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Complex Regulation of Heat Shock- and Glucose-Responsive Genes in Human Cells

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We have isolated a human genomic clone that encodes the glucose-responsive protein GRP78 and have used this cloned gene probe, together with a cloned *HSP70* gene, to study the expression of both stress-induced genes in response to inhibitors of cellular metabolism. On the basis of the effects of this group of chemicals on *GRP78* and *HSP70* expression, we have identified three classes of stress gene inducers. The first class induces *GRP78* expression and includes inhibitors of glycoprotein processing. The second class results in coordinate activation of both GRP78 and HSP70 synthesis and includes amino acid analogs and heavy metals. Chemicals in the third class coordinately induce *GRP78* and repress *HSP70* expression; this class includes the calcium ionophore A23187 and the glucose analog 2-deoxyglucose. Whereas induction of *GRP78* or *HSP70* expression is primarily due to transcriptional activation, chemicals that repress *HSP70* expression act through posttranscriptional regulation. These results reveal that the regulation of *GRP78* and *HSP70* expression is complex and may be dependent on the specificity and magnitude of physiological damage.

Eucaryotic cells respond to a transient stress such as heat shock by inducing the synthesis of a specific set of proteins known as the heat shock proteins. This physiological response to environmental extremes has been found in all species examined to date, and the size and structure of the induced proteins is highly conserved (8, 24). For example, the nucleotide sequences for the major heat shock gene *HSP70* are 60% identical among eucaryotes and 50% identical between the *Escherichia coli dnaK* protein and *HSP70* (3, 15). The *HSP70* gene is present in multiple copies in the *Saccharomyces cerevisiae*, *Drosophila melanogaster*, mouse, and human genomes (9, 17, 26, 51). The eucaryotic genome also contains sequences closely related to but not identical to those in HSP70. These cognate genes, first identified in the *Drosophila* sp. and *S. cerevisiae*, encode proteins which are related in sequence to *HSP70* but which appear to be regulated distinctly (16, 17).

Recent studies in mammalian cells have identified two proteins which share features of the HSP70 protein family, a 78,000-dalton (Da) glucose-regulated protein (GRP78) and a 72,000-Da protein with clathrin-uncoating ATPase activity, (6, 30). GRP78 was originally identified as a member of a second stress-responsive group of proteins, the glucose-regulated proteins (43, 50). The synthesis of glucose-regulated proteins is induced under a variety of conditions including glucose deprivation, anoxia, paramyxovirus infection, and treatment of cells with glycosylation inhibitors or the calcium ionophore A23187 (35, 36, 41, 43, 50, 53). Munro and Pelham have recently shown that the rat *GRP78* gene is closely related to the *HSP70* gene and that *GRP78* contains an amino-terminal signal sequence which directs this protein to the endoplasmic reticulum (30). Although *GRP78* appears to be functionally related to *HSP70*, little is known about how these two genes are regulated during various forms of stress.

In this study, we examined the expression of *GRP78* and *HSP70* in response to a wide range of metabolic inhibitors. We found that the inhibitors fell into three classes on the basis of their effects upon *HSP70* expression. All induce *GRP78* expression; the first class had no effect upon *HSP70* expression, the second class activated *HSP70* expression, and the third class repressed *HSP70* expression.

MATERIALS AND METHODS

Cell culture conditions and analyses of protein synthesis. HeLa and 293 (adenovirus-transformed human embryonic kidney) cells were maintained in Dulbecco modified Eagle medium with 5% calf serum. The cells were plated approximately 36 h before each experiment at the following densities: 7×10^5 cells per 60-mm dish, 1.5×10^6 cells per 100-mm dish, and 3×10^6 cells per 150-mm dish. Cells were treated with one of the following chemicals for the specified times: 10 mM 2-deoxyglucose (Sigma Chemical Co.), 7 μM A23187 (Calbiochem-Behring), 5 mM L-azetidine-2-carboxylic acid (Sigma), 10 μM cadmium sulfate (Fisher Scientific Co.), 50 μg of castanospermine (Genzyme Corp.) per ml, 0.5 μg of tunicamycin (Calbiochem) per ml, or 5 mM glucosamine (Sigma). Heat shock samples were prepared by incubating cells at 43°C for 2 h. At the indicated times after treatment, cells were pulse-labeled with 10 μCi of [^{35}S]methionine per ml in methionine-free medium for 30 min at 37°C. Cells were washed and harvested in phosphate-buffered saline, pelleted, and immediately frozen at −80°C.

