A novel DNA damage-inducible transcript, gadd7, inhibits cell growth, but lacks a protein product

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ABSTRACT

gadd7 cDNA was isolated from Chinese hamster ovary (CHO) cells on the basis of increased levels of RNA following treatment with UV radiation. The transcript for gadd7, as well as for four other gadd genes, was found to increase rapidly and coordinately following several different types of DNA damage and more slowly following other stresses that elicit growth arrest. Agents that induce gadd7 RNA include alkylating agents, such as methyl methanesulfonate (MMS), N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and mechlorethamine HCI (HN2), oxidizing agents, such as hydrogen peroxide, and growth arrest signals, such as medium depletion (starvation). Since growth arrest is a cellular consequence of many types of DNA damage in normal cells, it was thought that gadd7 may play a role in the cellular response to DNA damage. Indeed, overexpression of gadd7 led to a decrease in cell growth. Interestingly, gadd7 cDNA does not contain an appreciable open reading frame and does not appear to encode a protein product, but instead may function at the RNA level.

INTRODUCTION

gadd7 is one of many cDNA clones isolated from Chinese hamster ovary (CHO) cells on the basis of a rapid increase in mRNA expression following treatment with UV radiation. A survey of a variety of DNA damaging agents led to the grouping of these genes based on which agents caused induction (1). Five of these, the gadd7 genes, were induced by various types of stressful growth arrest signals, as well as by DNA damage (2). These genes are of interest since important cellular consequences of DNA damage include growth suppression and transient activation of cell cycle checkpoints.

Several of the gadd genes have been investigated extensively and have turned up an intriguing variety of functions. The human Gadd45 protein binds proliferating cell nuclear antigen (PCNA) and may be involved in both enhanced DNA repair and cell cycle arrest following DNA damage (3). The Gadd153 protein is a member of the C/EBP family of transcription factors (4) and may therefore modulate expression of C/EBP-regulated genes. A herpes virus gene with homology to gadd34 is involved in escape from apoptosis of infected neuroblastoma cells (5) and a gadd34 homolog, MyD116, was induced during myeloid differentiation of murine cells (6). gadd33 is the hamster homolog of the human cornifin gene, which is a structural component of a stratified envelope formed during keratinocyte differentiation (7). gadd34, gadd45 and gadd153 are coordinately regulated by DNA damage and growth arrest in nearly all human and rodent cells examined (2), while gadd33 may be more cell type or tissue specific (unpublished data).

To further investigate the regulation of gadd7 by DNA damage and its potential functions in both rodent and human cells a full-length cDNA was isolated. Surprisingly, this cDNA did not contain a large open reading frame (ORF) and did not hybridize with any transcript in several human cell lines. Following in vitro transcription and translation no protein product could be detected. In hamster cells the gadd7 transcript was inducible by various types of DNA damage and growth arrest signals. This transcript may, like gadd33, be cell type or tissue specific or may not contain enough homology to detect a low abundance transcript in heterologous hybridizations. Like other gadd genes (6), overexpression of gadd7 in both hamster and human cells led to a decrease in colony growth. The absence of a large ORF raises questions as to the function of this small, low abundance, DNA damage-inducible transcript.

MATERIALS AND METHODS

Cloning and sequence analysis

The original partial length cDNA from the subtraction library pA7 was used to screen a Zap XR cDNA library made from CHO mRNA (Stratagene) to isolate a cDNA corresponding to the full-length gadd7 RNA, which is ~900 nt. Several cDNA clones of ~780 bp were considered to be nearly full length when the poly(A) tail is taken into consideration. One of these, pXR7g, and another smaller cDNA, pXR7d, which were known to be independent clones, were sequenced. Sequencing of the hamster gene was done using the dideoxy chain termination method (7). Portions of some clones were sequenced by Lark Sequencing Technologies (Houston, TX). Sequence comparisons were made with the Genetics Computer Group Sequence Analysis Software Package (8) and the Blast program (9). Genomic Southern blots were run as previously described (10).

gadd7 overexpression

A gadd7 expression vector, pCMV7a, was made by ligating the 0.9 kb NotI–AvaI fragment from pXR7g into NotI/XhoI-cut pCMV.3 (6). This construct contains the entire gadd7, since NotI and

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Figure 1. Northern blot analysis of gadd7 RNA. Cells were treated as indicated and poly(A) RNA was isolated after 4 h and 0.5 µg for each sample was size separated as described in Materials and Methods. Lane 1, HEC, untreated control; lane 2, HEC, 100 µg/ml MMS for 4 h; lane 3, HEC, growth arrest (held at confluence for 2 days without refeeding); lane 4, CHO, untreated control; lane 5, CHO, 100 µg/ml MMS for 4 h; lane 6, CHO, growth arrest. The autoradiograph was intentionally overexposed to show the relative hybridization of HEC versus CHO. All lanes are from the same Northern blot membrane and same exposure.

