Transactivation of the grp78 Promoter by Malfolded Proteins, Glycosylation Block, and Calcium Ionophore Is Mediated through a Proximal Region Containing a CCAAT Motif Which Interacts with CTF/NF-I

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The 78-kDa glucose-regulated protein (GRP78) is a major endoplasmic reticulum (ER) protein that can form stable associations with a variety of proteins retained in the ER because of underglycosylation or other conformational changes. In this study, we provide evidence at the transcriptional level that a conformationally abnormal protein, an altered herpes simplex virus type 1 envelope protein that is retained in the ER of a mammalian cell line, transactivates the grp78 promoter. In contrast, the normal viral envelope glycoprotein does not elevate grp78 promoter activity. Using a series of 5' deletions, linker-scanning, and internal deletion mutations spanning a 100-bp region from −179 to −80, we correlate the cis-acting regulatory elements mediating the activation of grp78 by malfolded proteins, glycosylation block, and the calcium ionophore A23187. We show that they all act through the same control elements, suggesting that they share a common signal. We report here that the highly conserved grp element, while important for basal level and induced grp78 expression, is functionally redundant. The single most important element, by linker-scanning analysis, is a 10-bp region that contains a CCAAT motif. It alone is not sufficient for promoter activity, but a 40-bp region (−129 to −90) that contains this motif is essential for mediating basal level and stress inducibility of the grp78 promoter. We show that the transcription factor CTF/NF-I is able to transactivate the grp78 promoter through interaction with this CCAAT motif.

It has been proposed that cellular proteins of a class generally referred to as chaperones have the ability to bind to proteins for transmembrane targeting and may also participate in the assembly of oligomeric proteins (56). Included among the ATP-dependent chaperones is the 78-kDa glucose-regulated protein GRP78, which is a resident protein in the endoplasmic reticulum (ER). Although GRP78 was first discovered as a binding protein in the ER because of its association with the immunoglobulin heavy chains (15), it has become apparent that the repertoire of proteins to which GRP78 can stably bind is much broader (18). For example, GRP78 can associate with the immunoglobulin light chains in a nonsecreting B-cell myeloma line (40) and with a mutant form of influenza virus hemagglutinin that fails to assemble into trimers (11, 20). GRP78 can be considered a component of the secretary pathway, since it can regulate the transport and maturation of normal proteins (17, 44, 55). Proteins that are malfolded because of mutagenesis, underglycosylation, or other physiological stress conditions appear to form stable associations with GRP78 and as a consequence are retained in the ER (8, 10, 11, 20, 22, 40, 43).

It has been demonstrated that grp78 and another glucose-regulated protein gene, grp94, are stress inducible (27). In particular, reagents that block protein glycosylation or deplete intracellular calcium stores (such as the calcium ionophore A23187) can enhance the transcription of grp78 and grp94 by 10- to 20-fold (4, 9, 53, 62). Since a variety of stress conditions that induce the transcription of grp genes also interfere with protein glycosylation in the ER, we postulated that the accumulation of underglycosylated protein in ER would trigger a regulatory mechanism that eventually increase the transcription of the grp genes (27). Subsequently, it was shown that protein malfolding, in the absence of abnormal glycosylation, is also able to induce a higher synthesis rate of GRP78 and GRP94 (25), implying that the malfolded protein may be the proximal inducer of the grp genes.

GRP78 shares about 60% amino acid sequence identity with the heat shock protein HSP70 (38). Members of the HSP70 protein family have been implicated in the translocation of secretory and mitochondrial precursor polypeptides (6, 37). Cultured animal cells exposed to amino acid analogs or puromycin rapidly synthesize the HSPs as a response to the presence or catabolism of abnormal proteins (19). Further, activation of the hsp70 promoter was reported when denatured protein was coinjected with hsp genes into frog oocytes (2). Since the heat shock element was required for the activation, it was suggested that abnormal proteins and heat shock activate the hsp70 gene by a common mechanism. Interestingly, the yeast grp78 promoter contains the heat shock element and the gene is inducible by heat and tunicamycin treatment (45). In mammalian cells, however, the grp78 promoter is devoid of the heat shock element. Potent inducers of grp78 include the calcium ionophore A23187, tunicamycin, and the accumulation of abnormal proteins in the ER (3, 23, 25, 40, 53, 62).

GRP78 provides a model system to learn how a cell responds to the state of the ER and how this signal is

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transmitted to the nucleus. While it has been widely assumed that the accumulation of abnormal proteins in the ER generates a signal from the ER to the nucleus to activate the grp genes, there has been no direct demonstration that such proteins can transactivate the grp promoters. In addition, no information that correlates the existing regulatory elements mediating the response by malfolded proteins and that by the other stress conditions currently exists. Furthermore, very little is known about the types of transcription factors which mediate this form of regulation.

