 Nucleic Acids Research, 1999, Vol. 27, No. 15 **3111–3119**

# Post-transcriptional regulation of the DNA damage-inducible gadd45 gene in human breast carcinoma cells exposed to a novel retinoid CD437

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Received April 8, 1999; Revised and Accepted June 7, 1999

## ABSTRACT

**The biologically active synthetic retinoid CD437 (6-[3-adamantyl-4-hydroxyphenyl]-2-naphthalene, AHPN) and different human breast carcinoma (HBC) cell lines were used to examine the possible mechanism(s) of *gadd45* induction. Northern blot analysis of mRNA isolated from MCF-7, MDA-MB-468 and MDA-MB-231 HBC cell lines demonstrated a progressive increase in the 1.4 kb *gadd45* transcript after exposure to 1 μM CD437. Western blot analysis showed increased *gadd45* protein levels in MDA-MB-468 HBC cells following exposure to CD437. CD437 increased *gadd45* mRNA levels by ~20-fold in MDA-MB-468 cells, however, the transcriptional activity was increased ~2–3-fold as demonstrated by the human *gadd45* promoter–luciferase reporter construct and nuclear run-off assays. Sublines of MDA-MB-468 HBC cells expressing stably integrated GADD45 cDNA fragments were obtained and CD437-dependent induction of GADD45 analyzed. We report that ~300 nt located in the 5'-untranslated region (5'-UTR) of *gadd45* mRNA are involved in the CD437-dependent 4-fold enhanced stability of *gadd45* transcripts. MDA-MB-468 cells were stably transfected with either a plasmid having a CMV promoter-driven rabbit β-globin gene or plasmids having a CMV promoter-driven chimeric gadd45 5'-UTR–rabbit β-globin gene, where the entire *gadd45* 5'-UTR (from +1 to +298) or a 45 bp subfragment of the *gadd45* 5'-UTR (from +10 to +55) was positioned at the 5'-end of the rabbit β-globin gene. CD437 was found to up-regulate expression of both the chimeric *gadd45*–rabbit β-globin transcripts, suggesting that *cis* element(s) involved in the CD437-dependent enhanced stability of gadd45 mRNA are contained in the 45 nt of the 5'-UTR of the gadd45 mRNA.**

## INTRODUCTION

Growth arrest and DNA damage-inducible (GADD) genes belong to a subgroup of genes which are not only rapidly induced by DNA-damaging agents but are coordinately induced in growth-arrested cells (1). Five GADD genes are known to be coordinately expressed following treatment of cells with most DNA-damaging agents and during growth arrest conditions (2). *Gadd45* is a growth arrest and DNA damage-inducible gene that has been shown to be induced by both p53-dependent and -independent pathways in various cell lines (3,4). *Gadd45* was cloned by subtractive hybridization as a mRNA more abundantly expressed in growth-arrested cells or those with damaged DNA and its expression was found to be up-regulated by genotoxic insults *in vivo* (5,6). *Gadd45* mRNA expression has also been found to be induced *in vitro* by a range of stimuli including DNA damage caused by UV light, γ-irradiation and alkylating agents such as methyl-methane sulfonate (MMS) (7). The *gadd45* gene encodes a 165 amino acid protein that is highly conserved in mammals and is a cell cycle regulated nuclear protein that reaches maximal levels in $G_1$ phase of the cell cycle (7 and references therein). Recently gadd45 and the related proteins Myd118 and gadd45γ have been shown to bind and activate the MTK1 MAP kinase kinase kinase, which is upstream of both the p38 and JNK MAPKs (8). The gadd45-like proteins may, therefore, play an important role in activation of the p38 and JNK MAPK cascades in the processes of growth arrest and apoptosis induced by DNA damage and other environmental stresses.

We have previously described a novel retinoid, 6-[3-adamantyl-4-hydroxyphenyl]-2-naphthalene (CD437, AHPN)

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which has been shown to induce $G_1$ arrest and cause apoptosis in different cell types including human breast carcinoma (HBC) cell lines (9,10). CD437 appears to exert its action through retinoic acid nuclear receptor (RAR)/retinoid X nuclear receptor (RXR)-independent as well as p53-independent pathways in order to induce $G_0/G_1$ arrest and activate known downstream effectors of p53 in cells possessing a non-functional p53 (9,10).

CD437 has previously been shown to induce expression of gadd45 in a number of cell types (10–12). The objective of this study was to delineate the underlying molecular mechanism(s) of CD437-dependent induction of *gadd45* in HBC cell lines. We found that CD437-dependent enhancement of *gadd45* expression was accompanied by only minimal increases in *gadd45* transcriptional activity. In this report, we provide evidence that CD437 regulates *gadd45* expression by enhancement of *gadd45* message stability and identify a 45 nt sequence element of the 5'-UTR of *gadd45* mRNA which is responsible for CD437-dependent enhanced expression of gadd45 as well as heterologous rabbit β-globin mRNAs in the HBC cells.

## MATERIALS AND METHODS

### Cell lines and cell culture

The HBC cells MCF-7 and MDA-MB-231 were a gift from Dr Marc Lippman (Lombardi Cancer Center, Washington, DC). The MDA-MB-468 cells were provided by Dr Anne Hamburger (University of Maryland Cancer Center, Baltimore, MD). Cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 5% fetal bovine serum, 20 mM HEPES and 50 μg/ml gentamicin.

### Cloning of plasmid constructs

The 1.6 kb promoter fragment was cloned into the promoterless vector plasmid pGL-2 Basic (Promega) as previously described (13). A 1.8 kb fragment containing the promoter and exon 1 of the *gadd45* gene was excised from plasmid pHG45 (14) using the restriction endonuclease *Eco*RI. The excised *Eco*RI fragment was then subcloned in the sense orientation into the *Eco*RI site of the vector Phagemid pBluescript SK– (clone 5.1). Clone 5.1 was then digested with *Xma*I to obtain a 1.6 kb fragment of the *gadd45* promoter and exon I. The 1.6 kb *Xma*I fragment was then subcloned in sense orientation into the *Xma*I site of pGL-2 Basic (Promega), which is a promoterless luciferase reporter vector, to obtain the *gadd45* promoter–luc construct (clone 6.3).

