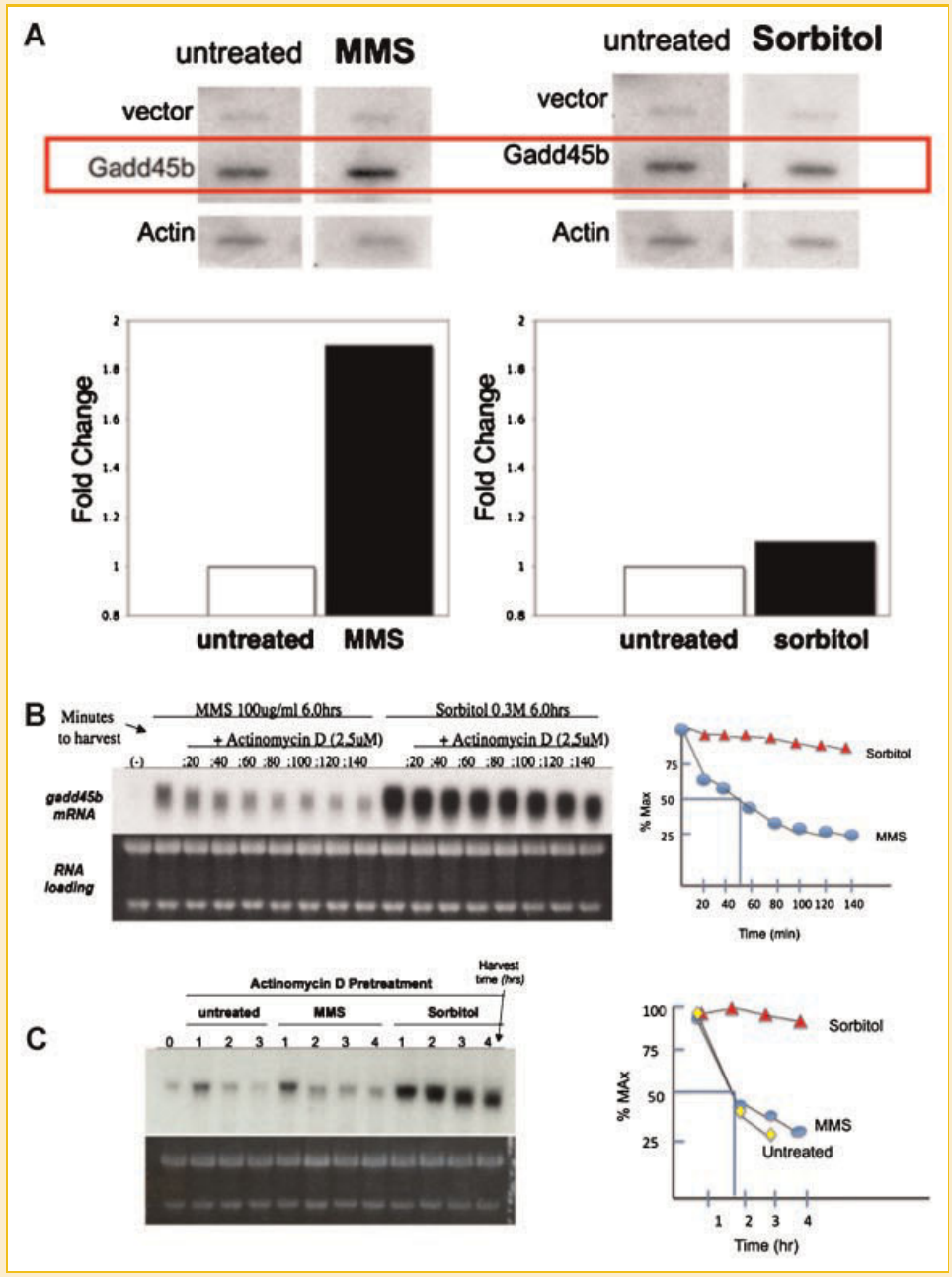

Fig. 5. gadd45b inducibility by MMS is regulated at the transcription level whereas sorbitol induces gadd45b by a non-transcriptional mechanism involving mRNA stabilization. A: Nuclear run-on assay measuring transcription of gadd45b prior to and following stimulation of RKO cells with either MMS or sorbitol. Band intensity was quantitated by band densitometry, and normalized to β-actin. B: Assessment of gadd45b-mRNA half-life by treatment of cells with MMS or sorbitol followed by inhibition of transcription with Actinomycin D. C: Assessment of gadd45b-mRNA half-life by pretreatment of cells with Actinomycin D (2.5 μM) followed by MMS or sorbitol treatment. Data shown in B and C were qauntitated by band densitometry, and normalized to mRNA loading. All data shown are representative of at least three independent experiments with reproducible results.