Cell pellets were solubilized in Laemmli sample buffer or urea sample buffer and analyzed by one- and two-dimensional gel electrophoresis (19, 31). For Western blot (immunoblot) analysis, unlabeled cell lysates were separated by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred to nitrocellulose (5). The filters were incubated with the monoclonal antibody 7.10 raised against *Drosophila* HSP70 (J. Velazquez and S. Lindquist, personal communication), followed by iodinated goat anti-mouse antibody.

Isolation of recombinant clones and Southern blot analysis. A human genomic library was screened for *HSP70*-related sequences as previously described (51). DNA from one (H14) of the three isolated recombinant clones homologous to *HSP70* sequences was prepared from phage particles banded by equilibrium cesium chloride centrifugation (27). Subclones of *Bam*HI and *Bam*HI-*Eco*RI fragments from H14

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were constructed in the vector pT7/T3 (Bethesda Research Laboratories, Inc.), and plasmid DNA was isolated by ethidium bromide-cesium chloride equilibrium centrifugation (25). DNAs were radioactively labeled by nick translation (38) in the presence of [α-^{32}P]deoxynucleotide triphosphates (800 Ci/mmol; Amersham Corp.).

The restriction enzyme digests of *GRP78*- and *HSP70*-subcloned DNAs were separated by horizontal agarose gel electrophoresis and transferred to nitrocellulose filters (44). The blots were prehybridized for 24 h at 65°C in 6× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 5× Denhardt solution (1× is 0.02% Ficoll [Pharmacia Fine Chemicals], 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), and 100 μg of single-stranded DNA per ml. The radiolabeled DNA was denatured, added to the prehybridization solution, and incubated for over 24 h at 65°C. Filters were washed as described in the figure legends.

mRNA levels, hybridization-selection, in vitro translation, and in vitro transcription analysis. Cytoplasmic RNA was isolated from cell lysates as described previously (7). For RNA dot blot analyses, the RNA was ethanol precipitated, suspended in 1× NaPF (1× NaPF is 1 M sodium chloride, 40 mM sodium phosphate [pH 7.0], and 6% formaldehyde), denatured by heating at 65°C for 5 min, and bound to nitrocellulose (Minifold; Scheicher & Schuell, Inc.). Filters were washed with 1 M sodium phosphate (pH 7.0), baked, and prehybridized for 7 to 12 h at 42°C in a solution containing 50% formamide, 6× SSC, 5× Denhardt solution, 0.1% SDS, and 50 μg of tRNA per ml. The denatured ^{32}P-labeled DNA was added to the prehybridization solution for an additional 12 to 24 h at 42°C. Filters were washed in 2× SSC and 0.2% SDS, followed by 0.2× SSC and 0.2% SDS at 65°C.

For Northern (RNA) blot analysis, cytoplasmic RNA was denatured by glyoxal (28), separated by electrophoresis on 1.2% agarose gels, and transferred to nitrocellulose (46). Filters were baked, hybridized, and washed as described above.

Gene-specific mRNAs were isolated by hybridization-selection with filter-bound DNA, as previously described (51). The following modifications were done. Plasmid DNAs were fragmented by sonication, denatured, bound to nitrocellulose, and hybridized with 15 μg of cytoplasmic RNA for over 12 h at 53°C. The filters were washed repeatedly in 0.1 M NaCl–10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA–0.5% SDS, followed by several washes in the same buffer without SDS.

Selected mRNAs or 5 μg of total cytoplasmic RNA were translated in vitro in micrococcal nuclease-treated rabbit reticulocyte lysates (2, 34) and analyzed by one- or two-dimensional gel electrophoresis.

In vitro runoff transcription (10) was performed in isolated HeLa cell nuclei as described previously (2). Radiolabeled RNA was hybridized to filter-bound DNA under the conditions used for RNA dot blot analysis.