The 1.4 kb *gadd45* cDNA was excised from plasmid pHUL45B2 (4) using the restriction endonucleases *Xba*I and *Xho*I and subcloned into the pCDNA3 vector plasmid (Invitrogen) as follows. First, plasmids pHUL45B2 and pCDNA3 were linearized with the restriction endonucleases *Xba*I and *Eco*RI, respectively. The linearized plasmid DNAs were then end-filled using Klenow DNA polymerase (13). The linearized, blunt-ended plasmid DNAs pHUL45B2 and pCDNA3 were then separately digested with the restriction endonuclease *Xho*I. The 1.4 kb *Xba*I(end-filled)–*Xho*I-digested *gadd45* cDNA fragment from plasmid pHUL45B2 was gel purified and ligated into the ~5.4 kb *Eco*RI(end-filled)–*Xho*I-digested, gel-purified vector plasmid pCDNA3. The resultant pCDNA3-*gadd45* clone 4.2 was obtained where *gadd45* cDNA is under the control of the CMV promoter.

Additional pCDNA3-*gadd45* constructs were derived by PCR amplification as follows. Approximately 1.1 kb of *gadd45* cDNA containing all of the *gadd45* protein open reading frame (ORF) and 3'-UTR was PCR amplified using oligos GADD45.2 (5'-CCGCTCGAGATGCAATTATTCAT-ACCAG-3', antisense, positions 1321–1340; 4) and GADD45.3 (5'-CGCAAGCTTCGACCTGCAGTTTGCAATAT-3', sense, positions 266–285; 4). In addition, ~0.5 kb of *gadd45* cDNA fragment containing ORF only was PCR amplified using oligos GADD45.3 and GADD45.1R (5'-CCGCTCGAGTTT-TCATTCAGATGCCATCA-3', antisense, positions 779–798; 4). The PCR-amplified *gadd45* cDNA subfragments were then digested with *Hin*dIII and *Xho*I and subcloned into *Hin*dIII–*Xho*I-digested vector plasmid pCDNA3. The procedures for cloning and screening of the recombinant plasmids were as described previously (13,15). The resultant pCDNA3-*gadd45* clone 2.1 contained an ~1.1 kb *gadd45* cDNA fragment having the *gadd45* protein-encoding ORF and 3'-UTR, while clone 3.1 contained an ~0.5 kb *gadd45* cDNA fragment having the *gadd45* protein-encoding ORF only. The *gadd45* cDNA fragments of clones 2.1 and 3.1 were sequenced to confirm the validity of the inserts.

An ~0.3 kb 5'-UTR fragment of *gadd45* cDNA was cloned at the 5'-end of the rabbit β-globin gene (16) as described below. First, an ~0.9 kb metallothionein promoter fragment of the vector plasmid pMEP4 (Invitrogen) was replaced with the CMV promoter to obtain construct 28.1 as follows. Plasmid pMEP4 was initially digested with *Xba*I and end-filled using the Klenow fragment of DNA polymerase essentially as described before (13). The linearized, end-filled plasmid pMEP4 was then digested with *Hin*dIII and ~9.4 kb of the vector plasmid was gel purified. The *Xba*I(end-filled)–*Hin*dIII-digested pMEP4 vector plasmid was then ligated with an ~0.65 kb *Nru*I–*Hin*dIII-digested and gel-purified CMV promoter fragment derived from plasmid pCDNA3 (Invitrogen) to obtain clone 28.1. Second, the CMV promoter-driven rabbit β-globin gene construct 29.6 was derived from clone 28.1 as follows. Clone 28.1 was digested with *Sal*I and end-filled as described above. An ~0.45 kb fragment containing the polylinker and SV40 polyadenylation signal sequence was removed by digesting the *Sal*I-linearized and end-filled plasmid DNA of clone 28.1 with *Hin*dIII. The vector plasmid of ~10.6 kb was gel purified in order to obtain *Sal*I(end-filled)–*Hin*dIII-digested linear vector plasmid 28.1. The plasmid pfRβ+3' (G.Brewer *et al*., submitted for publication) containing the entire rabbit β-globin gene (16) was first linearized with *Kpn*I, followed by blunting the *Kpn*I overhang using bacteriophage $T_4$ DNA polymerase (NEB) as per the manufacturer's guidelines. The *Kpn*I-digested, blunt-ended plasmid pfRβ+3' was then digested with *Hin*dIII to release an ~1.8 kb entire rabbit β-globin gene fragment. The *Kpn*I(blunt-ended)–*Hin*dIII-digested, gel-purified rabbit β-globin gene fragment was then ligated with the above described *Sal*I(end-filled)–*Hin*dIII-digested vector plasmid 28.1 to obtain clone 29.6. Third, plasmid 29.6 was linearized with *Hin*dIII and end-filled as described above. An ~0.3 kb 5'-UTR fragment of *gadd45* cDNA (positions +1 to +298; 4) was excised from plasmid pHUL45B2 (4) using the restriction enzymes *Eco*RI and *Hin*dIII, end-filled as described above and gel purified. The end-filled 0.3 kb 5'-UTR

fragment of *gadd45* cDNA was then ligated in the sense orientation into *Hin*dIII-linearized, end-filled vector plasmid 29.6 in order to obtain CMV promoter-driven *gadd45*–rabbit β-globin gene construct 8.5. In addition, a 45 bp subfragment of the *gadd45* 5'-UTR (positions +10 to +55; 4) was synthesized in the form of sense and antisense oligonucleotides with 5'-overhangs for *Hin*dIII. The sense and antisense oligos were phosphorylated, annealed and ligated into the *Hin*dIII site of clone 29.6 to obtain CMV promoter-driven *gadd45*–rabbit β-globin gene construct 11.1. Clone 11.1 thus contains the above 45 bp of the *gadd45* 5'-UTR positioned at the 5'-end of the rabbit β-globin gene in the sense orientation.

### RNA isolation and northern blot analysis

Total RNA isolation, gel electrophoresis and northern blot analysis were performed essentially as previously described (17,18).

