MOLECULAR AND CELLULAR BIOLOGY, July 1991, p. 3446–3453
0270-7306/91/073446-08$02.00/0

Vol. 11, No. 7

Competitive Inhibition of a Set of Endoplasmic Reticulum Protein Genes (*GRP78*, *GRP94*, and *ERp72*) Retards Cell Growth and Lowers Viability after Ionophore Treatment

XUAN LI AND AMY S. LEE*

Department of Biochemistry and Norris Cancer Hospital and Research Institute, University of Southern California School of Medicine, Los Angeles, California 90033

Received 6 February 1991/Accepted 4 April 1991

GRP78, a 78-kDa protein localized in the endoplasmic reticulum (ER), has been implicated in protein processing and stress protection. Its promoter contains a 36-bp region which is conserved among *GRP* genes across species and has the ability to compete for *trans*-acting factors mediating *GRP* gene expression. Integration of about 800 tandem copies of this sequence into the genome of a Chinese hamster ovary cell line (DG44) results in transfectants with the following phenotypes: (i) the induction level of *GRP78* by the calcium ionophore A23187 and tunicamycin is reduced 4- and 2-fold, respectively, (ii) the induction levels of two other ER luminal protein genes, *GRP94* and *ERp72*, are simultaneously down-regulated, (iii) the growth rate of these cells is half that of transfectants without the amplified sequence, and (iv) cell viability is decreased by 25-fold after A23187 treatment. These results provide new evidence that *ERp72* shares common *trans*-acting regulatory factors with the *GRP* genes and that a reduction of this set of ER proteins correlates with lower viability after ionophore treatment.

The endoplasmic reticulum (ER) consists of complex systems of intracellular membranes and is the site for the synthesis and processing of membrane and secretory proteins. Several resident ER proteins have been identified which share some interesting properties. GRP78, a 78-kDa protein also referred to as BiP, is known to associate transiently with nascent proteins, assisting in their assembly and transport (3, 16, 17). It can also block the transport of a variety of underglycosylated, improperly folded protein precursors by binding stably to them (10, 13, 15, 18, 20). GRP94, a 94-kDa protein, is a major ER glycoprotein. Both GRP94 and GRP78 have been identified as calcium-binding proteins (23). As such, the GRPs are implicated in the mobilization and regulation of sequestered calcium, resulting in the facilitation of ER protein transport (34) and cellular adaptation to sufficient rate of protein synthesis during chronic stress (5). Recently, a new ER luminal protein, ERp72, has been identified (30). This 72-kDa protein contains three copies of the active-site sequence of another ER protein, protein disulfide isomerase. While the function of ERp72 is not yet defined, protein disulfide isomerase is a multifunctional protein involved in the modification of nascent secretory proteins (14). Specific retention of these proteins may act through a receptor which recognizes the terminal peptide KDEL or its related sequence KEEL shared by these ER proteins. Recently, such a receptor has been isolated from yeast cells (27) and identified in mammalian cells (39).

The expression of GRP78 and GRP94 is regulated primarily at the transcriptional level (25). Under normal growth conditions, the *GRP* genes are transcribed at a basal level. During stress, the transcription rate of the *GRP* genes is greatly enhanced, resulting in a large increase of the GRPs in the ER (24). The most potent inducers of the *GRP* genes include the calcium ionophore A23187 (31) and tunicamycin, a nucleoside antibiotic which blocks N-linked glycosylation by inhibiting the formation of the core oligosaccharide. The GRPs are induced by A23187 through the depletion of intracellular, sequestered calcium (12). As a consequence of calcium leakage from the ER, protein glycosylation is blocked, and the *GRP* genes are transcriptionally activated (6). Interestingly, ERp72 is reported to be inducible by A23187 and tunicamycin (11).

A powerful approach to examining the physiological roles served by a gene in vivo is to study the consequences of deleting the gene. In yeast cells, GRP78 is encoded by the *KAR2* gene. Its deletion results in a recessive lethal mutation, showing that the gene is required for cell viability (33). In temperature-sensitive mutants, protein precursors accumulate on the cytoplasmic side of the ER membrane, apparently unable to translocate into the ER (40). In mammalian cells, conditional mutants which block protein glycosylation have been isolated, and GRP78 and GRP94 are overproduced (24, 26). By transfection of antisense *GRP78*, Chinese hamster cell lines have been established with two- to threefold reduction in GRP78 levels. These cells secrete higher levels of a recombinant protein which has been shown to associate with GRP78 (9).

Another approach is to modulate gene expression through competitive inhibition of transcription. A polymer of 40 tandem copies of the heat shock element is shown to inhibit expression of cotransfected heat shock genes (41). Further, by amplifying thousands of copies of the 5′ control region (spanning −250 to +470) of the *hsp70* gene, the heat-induced expression of the 70-kDa heat shock protein is much reduced, leading to the loss of thermal tolerance of these cells (19). Through 5′ deletion of the *GRP78* promoter, we have previously identified a 36-bp region spanning −135 to −170 which is important for both basal-level expression and inducibility by A23187 (32). This domain is conserved among *GRP* genes across species and contains protein binding sites. In gel mobility shift assays, a synthetic oligomer of this 36-bp

* Corresponding author.