RESULTS

Family of HSP70-related proteins in human cells. We have used two approaches to identify members of the HSP70 family. The first approach was to identify *HSP70*-related DNA sequences by Southern blot analyses of human genomic DNA and human-hamster somatic hybrid DNAs with cloned gene probes. These studies have identified multiple *HSP70* genes and several *HSP70*-related sequences in the human genome (14, 51). The second approach was to identify human proteins that are antigenically related to HSP70.

HeLa cell proteins sharing common epitopes with HSP70 were identified by Western blot analyses with the monoclonal antibody 7.10 raised against *Drosophila* HSP70 (provided by S. Lindquist and J. Velazquez). Extracts from HeLa cells maintained at 37°C contain three proteins of 78,000, 72,000,

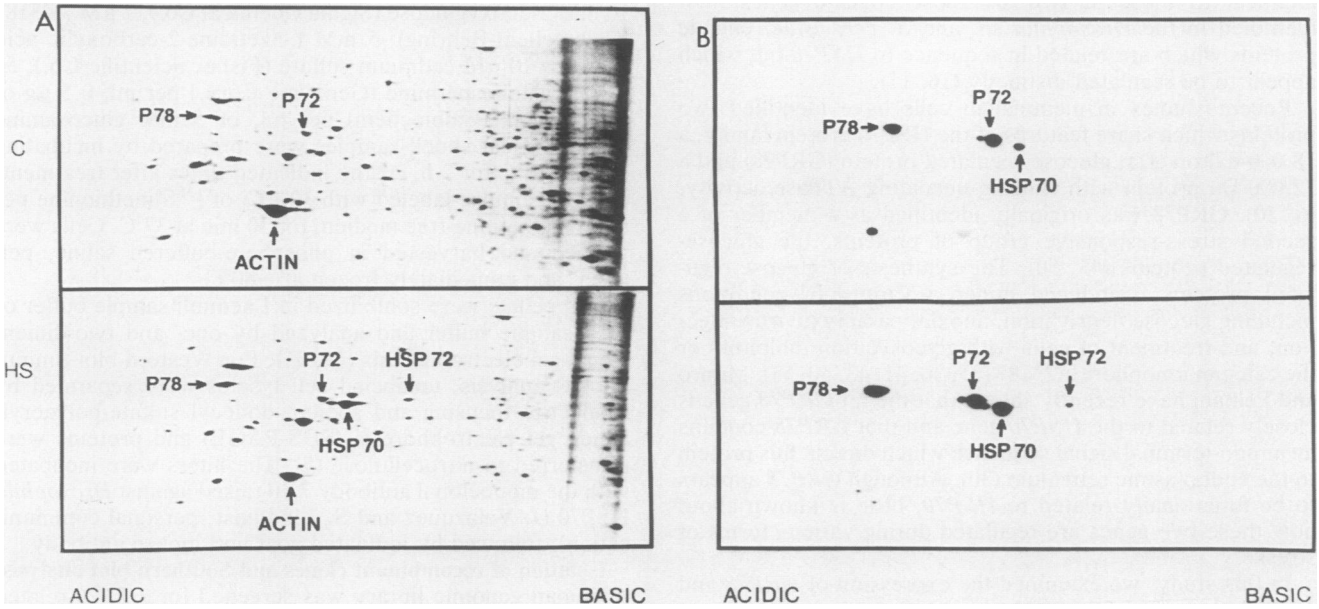


FIG. 1. Identification of human proteins antigenically related to HSP70. HeLa (JW36) cells were incubated at control (37°C) or heat shock (43°C) temperatures for 2 h. The samples were split into two portions; one set was pulse-labeled with [^{35}S]methionine, and the second set was used for Western blot analyses. Cell lysates were separated by isoelectric focusing in the first dimension, followed by SDS-PAGE. The panels correspond to an autoradiogram of (A) control and heat shock, [^{35}S]methionine-labeled proteins and (B) Western blot analysis of control and heat-shock proteins immunologically reactive to anti-HSP70 antibody 7.10.

