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Rat gene encoding the 78-kDa glucose-regulated protein GRP78: Its regulatory sequences and the effect of protein glycosylation on its expression

(promoter/leader peptide/calcium ionophore A23187/temperature-sensitive mutation/stress protein)

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ABSTRACT **The 78-kDa glucose-regulated protein GRP78 is a stress-inducible protein ubiquitously expressed in animal cells. In this paper we show that the first exon of this endoplasmic reticulum-localized protein consists of an 18 amino acid leader sequence rich in hydrophobic residues, followed by a highly acidic mature N-terminus and an 11 amino acid domain that is shared by members of the 70-kDa heat shock protein family. The end of this shared domain also marks the beginning of the first intron of this gene. A DNA region upstream of the promoter element important for induction by calcium ionophore and by a temperature-sensitive mutation was identified by deletion analysis. Our results indicate that a region spanning from 85 to 480 nucleotides upstream of the major transcription initiation site is important for both induction conditions. With evidence suggesting that perturbations in protein glycosylation may be one of the common stimuli involved in transcription activation of the GRPs, we measured the rate of glycosylation during A23187, glucose starvation, and temperature-shift induced conditions. The inverse correlation observed between the rate of glycosylation and the steady-state level of the GRP78 transcripts lends support to this hypothesis.**

Animal cells respond to glucose deprivation by synthesizing a set of proteins known as the glucose-regulated proteins, GRPs (1). These proteins were found to be induced by a variety of other stress conditions (2–4). In addition, treatment of cells with the calcium ionophore A23187 induced a 30-fold increase in the rate of transcription of the gene encoding the 78-kDa glucose/calcium-regulated protein GRP78 (5).

As a member of the stress protein family, GRP78 shares a common peptide domain near the N-terminal region with four proteins of the 70-kDa heat shock (HSP70) family (6). These proteins are related immunologically and possess the ability to bind ATP (3). Recent evidence has suggested that GRP78 may be identical to a protein known as BiP that is bound to immunoglobulin heavy chains in pre-B cells (7). Since GRP78 is found in many cell types (2, 5) and both GRP78 and BiP have been localized to the endoplasmic reticulum (ER) (8, 9), it has been postulated that although GRP78 may function to prevent aggregation of immunoglobulin heavy chains in pre-B cells, it may serve a more general role in the assembly and stabilization of secreted and membrane-bound proteins in the ER of many different cell types.

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We have been interested in the molecular control mechanism of the genes coding for GRPs, in particular GRP78, when cells in culture are subjected to glucose starvation (10) or calcium-ionophore treatment (5). Other studies in this laboratory have focused on induction of the GRPs in a temperature-sensitive (ts) hamster mutant cell line, K12, that overproduces these proteins at the nonpermissive temperature (11, 12). In these three cases, transcriptional regulation of the gene encoding GRP78 has been established. Gene-fusion experiments indicated further that DNA sequences upstream of the rat GRP78 gene could confer regulation to heterologous genes after glucose starvation (13), after incubation at elevated temperature (40°C) in K12 mutant cells (13), or in the presence of calcium ionophore (5, 14).

While the promoter of the rat GRP78 gene has been characterized as highly active and has enhancer-like properties (14), very little is known about the nature of the transacting regulatory elements involved in transcriptional activation of this gene. Several lines of evidence suggest that glycosylation may be involved. First, GRP78 was induced when cells were subjected to glucose starvation (1, 10), treatment by tunicamycin (15), or infection by glycoprotein-containing viruses (16, 17). These conditions all interfere with cellular glycoprotein synthesis and produce perturbations in the cell membrane. Calcium-ionophore treatment causes severe disruption of the intracellular calcium stores and this could also affect the process of glycosylation occurring in the ER. Moreover, the ts lesion in mutant K12 cells has been shown to affect the transfer of oligosaccharide cores from the lipid carrier to the nascent polypeptides in the ER, resulting in a much lower rate of protein glycosylation in K12 cells under nonpermissive conditions (18–20).