### Nuclear run-off transcription assay

MDA-MB-468 cells were treated with CD437 for 48 h. Isolation of nuclei from CD437-treated and untreated cells, transcription reactions in the presence of [α-$^{32}$P]UTP and isolation of newly synthesized labeled RNA were carried out according to previously published methods (17–19). Immobilization of 1 μg of each of the cDNA inserts of GADD45 (1.3 kb) and GAPDH (780 bp) (20) to Zeta-Probe GT membrane (Bio-Rad, Hercules, CA), followed by their hybridization with labeled newly transcribed RNAs and washing of the filters were essentially as described before (19). The filters were then exposed for autoradiography for a period of 7–10 days. The densitometric quantification of the bands on the autoradiograms was performed using a Molecular Dynamics Laser Densitometer (model PSD1) and the ImageQuant v.1.1 software program.

### Western immunoblotting

Twenty-five micrograms of the protein lysate from untreated and AHPN-treated MDA-MB-468 HBC cells were analyzed by SDS–PAGE and immunoblotted using gadd45 rabbit polyclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) essentially as per the previously published methods (19).

### Transient transfection and luciferase assay

MDA-MB-468 HBC cells were transiently transfected with a mixture of 10 μg *gadd45*-luc (clone 6.3) and 5 μg pCMV-β-gal essentially as per the methods described before (13,19). The methods for treatment of transfected cells with 1 μM CD437, their harvesting and lysis followed by the assays for luciferase and β-galactosidase activities were as published before (19).

### Stable transfections

MDA-MB-468 HBC cells were transfected independently with the pCDNA3-*gadd45* plasmids 2.1, 3.1 and 4.2 as described above. The cells containing stably integrated pCDNA3-*gadd45* plasmids were then selected in the presence of 500 μg/ml neomycin (G418) as described before (21). In addition, MDA-MB-468 cells were also transfected with plasmid clones 29.6 and 8.5 and stable sublines were obtained after selection in the presence of 400 μg/ml hygromycin. Several independent clones from each of the transfections were obtained and analyzed for expression of either the exogenously transfected *gadd45* mRNAs or chimeric *gadd45*–rabbit β-globin mRNAs by the northern blot hybridization methodology described above. The exogenously expressed *gadd45* transcripts were detected by hybridization of the northern blots with a radiolabeled cDNA fragment of the bovine growth hormone polyadenylation signal sequence obtained from the vector plasmid pCDNA3. The exogenously expressed chimeric *gadd45*–rabbit β-globin transcripts were detected by hybridization of the northern blots with a radiolabeled, *Nco*I–*Bam*HI-digested, gel-purified exon 2 subfragment of rabbit β-globin gene (16).

### Analysis of mRNA decay

Two or more independent clones containing stably integrated pCDNA3-*gadd45* plasmid 2.1, 3.1 or 4.2 which showed low to moderate levels of expression of exogenously transfected *gadd45* transcripts were selected for analysis of mRNA decay. CD437-treated or untreated clonal derivatives of MDA-MB-468 cells were cultured in the presence of the transcriptional inhibitor actinomycin D (4 μg/ml) for various times in order to study the rate of decay of the exogenously expressed CMV promoter-driven *gadd45* transcripts. Total cellular RNAs were extracted and 5 μg of each RNA electrophoresed on northern blots as described above. To measure mRNA decay rates, the data from autoradiograms were quantitated by densitometry as described above. For each sample, the concentration of exogenously expressed *gadd45* mRNA transcripts was normalized to an 18S ribosomal RNA standard. The half-lives of the exogenously expressed *gadd45* mRNAs were determined by least squares analysis of semi-logarithmic plots of normalized mRNA concentration as a function of time (22–24).

## RESULTS

### CD437 induction of *gadd45* mRNA

Northern blot analysis demonstrated that exposure of different HBC cell lines to 1 μM CD437 resulted in a progressive increase in *gadd45* mRNA expression (Fig. 1A). An ~5-fold increase in *gadd45* mRNA levels was noted within 8 h in p53-positive MCF-7 cells with a maximum ~14-fold increase at 72 h (Fig. 1B). Treatment of p53-negative MDA-MB-231 HBC cells with 1 μM CD437 resulted in a 3- and 9-fold increase in *gadd45* mRNA levels at 8 and 72 h, respectively (Fig. 1B). Another p53-negative HBC cell line, MDA-MB-468, when exposed to 1 μM CD437, also showed a 3- and 22-fold increase in *gadd45* mRNA levels at 8 and 72 h treatment, respectively (Fig. 1B). Taken together, the data in Figure 1 strongly suggest that exposure of HBC cells to 1 μM CD437 results in induction of *gadd45* mRNA expression independent of the p53 status of the cells.

### CD437 induction of *gadd45* protein

Exposure of p53-negative MDA-MB-468 HBC cells to 1 μM CD437 also resulted in an increase in *gadd45* protein levels. Western blot analysis was performed on cell lysates obtained after 24, 48 and 72 h exposure to CD437. As indicated in Figure 2A, western blots utilizing gadd45 rabbit polyclonal antibody reveal three closely migrating bands of gadd45 protein in the size range 18–19 kDa. A significant increase in all three immunoreactive bands of *gadd45* protein was noted at all the time points of CD437 treatment when compared to