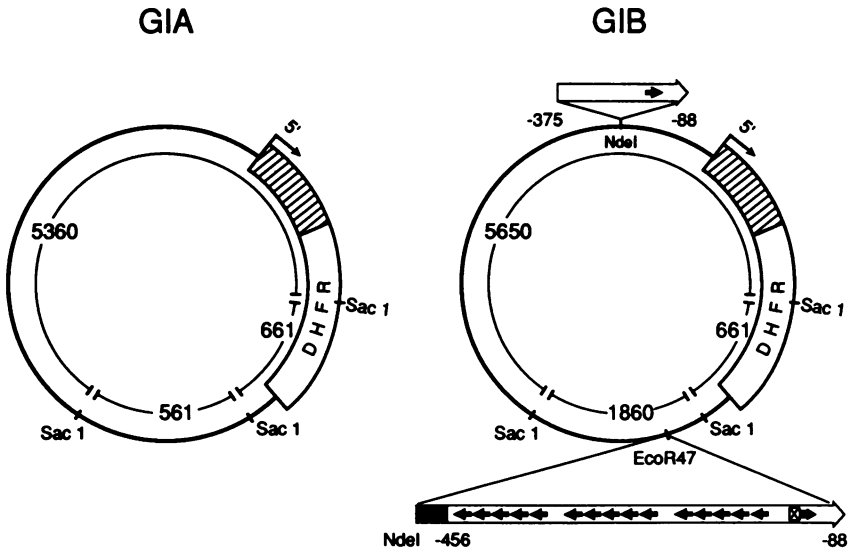

FIG. 1. Structure of the amplification plasmids. Plasmid GIA contains a dicistronic (adenosine deaminase [▨] and DHFR [□]) transcriptional unit. GIB is a derivative of GIA with *GRP78* promoter sequences inserted at the *Nde*I and *Eco*R47 sites. Each solid arrow represents one copy of the *GRP* core sequence (−135 to −170). The insert at the *Nde*I site contains one copy of the *GRP* core within the *GRP78* promoter spanning −88 to −375. The insert at the *Eco*R47 site is a 1,300-bp fragment containing 15 tandem copies of the *GRP* core in the orientation indicated, within a *GRP78* promoter fragment spanning −88 to −456. The hatched box represents a 10-bp mutation at −160 to −169. The stippled bar indicates vector sequence from plasmid pSV2CAT. The *Sac*I restriction sites and sizes (in base pairs) of the *Sac*I fragments are indicated.

sequence can compete for binding specific protein-DNA complexes formed by using *GRP78* or *GRP94* promoter fragments (7). In cotransfection studies, this same sequence has the ability to abolish the *trans*-acting factors mediating the transcription of the *GRP78* and *GRP94* promoters under both induced and noninduced conditions (7). On the basis of these observations, we tested whether amplification of this *GRP* conserved sequence can modulate expression of *GRP78* and possibly other coordinately regulated ER genes. We report here that integration of amplified tandem arrays of this sequence into the genome of a CHO cell line (DG44) reduced the transcript levels not only of *GRP78* but also of *GRP94* and *ERp72*. Compared with similar transfectants without the amplified *GRP* sequence, the cells exhibit a twofold reduction in growth rate and a severe drop in cell viability after A23187 treatment.

MATERIALS AND METHODS

Cell lines and culture conditions. DG44 is a double mutant of a Chinese hamster ovary cell line that contains no copies of the hamster dihydrofolate reductase (DHFR) gene (38). These cells, obtained from Lawrence Chasin (Columbia University), are maintained in minimal essential alpha medium containing L-glutamine, ribonucleotides, and deoxyribonucleosides (GIBCO Inc., Grand Island, N.Y.), supplemented with 10% fetal calf serum. The DHFR$^+$ transformants can be selected on the same medium without the nucleosides and with dialyzed serum (alpha-minus).

Plasmids. The DHFR expression plasmid, GIA, was kindly provided by Andrew Dorner and Randal Kaufman (The Genetics Institutes, Cambridge, Mass.). The construction of this plasmid, referred to as pMT2-ada, has been previously described (11, 21). The plasmid GIB is constructed by inserting a 288-bp *Stu*I-*Sma*I fragment of the *GRP78* promoter (29) into the *Nde*I site of GIA and a 1,300-bp *Stu*I-*Nde*I fragment containing 15 copies of the *GRP78* promoter sequence (−135 to −170) in a tandem array (Fig. 1). The tandem array was first generated by ligation and subcloning the synthetic oligomer (−135 to −170) bearing terminal *Xho*I and *Sac*I sites into the vector pUC13X as previously described (41). This array was then inserted into the *Sma*I site (−375) of a *GRP78*(−456)-CAT fusion gene bearing a 10-bp linker-scanning mutation at −160 to −170. This 10-bp mutation affected only slightly the *GRP78* promoter activity (40a). The orientation of the *GRP* inserts was determined by DNA sequencing. Digestion of this CAT fusion gene with *Stu*I and *Nde*I generated the 1,300-bp fragment described above.

The cDNA plasmids, p3C5, p4A3, and p3A10 encoding hamster GRP78, GRP94, and an invariant transcript, respectively, have previously been described (25). The cDNA plasmid, pGEM72, a pGEM plasmid containing the coding region of ERp72 (30), was kindly provided by Michael Green (St. Louis University, St. Louis, Mo.).

Selection and maintenance of stable transformants. For the selection of DHFR$^+$ transformants, 10 μg of GIA or GIB was transfected into DG44 cells by using the calcium phosphate precipitation method (31). The next day, the medium was changed to alpha-minus medium. After 2 weeks, surviving colonies were observed. Single colonies were picked and expanded, and 0.005 μM methotrexate (MTX; Sigma, St. Louis, Mo.) was added. Surviving colonies which appeared after 2 weeks were picked, expanded, and subjected to 0.01 and then 0.05 μM MTX. The subsequent selections at 0.1, 0.5, 1, 2, 5, 10, 50, 200, and 500 μM MTX were carried out with pooled colonies. DHFR$^+$ transfectants were maintained in alpha-minus medium with MTX.

Genomic DNA hybridization. The preparation of high-molecular-weight genomic DNA and the conditions for the blot hybridizations have been described previously (28). Here, 3 μg of each DNA sample was used. The probe for the

GRP78 promoter sequence is a 1,000-bp fragment spanning −88 to −375 and contains 10 copies of the synthetic oligomer (−135/−170) inserted at −160. The probe for the DHFR coding sequence is a 767-bp *Eco*RI-*Hep*I fragment from pMT21 (11). Both fragments were labeled by the hexamer method to specific activities of 1×10^8 to 2×10^8 cpm/μg.