We have investigated the mechanism of activation of the mammalian grp78 gene by malfolded proteins and the relationship of this induction process with those caused by other stress conditions currently exists. Furthermore, because it is a very little is known about the types of transcription factors. It has served as a model system for studying the effects of protein glycosylation block on the expression of the GRPs and their formation of complexes with cellular proteins. Also, we have shown previously that a 422-bp fragment spanning -456 to -34 contains cis-regulatory elements for basal-level expression, as well as all of the elements required for induction under the various stress conditions. Furthermore, deletions of the promoter region from -208 to -104 abolish basal-level expression as well as induction by the calcium ionophore A23187 (S4). Here, using a series of linker-scanning (LS) and internal deletion mutants, we further define and correlate the sequence requirements for induction by abnormal proteins, glycosylation block, and A23187. We show that all three stimuli act through the same cis-acting elements. In addition, we identify a 22-bp region which contains a CCAAT motif that mediates both basal level and stress inducibility of grp78. We demonstrate that the CCAAT-binding factor CBF (34), which has the ability to transactivate the human hsp70 promoter in COS cells, is unable to stimulate the grp78 promoter. In contrast, the CCAAT transcription factor/ nuclear factor I CTF/NF-1 (S8) is proficient at transactivating grp78 promoters through the C1 motif.

**MATERIALS AND METHODS**

**Cells and culture conditions.** K-12 cells are grown in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal calf serum as previously described (28). The COS-7 cells are derived from an African green monkey kidney cell line and produce a simian virus 40 (SV40) large-T antigen (12). They are maintained at 37°C in Dulbecco’s modified Eagles medium supplemented with 10% fetal calf serum and 2 mM glutamine.

**grp78 promoter mutant constructions.** The wild-type plasmid, previously referred to as PE43, contains a 422-bp fragment of the rat grp78 promoter (-456 to -34) fused 5’ to the chloramphenicol acetyltransferase (CAT) gene as previously described (4). The wild-type grp78 promoter sequence has been published (4). Each LS mutant replaces 10 bp of the rat grp78 promoter (Fig. 1). All replacement sequences contain a HindIII site. The LS mutations were constructed by either site-directed oligonucleotide mutagenesis (LS140, LS150, and LS160) or polymerase chain reaction-generated mutagenesis. LS primers were synthesized with the 5’-nucleotide (nt) mutation, flanked on either side by 15 nt of wild-type grp78 promoter sequences for proper hybridization. The LS95/98 primer was 20 nt in length, spanning -101 to -82 of the grp78 promoter, and mutates the sequences from -98 to -95. For site-directed oligonucleotide mutagenesis, plasmid PE43 (E43:800) was used. This plasmid contains a 775-bp HaeIII-EcoRI fragment containing from -456 to -34 of the rat grp78 promoter inserted into a Smal-EcoRI-digested PE43:18U vector. LS and universal primers for PE43:18U were 5’ phosphorylated and hybridized to denatured PE43:18U. The primers were extended and ligated with Klenow enzyme and T4 DNA ligase. These were transformed into Escherichia coli HB101, and the colonies were screened. The LS mutations were then cloned back into PE43 by using the unique Ndel and SstI sites. The rest of the LS mutations were constructed by polymerase chain reaction mutagenesis in a two-step synthesis procedure. LS primers and a CAT 22-nt oligonucleotide (5’-ATATCAACG TGTGTTATATTCCAG-3’, the 5’ end is located 69 nt downstream of the HindIII site of pSVOCAT) were used with -456 (E43) as a template to make a 3‘ promoter fragment. This fragment was then used as a primer to synthesize the full-length mutated grp78 promoter with an upstream CAT primer (5’-CGCATCTGTCGCGATT-3’, located 30 nt upstream of the Ndel site). The Ndel-Stul fragments containing the LS mutations were cloned into the plasmid -456 to replace the wild-type promoter.

Internal deletions were created by combining BamHI-HindIII fragments obtained from the LS CAT constructs. For example, the construct M -140 to -169 was created by combining fragments from LS140 and LS160. The LS140 fragment contains the 3’ end of the grp78 promoter (from -139) and the CAT gene, ending at the unique BamHI site. The LS160 fragment contains pBR sequences from the CAT plasmid, including the Amp’ gene, and the 5’ part of grp78 to
-170. This construct contains 9 nt of the LS mutations, regenerating the HindIII site. The rest of the internal deletions are constructed similarly.

The 5' deletion constructs, except for -169, have been described (54). The -169 construct was created by digestion of LS170 with NdeI and HindIII to remove sequences upstream of -169. Schematic diagrams for the grp78 5' deletion and LS promoter constructs are presented in Fig. 4. The sequences of all the constructs were confirmed by DNA sequencing.

**Cotransfection with the HSV gB expression vector.** The construction of the HSV gB expression plasmids pRB9221 encoding wild-type HSV-1 gB and pSB479 encoding gB-(Lk479) have been described (49, 52). The plasmid vector used was pMT2 modified from p91023 (66). The procedures for transient transfection of DNA into K-12 cells, preparation of cell extracts, measurement of protein concentration, and assay for CAT activity were carried out as previously described (53). In all the cotransfection experiments, 10 μg of the test plasmids was cotransfected with 5 μg of pMT2, pSB479, or pRB9221 per dish. In each transfection mixture, 3 μg of HeLa carrier DNA was added. The cell extracts were harvested 40 h after transfection. Sixteen hours prior to harvesting, the transfectants were all changed to fresh medium and were incubated at 35°C, supplemented with 7 μM A23187, or shifted to 39.5°C. Each construct was transfected independently from 2 to 6 times, and each transfection was performed in duplicate sets. The CAT activities were measured within the linear range of the assay. The percentage of chloramphenicol conversion was quantitated by an AMBIS Radioanalytic Imaging System (Ambis Systems, San Diego, Calif.).