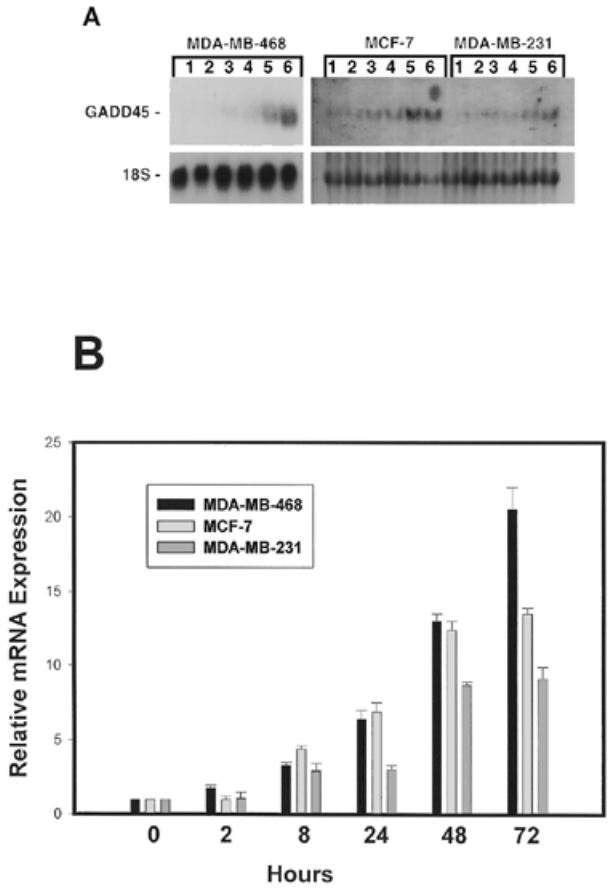



**Figure 1.** Expression of gadd45 in different breast carcinoma cells. (**A**) Northern blot of MDA-MB-468, MCF-7 and MDA-MB-231 cells treated with 1 μM CD437. Cells were harvested at different time points, total cellular RNAs prepared and electrophoresed on 1% alkaline agarose gels followed by their transfer to nitrocellulose membranes and hybridization with radiolabeled *gadd45* and 18S ribosomal RNA probes as described in Materials and Methods. Lanes 1, control untreated cells; lanes 2–6, cells treated with CD437 for 2, 8, 24, 48 and 72 h, respectively. (**B**) Histogram showing relative expression of *gadd45* mRNA at various time points of CD437 treatment. Columns represent the mean of two independent northern blot experiments expressed relative to the *gadd45* levels in untreated controls, which were arbitrarily defined as 1. The error bars represent the standard errors of the mean.

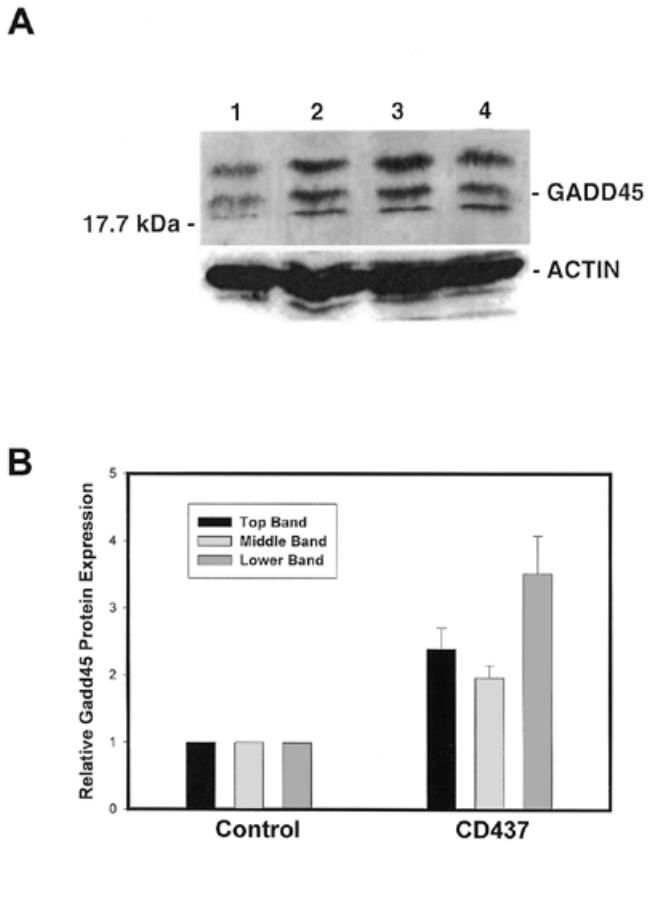



**Figure 2.** *Gadd45* protein expression after CD437 treatment. (**A**) MDA-MB-468 cells were treated with 1 μM CD437 as described in the text. The cell lysate (150 μg/lane) was electrophoresed on 12% SDS–polyacrylamide gels, transferred to nitrocellulose membrane and analyzed for gadd45 protein expression as described in Materials and Methods. The same western blot was subsequently analyzed for actin protein expression to confirm equal loading in all the lanes. Lane 1, control untreated cells; lanes 2–4, cells treated with CD437 for 24, 48 and 72 h. (**B**) Histogram showing relative expression of *gadd45* protein in control and CD437-treated cells. Each of the three gadd45 immunogenic bands in the western blot of (A) were separately quantitated in order to determine their respective modulation in control versus CD437-treated lanes. Each column for CD437-treated samples represents the mean of the values obtained for all three time points for a particular immunogenic band. The untreated controls for each of the immunogenic bands were arbitrarily defined as 1. The error bars represent the standard errors of the mean.

untreated controls (Fig. 2B). Taken together, CD437 treatment of HBC cells caused a cumulative 6–8-fold increase in expression of gadd45 protein. The identity of these three bands as gadd45 was also confirmed in other cell lines using this antibody (M.S.Sheikh, unpublished observation).

### CD437 modulation of *gadd45* transcription

We investigated whether CD437-mediated enhancement of *gadd45* mRNA levels was due to enhanced transcriptional activity. A human *gadd45* promoter-driven luciferase construct (clone 6.3; Fig. 3A) was transiently transfected into MDA-MB-468 cells in either the presence or absence of 1 μM CD437. Although exposure of MDA-MB-468 cells to CD437 for a period of 48 h resulted in an ~13-fold increase in *gadd45* mRNA levels (Fig. 1B), a <3-fold increase in *gadd45* promoter-mediated gene transcription was noted following exposure to CD437 (Fig. 3B).

It is possible that a moderate induction of CD437-dependent *gadd45* promoter activity when compared to the CD437-dependent increase in gadd45 message could be due to the CD437-responsive element(s) being upstream or downstream of the 1.6 kb *gadd45* promoter construct utilized. We therefore performed nuclear run-off transcription assays to determine CD437-mediated modulation of *gadd45* transcription. As shown in Figure 4, exposure of MDA-MB-468 cells to CD437 for a period of 48 h produced a 2–3-fold increase in the *gadd45* transcription rate over untreated cells. This increase in transcriptional activity agrees with the CD437-mediated increase