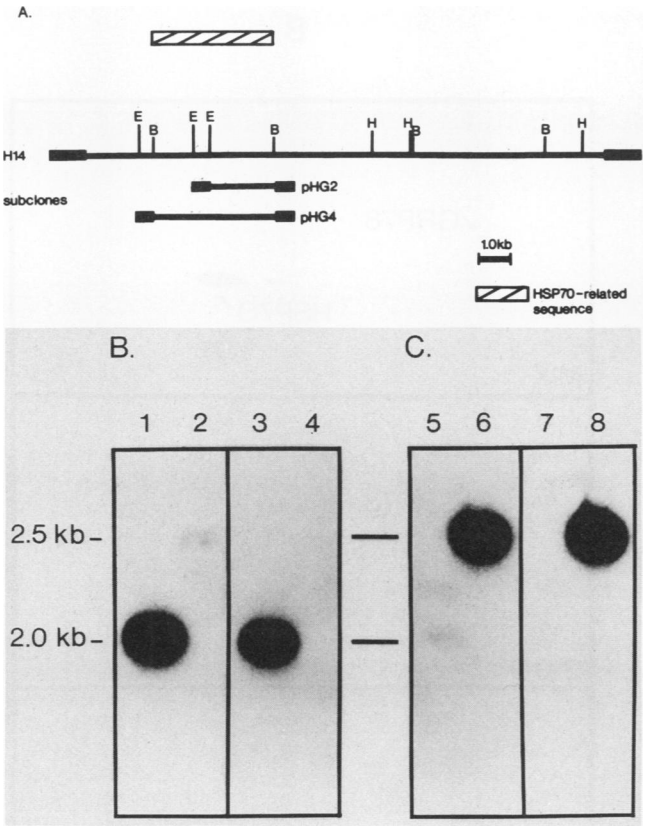


FIG. 2. Restriction map of H14 DNA and Southern blot hybridization of the human *GRP78* and *HSP70* genomic subclones. (A) Cleavage sites for the restriction enzymes *Bam*HI (B), *Hin*dIII (H), and *Eco*RI (E) were mapped. The subcloned *Bam*HI and *Bam*HI-*Eco*RI fragments are indicated, and the *HSP70*-related sequences are shown by the hatched box. (B and C) pHG2 DNA was digested with *Bam*HI and *Eco*RI (lanes 1, 3, 5, and 7) and pH2.3 DNA was digested with *Bam*HI and *Hin*dIII (lanes 2, 4, 6, and 8) to separate insert and vector sequences. DNA was separated on agarose gels and blotted to nitrocellulose. Blots were hybridized with either ^{32}P-labeled pHG4 coding sequences (lanes 1 through 4) or with ^{32}P-labeled pH2.3 coding sequences (lanes 5 through 8). The filter wash conditions were low stringency (6× SSC, 65°C) for lanes 1, 2, 5, and 6 and high stringency (0.2× SSC, 65°C) for lanes 3, 4, 7, and 8. The positions of the insert fragments of pHG2 (2.0 kb) and pH2.3 (2.5 kb) are indicated.

and 70,000 Da that cross-react with antibody 7.10 (Fig. 1B). All three proteins are constitutively synthesized in HeLa cells, as detected by pulse-labeling with [^{35}S]methionine (Fig. 1A). The 78,000-Da protein (pI, 5.2) comigrates with GRP78, a protein induced by glucose deprivation, glycosylation inhibitors, and the calcium ionophore A23187 (36, 43, 50). The 72,000-Da protein (P72) comigrates with a protein recently suggested to be the clathrin-uncoating ATPase (6, 47). The 70,000-Da protein (HSP70) is serum stimulated (52) and cell cycle regulated (29) and is the major heat-inducible protein (48).

After heat shock, the induced synthesis and accumulation of two proteins was detected, including HSP70, the major heat shock protein, and HSP72, a protein whose expression is entirely dependent upon heat shock (Fig. 1). GRP78 and P72 were constitutively expressed and slightly heat shock induced (Fig. 1). Although these four proteins are antigenically related and share common biochemical features, they are biochemically distinct proteins (6, 49) and are encoded by distinct mRNAs, as demonstrated by in vitro translation (data not shown).

Isolation of human genomic sequences which encode GRP78. A human genomic recombinant clone containing sequences that encode the 78,000-Da glucose-responsive protein (GRP78) was isolated by screening a human fetal liver DNA library (21) at low stringency with the *Drosophila HSP70* gene (plasmid 232.1) (25). We had previously isolated three genomic clones with distinct restriction maps, one of which (H3-1) was shown to contain the major heat shock-inducible *HSP70* gene (51). The other two recombinant clones, one of which is described here, have since been characterized.