**grp78 promoter activity measurements.** The CAT plasmids were transfected into K-12 cells, and the CAT activity was assayed in cells grown in Dulbecco’s modified Eagle medium at 35°C, cells treated with 7 μM A23187 for 16 h, or cells shifted to 39.5°C for 16 h as previously described (53). Each construct was assayed multiple times, up to 12 times for some key constructs. Each condition was performed in duplicate to ensure reproducibility. The standard deviation analysis was performed by using the program Sigma-Plot (Jandel Corp., Corte Madera, Calif.). The plasmid pSV2 CAT (13) was used as a negative control. To standardize for transfection efficiencies, 3 μg of the plasmid pCH110 (a gift from Peter Vogt), containing the bacterial β-galactosidase gene driven by the SV40 promoter (16), was cotransfected with the CAT plasmids.

**Assays for β-galactosidase activities.** Extracts from cells cotransfected with pCH110 were assayed for β-galactosidase activity as previously described (57) with the following modifications. Equal amounts of extract (~15 μg) were adjusted to 200 μl (0.25 M Tris, pH 7.8) and assayed in duplicate. Each sample was mixed with 500 μl of solution 1 (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgCl2, 50 mM 2-mercaptoethanol) and 100 μl of ONPG solution (60 mM Na2HPO4, 40 mM NaH2PO4, 2 mg of o-nitrophenyl-β-D-galactopyranoside per ml). The samples were incubated for 20 min at 37°C until a yellow color appeared and then stopped with the addition of 500 μl of 1 M Na2CO3. The optical density at 420 nm readings were taken. Purified β-galactosidase enzyme was used to ensure linearity of the assay. Equal β-galactosidase units were then used for CAT assays.

**Cotransfection with the CBF expression vector.** Three micrograms of the test plasmids ([(-456)CAT, LS90CAT, and Hsp70CAT] was cotransfected into COS-7 cells with 3 μg of either pMT2 (the vector alone) or pMT2-CBF (the CBF expression vector). The plasmids Hsp70CAT, pMT2, and pMT2-CBF have been described (34). The conditions for transfections and CAT assays were performed as described for the K-12 cells.

**Cotransfection with the CTF/NEF-I expression vectors.** The transfection conditions for SL-2 cells have been described (7). Essentially, the SL-2 cells were plated on the day of transfection in 6-cm dishes at a density of 10⁷ cells per 5 ml of medium and allowed to settle. Aliquots of 300 μl of 0.25 M CaCl2, containing 5 μg of test plasmid DNA and an additional 15 μg of expression plasmid or vector alone, were
added dropwise to an equal volume of 2× IBS + HEPES [275 mM NaCl, 10 mM KCl, 1.5 mM NaHPO₄, 0.22% glucose, and 45 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; pH 7.1)] with aeration. No HeLa carrier DNA was used. The CaCl₂-IBS solution was allowed to stand for 10 h at room temperature. Aliquots of 600 μl were added directly to the SL-2 culture. The cells were harvested 36 to 48 h after transfection by scraping the dishes with a rubber policeman to loosen any attached cells. The cells were transferred to a 15-ml centrifuge tube and were pelleted in an IEC Centra-7R centrifuge at 1,000 rpm for 2 min. The pellet was resuspended in 1,000 μl of phosphate-buffered saline (PBS) and transferred to a 1.5-ml microtube. The cells were pelleted (2 min in a microfuge) and washed with another 1,000 μl of PBS. The CAT assays were performed as previously described (29, 53). The constructs pPADH (vector), pPADH-CTF1, pPADH-CTF2, α55CAT, and α87(+3 Ad)CAT were a gift from R. Tjian (36, 58).

RESULTS

Transactivation of the grp78 promoter by malfolded protein. HSV-1 gB is a membrane-anchored glycoprotein, 874 amino acids in length after cleavage of the signal sequence. It contains an amino-terminal hydrophilic ectodomain containing six potential N-linked glycosylation sites, a hydrophobic transmembrane region, and a charged intracellular carboxy terminus (Fig. 2) (48). The mutant gB-(Lk479) contains an insertion of four amino acids (Glu Asp Leu Pro) at position 479 which results in a conformational change that precludes transport of the mutant from the ER. By using monoclonal antibodies to gB and monospecific antiserum against GRP78 and GRP94 in immunoprecipitating assays, it has been shown in COS cells that this mutant glycoprotein and precursors of wild-type gB formed complexes with GRP78 and GRP94 (41, 50).

To investigate the effect of the mutant gB on the transcriptional activation of the grp78 promoter, we cotransfected the hamster fibroblast cell line K-12 with expression plasmids for the HSV-1 glycoproteins and grp78 promoter linked to the CAT reporter gene. After transfecting into the K-12 cells, we confirmed by immunoprecipitation that the previous observations (41, 50) that the wild-type gB and the mutant gB-(Lk479) were efficiently expressed and that the mutant gB but not the wild-type gB complexed to GRP78 (42).