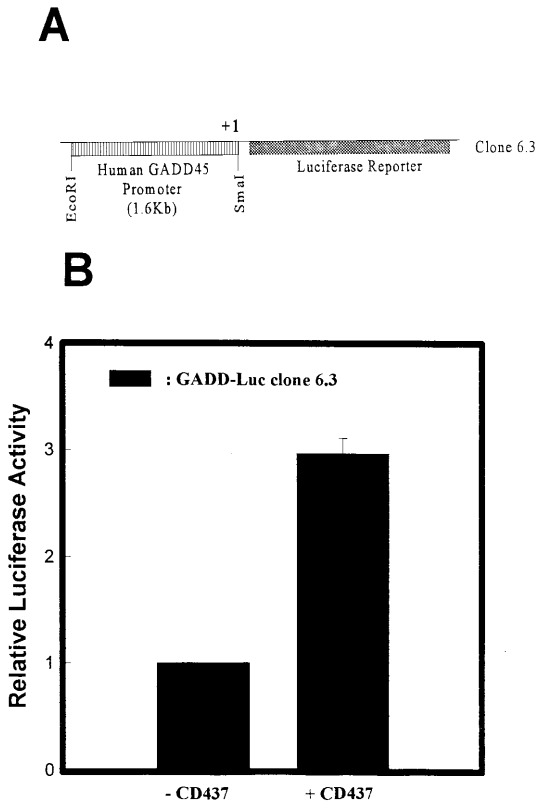



**Figure 3.** Effect of CD437 on *gadd45* promoter activity in MDA-MB-468 cells. (**A**) Schematic diagram of human *gadd45* promoter–luciferase reporter construct 6.3. (**B**) MDA-MB-468 cells were transiently transfected with construct 6.3 and luciferase activity was measured 40 h post-transfection. The luciferase activity is expressed as light units and normalized to β-galactosidase activity expressed as absorbance. Columns represent the means of three independent experiments expressed relative to the luciferase activities obtained for untreated cells (–CD437), which were arbitrarily defined as 1. The error bars represent the standard errors of the mean.

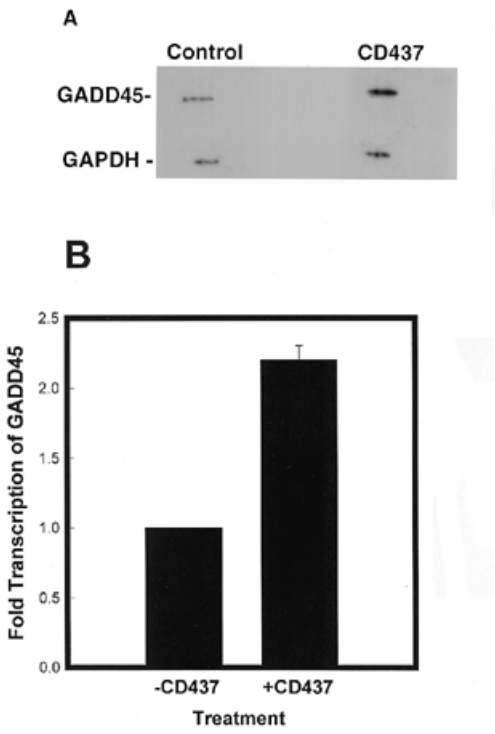



**Figure 4.** Transcriptional run-off analysis of *gadd45* expression in CD437-treated MDA-MB-468 cells. Cells were either untreated or incubated with 1 μM CD437 for 48 h and the nuclear run-off experiment was performed as described in the text. The intensity of each band was scanned using laser densitometry and values were normalized with respect to GAPDH signals. Data are expressed as the means of two independent experiments. (**A**) A representative autoradiograph of a nuclear run-off assay. (**B**) Quantitation of two independent experiments. Bar, SE.

in promoter activity noted in MDA-MB-468 cells transiently transfected with clone 6.3 (Fig. 3).

### Induction of *gadd45* mRNA stability by CD437

Significantly increased *gadd45* mRNA levels were found in the presence of moderate induction of *gadd45* gene transcription. We therefore examined whether exposure of MDA-MB-468 cells to CD437 increased *gadd45* mRNA stability. First, MDA-MB-468 cells were stably transfected with each of the pCDNA3-*gadd45* constructs 2.1, 3.1 and 4.2 (Fig. 5). Next, two or more of the independent subclones of MDA-MB-468 cells derived after transfection of pCDNA3-*gadd45* constructs were incubated in the presence or absence of CD437 for 40 h. Actinomycin D (4 μg/ml) was then added to the cells and *gadd45* mRNA levels were determined at various time intervals. The mRNA decay plotting and calculation of the mRNA half-life ($t_{½}$) were carried out as described before (21,22).

The rate of decay of the transfected *gadd45* transcript was analyzed by utilizing three independent subclones of MDA-MB-468 cells expressing pCDNA3-*gadd45* construct 4.2. The rate of decay was determined either in the absence or presence of