In this paper, we report the sequence of the promoter and first exon of the rat GRP78 gene. Direct comparison of the amino acid sequence deduced from DNA sequencing of the putative GRP78 genomic clone (13) and that previously obtained by direct amino acid sequencing of the N-terminus of the hamster GRP78 (4) confirmed the authenticity of the genomic clone. An intron is located immediately after the short peptide domain shared by several stress proteins from various species (6). In addition, a DNA region upstream of the promoter element that was important for calcium-ionophore and K12 ts inductions was identified by deletion analysis. From comparisons of the rates of protein glycosylation during several induced and noninduced conditions, we

Abbreviations: CAT, chloramphenicol acetyltransferase; ER, endoplasmic reticulum; GRP, glucose-regulated protein; GRP78, 78-kDa GRP; HSP70, 70-kDa heat shock protein; ts, temperature-sensitive; nt, nucleotide(s).

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postulate that under various induction conditions the block of glycosylation may generate a common signal resulting in enhanced transcription of the GRP78 gene.

MATERIALS AND METHODS

Cells and Culture Conditions. Culture conditions for K12, a ts mutant derived from Chinese hamster lung fibroblast line Wg1A, have been described (21, 22).

Plasmids. *pI10.* The construction of plasmid pI10 has been described (5). Essentially, a 1.25-kilobase (kb) *Pst* I–*Bssh*II fragment of the 5′ flanking sequence of the rat GRP78 gene was fused 5′ to the bacterial chloramphenicol acetyltransferase (CAT) gene contained within pSV0cat.

pK, pA2, and pE43. These plasmids were constructed in the same way as pI10, but with a longer BAL-31 digestion (5). Essentially, the pUC6.2 plasmid (13) was treated with BAL-31 nuclease at 37°C for 2 min in the case of pK, for 15 min in the case of pA2 and pE43.

p3K, p2A, and p3H. These plasmids were derived from pA2, which contains 740 nucleotides (nt) of 5′ flanking sequence of the rat GRP78 gene fused to the CAT gene. To delete further into the 5′ flanking sequence, pA2 was first digested with *Sma* I (Fig. 3) and then treated with BAL-31. To eliminate the sequence upstream of the *Sma* I site, the plasmids were further treated with *Nde* I, which has a recognition site located within pSV0cat. Fragments containing various 5′ deletions and the CAT gene were recovered from low-melting-point agarose gels and purified with Elutip-d columns (Schleicher & Schuell). After treatment with the Klenow fragment of DNA polymerase I and bacteriophage T4 DNA ligase, these recircularized plasmids were used to transform *Escherichia coli* HB101 cells (23). The lengths of the 5′ flanking sequence of the rat GRP78 gene contained within the CAT constructs were determined by restriction mapping. The 3′ endpoint for all these constructs was the *Bssh*II site, which is located about 20 nt downstream of the TATA sequence and 40 nt upstream from the major transcription initiation site of the rat GRP78 gene (see Fig. 1).

These plasmids were used to transfect K12 cells. Conditions for transfection and assay for CAT activity have been described (5, 20, 24).

DNA Sequencing. DNA was sequenced by the Maxam–Gilbert chemical method (25) and the dideoxy method of Sanger *et al.* (26). The DNA fragments used were isolated from a subclone of the rat GRP78 gene, pUC6.2 (13), and represented overlapping regions for accuracy (27).

Glycosylation Measurements. The rate of incorporation of radioactive fucose into glycoproteins was measured as described (19, 28), with the following modifications. The cells were seeded in 30-mm dishes at 35°C in Dulbecco's modified Eagle's medium (DMEM). For cells subjected to A23187 and elevated-temperature treatments and the corresponding control cells, cultures that had reached 90% confluence were used. For glucose-starvation treatment (since a longer test period was required), both control and experimental cells were at 70% confluence at the beginning of the treatment (time 0). At this point, sets of cells were changed to either fresh DMEM (1 ml per dish), fresh DMEM with 7 μM A23187, or glucose-free medium (10). One set of cells in DMEM was shifted to 40°C. At various times thereafter, [6-^{3}H]fucose (New England Nuclear, specific activity 86.3 Ci/mmol; 1 Ci = 37 GBq) was added (2 μCi per dish) and the incubation was continued for 1 hr. The cells were then washed twice with phosphate-buffered saline and resuspended in 1 ml of 0.1 M Tris·HCl, pH 6.8/10% glycerol/2% $NaDodSO_4$. Total radioactivity in the lysate was determined by direct counting of an aliquot of the suspension. For determination of acid-precipitable counts, trichloroacetic acid was added to a final concentration of 10% (wt/vol). After 10 to 15 min on ice, the precipitate was collected on Whatman GF/C filters and washed with 10% trichloroacetic acid and 95% ethanol. Radioactivity on the dried filters was measured in a scintillation counter. The incorporation of the labeled fucose into glycoprotein was determined by the fraction of radioactivity recovered in acid-precipitable material.