AvaI cut in the polylinker region of the pBluescript vector. For colony formation during Gadd7 overexpression RKO and CHO cells were plated at 1 × 10⁶ cells/100 mm tissue culture dish. Once cells were attached they were transfected using the calcium phosphate co-precipitation method with 10 µg pCMV.7a, pCMV45 or pCMV.3 and 1 µg pSV2neo (6). pCMV45 is a human GADD45 mammalian expression vector (6). Cells were grown for 2–3 weeks in 500 µg/ml G418 and then fixed and stained with crystal violet.

RNA and DNA

For RNA isolation cells were rinsed briefly with phosphate-buffered saline and were then immediately lysed in situ with 5 ml/150 cm² tissue culture plate 4 M guanidine thiocyanate. Total cellular RNA was isolated by the acid phenol method (11). Poly(A) RNA was isolated by oligo(dT) cellulose chromatography (12). Poly(A) RNA was blotted onto Nytran (Schleicher & Schull) nylon membranes or electrophoresed and transferred as described previously (13). For a β-actin probe a Chinese hamster cDNA clone, pA2, was used (1). cDNA probes were labeled with [32P]dCTP using random hexamer primers and Klenow fragment and hybridized as previously described (1).

The poly(A) content of all RNA samples was estimated by hybridization to a labeled polythymidylic acid probe (13). Hybridization was quantified by counting radioactivity on a Betagen 603 blot analyzer. Relative hybridization was then determined by normalizing to the result with RNA from untreated cells using the computer program RNA Analysis, which generated a standard curve for dilutions of RNA from untreated cells (13). Values for other samples were then compared with these standard curves. The amount of RNA from untreated cells was divided by the amount of RNA from treated cells which gave the same radioactive counts on the standard curve; this result gave the relative abundance of gadd7 RNA in a particular sample compared with the untreated sample (13).

Genomic DNA was isolated from hamster (CHO), mouse (J1-ES) and human colon carcinoma (RKO) cells as described previously (10). Southern blots were hybridized with a cDNA probe made from XR7g.

In vitro translation

For in vitro transcription and translation circular and linearized pX7d, pXR7g, pXR7i and pXR7j and control RNA Xef-1 were sequentially transcribed and translated in vitro with rabbit reticulocytes lysate (mMessage mMACHINE and Retic Lysate IVT; Ambion, Austin, TX). Due to the small size of the predicted proteins, either [35S]methionine (320 mCi/mmol) or [14C]leucine (320 mCi/mmol) (ICN) were used to label the in vitro translated proteins. After translation, mRNA was digested with an RNase A/RNase T1 mixture (Ambion) and translation products were run on a 10% (Xef-1) or 20% (gadd7) polyacrylamide gel. Following separation, the gel was soaked in a fluorographic solution (Amplify; Amersham), dried and exposed to X-ray film. In vitro transcription products were lightly labeled with [32P]UTP and were gel electrophoresed to verify the presence of the correct size products.

RESULTS

RNA analysis after various DNA damaging and other treatments was done to determine the scope of agents which could increase gadd7 expression. Both the partial length gadd7, pA7, and full-length pXR7g hybridized to an ~0.9 kb band on Northern blots using mRNA from CHO cells (Fig. 1). Increased expression of the gadd7 transcript was seen following treatment of cells with various agents which damage DNA, but not with agents which are similarly toxic yet do not damage DNA, such as 12-O-tetradecanoylphorbol...
gadd7 expression showed a dose–response relationship following methylnethanesulfonate (MMS) treatment, although at very high doses there was no additional increase, perhaps due to cell death (Fig. 2). Induction by MMS was inhibited by the RNA polymerase inhibitor actinomycin D.