RNA blot hybridization. Total cellular RNA was isolated by using the guanidium thiocyanate method as previously described (8). Conditions for the RNA blot hybridizations have been described previously (25). The cDNA fragments used as probes were GRP78, a 2,968-bp *Sal*I-*Hin*dIII fragment from p3C5 (37); GRP94, a 1,000-bp *Eco*RI-*Bam*HI fragment from p4A3 (25); ERp72, a 700-bp *Bam*HI-*Eco*RI fragment from pGEM7 (15a); and p3A10, the entire plasmid (25). The DNA fragments were labeled by the hexamer method to the specific activities of 1×10^8 to 2×10^8 cpm/μg. The band intensities of the autoradiograms were quantitated by using an LKB ultrascan XL-enhanced laser densitometer.

Protein immunoblot. The protein was probed with the anti-GRP78 peptide serum at a 1:200 dilution as previously described (28).

Growth rate and survival analysis. For measurement of growth rate in normal culture medium, 2.7×10^5 cells were seeded on each 100-mm diameter dish in duplicates. Every 24 h, the cells were trypsinized and counted on a hemacytometer for cell number. For survival analysis, 2,000 cells were seeded on each 100-mm-diameter dish in duplicates. After 24 h, the cells were treated with A23187 for 16 h. The cells were then changed to fresh alpha medium and allowed to grow for 10 to 15 days until colonies were formed. The colonies were fixed with 100% methanol and stained with Giemsa stain solution.

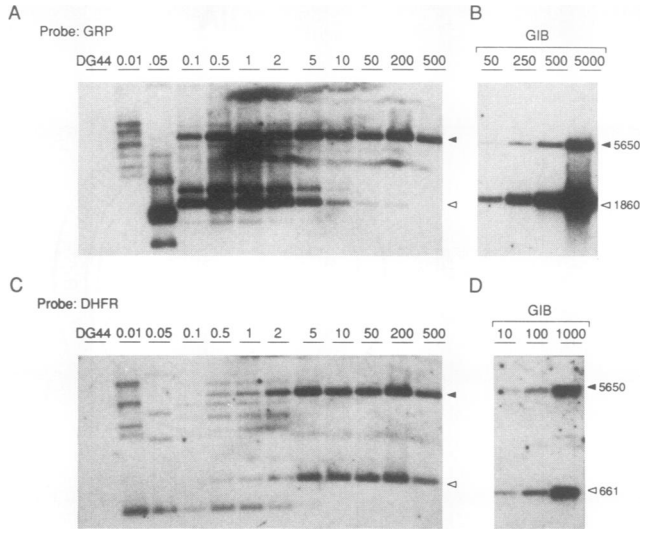

FIG. 2. Amplification of the transfected genes in DG44 cells. (A and C) Genomic DNA was isolated from DG44 cells or from GIB transfectants selected by increasing concentrations (0.01 to 500 μM) of MTX. The DNA samples were digested with *Sac*I, size separated on agarose gels, and blotted on filters for DNA hybridization. (B and D) GIB DNA was digested with *Sac*I and similarly blotted. In panel B, amounts equivalent to 50, 250, 500, and 5,000 copies of the core were applied; in panel D, amounts equivalent to 10, 100, and 1,000 copies of the DHFR gene were applied. A radiolabeled *GRP78* promoter probe (A and B) and a DHFR cDNA probe (C and D) were used. Sizes of the bands (in base pairs) are indicated at the right.

RESULTS

Construction of the amplification vector. To test whether amplification of the conserved *GRP78* domain spanning −135 to −170 (referred to below as the *GRP* core) would affect transcription of the endogenous *GRP78* gene, we devised constructs which would offer maximum competition for the *trans*-acting factors when introduced into the host cells. For amplification, we used the well-studied dihydrofolate reductase (DHFR) system (21). The structure of the DHFR expression vector (GIA) is shown in Fig. 1. The DHFR transcription unit is driven by the adenovirus major late promoter. Transcription from this vector generates a dicistronic mRNA with the 5′ open reading frame encoding adenosine deaminase and the 3′ open reading frame encoding DHFR (11, 21). Compared with a monocistronic mRNA with a more efficient translational start site, this vector requires extra amplification to produce high amounts of functional DHFR.

Next, the *GRP* sequence was inserted into this plasmid. Initially, a *GRP* promoter fragment spanning −88 to −375 was inserted at the unique *Nde*I site 5′ to the DHFR transcription unit (Fig. 1). We previously determined that this region contains most of the basal and regulatory elements for the *GRP78* promoter, including one copy of the core (22, 29). Transfection of this plasmid into a DHFR-defective cell line (DG44) (38) followed by methotrexate (MTX) selection resulted in transfectants with amplified copies of the *GRP78* promoter sequence. In these transfectants, the induced level of *GRP78* was only slightly reduced (data not shown). At the same time, we constructed another plasmid, GIB (Fig. 1), which contains an extra insertion at the *Eco*R47 site 3′ to the DHFR transcriptional unit. This extra insertion contains 15 copies of the *GRP* core in direct tandem arrays embedded within a *GRP* promoter fragment spanning −88 to −456. The rationale for this construct is threefold. First, tandem arrays of control elements are known to enhance factor binding, particularly if cooperative binding is involved. Second, introduction of 15 extra copies of the core into the plasmid would enhance the amplification number of the core sequence 15-fold. Third, by insertion at the 3′ end of the DHFR unit instead of the 5′ end, the extra sequence would offer less interference to DHFR transcription, which is needed for the selection. This plasmid was transfected into DG44 cells for further analysis.