It was previously shown that the rat grp78 promoter, containing the 5′ sequence up to −456, is highly sensitive to treatment with A23187. The induction is triggered by depletion of intracellular calcium stores with a concomitant block in protein glycosylation (4, 9). Our experiments showed that about the same magnitude of induction (fivefold) of grp78 promoter activity can be observed in cells cotransfected with the mutant gB (Fig. 3A and B). In contrast, there was no stimulation but a slight decrease of the grp78 promoter activity by wild-type gB (Fig. 3C). No stimulation was observed for the plasmid vector alone, and the same results were observed in the presence or absence of a β-galactosidase plasmid serving as internal reference for transfection efficiencies (30). Next we identify the grp78 promoter sequence required for the transactivation by mutant protein and correlate it with the rat promoter elements required for stress induction.

5′ deletion analysis. Close examination of the grp78 promoter sequence 500 bp upstream of its TATA element reveals a series of repeated elements containing CCAAT-like motifs flanked by GC-rich sequences, referred here as C5 through C1 (Fig. 1A). Further, HeLa and B-cell extractive extracts give footprints within the C5, C4, C3, and C1 regions (39). It has been determined previously from 5′ deletion mutants of the grp78 promoter that the region between −208 and −154 is required for high basal-level expression and the region between −154 and −104 is required for both basal-level and A23187 inducibility (54). To delimit the cis elements required for glycosylation block inducibility, we created a new 5′ deletion construct at −169 and examined the series of 5′ deletions (Fig. 4A) for their promoter activities under basal, A23187 and K12 ts mutation-induced conditions. These 5′ deletion mutants were linked to the CAT reporter gene and the promoter activities were examined after transfection into the K12 cells as described (54). The results, summarized in Table 1, show that sequences spanning −456 to −169 contribute significantly to basal-level expression of the grp78 promoter but have minor effects on its inducibility. The cis elements for A23187 and K-12 ts mutation inducibility appear to reside within the promoter region spanning −154 to −104. These results also indicate that the promoter containing the C1 CCAAT motif alone (−104) retains less than 10% of the basal-level promoter activity and has lost inducibility by both A23187 and glycosylation block. The 1.5-fold induction observed is similar to that of the control plasmid pSV2CAT (Table 1). Using equivalent amounts of protein extracts for each CAT assay, we routinely observed about a 1.5-fold increase in pSV2CAT activity in stressed cells.
FIG. 3. Transactivation of the grp78 promoter. The test plasmid used in all panels is grp78 (~456)CAT. Test plasmid was cotransfected with the plasmid vector pMT2 (MT2) (A) and with the expression vector pSB479 (gBm) (B). The effects of calcium ionophore were tested by adding 7 μM A23187 to transfectants for 16 h (A23187). (C) Test plasmid was cotransfected with either MT2 or HSV-1 gB (gBwSt). Duplicate samples are shown for each condition. (A B) Protein extract (2.5 μg) was used for each CAT assay; (C) for each assay, 10 μg of protein extract were used. Autoradiograms are shown. The positions of acetylated (Ac) and nonacetylated chloramphenicol (Cm) are shown. The percent chloramphenicol conversion for each reaction is indicated below.

To identify the grp78 promoter sequence required for transactivation by the mutant gB, we cotransfected the expression vector for the mutant gB into K-12 cells along with 5' deletion mutants of the grp78 promoter. The results, as shown in Fig. 5A and summarized in Table 1, indicate that the region important for the transactivation resides within a 50-bp region spanning −154 to −104. This conclusion is based on the observation that full inducibility was still observed when the grp78 promoter was reduced from −456 to −154. Further reduction from −154 to −130 and then to

FIG. 4. Structure of 5' deletion and LS mutants. Locations of the 5' deletions (A) and LS mutations (B) are aligned underneath the schematic drawing of the grp78 promoter. Locations of the CCAAT elements (C5 through C1 [Fig. 1]) and the TATA element are shown. The footprinted regions of the grp78 promoter (39) are bracketed. The endpoints of each 5' deletion and LS mutation (solid bar) are indicated. For example, LS170 replaces −179 to −170.
since the mutant protein was unable to stimulate promoter activity of viral promoters from SV40 and the Rous sarcoma virus (Table 1) (30).

**LS mutation analysis.** To determine the contributions of C5 through C1 and their intervening sequences towards the basal-level and induced transcription of grp78, we examined the promoter activities of LS mutants (10-bp replacements spanning −179 to −90 [Fig. 4B]). The 5′ endpoint for all the LS mutants is −456, since this construct provided us with a strong basal promoter and inducibility under the various stress conditions. The results of the LS mutants, summarized in Table 1, show a striking lack of effect of the LS mutations with the exception of LS90, which mutates the C1 element (Fig. 1B). This 10-bp mutation reduces the basal-level expression to 35% of the wild-type promoter, reduces the inducibility by A23187 and the K-12 ts mutation. To determine whether this same element is needed for transactivation by misfolded proteins we cotransfected LS90 into K-12 cells along with the plasmid encoding mutant gB. The mutant gB was unable to stimulate LS90 promoter activity, even though LS90 contains all the upstream regulatory elements as the (−456) construct (Fig. 5B). Therefore, this mutation at −99 to −90 blocks transactivation by the mutant gB.

To determine if the LS90 effect was due to the CCAAT element, we created another construct, LS95/98. This plasmid mutates the CCAAT motif but not the flanking regions (Fig. 1B). This mutation had similar results to that of LS90 (Table 1B). Thus, the effects are primary because of the CCAAT element mutation. In contrast, LS100, which mutates the 10-bp sequence adjacent to C1 and does not affect a CCAAT element, exhibits a promoter activity resembling that of the wild type. Mutation of C3 in LS130 results in 45% of the wild-type basal-level activity, while the inducibility levels were only slightly affected. Mutations in other elements (C5, C4, and C2) resulted only in very minor effects.