The recombinant clone H14 was plaque purified, and the phage DNA was isolated and shown to contain a 17-kilobase (kb) insert from analysis of restriction enzyme digestion patterns. The *HSP70*-related sequences in H14 were identified by Southern blot hybridization with the *Drosophila HSP70* gene, plasmid 229 (25), and the human *HSP70* gene pH2.3 (51). Southern blots of restriction digests of H14 DNA hybridized with either *HSP70* gene probe-identified sequence-related 2.0-kb *Bam*HI-*Eco*RI or 4.0-kb *Bam*HI fragments (Fig. 2A).

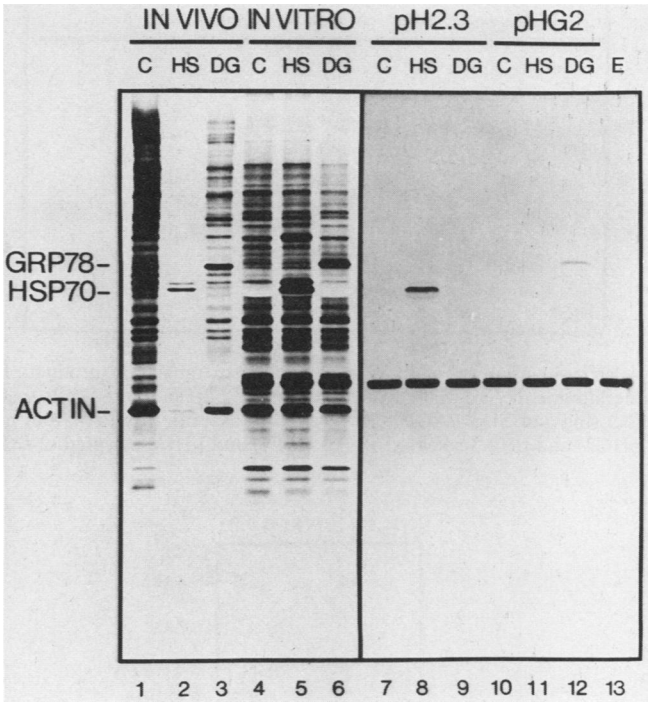


FIG. 3. Identity of pHG2 sequences by hybridization-selection and in vitro translation. HeLa cells were maintained at 37°C as a control (lane C), heat shocked for 2 h at 43°C (lane HS), or treated with 10 mM 2-DG for 8 h (lane DG). After treatment, proteins were labeled in vivo with [^{35}S]methionine, and cytoplasmic RNA was isolated from a duplicate set of cells for hybrid-selection and translation in rabbit reticulocyte lysates. Lanes: 1 to 3, in vivo patterns of protein synthesis in control cells (lane 1), heat-shocked cells (lane 2), and 2-DG-treated cells (lane 3); 4 to 6, in vitro translations of 5 μg of cytoplasmic RNA from control RNA (lane 4), heat-shocked RNA (lane 5), and 2-DG RNA (lane 6); 7 to 12, translation products of plasmid DNA hybrid-selected mRNAs in pH2.3 plus control RNA (lane 7), pH2.3 plus heat-shocked RNA (lane 8), pH2.3 plus 2-DG RNA (lane 9), pHG2 plus control RNA (lane 10), pHG2 plus heat-shocked RNA (lane 11), pHG2 plus 2-DG RNA (lane 12). Lane 13 (E) corresponds to endogenous background in the lysate.