Amplification of the *GRP* core and flanking sequence. To control for possible complications involving the effect of MTX on the phenotypes of the transfectants, DG44 cells were transfected with GIB and GIA, which is the parental plasmid without the *GRP* inserts. After selection by stepwise increases in MTX concentration, resistant colonies were picked and expanded. DNA was extracted from these cells and assayed for the copy number of the *GRP* promoter and of the DHFR gene (Fig. 2). Starting at 0.1 μM MTX, stable amplification of the *GRP* sequence as contained within GIB occurred, as evidenced by the emergence of the two *Sac*I bands (5,650 and 1,860 bp) expected from *Sac*I digestion of GIB (Fig. 1). Identical blot results were obtained with use of the synthetic core sequence (−135 to −170) as a probe (data not shown). Comparison of the band intensities of the genomic blots with plasmid GIB (Fig. 2A and B) shows that the copy numbers of the core for the transfectants selected at 0.1, 0.5, 1.0, 2.0, and 5.0 μM MTX are 450, 800, 650, 300, and 200, respectively. Therefore, the transfectants selected at 0.5 μM MTX harbor the highest copy number of GIB with

the intact tandem array of the core sequence. With increasing MTX concentration, the surviving transfectants began to lose selectively the *GRP* fragment containing 15 copies of the core (the 1,860-bp *Sac*I band; Fig. 2A), while the DHFR gene sequence showed a steady increase from 0.05 to 5.0 μM. Between 5 and 200 μM MTX, a plateau level of about 500 copies of the DHFR gene was observed, with a slight decrease at 500 μM (Fig. 2C and D). In the 0.5 μM MTX-selected transfectants, the DHFR gene was amplified about 50 times and the *GRP* core was amplified about 800 times.

Relative levels of GRP78 in MTX-resistant cell lines. Next, we examined the *GRP78* transcript levels in DG44 cells selected by MTX. Total RNA was prepared from control cells and cells treated with A23187. Examples of the RNA blot hybridizations are shown in Fig. 3A. After quantitation of the band intensities by laser densitometry and normalization against the level of an invariant transcript p3A10 (25), the relative induced mRNA levels were plotted against the *GRP* core copy number (Fig. 3B). An inverse correlation was observed between the *GRP78* core copy number and the induced *GRP78* mRNA level. Comparison between the parental DG44 cell line and all transfectants examined (Fig. 3A and data not shown) shows that the induced level of the *GRP78* transcript was lowest in transfectants selected at 0.5 μM MTX, which contained the highest number of core sequences. Correspondingly, the GRP78 protein level was decreased in MTX-selected transfectants harboring several hundred copies of the *GRP* core sequences (Fig. 3C). We noted that while the basal level of *GRP78* mRNA in the MTX-selected transfectants did not appear to be much lower than in the parental cells, the GRP78 protein levels in nontreated MTX-selected transfectants were lower than in the parental DG44 cells. This finding could be due to difficulties in comparing very low basal RNA levels or to the slight loss of the core sequence, since the cells used for the RNA extraction had been passaged about 7 to 10 days longer than the cells used for DNA and protein extraction. Thus, the competing core sequences reduce the overall noninduced and induced levels of GRP78.

To determine whether other genes were negatively affected in the DG44 transfectants, we tested the transcript levels of actin and histone H3.2 (1) in these same cells (Fig. 3B). The expression patterns of these transcripts were identical in the parental and MTX-selected DG44 transfectants. The level of actin mRNA in these cells remained relatively constant in both A23187-treated and nontreated cells. On the other hand, the histone mRNA levels were similar in all noninduced cells; however, in all A23187-treated cells, the histone mRNA levels dropped to 25% as the cells were arrested in G_1 (Fig. 3B and data not shown). These results suggest that the amplification of the *GRP* core sequence does not have a general negative effect on the regulated expression of other genes.

Next we examined the relative levels of *GRP78* transcripts in tunicamycin-treated cells. The DG44 transfectants were further propagated in culture containing MTX, and total RNAs were isolated. As in the case of the A23187-treated cells, the induced level of *GRP78* by tunicamycin was lowest in the transfectants selected at 0.5 μM MTX, decreasing to about half that of DG44 and cells transfected with GIA (data not shown).

Simultaneous reduction in *GRP94* and *ERp72* transcript levels. Since the *GRP78*, *GRP94*, and *ERp72* genes are all induced by A23187 and tunicamycin in DG44 cells, it is possible that the three genes are regulated by common