Table 1. Regulation of expression of gadd7 RNA in CHO cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (h)</th>
<th>Relative RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µg/ml MMS</td>
<td>4</td>
<td>6.4</td>
</tr>
<tr>
<td>5 µg/ml ActD + 100 µg/ml MMS</td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>30 µM MNNNG</td>
<td>4</td>
<td>2.3</td>
</tr>
<tr>
<td>40 µM HN2</td>
<td>2</td>
<td>2.4</td>
</tr>
<tr>
<td>400 µM H2O2</td>
<td>1</td>
<td>3.8</td>
</tr>
<tr>
<td>Growth arrest</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>0.5% serum</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>20–1600 ng/ml TPA</td>
<td>4</td>
<td>0.8–0.9</td>
</tr>
</tbody>
</table>

- MMS, methylnethanesulfonate; MNNNG, N-methyl-N-nitro-N-nitrosoguanidine; HN2, methotrexate; H2O2, hydrogen peroxide; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.
- Cells were treated in logarithmic growth phase and RNA prepared and measured by quantitative RNA dot blot analysis as previously described (1). Cells were harvested 4 h after initiation of treatment except as indicated. Relative RNA is the amount of RNA in treated cells divided by the amount of RNA in untreated cells (13).
- Cells were grown to confluence then held without refeeding for an additional 48 h. Doses of 20, 80, and 1600 ng/ml did not lead to gadd7 induction.

Sequence comparison of the three gadd7 clones showed that the partial 3′-end clone, pA7, and the two full-length cDNA, pXR7g and pXR7d, matched exactly in regions where they overlapped. This cDNA sequence did not match any sequence in the databases with the exception of a hydrogen peroxide-induced hamster message, adapt 15-P8 (accession no. U26834), of unknown function. An ORF analysis of the full-length cDNA revealed that gadd7 does not have a large ORF, but does have three small ones, which would encode proteins of 38, 37 or 43 amino acids (Fig. 3, ORFs 1, 2 and 3 respectively). None of these potential proteins has any homology with any protein in the databases and none show homology with the consensus translation initiation sequence (14). The sequence of gadd7 exactly matched the sequence of adapt 15-P8 with the exception of a 1 nt insertion in the adapt 15-P8 sequence near the 3′-end of the message. This difference could be due to a sequencing error, since at this position the gadd7 cDNA has a run of four thymidine residues where adapt 15-P8 has a run of five.

Since gadd7 is expressed coordinately with several other gadd genes during certain types of growth arrest (C), it was thought that overexpression of Gadd7 might lead to growth arrest in cell cultures. Following transfection of both hamster and human cells with a gadd7 eukaryotic expression vector, a decrease in the colony formation of these cells was observed (Fig. 4). This decrease was
Figure 4. Overexpression of gadd7 leads to a decrease in colony formation. Cells were transfected as described in Materials and Methods. Each set of three bars represents three plates transfected with the same plasmid as indicated. The cell line is shown underneath the transfected plasmid designation.

Figure 5. In vitro transcription and translation of four different gadd7 clones indicate that no protein product is made: Lane 1, the 50 kDa Xef-1 translation product electrophoresed by 10% PAGE; lane 2, no RNA control, 10% PAGE; lanes 3–6, translation reactions for pXR7d, pXR7g, pXR7i and pXR7j, 20% PAGE; lane 7, no RNA control, 20% PAGE.

most pronounced in CHO cells (85% decrease), but was also seen in human RKO cells (58% decrease).

Since gadd7 has no large ORF, an in vitro translation was done to determine if there were any protein products from any of the smaller ORFs. There were no detectable protein products made from any of the four gadd7 clones, all of which are nearly full-length, based on their size (Fig. 5). A protein product was seen from a control transcript, Xef-1.

DISCUSSION

The gadd7 cDNA was originally isolated from CHO cells on the basis of increased expression following UV irradiation (1). In contrast to gadd33, gadd34, gadd45 and gadd153, the full-length gadd7 cDNA does not hybridize to RNA from non-hamster cell lines. This may be due to a lack of conservation between species, since hybridization with labeled pXR7g has not been seen in human HeLa, lymphoblast or fibroblast cell lines or in mouse 3T3 cells. gadd7 transcripts, which were inducible by DNA damage, were seen in two Chinese hamster lines derived from ovary and lung. An MMS-inducible hybridizing band on Northern blots was also seen with mRNA from HEC cells, which are a Syrian hamster cell line. However, the hybridization signal was much less than with CHO mRNA, suggesting that the gadd7 gene has diverged even in these closely related species or that transcript levels vary between cell lines (Fig. 1). Since the gadd7 transcript is of low abundance, lower hybridization signals due to sequence differences might limit the detection of gadd7 in non-hamster cell lines. On genomic Southern blots only hamster DNA hybridized with a gadd7 cDNA, suggesting that this gene is not well conserved or is not present in mouse and human cells.