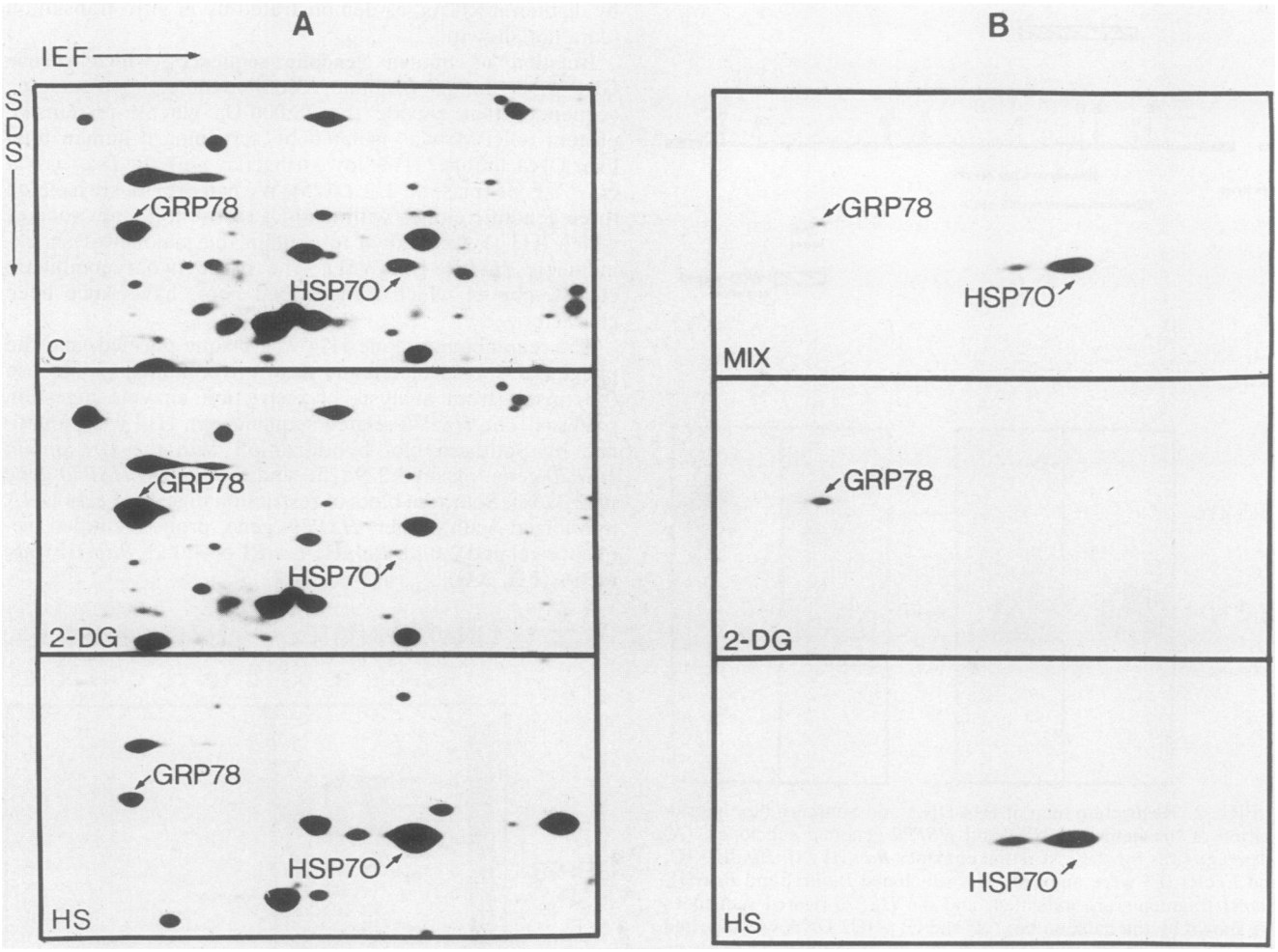
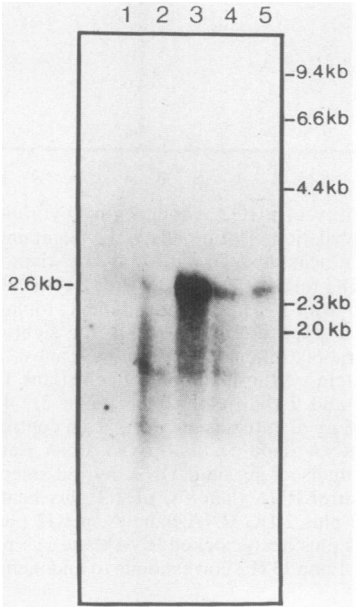


FIG. 4. Analysis of in vivo and in vitro translation products by two-dimensional gel electrophoresis. (A) Proteins labeled with [^{35}S] methionine in vivo in control cells (panel C), 2-DG treated cells (panel 2-DG), or heat-shocked cells (panel HS) were separated by isoelectric focusing and SDS-PAGE. (B) In vitro translation products from hybrid-selection experiments, including a mix of proteins translated from pHG2- and pH2.3-selected RNAs (MIX) and pHG2-selected (2-DG) pH2.3-selected (HS) translation products.