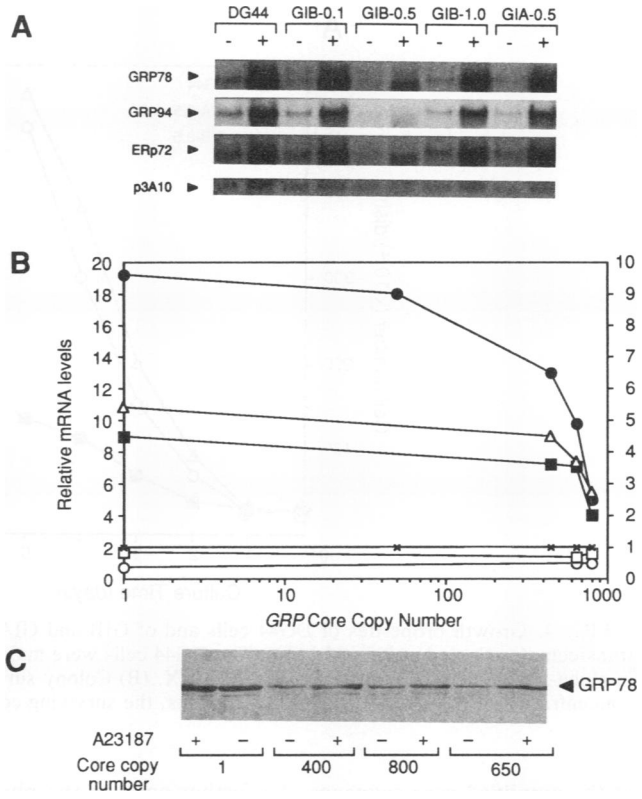

FIG. 3. (A) Relative mRNA levels of ER protein genes after A23187 treatment. Total RNA was isolated from DG44 cells, GIB transfectants were selected at 0.1, 0.5, and 1.0 μM MTX, and GIA transfectants were selected at 0.5 μM MTX. The DG44 cells were grown in alpha medium; the GIB and GIA transfectants were grown in alpha-minus medium plus MTX. At 16 h prior to RNA extraction, the cells were changed either to fresh medium (−) or to fresh medium containing 7 μM A23187 (+). Autoradiograms for the RNA blot hybridizations are shown. (B) Correlation between *GRP* core copy number and induced mRNA levels. The autoradiograms shown in Fig. 2A and 3A were quantitated by scanning densitometry. The induced mRNA levels for *GRP78* (●) are indicated by the scale on the left; those for *GRP94* (△), *ERp72* (■), p3A10 (×), actin (□), and histone H3.2 (○) are indicated by the scale on the right. The numbers 1, 50, 450, 650, and 800 correspond to the *GRP* core number in the parental DG44 cells, the 0.5 μM MTX-selected revertant, and the 0.1, 1.0, 0.5 μM MTX-selected transfectants, respectively. (C) Immunoblot of protein extracts from noninduced cells (−) and cells treated with 7 μM A23187 for 16 h (+) with antibody specific for GRP78 (28). Equal amounts (20 μg) of protein extracts were applied to all lanes.

trans-acting factors interacting with the *GRP* core sequence. To test this hypothesis, the levels of *GRP94* and *ERp72* transcripts were examined in the transfectants described above. The RNA blots, after removal of the *GRP78* probe, were rehybridized with cDNA probes encoding *GRP94* and *ERp72*. The mRNA profiles for *GRP94* and *ERp72* resembled that for *GRP78*, with the lowest induction level by A23187 and tunicamycin observed in the transfectants selected at 0.5 μM MTX (Fig. 3A and data not shown). Therefore, our results suggest that amplification of tandem arrays of the core and its flanking sequence affects not only the expression of *GRP78* but also the stress-induced expression of two other ER luminal protein genes.

Correlation between regain of A23187 inducibility and loss

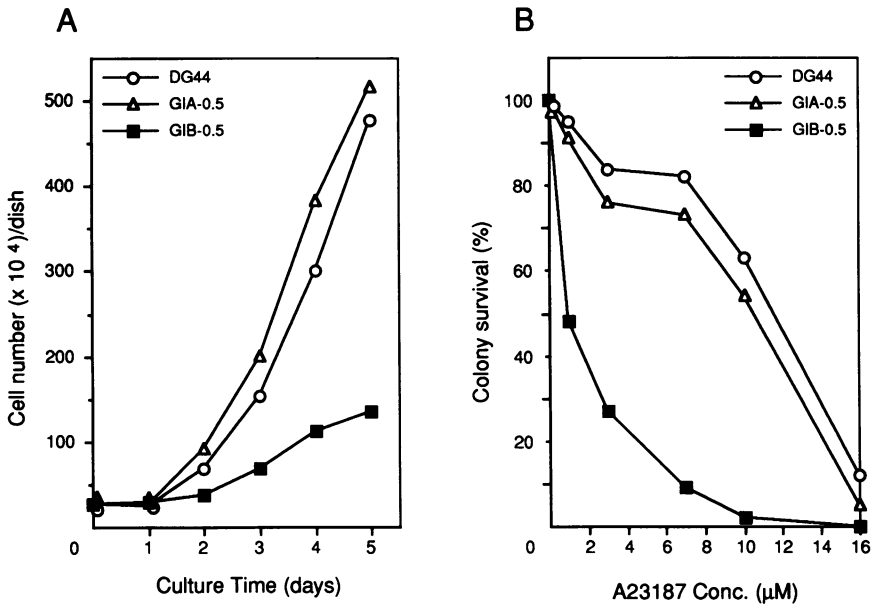

FIG. 4. Growth properties of DG44 cells and of GIB and GIA transfectants. (A) Growth curves for DG44 cells and for GIB and GIA transfectants selected at 0.5 μM MTX. The DG44 cells were maintained in alpha medium; the GIB and GIA transfectants were maintained in alpha-minus medium containing 0.5 μM MTX. (B) Colony survival analysis. The cells described above were treated with the indicated concentration of A23187 for 16 h. After 2 weeks, the surviving colonies were stained and counted.

of the amplified core sequence. To further analyze the phenotypes of the transfectants, we first attempted to obtain pure clonal cell lines derived from individual colonies of the 0.5 μM MTX-selected transfectants. To our surprise, every surviving colony that we picked and expanded regained the wild-type level of A23187 inducibility, even though the cultures were always maintained in 0.5 μM MTX during the several weeks of culture. To avoid the stress conditions within a cell colony, we next propagated the transfectants selected at 0.5 and 1.0 μM MTX as pools, with frequent medium changes to ensure that the cells were not subjected to glucose and oxygen deprivation. After eight trypsinization steps (approximately 40 days in culture), the pooled cells had regained a high level of *GRP78* inducibility and retained only about 50 copies of the core (Fig. 3B and data not shown).

Amplification of the core sequence confers a growth disadvantage. To examine whether the selective loss of the cells harboring amplified copies of the core was caused by a slower growth rate of these cells under normal growth conditions, the growth rates for DG44 cells and of early-passage GIA and GIB transfectants selected at 0.5 μM MTX were measured. The DG44 cells were grown in alpha medium, whereas the GIA and GIB transfectants were grown in alpha-minus medium with 0.5 μM MTX. As shown in Fig. 4A, the growth rates for DG44 cells and for GIA transfectants were similar, with a 24-h doubling time, showing that 0.5 μM MTX does not negatively affect the growth rate of GIA transfectants. For GIB transfectants, there was a long lag period after seeding, and the doubling time was twice as long. By day 5, the difference in cell number in comparison with GIA transfectants was fivefold. We noted that in cells that had lost the amplified core sequence, the growth rate reverted to normal (data not shown).