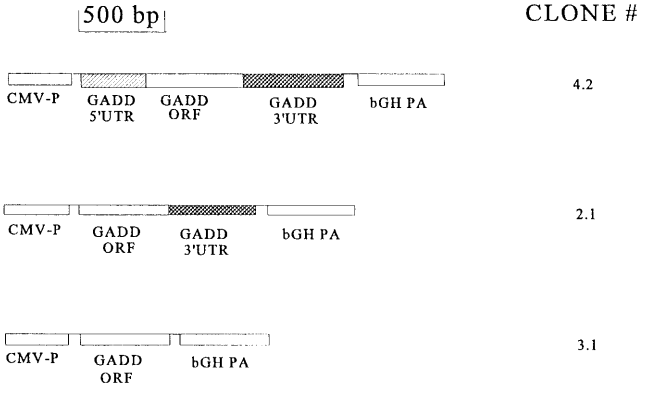
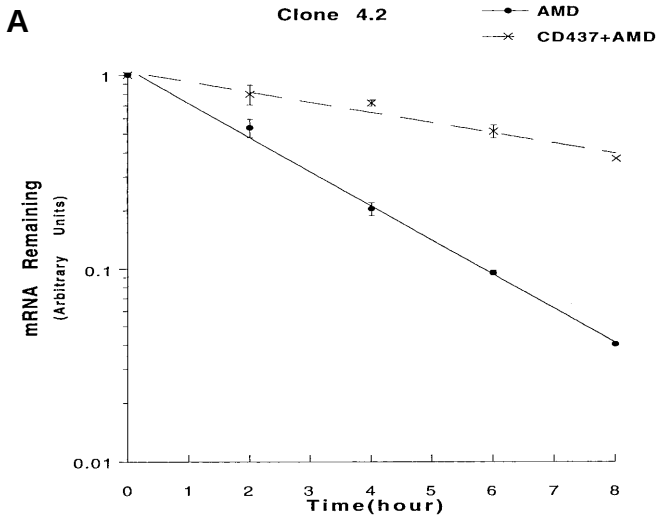
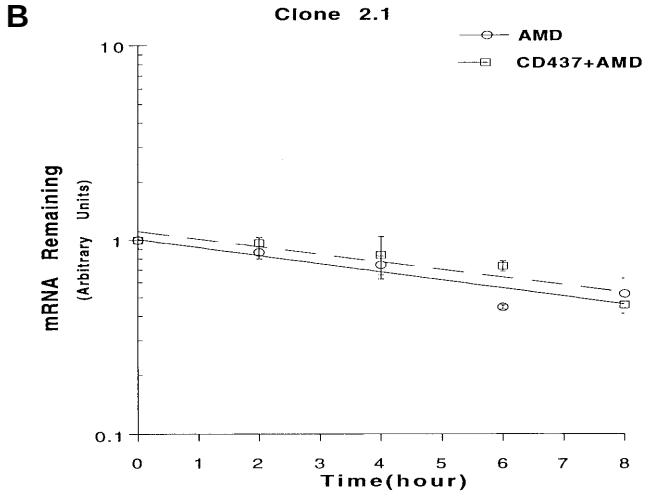
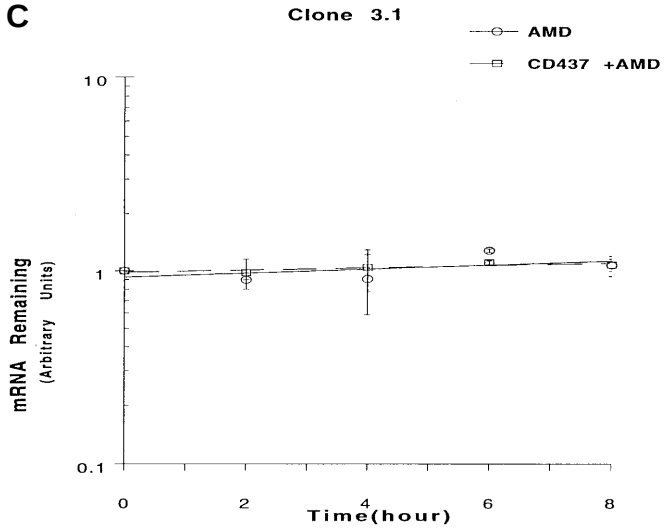



**Figure 5.** Schematic diagram of various pCDNA3-*gadd45* cDNA constructs. CMV-P, CMV promoter; UTR, untranslated region; ORF, protein-encoding open reading frame; bGHPA, bovine growth hormone polyadenylation signal sequence.

CD437. Figure 6A shows that the presence of CD437 significantly enhanced (~4.0-fold) the stability of the transfected *gadd45*




mRNA in all the subclones derived from transfection of construct 4.2.

In order to further delineate the region of *gadd45* mRNA involved in CD437-dependent enhancement of stability, additional subclones of MDA-MB-468 cells expressing pCDNA3-*gadd45* constructs 2.1 and 3.1 were utilized. Figures 6B and C show that the stability of the transfected *gadd45* mRNAs in the different subclones expressing either construct 2.1 or 3.1 was not significantly modulated in the presence of CD437. Taken together, the data in Figure 6 demonstrate that CD437-dependent induction of *gadd45* mRNA stability involves ~300 nt of the mRNA sequences located at the 5'-end. Further, Figure 6 also shows that the rate of decay of the transfected *gadd45* message derived from construct 2.1 was significantly faster when compared to the rate of decay of the transfected *gadd45* message derived from construct 3.1. Thus, the data in Figure 6 would also suggest that ~560 nt of the mRNA sequences located at the 3'-end of the transfected *gadd45* mRNA (construct 2.1) contribute towards the faster rate of message decay.

### CD437-dependent up-regulation of gadd45–rabbit β-globin chimeric mRNA

CD437-dependent up-regulation of *gadd45* mRNA was further investigated by utilizing MDA-MB-468 HBC sublines stably expressing either CMV promoter-driven rabbit β-globin gene clone 29.6 or CMV promoter-driven chimeric *gadd45*–rabbit β-globin gene clone 8.5 or CMV promoter-driven chimeric *gadd45*–rabbit β-globin gene clone 11.1 (Fig. 7A). Two independent sublines expressing either clone 29.6 or 8.5, and three independent sublines expressing either 29.6 or 11.1, were incubated for 48 h in either the presence or absence of CD437 as described above. As shown in Figure 7B and C, CD437 causes 3–4-fold up-regulation of chimeric *gadd45*–rabbit β-globin mRNA derived from clones 8.5 and 11.1 when compared to rabbit β-globin mRNA derived from clone 29.6. The data in Figure 7C therefore strongly suggest that 45 nt of the 5'-UTR of *gadd45* mRNA (positions +10 to +55; 4) contain RNA *cis* elements responsible for CD437-dependent up-regulation of chimeric *gadd45*–rabbit β-globin mRNA. Taken together, the data in Figures 6 and 7 demonstrate that 45 nt located at the 5'-end of *gadd45* mRNA participate, independent of their

**Figure 6.** Effect of CD437 on *gadd45* mRNA stability in MDA-MB-468 cells. MDA-MB-468 cells were stably transfected with pCDNA3-*gadd45* cDNA construct 4.2, 2.1 or 3.1. Several independent neomycin-resistant stable sublines for each of the constructs were obtained as described in the text. Two or more independent sublines derived from transfection of each of the above pCDNA3-*gadd45* cDNA constructs were cultured in the absence or presence of 1 μM CD437 and exposed to the transcriptional inhibitor actinomycin D for 2, 4, 6 and 8 h, total cellular RNAs prepared and expression of CMV promoter-driven *gadd45* mRNAs analyzed by northern blot hybridization as described in Materials and Methods. Levels of RNA loading in each lane were assessed by the signal from the 18S ribosomal RNA bands. (**A**) Semi-logarithmic plot of the decay of *gadd45* mRNA derived from construct 4.2 in either the presence or absence of 1 μM CD437. (**B**) Semi-logarithmic plot of the decay of *gadd45* mRNA derived from construct 2.1 in either the presence or absence of 1 μM CD437. (**C**) Semi-logarithmic plot of the decay of *gadd45* mRNA derived from construct 3.1 in either the presence or absence of 1 μM CD437. CMV promoter-driven *gadd45* mRNA levels at time 0 in either the absence or presence of CD437 were arbitrarily defined as 1. Data represent the means from three independent clones for (A) and two independent clones for each of (B) and (C). Bar, SE.