RESULTS

The 5′ Sequence of the Rat GRP78 Gene. The DNA sequence of the promoter region, the 5′ untranslated sequence, and the first exon of the rat GRP78 gene is shown in Fig. 1. As in other eukaryotic genes, there is a "TATA" promoter element, but, in this case, it is flanked by four CCAAT sequences within 300 nt upstream. Besides having strong promoter activity, the 291-nt *Sma* I–*Stu* I fragment immediately 5′ to the TATA sequence can act as an enhancer to increase the transcriptional activities of heterologous promoters (14). The transcription initiation site was mapped by primer extension (14) using a synthetic oligonucleotide (24 nt) homologous to a region within the first exon and was further defined by using another 17-nt synthetic primer within the 5′ untranslated region (ref. 27 and data not shown).

Estimating from this transcription initiation site, the rat GRP78 transcript has a 5′ untranslated region of about 200 nt. The first 18 amino acid sequence consisted of a leader sequence rich in hydrophobic residues. Recent data indicate that this leader sequence is functional and may target the mature protein into the ER (7).

The cleavage point for the signal peptidase occurred between alanine and glutamic acid, generating an N-terminal

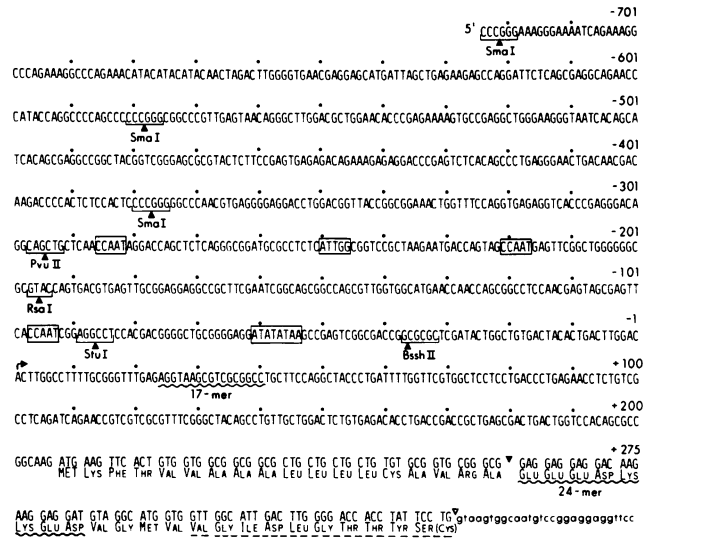

FIG. 1. Sequence of the GRP78 promoter and its first exon. The sequence is numbered starting from the major mRNA cap site (↷) as +1. Bases downstream of this site are numbered positively and bases upstream are numbered negatively. The wavy lines show the 17-mer and 24-mer used in primer-extension analyses (14, 27) to delineate the cap site. Some restriction sites of importance are noted. The CAATT and TATAA regions are boxed. The first exon of the GRP78 protein sequence is translated from the DNA sequence. Black triangle (▼) indicates the proteolytic cleavage site that excises the 18 amino acid leader sequence and produces the N-terminus of the mature protein. Open triangle (▽) indicates the 5′ splice site of the first exon/intron junction. Dashed line shows the conserved peptide shared with four other stress-induced proteins (6). The last amino acid prior to the intron junction is likely to be cysteine, which is found in other stress proteins and is compatible with TG as the first two bases of the codon. Intron sequence is given in lowercase.

sequence homologous to that previously determined by direct amino acid sequencing of the mature hamster GRP78 (4) and that derived from cDNA sequencing (7). This directly confirmed that this genomic clone encodes the rat GRP78 protein. Comparing these sequences, we detected a base transition, from cytosine to thymine at nucleotide 292, changing the predicted amino acid from threonine to methionine. However, this change could have arisen during cloning.

The N-terminal sequence of the mature GRP78 protein is highly acidic, with six of eight residues either glutamic acid or aspartic acid. Immediately following this sequence, there is a stretch of 11 residues that is shared with stress-inducible proteins related to the heat shock gene family (6). The end of this shared domain marks the beginning of the first intron of the rat GRP78 gene (Fig. 1). This strongly suggests that the shared domain in the first exon serves a common function among the stress-inducible proteins.

