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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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 humangadd45 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 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 methylmethane 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 G1 phase of the cell cycle (7). 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 G1 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 G1/G0 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 pG-L2 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 EcoRI. The excised EcoRI fragment was then subcloned in the sense orientation into the EcoRI site of the vector Phagemid pBluescript SK– (clone 5.1). Clone 5.1 was then digested with XmaI to obtain a 1.6 kb fragment of the gadd45 promoter and exon I. The 1.6 kb XmaI fragment was then subcloned in sense orientation into the XmaI 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 XhoI and Xhol and subcloned into the pCDNA3 vector plasmid (Invitrogen) as follows. First, plasmids pHUL45B2 and pCDNA3 were linearized with the restriction endonucleases XhoI and EcoRI, 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 Xhol. The 1.4 kb Xhol(end-filled)–Xhol-digested gadd45 cDNA fragment from plasmid pHUL45B2 was gel purified and ligated into the ~5.4 kb EcoRI(digested)-Xhol-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'-CCGCTCGAGATGCAATTATTCATCA-3', antisense, positions 1321–1340; 4) and GADD45.3 (5'-CGCAAGCTTCGCATCTTGATGCTAT-3', sense, positions 266–285; 4). In addition, ~0.5 kb of gadd45 cDNA fragment containing ORF only was PCR amplified using oligos GADD45.4 and GADD45.1R (5'-CCGCTCGAGTTTAGTCACGAGATGCTAT-3', antisense, positions 779–798; 4). The PCR-amplified gadd45 cDNA subfragments were then digested with HindIII and Xhol and subcloned into HindIII–Xhol-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 XbaI 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 HindIII and ~9.4 kb of the vector plasmid was gel purified. The XbaI(end-filled)–HindIII-digested pMEP4 vector plasmid was then ligated with an ~0.65 kb Neul–HindIII-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 SalI and end-filled as described above. An ~0.45 kb fragment containing the poly linker and SV40 polyadenylation signal sequence was removed by digesting the SalI-linearized and end-filled plasmid DNA of clone 28.1 with HindIII. The vector plasmid of ~10.6 kb was gel purified in order to obtain SalI(end-filled)–HindIII-digested linear vector plasmid 28.1. The plasmid pRβ3'–(G.Brewer et al., submitted for publication) containing the entire rabbit β-globin gene (16) was first linearized with KpnI, followed by blunting the KpnI overhang using bacteriophage T4 DNA polymerase (NEB) as per the manufacturer’s guidelines. The KpnI-digested, blunt-ended plasmid pRβ3'– was then digested with HindIII to release an ~1.8 kb entire rabbit β-globin gene fragment. The KpnI(blunt-ended)–HindIII-digested, gel-purified rabbit β-globin gene fragment was then ligated with the above described SalI(end-filled)–HindIII-digested vector plasmid 28.1 to obtain clone 29.6. Third, plasmid 29.6 was linearized with HindIII 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 EcoRI and HindIII, end-filled above and gel purified. The end-filled 0.3 kb 5'-UTR
fragment of gadd45 CDNA was then ligated in the sense orientation into HindIII-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 HindIII. The sense and antisense oligos were phosphorylated, annealed and ligated into the HindIII 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-32P]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 300 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, NcoI–BamHI-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
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...
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_1/2) 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 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 \textit{gadd45} mRNA involved in CD437-dependent enhancement of stability, additional subclones of MDA-MB-468 cells expressing pCDNA3-\textit{gadd45} constructs 2.1 and 3.1 were utilized. Figures 6B and C show that the stability of the transfected \textit{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 \textit{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 \textit{gadd45} message derived from construct 2.1 was significantly faster when compared to the rate of decay of the transfected \textit{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 \textit{gadd45} mRNA (construct 2.1) contribute towards the faster rate of message decay.

**CD437-dependent up-regulation of \textit{gadd45}–rabbit β-globin chimeric mRNA**

CD437-dependent up-regulation of \textit{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 \textit{gadd45}–rabbit β-globin gene clone 8.5 or CMV promoter-driven chimeric \textit{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 \textit{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 \textit{gadd45} mRNA (positions +10 to +55; 4) contain RNA \textit{cis} elements responsible for CD437-dependent up-regulation of chimeric \textit{gadd45}–rabbit β-globin mRNA. Taken together, the data in Figures 6 and 7 demonstrate that 45 nt located at the 5’-end of \textit{gadd45} mRNA participate, independent of their...
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 G1 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 p21WAF1/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 p21WAF1/CIP1 mRNA. This CD437-dependent increase in p21WAF1/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 p21WAF1/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 p21WAF1/CIP1 expression has been shown to utilize elements located in a 1.0 kb segment of the 3′-UTR of p21WAF1/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 p21WAF1/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 mRNAs 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-α, 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 shown to have 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.

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