The morphology of these cells under stressed and nonstressed conditions was examined. The same three cell lines were treated with A23187 and examined under the light microscope. The GIB transfectants selected at 0.5 μM MTX were much more elongated after A23187 treatment than were DG44 cells and GIA transfectants (Fig. 5B). The stretched cell shape resembled that of the parental cells treated with extremely high doses of A23187 (our unpublished observation). We tested by colony survival analysis the resistance of the three cell lines to A23187 treatment (Fig. 4B and 5A). While DG44 cells and GIA transfectants showed similar levels of tolerance to increasing dosages of A23187, GIB transfectants were much more sensitive to the drug treatment. At 7 μM A23187, the survival rate of GIB transfectants was 8-fold lower; at 10 μM, the rate dropped to 25-fold lower than that of DG44 cells and GIA transfectants. These results show that the GIB transfectants have lower growth rates and lower tolerance to A23187 treatment.

DISCUSSION

While evidence has accumulated that GRP78 has the ability to bind to a variety of proteins processed through the ER in mammalian cells (10, 15, 16, 18), little is known about its contributions to cell growth and protection during stress. Elegant genetic selection systems have been used to dissect functional genes involved in protein transport and modification in yeast cells (2). However, the creation of specific conditional mutations in diploid mammalian cells affecting ER function is difficult. Since the gene encoding GRP78 is present in single copies in both human and rat cells (36), it is possible to eliminate this gene through homologous recombination, allowing one to examine whether the function normally served by GRP78 in mammalian cells is essential or is replaceable. However, given the lethality observed for yeast *GRP78* deletion mutants (33), this approach may also result in nonviable cells, thus precluding any further analysis of the phenotypes of the cells.

In this report, we show that amplified, tandem arrays of this sequence, along with some flanking promoter sequences, can reduce the A23187- and tunicamycin-induced

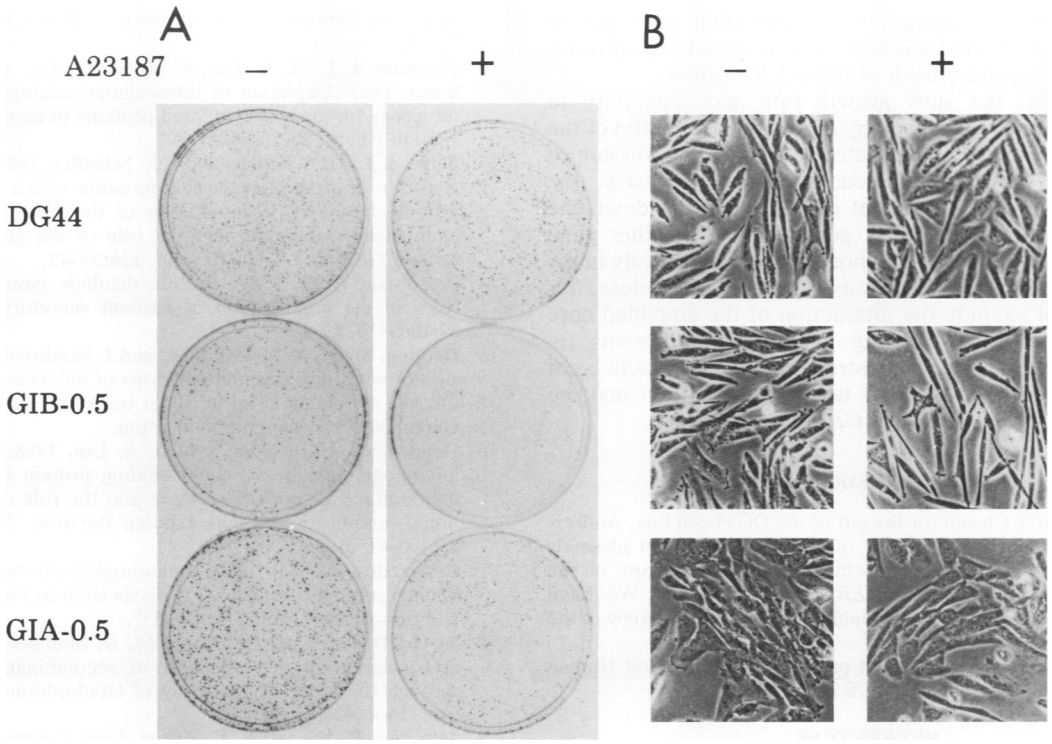

FIG. 5. Cell survival after A23187 treatment. (A) The seeded cells were grown either in normal culture medium (−) or in medium containing 7 μM A23187 for 16 h (+). The fixed and stained colonies formed after 10 to 15 days are shown. (B) The same cells were photographed 16 h after A23187 treatment.

level of *GRP78*. At the same time, the endogeneous transcript level of *GRP94*, another ER protein gene which is coordinately regulated with *GRP78* (7, 35), is similarly affected. Most interestingly, the endogenous transcript level of yet another ER protein gene, that encoding ERp72, is down-regulated in a similar manner. The correlation between the presence of the amplified copies of the *GRP* promoter sequence and the reduction in induced transcript levels was observed for all three ER genes. Our results confirm the recent finding that *ERp72* is inducible by A23187 (11) and further suggest that these ER proteins, GRP78, GRP94, and ERp72, are all regulated by *trans*-acting factors that interact with the conserved *GRP78* core sequence. The promoter sequence of *ERp72* is unknown. On the basis of these results, it is tempting to speculate that the *ERp72* promoter is similar in sequence to the *GRP* core and that common transcription factors exist that can activate or suppress sets of ER protein genes, responding to the state of the ER. Since amplification of the *GRP* promoter sequence results in reduced induction and loss of the amplified sequence restores inducibility, the common protein factors which are competed for are likely to be activators of promoter activity.