Deletion Analysis of the 5′ Flanking Sequence. To identify the DNA region that is important for calcium-ionophore regulation of the rat GRP78 gene, we constructed 5′ deletions of the promoter region by treatment with the nuclease BAL-31. The 3′ endpoint of all the deleted fragments was the *Bss*hII site, which is about 20 nt downstream from the TATA sequence (Fig. 1). Fusion of these 5′ regions of various lengths to the bacterial CAT gene yielded a series of recombinants containing from 1250 to 45 nt of the 5′ flanking region upstream of the *Bss*hII site of the rat GRP78 gene. These recombinants were transfected into hamster K12 cells. After 28–32 hr, the cells were either treated with 7 μM calcium ionophore A23187 or maintained in the regular culture medium. Cell extracts were prepared and assayed for CAT enzyme activity (Fig. 2). As the 5′ sequences were deleted, both the basal and A23187-induced levels decreased. The percent conversion of [^{14}C]chloramphenicol to its acetylated forms was quantitated (Fig. 3). By comparing the percent of conversion at the induced state with the basal level, the induction ratio can be calculated for each of the deletion constructs (Table 1). Our data indicate that a region 500 nt upstream of the *Bss*hII site was most important for A23187 induction: deletion mutants within this region resulted in a factor-of-8 decrease in the A23187-inducible CAT activity.

It is also evident that deletions in the 5′ region reduced the basal level of the promoter activity. A 90% reduction in the basal expression was detected as the 5′ flanking sequence of the rat GRP78 was reduced from 1250 nt to 45 nt. This reduction is not likely due to the pBR322 sequences brought into closer proximity to the CAT gene, since this effect was not observed in deletion constructs with another eukaryotic promoter (S. Wells and A.S.L., unpublished results). In addition, since the 291-nt *Sma* I–*Stu* I fragment (Fig. 3) has been shown to contain enhancer-like activities (14), the reduction in both basal and inducible promoter activity can be partially explained by the removal of this element. The shortest construct, p3H, which retained weak but detectable promoter activity but did not respond to either A23187 or temperature induction (see below), contained the TATA sequence but not CCAAT sequences.

When the hamster fibroblast ts mutant K12 is incubated at the nonpermissive temperature (40°C), the transcriptional rate of the GRP78 gene is increased about 10-fold within several hours (12). To test whether the K12 ts mutation and the calcium-ionophore treatment involved the same regulatory mechanisms, we compared the DNA sequences in the promoter region that were involved in the two different induction responses. The deletion constructs described above were transfected into K12 cells, and the levels of CAT activity at the permissive (35°C) and nonpermissive (40°C) temperatures were determined (Figs. 2 and 3). By comparing the increase over the basal level, a region ranging from −480 to −85 nt was found to be most important for A23187 and K12 ts induction (Table 1).

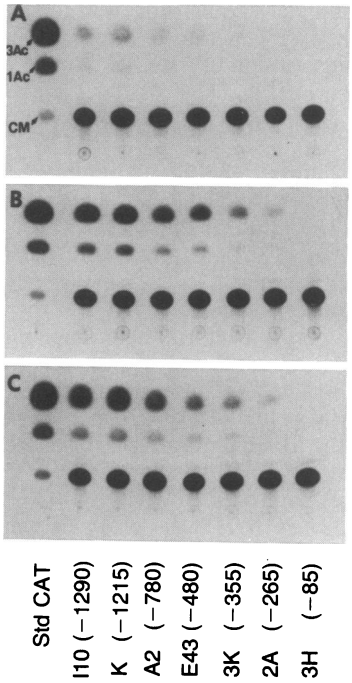

FIG. 2. Promoter activities of the 5′ deletion CAT constructs. K12 cells were transfected with 3 μg of cesium chloride gradient-purified pI10, pK, pA2, pE43, p3K, p2A, or p3H, which contained various lengths of the 5′ flanking sequence of the rat GRP78 gene fused 5′ to the CAT gene (5, 20, 24). (*A*) Cells were continuously incubated at 35°C in regular DMEM. (*B*) Twenty-eight to thirty-two hours after transfection, DMEM supplemented with 7 μM A23187 was added to the cells and incubation was continued at 35°C for 16 hr. (*C*) Twenty-eight to thirty-two hours after transfection, the cells were shifted to 40°C and incubated for 16 hr. Cell extracts were prepared 48 hr after transfection. Equal amounts of protein (75 μg) from each sample was assayed for CAT activity (5). The autoradiograms are shown. Assay performed with 0.08 unit of CAT (P-L Biochemicals) is also shown. Positions of chloramphenicol (CM) and its acetylated forms (3Ac and 1Ac) are indicated.