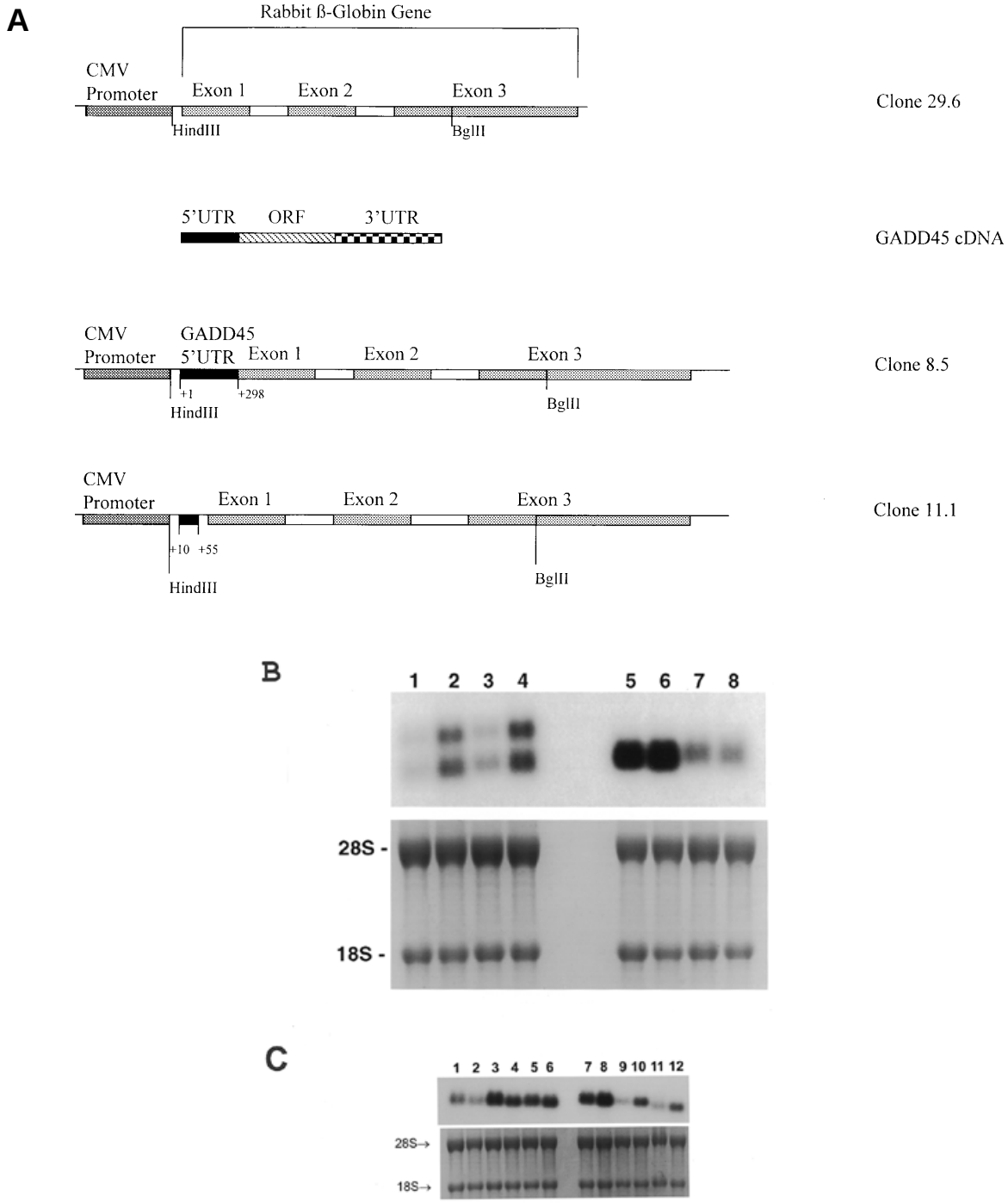



**Figure 7.** Effect of CD437 on chimeric *gadd45* 5'-UTR–rabbit β-globin gene expression in MDA-MB-468 cells. (**A**) Schematic diagrams of CMV promoter-driven rabbit β-globin gene construct 29.6 and CMV promoter-driven chimeric *gadd45* 5'-UTR–rabbit β-globin gene constructs 8.5 and 11.1. (**B**) MDA-MB-468 cells were stably transfected with construct 29.6 or 8.5. Several independent hygromycin-resistant stable sublines for each of the constructs were obtained as described in the text. Two independent sublines derived from transfection of each of the constructs in (A) were grown in either the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of 1 μM CD437 for 48 h, total cellular RNAs prepared and expression of rabbit β-globin transcripts analyzed by northern blot hybridization as described in Materials and Methods. Equal levels of RNA loading in each lane were assessed by signals from 28S and 18S ribosomal RNA bands. Lanes 1 and 2, clone 8.5/A5; lanes 3 and 4, clone 8.5/A10; lanes 5 and 6, clone 29.6/B13; lanes 7 and 8, clone 29.6/B1. (**C**) MDA-MB-468 cells were stably transfected with construct 29.6 or 11.1. Several independent hygromycin-resistant stable sublines for each of the constructs were obtained as described in the text. Three independent sublines derived from transfection of each of the constructs in (A) were grown in either the absence (lanes 1, 3, 5, 7, 9 and 11) or presence (lanes 2, 4, 6, 8, 10 and 12) of 1 μM CD437 for 48 h, total cellular RNAs prepared and expression of rabbit β-globin transcripts analyzed by northern blot hybridization as described in Materials and Methods. Equal levels of RNA loading in each lane were assessed by signals from 28S and 18S ribosomal RNA bands. Lanes 1 and 2, clone 29.6/B1; lanes 3 and 4, clone 29.6/B16; lanes 5 and 6, clone 29.6/B19; lanes 7 and 8, clone 11.1/B8; lanes 9 and 10, clone 11.1/B9; lanes 11 and 12, clone 11.1/A4.

context, in CD437-dependent up-regulation of *gadd45* expression in the breast carcinoma cells.