The transfectants harboring amplified copies of the *GRP* promoter sequence grew slowly and did not survive well when the cells were treated with A23187. While it is intriguing that a severalfold reduction in GRP78 could cause such effects, one explanation is that the ability to maintain and induce a certain threshold level of GRP78, and presumably the other two ER proteins, GRP94 and ERp72, is crucial for a normal growth rate as well as for conferring protection against disturbance of the intracellular calcium distributions. Calcium may also play a regulatory role in the activity of one or more of the GRPs. If reduced ER calcium leads to reduced GRP function and the cells cannot respond by increasing GRP levels, A23187 might well be more toxic to the transfectants harboring amplified copies of the *GRP* core. The lowest reduction in mRNA levels that we observed in several rounds of selection is around four- to fivefold. It is possible that further reduction in these proteins will lead to the loss of cell viability, thus precluding their selection. Consistent with this hypothesis is the finding that the maximum number of copies of the *GRP* core sequence is reached at much lower levels of MTX while copies of the DHFR gene continue to increase. If this is true, there may be a limit as to how low one can manipulate the endogenous levels of *GRP78* and other ER gene transcripts.

These transformants treated with A23187 are distinctly different in the morphology from untreated or control cells, assuming a much more elongated shape resembling that of normal cells treated with a high dose of the ionophore. It is known that mobilization of sequestered intracellular calcium with calcium ionophores depresses rates of translational initiation in mammalian cells (4). A correlation is observed between the nascent synthesis of GRP78 and the regain of protein synthesis in the cells following A23187 treatment, and antisense oligodeoxynucleotides directed against *GRP78* mRNA suppressed this adaptation, implying that GRP78 is critical for this accommodation mechanism (5). On the other hand, GRP94 has been identified as a high-capacity calcium-binding protein in the ER (23). It is therefore possible that during A23187 treatment, the drainage of sequestered calcium disrupts translation and protein glycosylation. The arrest of translation and the accumulation of underglycosylated proteins require the presence of high levels of one or more of the affected ER proteins for survival. Use of specific

reagents which will recognize the individual promoter or coding sequence would allow us to differentiate the physiological roles played by each of these ER proteins.

Alternatively, the slow growth rate and sensitivity to A23187 stress may be caused by an unknown effect(s) of the amplified core sequence unrelated to reduced expression of the three ER genes examined. If this is the case, the amplified core sequence is not globally shutting down the normal regulation of cellular genes, as three other gene transcripts examined behaved normally and identically in the parental and MTX-selected transfectants. Nonetheless, our studies do not exclude the interaction of the amplified core sequences with other limiting DNA-binding proteins required for normal growth and stress viability. It would be of interest to identify additional target genes which may be coordinately regulated by the *GRP* core sequence.

ACKNOWLEDGMENTS

We thank Larry Chasin for the gift of the DG44 cell line, Andrew Dorner and Randal Kaufman for the DHFR expression plasmids pMT2-ada and pMT2-adaGRE (which contains one copy of the core), and Michael Green for the *ERp72* cDNA plasmid. We thank Robert Stellwagen and Reinhold Gahlmann for critical review of the manuscript.

This research was supported in part by the Health and Human Services grant R37 CA27607 to A.S.L.

REFERENCES

1. **Artishevsky, A., A. M. Delegeane, and A. S. Lee.** 1984. Use of a cell cycle mutant to delineate the critical period for the control of histone mRNA levels in the mammalian cell cycle. Mol. Cell. Biol. **4**:2364–2369.
2. **Bernstein, M., F. Kepes, and R. Schekman.** 1989. *SEc59* encodes a membrane protein required for core glycosylation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9**:1191–1199.
3. **Bole, D. G., L. M. Hendershot, and J. F. Kearney.** 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. **102**:1558–1566.
4. **Brostrom, C. O., and M. A. Brostrom.** 1990. Calcium-dependent regulation of protein synthesis in intact mammalian cells. Annu. Rev. Physiol. **52**:577–590.
5. **Brostrom, M. A., C. Cade, C. R. Prostko, D. Gmitter-Yellen, and C. O. Brostrom.** 1990. Accommodation of protein synthesis to chronic deprivation of intracellular sequestered calcium. J. Biol. Chem. **263**:20539–20546.
6. **Chang, C. S., S. Wooden, T. Nakaki, Y. K. Kim, A. Lin, L. Kung, J. W. Attenello, and A. S. Lee.** 1987. Rat gene encoding the 78-kDa glucose-regulated protein GRP78: its regulatory sequences and effect of glycosylation on its expression. Proc. Natl. Acad. Sci. USA **84**:680–684.
7. **Chang, S. C., A. Erwin, and A. S. Lee.** 1989. The glucose protein genes (*GRP94* and *GRP78*) share common regulatory domains and are coordinately regulated by common *trans*-acting factors. Mol. Cell. Biol. **9**:2153–2162.
8. **Chirgwin, J. J., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
9. **Dorner, A. J., M. G. Krane, and R. J. Kaufman.** 1988. Reduction of endogenous GRP78 levels improves secretion of a heterologous protein in CHO cells. Mol. Cell. Biol. **8**:4063–4070.
10. **Dorner, A. J., L. C. Wasley, and R. J. Kaufman.** 1989. Increased synthesis of secreted proteins induced expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. J. Biol. Chem. **264**:20602–20607.
11. **Dorner, A. J., L. C. Wasley, P. Raney, S. Haugejorden, M. Green, and R. J. Kaufman.** 1990. The stress response in Chinese hamster ovary cells: regulation of ERp72 and protein disulphide isomerase expression and secretion. J. Biol. Chem. **265**:22029–22034.
12. **Drummond, I., A. S. Lee, E. Resendez, Jr., and R. A. Steinhardt.** 1987. Depletion of intracellular calcium stores induces the genes for glucose regulated proteins in hamster fibroblasts. J. Biol. Chem. **262**:12801–12805.
13. **Feige, J. J., G. A. Keller, and I. E. Scheffler.** 1988. Temperature-sensitive Chinese hamster cell mutation with a defect in glycoprotein synthesis: accumulation of the EGF receptor in the endoplasmic reticulum and the role of the glucose regulated protein *GRP78*. J. Cell. Physiol. **136**:33–42.
14. **Freedman, R. B.** 1989. Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. Cell **57**:1069–1072.
15. **Gething, M.-J., K. McCammon, and J. Sambrook.** 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. Cell **46**:939–950.

15a. **Green, M.** Personal communication.