Correlation Between Rate of Protein Glycosylation and GRP78 mRNA Levels. Previous studies have established that when K12 cells are incubated at 40°C, the incorporation of radioactive sugars into glycoproteins is drastically reduced (19). The K12 ts lesion has recently been shown to affect the transfer of oligosaccharide cores from the lipid-oligosaccharide intermediates to the nascent polypeptide chain (20).

To test directly whether glycosylation is blocked under conditions that induce the GRP78 gene, the rate of protein glycosylation was monitored during induction by A23187, K12 ts mutation, and glucose starvation. As in previous studies, the incorporation of fucose into glycoprotein was monitored because of its direct incorporation into glycoproteins (29). At the same time, the kinetics of induction of GRP78 transcripts were determined. Our results (Fig. 4) confirmed previous observations that elevated temperatures (40°C) reduced the rate of fucose incorporation into glycoprotein by a factor of about 3 within 3 hr. While we also confirmed the previous observation that the GRP78 transcripts accumulated to various levels with different kinetics under the three induction conditions, there was an inverse correlation between the rate of glycosylation and the steady-state level of GRP78 transcripts. In each case, we observed that the change in glycosylation preceded the change in GRP78 mRNA concentration. In contrast, neither GRP78 mRNA levels nor the rate of glycosylation was affected in control cells incubated continuously at 35°C (Fig. 4).

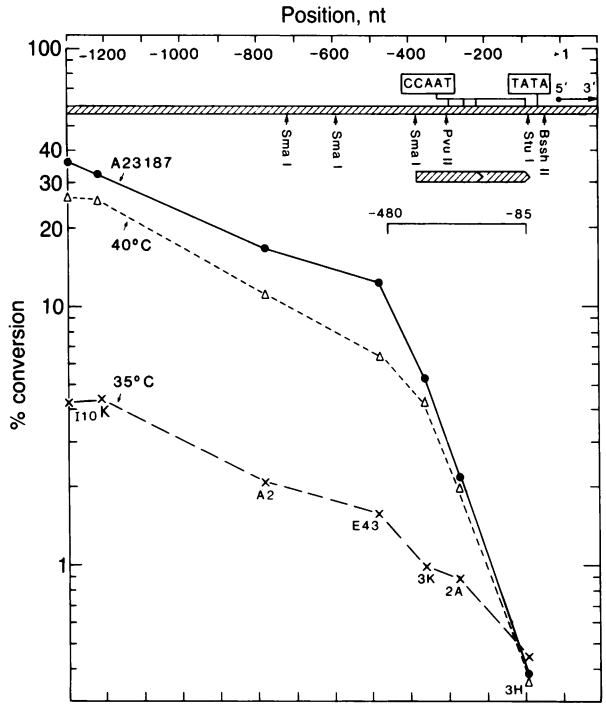

FIG. 3. Regions of the rat GRP78 promoter important for A23187 and K12 ts mutation induction. Percent conversion of [^{14}C]chloramphenicol to its acetylated forms in various 5′ deletion CAT constructs presented in Fig. 2 were quantitated as described (5). These values were plotted against the position of each of the 5′-deletion constructs along the promoter sequence. X, Incubation at 35°C; ●, incubation in DMEM containing A23187; △, incubation at 40°C. Results are summarized in Table 1. The *Sma* I–*Stu* I fragment previously identified as an enhancer element with two repeat domains that could confer partial A23187 inducibility to a heterologous promoter and marker gene is represented by two hatched arrows below the restriction map. Region important for A23187 and K12 ts mutation induction is bracketed (−480 to −85).