Since C2 is not a footprinted region (Fig. 4A), its lack of function is not surprising. The fact that C5 and C4, shown to be functional by 5′ deletions and in vivo competition (3, 31, 33a), can be eliminated without much effect suggests that

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**TABLE 1. Relative CAT activities of the grp78 promoter constructs**

<table>
<thead>
<tr>
<th>Constructa</th>
<th>Basal CAT activity level</th>
<th>Increase (fold)b in CAT activity with:</th>
<th>A23187 treatment</th>
<th>K-12 ts mutation</th>
<th>Stimulation by gBc</th>
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<tr>
<td>5′ Deletion</td>
<td></td>
<td></td>
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<tr>
<td>−456</td>
<td>100</td>
<td>503 ± 40 (5.0)</td>
<td>385 ± 40 (3.9)</td>
<td>(5.0)</td>
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<td>−169</td>
<td>27 ± 5</td>
<td>135 ± 11 (5.0)</td>
<td>92 ± 11 (3.4)</td>
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<td>−154</td>
<td>23 ± 5</td>
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<td>90 ± 9 (3.9)</td>
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<td>−130</td>
<td>10 ± 3</td>
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<td>pSV2CAT</td>
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<td>213 ± 24 (1.8)</td>
<td>188 ± 24 (1.6)</td>
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* a The CAT activities of all constructs (with their standard deviations) are expressed relative to that of the −456 wild-type promoter CAT construct at the noninduced condition, which is set at 100. The plasmid pSV2CAT is used as a negative control.

* b The 5′ deletion constructs and LS mutations are diagrammed in Fig. 4.  

* c Fold of induction is as a ratio induced to basal level.

* d For stimulation of gB, K-12 fibroblasts were cotransfected with plasmid pSB479, encoding gB (Lk479), and with plasmids encoding grp78 promoter sequences or the SV40 promoter. Fold increase is relative to transfection with vector alone.

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FIG. 5. Transactivation of the promoter mutants of grp78. (A) 5′ deletion mutants at −154, −130, and −104 were used as test plasmids. (B) LS mutant LS90 with 5′ endpoint at −456 and containing a 10-bp mutation at −99 to −90, was used as the test plasmid. Cotransfections were performed and abbreviations are as described in the legend to Fig. 3. The percent chloramphenicol conversion for each reaction is shown below.
these control elements are functionally redundant in the rat grp78 promoter. While the 5′ deletion analysis demonstrated the functionality of these sequences, it appears that elimination of C5 and C4 is compensated by duplicated or similar elements still present in the −456 promoter. Upon detailed reexamination of the grp78 promoter sequence, we discovered that in addition to the five C elements, the regions containing the C5 and C4 motifs are directly repeated about 75 bp upstream.

**Internal deletion analysis.** Since the elements controlling grp78 expression appear to be redundant, we constructed a series of internal deletion mutants spanning −169 to −80 to define further the functions of C5 through C1. The promoter activities of all the mutant constructs tested are summarized in Table 2. Compared with the wild-type promoter, the internal deletion mutant Δ(−140/−169), which removes both C5 and C4, was largely not affected. In contrast, the internal deletion Δ(−130/−149), which removes C3 and C4, reduces the basal level to 35% and retains some inducibility by A23187 and the glycosylation block. This result is consistent with the loss of basal-level activity in LS130, which mutates the C3 element. The elimination of C4, C3, and C2 in Δ(−110/−149) results in a partially active promoter. Previously, 5′ deletions between −154 and −104 abolished promoter activity. This indicates that while C4, C3, and C2 contribute to promoter strength, element C1 in combination with C5 and upstream enhancers can function and induce transcription under stress conditions, although at a reduced level. Comparison between Δ(−90/−149) and Δ(−110/−149) revealed that the elimination of the 20-bp region between −90 and −110 (containing element C1) reduces basal level to 30% and abolishes both A23187 and glycosylation block inducibility. This confirms the LS90 result that C1 and its flanking sequence are important in mediating basal level and stress inducibility of the grp78 promoter. The deletion Δ(−90/−129), which eliminates C3, C2, and C1, exhibits a promoter activity similar to that of the largest internal deletion mutant Δ(−80/−149), which has low basal level activity. Notably, Δ(−90/−129) is the smallest deletion which is no longer inducible by both A23187 treatment and glycosylation block. Therefore, this 40-bp region (−129 to −90) is essential for mediating basal level and stress inducibility of the grp78 promoter. Similarly, the same regions needed for stress inducibility are required for transactivation by the mutant viral glycoprotein (Table 2).

Analysis of the basal promoter activities of LS90, LS130, and Δ(−130/−149) over multiple independent transfection experiments shows that the reduction is reproducibly observed among the mutants. To ensure that the observed drop in basal levels in these constructs were not due to differences in transfection efficiencies, we cotransfected these plasmids with a plasmid which contains the gene for β-galactosidase. Cell extracts from the transfectedants were assayed for β-galactosidase activities to normalize for transfection efficiencies. CAT activities were measured by using extracts containing equal β-galactosidase units. Compared with the wild-type promoter, the mutated LS90, LS130, as well as the internal deletion Δ(−130/−149) all retained about 30% of basal-level activity (68). These results confirmed that the reduction in basal levels in LS90 and LS130 are due to the mutated promoter sequences in −99 to −90 and −139 to −130, respectively.