16. **Hendershot, L., J. Ting, and A. S. Lee.** 1988. Identity of the immunoglobulin heavy-chain binding protein with the 78,000-dalton glucose-regulated protein and the role of posttranscriptional modifications in its binding function. Mol. Cell. Biol. **8**:4250–4256.
17. **Hendershot, L. M.** 1990. Immunoglobulin heavy chain and binding protein complexes are dissociated in vivo by light chain addition. J. Cell Biol. **111**:829–837.
18. **Jarvis, D. L., B. C. Oker, and M. D. Summers.** 1990. Role of glycosylation in the transport of recombinant glycoproteins through the secretory pathway of lepidopteran insect cells. J. Cell. Biol. **42**:181–191.
19. **Johnston, R. N., and B. L. Kucey.** 1988. Competitive inhibition of hsp70 gene expression cause thermosensitivity. Science **242**:1551–1554.
20. **Kassenbrock, C. K., P. D. Garcia, P. Walter, and R. B. Kelly.** 1988. Heavy-chain binding protein recognizes aberrant polypeptides translocated *in vitro*. Nature (London) **333**:90–93.
21. **Kaufman, R. J.** 1990. Vectors used for expression in mammalian cells. Methods Enzymol. **185**:487–511.
22. **Kim, Y. K., and A. S. Lee.** 1989. Cooperative interactions between the *GRP78* enhancer and promoter elements. Gene **77**:123–131.
23. **Koch, G., M. Smith, D. Macer, P. Webster, and R. Mortara.** 1986. Endoplasmic reticulum contains a common, abundant calcium-binding glycoprotein, endoplasmin. J. Cell Sci. **86**:217–232.
24. **Lee, A. S.** 1987. Co-ordinated regulation of a set of genes by glucose and calcium ionophore in mammalian cells. Trends Biochem. **12**:20–24.
25. **Lee, A. S., A. M. Delegeane, V. Baker, and P. C. Chow.** 1983. Transcriptional regulation of two genes specifically induced by glucose starvation in a hamster mutant fibroblast cell line. J. Biol. Chem. **258**:597–603.
26. **Lee, A. S., S. Wells, K. S. Kim, and I. E. Scheffler.** 1986. Enhanced synthesis of the glucose/calcium regulated proteins in hamster cell mutants deficient in transfer of oligosaccharide core to polypeptides. J. Cell. Physiol. **129**:277–282.
27. **Lewis, M. J., D. J. Sweet, and H. R. B. Pelham.** 1990. The *ERD2* gene determines the specificity of the luminal ER protein retention system. Cell **61**:1359–1363.
28. **Li, X., D. W. Warren, J. Gregoire, R. C. Pedersen, and A. S. Lee.** 1989. The rat 78,000 dalton glucose-regulated protein (GRP78) as a precursor for the rat steroidogenesis-activator polypeptide (SAP): the SAP coding sequence is homologous with the terminal end of GRP78. Mol. Endocrinol. **3**:1944–1952.
29. **Lin, A. Y., S. C. Chang, and A. S. Lee.** 1986. A calcium ionophore-inducible cellular promoter is highly active and has enhancerlike properties. Mol. Cell. Biol. **6**:1235–1243.
30. **Mazzarella, R. A., M. Srinivasan, S. M. Haugejorden, and M. Green.** 1990. ERp72, an abundant luminal endoplasmic reticulum protein, contains three copies of the active site sequences of protein disulfide isomerase. J. Biol. Chem. **265**:1094–1101.
31. **Resendez, E., Jr., J. W. Attenello, A. Grafsky, C. S. Chang, and A. S. Lee.** 1985. Calcium ionophore A23187 induces expression

of glucose-regulated genes and their heterologous fusion genes. Mol. Cell. Biol. **5**:1212–1219.

32. **Resendez, E. R., Jr., S. K. Wooden, and A. S. Lee.** 1988. Identification of highly conserved regulatory domains and protein binding sites in the promoters of the rat and human gene encoding the stress-inducible 78-kilodalton glucose-regulated protein. Mol. Cell. Biol. **8**:4579–4584.
33. **Rose, M. D., L. M. Misra, and J. P. Vogel.** 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. Cell **57**:1211–1221.
34. **Sambrook, J. F.** 1990. The involvement of calcium in transport of secretory protein from the endoplasmic reticulum. Cell **61**:197–199.
35. **Scharff, D. J., A. Delegeane, and A. S. Lee.** 1982. Characterization of a cell cycle mutant derived from hamster fibroblasts: reversion analysis. J. Cell Biol. **92**:629–633.
36. **Ting, J., and A. S. Lee.** 1988. The human gene encoding the 78,000 dalton glucose-regulated gene and its pseudogene: structure, conservation and regulation. DNA **7**:275–286.
37. **Ting, J., S. K. Wooden, R. Kriz, K. Kellcher, R. J. Kaufman, and A. S. Lee.** 1987. The nucleotide sequence encoding the hamster 78 kilodalton glucose-regulated protein (GRP78) and its conservation between the hamster and rat. Gene **44**:147–152.
38. **Urlaub, G., P. J. Mitchell, E. Kas, L. A. Chasin, V. L. Funanage, T. T. Myoda, and J. Hamlin.** 1986. Effect of gamma rays at the dihydrofolate reductase locus: deletions and inversions. Somatic Cell Mol. Genet. **12**:555–566.
39. **Vaux, D., J. Tooze, and S. Fuller.** 1990. Network antibodies identify an intracellular membrane protein that recognizes the mammalian ER retention signal. Nature (London) **345**:495–502.
40. **Vogel, J. P., L. M. Misra, and M. D. Rose.** 1990. Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. J. Cell Biol. **110**:1885–1895.

40a. **Wooden, S. K., and A. S. Lee.** Unpublished data.

41. **Xiao, H., and J. T. Lis.** 1986. A consensus sequence polymer inhibits in vivo expression of heat shock genes. Mol. Cell. Biol. **6**:3200–3206.