manner. MMS, a DNA alkylating agent, induces gadd45b transcriptionally through a cohort of constitutive and inducible bound factors, including NFY, Sp1, and of Egr1. In a hyperosmotic environment generated with sorbitol, however, gadd45b mRNA is induced by mRNA stabilization and not by increased transcription. Taken together, this study demonstrates insight into the complex and dynamic nature of the cellular stress-response and provides a basis for future studies in this area.

While this work was in progress it was documented that Egr-1 also participates in the regulation of UVB induced expression of gadd45a and gadd45b [Thyss et al., 2005]. Evidence for the regulation of gadd45a by NFY and Egr-1 following MMS stimulation has been documented as well [Jin et al., 2001; Takahashi et al., 2001; Hirose et al., 2003; Thyss et al., 2005]. However, unlike what was observed for gadd45b where elimination of the Oct1 binding site, located at position −1,569/−1,582 bp, had no effect on

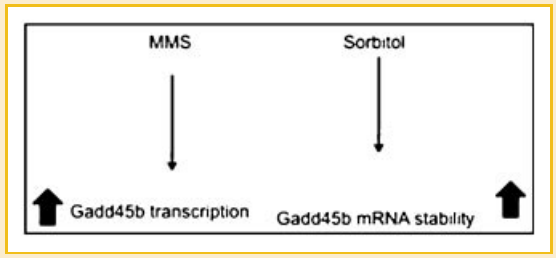

Fig. 6. Schematic diagram indicating that distinct mechanisms, which operate at the level of either RNA transcription or mRNA stability regulate induction of Stress Sensor gadd45b by MMS as opposed to sorbitol, respectively.

MMS induction (Fig. 2C), MMS-induced expression of gadd45a appeared to be dependent also on Oct1 [Jin et al., 2001; Takahashi et al., 2001; Hirose et al., 2003; Thyss et al., 2005]. Taken together, these observations and the present study demonstrate that gadd45 induction by different stressors may involve co-operation among common and distinct transcription factors.

Our present data add to this complexity of gadd45 gene induction showing that under the same experimental setting different stressors, such as MMS and sorbitol, utilize distinct mechanisms which operate at the transcription or post-transcriptional, respectively, to rapidly induce gad45b expression. Stress mediated induction of gadd45 by post-transcriptional mechanisms, notably gadd45a has been documented previously [Jackman et al., 1994; Abcouwer et al., 1999; Sakaue et al., 1999; Yoshida et al., 2005; Lal and Gorospe, 2006; Zhang et al., 2006]. Our study, nevertheless, is the first to show that gadd45b induction, in the same cell type and under the same experimental settings, by two different stressors, is distinctly regulated at level of either RNA transcription or RNA stability.

It is plausible that in different cell types the same stressors may utilize different mechanisms to induce gadd45 gene expression. It is also likely that gaddd45b (and other gadd45 genes) stress mediated induction may involve combinatorial regulation at the transcriptional and post-transcriptional levels. Clearly, in future experiments it will be of interest to further determine under comparable experimental settings, as done in this study, the mechanisms utilized by distinct stress agents to induce gadd45 gene expression in one cell type as opposed to others. Also of interest will be to elucidate stress–response signaling cascades that dictate gadd45b induction at the level of RNA transcription as opposed to mRNA stabilization.

To conclude, our data provide evidence for the novel observation that gadd45b induction by distinct stressors are regulated differentially at the level of mRNA transcription or mRNA stability. Importantly, data obtained provide the groundwork to further examine the regulation of gadd45b expression in an in vivo setting using animal models and tissues obtained from normal individuals and cancer patients prior to and after irradiation and/or chemotherapeutic intervention.

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