Since all five gadd genes showed similar regulation in CHO cells, it was thought that they might also have similar functions, perhaps in the cellular response to DNA damage or in growth control, which is a consequence of many types of DNA damage. In support of this, Gadd34, Gadd45 and Gadd153 proteins, when overexpressed following transfection, lead to growth inhibition of cells in culture (6). All of the gadd genes, including gadd7, have very low basal expression of transcripts. This may be important if overexpression, such as occurs following DNA damage, is involved in growth inhibition. In both hamster and human cells, stable transfection of a gadd7 expression vector led to fewer colonies than did the vector alone. This inhibition was similar to that seen following overexpression of the other gadd genes (6). A striking 85% decrease in CHO cells suggests that increased expression of Gadd7 may lead to growth arrest. Following various types of DNA damage a transient increase in gadd7 might contribute to a transient growth inhibition. In human cells a 58% decrease in colony formation was observed. This is surprising in the light of the lack of hybridization of gadd7 with any human gene or transcript. It is possible that human cells contain a transcript with a similar function that does not contain enough homology to hybridize with the hamster probe.

Some of the functions of three of the gadd genes are now known. Gadd45 binds to PCNA and may be involved in both growth arrest and DNA repair following DNA damage (3). Gadd153 is a member of the C/EBP family of transcription factors (4) and a translocation involving this gene, which disrupts its growth suppressive properties, is associated with myxoid liposarcomas (15).
Constitutive overexpression of GADD153 was found in a human drug-resistant cell line (16). Like DNA damage, differentiation of most cell types also leads to growth arrest. gadd33, gadd34, gadd45 and gadd153 were found to be induced during in vitro differentiation, as well as by DNA damaging agents in certain cell types (6,17). In fact, gadd33 is known to be the hamster homolog of human cornifin, a structural component of the envelope formed during keratinocyte differentiation (7). Why all these genes are induced coordinately is unknown, but it is clear that the mammalian response to DNA damage is complex. Further investigation may uncover surprising functions for the gadd7 gene as well.

Since gadd7 RNA can be hybridized following oligo(dT)–cellulose selection, the hamster gadd7 transcript is polyadenylated. The lack of a large ORF in the full-length gadd7 cDNA, pXR7g, is rather rare in a transcript of this length (Fig. 2). The lack of a consensus translation initiation sequence (14) is not in itself evidence for lack of a protein product, since the other gadd genes also do not show homology with this consensus sequence at their translation initiation sites (2,18; unpublished data). Frameshifting during translation (19–21), a rare occurrence, could result in a protein of 78 amino acids from the two slightly overlapping ORFs 2 and 3. However, in vitro translation of four different gadd7 cDNAs failed to show a protein product, suggesting that there may not be a protein product for gadd7. There are several reported polyadenylated, spliced RNAs which do not appear to encode proteins. Three mammalian RNAs, the products of the H19, xist and his-I genes, are conserved between rodents and human at the nucleotide level, but have no apparent protein products due to multiple small ORFs (22–24). These three transcripts are suggested to function in the absence of translation. The transcription of H19 regulates expression of another gene, IGF2, by competing for a common regulatory signal (22). In addition, H19 expression is decreased in many transformed and tumor cells, suggesting that H19 may also have growth inhibitory functions (25,26). XIST RNA has been suggested to be involved in X chromosome inactivation, since it is the only gene transcribed from the inactive X chromosome in murine females (23). The his-I gene is activated following viral insertion in murine myeloid leukemias, but is not expressed in normal tissues (24). A Drosophila RNA, hsr 1a also does not have any conserved ORF, but translation of one of the small ORFs is suggested to play a role in translational control following heat shock (27). It is possible, therefore that gadd7 RNA may also have a unique function as an RNA molecule, perhaps in the regulation of other genes following DNA damage.

REFERENCES