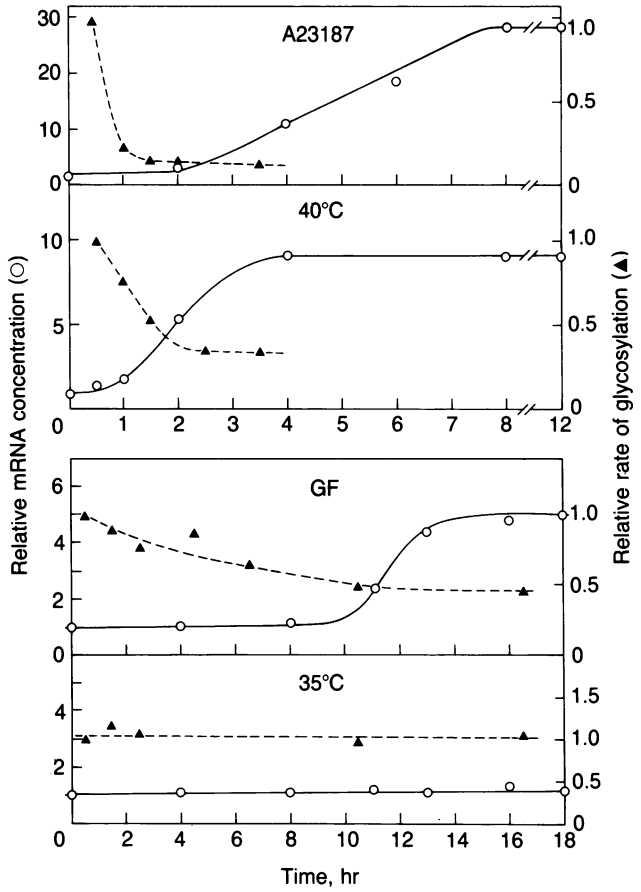

FIG. 4. Correlation between protein glycosylation and GRP78 mRNA levels in K12 cells. K12 cells were subjected to four different culture conditions, and the rates of glycosylation (▲) and levels of GRP78 mRNA (○) were determined. At various times during treatment, total cytoplasmic RNA was extracted. Ten micrograms of RNA from each time point was applied to denaturing (formaldehyde/formamide) agarose gels, electrophoresed, and blotted onto nitrocellulose filters (5, 10). Filters were probed with nick-translated p3C5, a cDNA clone coding for the hamster GRP78 protein (11, 12). Filters were then washed, dried, and exposed to Kodak X-Omat AR films at −70°C. The autoradiograms, exposed within the linear range of the film, were quantitated by densitometry. The relative GRP78 mRNA levels were plotted against the time of treatment. For the glycosylation measurements, the incorporation of [6-^{3}H]fucose into trichloroacetic acid-precipitable glycoproteins was measured as relative values of acid-precipitable radioactivity divided by total radioactivity. Points are plotted at the midpoint of the labeling period. Value obtained for the first time point was set as 1. Treatment conditions are indicated.

DISCUSSION

Current investigation into the molecular control mechanisms of the GRP78 (or BiP) gene has provided clues concerning the cis-regulatory elements and the possible role played by glycosylation in its induction in fibroblasts, where its expression is as abundant as in the case of plasma cells (ref. 7; T.N., R. J. Deans, and A.S.L., unpublished data).

At the protein level, it has been established that GRP78 shares a common peptide domain with other members of the 70-kDa stress protein family at the N-terminus (6). However, GRP78 is also unique in that it possesses a leader peptide. This leader sequence was predicted from *in vitro* translation of the cytoplasmic RNA (11). It has now been confirmed in this study by direct sequencing of the rat GRP78 genomic clone and in another study (7) where an independently isolated rat cDNA clone likely to code for GRP78 was sequenced. The latter study further showed that the leader sequence was functional and might target the GRP78 into the ER. If GRP78 and the immunoglobulin heavy chain binding protein BiP are similar or identical proteins, the absence of a hydrophobic transmembrane sequence within the predicted GRP78 mature protein sequence (7) agrees with the finding that BiP is a soluble protein within the ER (9). The occurrence of an intron immediately following the short shared N-terminal domain suggests that a special function served by this domain may be common to the stress proteins such as the glucose/calcium-regulated proteins and the heat shock proteins. It is notable that while the HSP70 gene shares about 60% sequence homology with the GRP78 gene (7), the former is devoid of intervening sequences. In contrast, the rat GRP78 gene contains several introns (13). Conceivably, a block in RNA splicing would not affect the synthesis of HSP70 but would affect the synthesis of GRP78. Since GRP78 itself does not contain the sequence required for