**Simultaneous induction of the grp promoters by malfolded protein and other stress inducers.** As summarized in Tables 1 and 2, our experiments demonstrated that the grp78 promoter sequences required for transactivation by the mutant gB and induction by A23187 and glycosylation block are nearly identical. The level of stimulation by these three stress conditions is also similar, at about four- to fivelfold. These data suggested that all three inducers may act through a common mechanism. For example, both A23187 and the K-12 ts mutant contain a gB protein which stimulates the grp78 promoter. Knowing that the malfolded protein is the most proximal inducer of the grp genes (25), there would be no additive effect when both the abnormal protein and the other, more distal, inducers are presented together to the grp promoter.

To test this hypothesis, we compared the level of induction of the grp78 promoter by the mutant gB protein alone and in combination with A23187. An example of coinduction of the grp78 promoter by A23187 and mutant protein is shown in Fig. 3. In the case of coinduction by gB and A23187, we observed a seven- to eightfold induction as compared with the fivelfold induction for the mutant gB alone or A23187 treatment alone. Thus, there is a slight but detectable additive effect by these two inducers. Nonetheless, the inducers act through the same grp78 promoter elements, and the C1 CCAAT element is essential.

**Effects of the hsp CBF on the grp78 promoter activity.** Next, we searched for known CCAAT transcriptional factors capable of transactivating the grp78 promoter in vivo. Since the hsp70 promoter contains a CCAAT motif at similar locations with reference to the transcriptional initiation (14, 65) as the C1 element in grp78 and that motif is crucial in mediating the expression of hsp70, we tested whether the

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**TABLE 2. Relative CAT activities of internal deletion mutants**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Presence of CCAAT motif within:</th>
<th>Basal CAT activity level</th>
<th>Increase (fold) in CAT activity with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C5</td>
<td>C4</td>
<td>C3</td>
</tr>
<tr>
<td>−456</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ(−140/−169)</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Δ(−130/−149)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Δ(−110/−149)</td>
<td>−</td>
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<td>Δ(−90/−149)</td>
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<tr>
<td>Δ(−90/−129)</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>Δ(−80/−149)</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pSV2CAT</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* All internal deletion mutants have 5′ endpoints at −456 and 3′ endpoints at −34. The positions of C5 through C1 are shown in Fig. 4A. Analyses and conditions are as described in the footnotes to Table 1.

* a, CCAAT motif is present; b, CCAAT motif is lost.
FIG. 6. CBF cotransfection assays. The sequences around the proximal CCAAT site in the promoters of grp78 (−456 and LS90 constructs) and hsp70 are shown. The CCAAT motifs are in boldface, and the positions in the respective promoters are indicated. The LS90 mutation is in lowercase. The regions of the LS90 mutation are underlined. The autoradiograms for the CAT assays of the cotransfection experiments are shown. Extracts (100 μg) were assayed for CAT enzyme activity from COS-7 cells transfected with (−456) CAT (lanes 1 and 2), LS90CAT (lanes 3 and 4), or Hsp70CAT (lanes 5 and 6). The reporter constructs were cotransfected with either the vector pMT2 (−) or with the expression vector pMT2-CBF (+).

recently cloned human CBF is able to transactivate the grp78 promoter. For this purpose, the expression vector either for CBF (pMT2-CBF) or pMT2 (the vector alone) was cotransfected into COS cells with either (−456)CAT or the Hsp70CAT, the latter contains the human hsp70 promoter sequence linked to the CAT reporter gene and serves a positive control (34). We confirmed that CBF is a transactivator of the hsp70 promoter. However, it is unable to stimulate the grp78 promoter activity and instead exhibits a mild inhibitory effect (Fig. 6). One explanation is that CBF is able to bind to the grp78 CCAAT motif at −95 but is unable to transactivate the promoter because this factor lacks the proper activating domain to stimulate the grp78 promoter. The drop in the grp78 promoter activity is probably because of steric hindrance. To test this hypothesis, we examined the effects of CBF on the LS mutant LS90. In LS90, the grp78 promoter sequence is identical to that of the wild-type −456 promoter with the exception of a 10-bp mutation which eliminates the CCAAT motif at −95 (Fig. 6). We observed that when LS90CAT was used, the inhibitory effect of CBF was removed (Fig. 6). At the same time, there was no activation. Similar results were observed with COS cells stimulated with A23187 (68). Therefore, it is unlikely that CBF is involved in the basal or induced expression of grp78, although we cannot exclude the possibility that the amount of the endogenous CBF present in COS cells is sufficient to promote maximum expression of the grp78 promoter and that excess amounts would be detrimental.

Effects of CTF/NF-1 on the grp78 promoter. Another factor that can bind to a CCAAT motif is the CTF/NF-1. This factor is composed of a family of proteins (from 52 to 66 kDa) which arise from alternatively spliced products of the CTF/NF-1 gene (58). We have obtained the expression vectors for this gene and assayed their effects on the grp78 promoter.