The HSP70-related sequences in H14 were subcloned (pHG2 and pHG4 in Fig. 2A) for further studies. To demonstrate the extent of identity between HSP70 genomic sequences and H14 sequences, we performed Southern blotting experiments with homologous (pHG2) and heterologous (pH2.3) gene probes (Fig. 2B and C). We initially used nonstringent DNA-DNA hybridization conditions (6× SSC, 65°C) during library screening and restriction mapping to identify the HSP70-related sequences. When more stringent hybridization conditions were applied (0.2× SSC, 65°C), we no longer detected the hybridization between HSP70 and the H14 subcloned fragments (Fig. 2B and C). The extent of mismatch revealed from the conditions required for melting of the heterologous hybrids suggested that pHG2 sequences

FIG. 5. Northern blot analysis of RNA from 2-DG-treated HeLa cells. Total cytoplasmic RNA was isolated from untreated cells (lane 1) or cells treated with 10 mM 2-DG for 8, 12, 16, or 24 h (lanes 2 through 5). The RNA was glyoxylated, separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pHG2 DNA. The filters were washed with 0.2× SSC at 65°C. The positions of *GRP78* mRNA (2.6 kb) and molecular size markers are indicated.

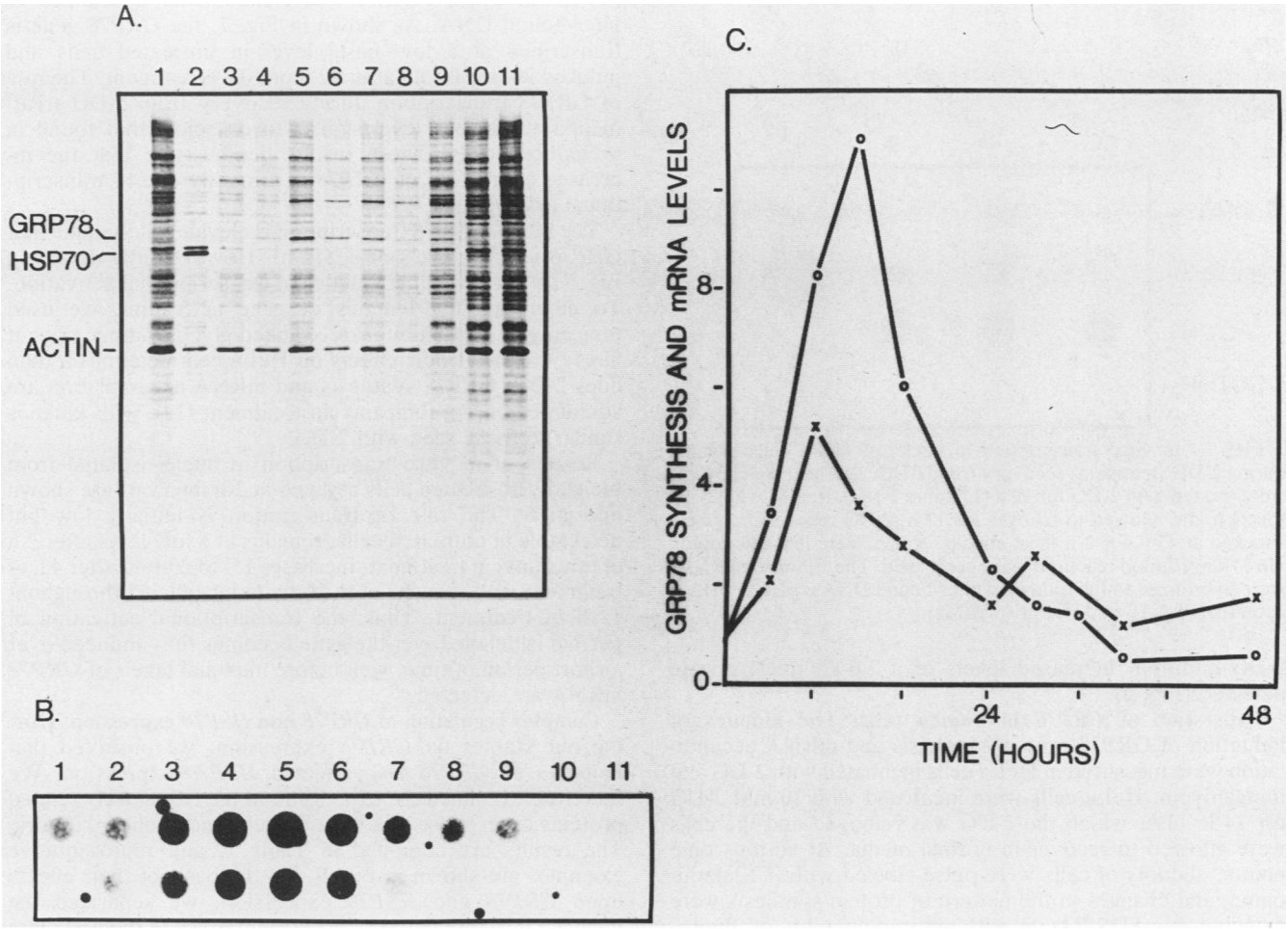


FIG. 6. Induction of GRP78 synthesis and mRNA levels during 2-DG treatment. (A) Autoradiogram of [^{35}S]methionine-labeled proteins synthesized by HeLa cells during 2-DG treatment or during heat shock. HeLa cells were untreated (lane 1), incubated at 43°C for 2 h (lane 2), treated with 2-DG for 2, 4, 8, 12, 16, or 24 h (lanes 3 through 8), or treated with 2-DG for 24 h and allowed to recover for 4, 12, or 24 h (lanes 9 through 11). The cells were pulse-labeled with [^{35}S]methionine and analyzed by SDS-PAGE. Approximately equal numbers of cells were loaded per lane. (B) Analysis of *GRP78* mRNA levels during 2-DG treatment or heat shock. Cytoplasmic RNA was isolated from untreated HeLa cells (column 1), cells incubated at 43°C for 2 h (column 