Table 1. Effect of 5′ deletions on CAT activity

Plasmid	Deletion endpoint	Fold increase over basal level	
		A23187	40°C
pI10	−1290	8.6	6.4
pK	−1215	8.2	6.3
pA2	−780	7.9	5.2
pE43	−480	7.8	4.0
p3K	−355	5.2	4.5
p2A	−265	2.2	2.4
p3H	−85	0.78	0.78

N-linked glycosylation (7) and is not labeled by radiolabeled sugars (3), it is probably not glycosylated. Therefore, a block in glycosylation may not affect its own synthesis directly. These salient features may define the roles played by these proteins under different stress conditions.

At the gene level, the promoter region of the rat GRP78 contains features common with other eukaryotic promoters, with the presence of the TATA and CCAAT sequences and a preferred transcription initiation site. Therefore, although GRP78 is expressed in many cell types (2, 5), it differs from the general characteristics of some housekeeping genes in that these genes lack TATA and CCAAT sequences and/or have multiple transcription start sites (30, 31).

The promoter region of the rat GRP78 gene has enhancer-like properties (14). It contains tandem-repeat domains and shares sequence homologies to other cellular and viral enhancers. Two blocks of sequence (11 nt) are also common between the rat GRP78 and human HSP70 promoters (14, 32, 33). Recently, enhancer activity was also detected for the *Drosophila* heat shock element (34). When a 291-nt subfragment from the rat GRP78 promoter is fused to a heterologous marker gene, it can substantially increase the basal level expression of this gene. Upon treatment with A23187 and shift to 40°C in K12 cells, this fragment can cause further increase in the transcript levels of the marker gene. Our deletion analysis into the promoter provides further evidence that essentially the most important control regions for these two induction conditions occur within the 291-nt fragment.

As for the regulation by glucose starvation, a strong response was observed in stable transformants harboring a hybrid gene containing about 6 kb of the GRP78 gene including some of the intervening sequences (13). In later constructs with the CAT gene as described here, we observed that the glucose-starvation response in transient transformants was at most 2-fold above the basal level (S.C.C. and A.S.L., unpublished results). In stable transformants of the CAT constructs, enhanced levels of CAT mRNA were observed as long as about 480 nt of the 5′ flanking sequence was present (S. Wells, A. Grafsky, J.W.A., and A.S.L., unpublished results). Based on these observations, it is most likely that regulatory elements induced by A23187 treatment, the K12 ts mutation, and glucose starvation interact with overlapping, if not identical, domains at the promoter region, suggesting that at least some of the regulatory factors involved may be common to all three stress conditions.

If such common factors do exist, it is possible that blockage in glycosylation may trigger the synthesis of a regulatory element which in turn increases the transcriptional activities of the GRPs (35). Both biochemical and genetic evidence lend support to this view. First, it has been shown that GRP78 was induced to variable extents upon treatment with 2-deoxyglucose or tunicamycin or upon paramyxovirus infections (7, 15–17), all of which result in blocking normal cellular glycosylation. We also observed a large increase in the steady-state level of p3C5 transcripts when cells were treated with tunicamycin (Y.K.K. and A.S.L., unpublished results). Second, two mutant cell lines defective in glycosylation have now been shown to overproduce the GRPs. One is the hamster fibroblast line K12 used in this study; it is known to be defective in the transfer of oligosaccharide cores to polypeptides (20). Another is a mutant mouse cell line which is unable to synthesize *N*-acetylglucosamine needed for the oligosaccharide core (36). Moreover, revertants of K12 cells, or cell hybrids that no longer overproduced the GRPs, also reverted their block in glycosylation at 40°C. Other induction conditions, such as treatment with 2-mercaptoethanol (37), also block glycosylation (unpublished results).

These observations support the hypothesis that the presence of underglycosylated proteins caused by either glucose starvation, calcium-ionophore treatment, or the K12 ts mutation may generate a signal leading to increased transcriptional activity of the GRP78 gene. To define the role of glycosylation in the transcriptional activation of the GRP genes, it will be necessary to isolate and characterize the trans-acting factors mediating the induction response. The fact that the 291-nt *Sma* I–*Stu* I fragment from the rat GRP78 promoter could compete for the regulatory factors *in vivo* during A23187 and K12 ts induction (14) suggests the existence of at least a positive stimulatory mechanism.

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