These expression vectors were from a Drosophila system, since it is reported that the Drosophila cell line Schneider SL-2 is devoid of Sp1 and these CCAAT transcriptional factors, which are ubiquitous in vertebrates (58). Because the Schneider SL-2 cell line was used as recipient cells, the possibility of CTF/NF-1 being in excess can be discounted, and if the CTF factor can transactivate the promoter of interest, its effects will be easily detectable. Using a CAT fusion gene driven by the α-globin promoter with an additional three CCAAT motifs from the adenovirus major late promoter [a87(+3 AdCAT)], we confirmed the previous observation (36) that CTF-1 and CTF-2 can transactivate this promoter by 6- to 10-fold, respectively (Fig. 7). The grp78 promoter was transactivated by both CTF proteins by about three- to sixfold (Fig. 7). This transactivation presumably occurs at the C1 element since LS90CAT, which contains a mutation at this motif, cannot be stimulated by the CTF factors (Fig. 7). In contrast, the human thymidine kinase promoter (TK-CAT), which also contains several CCAAT elements (24), is not transactivated by the CTF/NF-1 proteins (68).

DISCUSSION

The emergence of a class of cellular proteins that have affinities for nascent or abnormal proteins provides important information about the process of protein targeting across membranes and about the assembly, oligomerization, and eventual export of proteins to the cell surface (6, 56). Among this class of chaperone proteins, GRP78 is one of the better characterized with regard to its binding activities, as there are many examples of its specific association with a wide variety of cellular and viral proteins (8, 10, 11, 18, 19, 22, 40, 43, 44).

In examining the transcriptional regulation of the grp78 gene, we and others have noted that a wide spectrum of stress conditions can enhance its mRNA level (25, 27, 63). A common consequence of these conditions is the production of underglycosylated or simply unfolded proteins. At the same time, GRP78 is found in association with many of these proteins. We have proposed (40) that the transcription of the grp78 gene is primarily determined by the accumulation of transport-defective proteins in the ER and is not simply a reflection of the level of protein traffic through the ER.

In this study, we examined the effects of the production of wild-type HSV-1 gB and a conformation-defective mutant gB (LK479) on grp78 promoter activity. Our results are consistent with the hypothesis that the production of the mutant protein, not the wild-type glycoprotein, triggers a higher rate of grp78 transcription. While it has not been established that the transcriptional activation of the grp78 gene is a direct consequence of complex formation between the mutant protein and GRP78, we have previously observed that GRP78 exists in two forms, a phosphorylated and adenylated form and a nonmodified form. Only the nonmodified form of GRP78 is found in association with the ER proteins (18). We provide here direct evidence that the grp78 promoter undergoes transactivation by the production of a
mutant glycoprotein. We demonstrate that a 50-bp region spanning -154 to -104 of the rat grp78 promoter contains the regulatory elements for this induction, which in turn requires a 10-bp region spanning -99 to -90 for mediating the response. These results, coupled with our analysis on grp78 induction by calcium ionophore A23187 and glycosylation block, established that all three inducers recognize common cis-regulatory sequences.

The nucleotide sequences of the grp78 promoter required for induction contain interesting features, such as several arrays of CCAAT-like elements flanked by GC-rich regions. Part of the sequence is highly conserved among grp promoters across different species (33a, 54, 62) and is involved in the coordinated regulation of other ER protein genes (31). However, the heat shock element conserved among the heat shock gene promoters (1, 69) is not found. Therefore, although GRP78 shares partial amino acid sequence identity with HSP70 and both sets of proteins belong to the family of chaperone proteins, the protein factors mediating their induction in response to malfolded proteins are likely to be different.

Furthermore, in this report, we discovered through LS and internal deletion analysis that the grp78 promoter contains multiple control elements. In particular, the highly conserved grp core element containing C5 and C4 shown to be important for both basal level and induced expression by S' deletion and in vivo competition analysis, is functionally redundant. We speculate that since grp78 is a single-copy gene (62, 67) and is essential for survival (31, 55), these regulatory elements may have duplicated during evolution to ensure grp78 expression.

While the general lack of effect of the LS mutants suggests that no one element is essential, the LS90 mutation that disrupts the sequence of the C1 region results in a drop in both basal level and stress inducibility, with its most severe effects on the glycosylation block induction and transactivation by malfolded proteins. The CCAAT element at this region is the closest to the TATA element. The next notable mutation is the -139 to -120 region encompassing C3 and its flanking sequence.

We present two models for how the transcriptional activation of grp78 is mediated (Fig. 8). Evidence accumulated so far suggests that multiple, redundant upstream regulatory elements exist in the grp78 promoter. However, the 10-bp region between -99 and -90, which contains a CCAAT element, appears to mediate both high-basal-level activity and stress inducibility. It is also apparent, from deletion studies, that this element alone is not functional. Therefore, we favor model A, in which upstream accessory proteins act in concert with the CCAAT-binding factor at -95 to provide expression of grp78 under noninduced and induced conditions. Since C1 itself is not sufficient for promoter activation and mutation in C1 alone reduces the promoter activity in spite of the presence of intact arrays of upstream elements, factors binding to C1 and upstream regulatory elements are unlikely to communicate separately with the transcriptional machineries at the TATA element, as model B depicts. However, because of the existence of the multiple C elements, it is possible that C1 is not obligatory for grp78 regulation and that a sequence sufficiently similar to C1, positioned similarly to the TATA element, can function in its place.