## DISCUSSION

Retinoic acid (RA) and its derivatives (retinoids) have been found to inhibit the proliferation of a wide variety of both normal and malignant cell types (25). Retinoids modulate activity of numerous genes through binding to the nuclear receptors RARs and RXRs (25). We have previously described a novel retinoid, CD437 which causes $G_0/G_1$ arrest and apoptosis of different human breast carcinoma cells. Although CD437 selectively binds to the RARγ nuclear receptor, it has been reported to induce apoptosis and growth arrest in both RA-sensitive and RA-resistant breast carcinoma cell lines (9). CD437 has also been shown to induce growth arrest and apoptosis of HL-60 human leukemia cells (10). CD437-mediated growth arrest and apoptosis appear to utilize a unique pathway(s) which is independent of the retinoid nuclear receptors and cellular p53 (9,10).

CD437 has been previously shown to induce cellular expression of $p21^{WAF1/CIP1}$ mRNA in a variety of breast carcinoma cells (9). CD437-treated MDA-MB-468 breast carcinoma cells were found to express ~10-fold elevated levels of $p21^{WAF1/CIP1}$ mRNA. This CD437-dependent increase in $p21^{WAF1/CIP1}$ expression was subsequently found to utilize post-transcriptional stability mechanisms (19). In this report we describe that CD437 also causes elevated expression of the DNA damage-inducible gene *gadd45* in a variety of breast carcinoma cell lines. We further demonstrate that, like $p21^{WAF1/CIP1}$ expression, CD437-mediated induction of *gadd45* gene expression is also accomplished, predominantly, via post-transcriptional stability mechanisms. CD437-dependent post-transcriptional up-regulation of $p21^{WAF1/CIP1}$ expression has been shown to utilize elements located in a 1.0 kb segment of the 3'-UTR of $p21^{WAF1/CIP1}$ mRNA (19). The data presented here also demonstrate, for the first time, that CD437-dependent post-transcriptional up-regulation of *gadd45* expression utilizes sequence elements housed in 45 nt of the 5'-UTR of the *gadd45* mRNA. It remains to be determined whether the mechanism(s) underlying CD437-dependent increased stabilization of the gadd45 mRNA involving the above 45 nt subfragment of the 5'-UTR are similar to those utilized by CD437 to enhance the stability of $p21^{WAF1/CIP1}$ mRNA.

Intracellular stability of eukaryotic mRNAs has been shown to be influenced by a number of factors, including *cis* determinants and their corresponding *trans* factors, primary and secondary structure, translation rate and intracellular location of the mRNAs (22,23). The *cis* determinants of mRNA stability include poly(A) tails in the case of a vast majority of cellular RNAs and specific sequence motifs located either in the 3'-UTR, protein-encoding ORF or the 5'-UTR of different mRNAs. For example, stability of histone mRNAs is regulated by a 3'-terminal stem–loop structure, while specific *cis* elements called iron-responsive elements located in the 3'-UTR of transferrin receptor mRNA regulate message stability in the presence of iron. Similarly, specific 3'-UTR *cis* elements are also known to regulate stability of mammalian ribonucleotide reductase mRNA. In addition, the presence of an AUUUA pentamer(s) in the 3'-UTR of several labile mRNAs including $TNF_\alpha$, GMCSF, c-myc and c-fos is known to regulate stability of these mRNAs. Further, *cis* elements located in the protein-encoding region are known to regulate stability of c-myc, c-fos and β-tubulin mRNAs, while a single iron-responsive element located in the 5'-UTR of ferritin mRNA is involved in the regulation of its stability in the presence of iron (22 and references therein). The half-life of a mRNA can also be affected by how the 5'-UTR influences its translation, since introduction of a translation-inhibiting stem–loop in the 5'-UTR can alter mRNA half-life (26). The presence of a mRNA cap at the 5'-end is also known to influence the stability of eukaryotic mRNAs. By utilizing deletion and transfection analyses, this report demonstrates that the 5'-UTR of *gadd45* mRNA is involved in the regulation of its stability in HBC cells. Since retinoid (CD437)-dependent enhanced message expression is noted for both the *gadd45* and rabbit β-globin transcripts containing *gadd45* 5'-UTR sequences, it would appear that *cis–trans* interactions rather than mRNA structure play an important role in CD437-dependent stability enhancement effects. The precise mechanism(s), including specific 5'-UTR *cis–trans* interactions, of the CD437-dependent stability increase of *gadd45* mRNA is currently under investigation.

The stability of *gadd45* mRNA is also known to be influenced by the alkylating agent MMS (27). We utilized various *gadd45*-expressing MDA-MB-468 transfectants (clones 4.2, 2.1 and 3.1; Fig. 5) to determine the sequences responsible for MMS-dependent enhanced stability of *gadd45* mRNA. Again, transfectants expressing clone 4.2 were found to show an ~4.0-fold enhanced stability of *gadd45* transcripts when compared to the transfectants expressing either clones 2.1 or 3.1 in the presence of MMS and the transcriptional inhibitor actinomycin D (data not shown), suggesting involvement of the *gadd45* 5'-UTR in MMS-dependent enhancement of message stability. However, the transfectants expressing the chimeric *gadd45* 5'-UTR–rabbit β-globin mRNA derived from clone 8.5 (Fig. 7A) failed to show a MMS-dependent increase, suggesting that additional sequences located in either the coding frame or the 3'-UTR are involved and, possibly, co-operate with the 5'-UTR in eliciting a MMS-dependent increase in *gadd45* message stability in breast carcinoma cells. The exact nature and location of such sequences remain to be determined.

## ACKNOWLEDGEMENTS

We thank Jordan Denner and Richard Milanich for their expert assistance with the photographic work. Part of this work was carried out at Greenebaum Cancer Center (University of Maryland, Baltimore, MD) and was supported by a University of Maryland Institutional Research grant (A.K.R.). Additional support was provided by the medical research services of the Department of Veterans Affairs (J.A.F.) and by NIH grants CA-63335 (J.A.F.) and CA51993 (M.I.D.)

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