Recent in vitro DNase footprinting with a larger promoter

![Diagram](http://mcb.asm.org/)
fragment of the rat grp78 promoter revealed that in addition to C5 and C4, the regions around C3 and C1 are also protected (39). To search for the factor which interacts with the most essential C1 region, we focused on CCAAT-binding factors that have been identified. These include the CBF, CTF/NF-I, the CCAAT/enhancer factor (C/EBP), CP1, and CP2 (5, 26, 34, 58). The human CBF is distinct from CTF and C/EBP, but its relationship to CP1 and CP2 is unknown (34). Of these, the C/EBP protein has been the best characterized. It has been implicated in the regulation of genes involved in gluconeogenesis (35, 47). Thus, when blood glucose levels

C/EBP, but its relationship to CP1 and CP2 is unknown (34).

CTF/NF-I, and glucose starvation, the HSPs are induced with

lack of a proper activating domain for this

unable to transactivate the grp78 promoter because of the

prints the adenovirus origin of

65). By using a human clone of the CBF factor, an

expression vector for C/EBP has been unable to transac-

be induced differentially, simultaneously, and reciprocally

HSP70

interacts with a factor known as CBF (34). We tested the

sion while simultaneously promoting the

simultaneous deinduction of the GRPs (61).

It is now known that the human hsp70 CCAAT motif

CTFINF-I

motif. We tested the

ability of the cloned human CBF to stimulate grp78 promoter

activity. It has been shown previously that GRP78 and

HS70 are two subsets of eukaryotic stress proteins that can

be induced differentially, simultaneously, and reciprocally

(63, 64). Interestingly, as in the case of grp78, the regulated

basal and induced expression of the human hsp70 promoter

is mediated primarily through a CCAAT element at 

14, 65. By using a human clone of the CBF factor, an

inhibition of the grp78 promoter activity was observed. We

speculate that while this factor can bind to the C1 CCAAT element

which is similarly located within the grp78 promoter, it is

unable to transactivate the grp78 promoter because of the

lack of a proper activating domain for this promoter. Thus,

CBF, in vivo, could serve as an inhibitor for grp78 expres-

sion while simultaneously promoting the expression of

hsp70. For example, during recovery from both anaerobiosis and

glucose starvation, the HSPs are induced with the

simultaneous deinduction of the GRPs (61).

CTF/NF-I transactivates the a-globin promoter and foot-

prints the adenosine origin of replication (58). We now

show that the grp78 promoter can be transactivated with

CTF/NF-I through the C1 CCAAT motif. Assuming that this

common CCAAT-binding protein, which can bind to degener-

ative sequence elements, is the in vivo mediator for the

grp78 expression, this factor could interact with other acces-

sory proteins or coactivators (51) and account for the stress

inducibility specific for grp78. Since CTF/NF-I factors have

been shown to be glycosylated (21), this and other forms of

posttranslational modifications may explain for its specific

activity towards the grp78 promoter. The discovery of the

C1 element as an essential mediator for grp78 induction by

malfolded protein, glycosylation block, and A23187 and the

CTF/NF-I factor as a transactivator of the grp78 promoter

provides the first clue as to how the transcription of this

ER-localized protein may be regulated.

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expression plasmids and plasmid a87(+3 Ad)CAT, and S. McKeon

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REFERENCES

1. Amin, J., J. Ananthan, and R. Voellmy. 1988. Key features of


proteins serve as eukaryotic stress signals and trigger the


regulated protein (GRP78 and GRP87) genes share common

regulatory domains and are coordinately regulated by the


the 78-kDa glucose-regulated protein GRP78: its regulatory

sequences and the effect of protein glycosylation on its expres-


1988. Human CCAAT-binding proteins have heterologous sub-


6. Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A.


facilitates translocation of secretory and mitochondrial precursor


Acad. Sci. USA 80:7095-7098.


synthesis of secreted proteins induces expression of glucose-

regulated proteins in butyrate-treated Chinese hamster ovary


Steinhardt. 1987. Depletion of intracellular calcium stores by

calcium ionophore A23187 induces the genes for glucose-regu-

lated proteins in hamster fibroblasts. J. Biol. Chem. 262:12801-

12805.


sensitive Chinese hamster cell mutant with a defect in glycopro-

tein synthesis: accumulation of the EGFl receptor in the endo-

plasmic reticulum and the role of the glucose-regulated protein


sion of wild-type and mutant forms of influenza hemagglutinin:


12. Gluzman, Y. 1981. SV40-transformed simian cells support the


genomes which express chloramphenicol acetyl-


TATA-independent function of the basal and heat shock ele-

ments of a human hsp70 promoter. Mol. Cell. Biol. 10:1319-

1328.


Expression and regulation of Escherichia coli lacZ gene fusions


17. Hendershot, L. M. 1990. Immunoglobulin heavy chain and

binding protein complexes are dissociated in vivo by light chain


immunoglobulin heavy-chain-binding protein with the 78,000-

dalton glucose-regulated protein and the role of postransla-


4:4250-4256.


acid analogues or puromycin rapidly synthesize several poly-


20. Hurtley, S. M., D. G. Bole, H. Hoover-Litty, A. Helenius, and