2), cells treated with 2-DG for 4, 8, 12, 16, or 24 h (columns 3 through 7), or cells treated for 24 h and allowed to recover for 4, 8, 12, or 24 h (columns 8 through 11). The two rows correspond to 2 μg (top) and 1 μg (bottom) of total cytoplasmic RNA bound to nitrocellulose and hybridized to ^{32}P-labeled pHG2. The filters were washed in 0.2× SSC at 65°C. (C) Quantitation of GRP78 synthesis (×) and *GRP78* mRNA (○) levels in 2-DG-treated cells. Protein synthesis (panel A) and mRNA levels (panel B) were quantitated by densitometry; synthesis, and accumulation levels are expressed relative to control levels.

are less than 75% identical to the *HSP70* sequences. Comparison of a partial nucleotide sequence of pHG2 to those of *HSP70* reveals sequences which are 70% identical (S. Watowich, C. Hunt, and R. Morimoto, data not shown). The *HSP70*-related sequences in H14 are more divergent than might be expected for an *HSP70* gene, suggesting that the H14 sequences may encode a cognate protein related to but not identical to HSP70. This possibility was confirmed by comparison of pHG2 sequences to sequences for the rat GRP78 gene (30) which are 90% identical.

Identity of the sequences in H14 was directly established by in vitro translation of mRNA selected by hybridization to filter-bound DNAs and comparison to proteins synthesized in vivo using one- and two-dimensional gel electrophoresis. Cytoplasmic RNAs were prepared from HeLa cells incubated at 43°C for 2 h, treated for 8 h with 2-deoxyglucose (2-DG), or maintained in normal media at 37°C. The RNAs were hybridized to filter-bound DNAs from the human *HSP70* gene (pH2.3), the H14 subclone (pHG2), and a plasmid control (pAT153). The hybrid selected mRNAs were eluted and translated in vitro in rabbit reticulocyte extracts (Fig. 3). pHG2 specifically hybridizes to an mRNA induced after treatment with 2-DG which translates a 78,000-Da protein that comigrates with in vivo-synthesized GRP78 (Fig. 3, lane 12 and Fig. 4). We further demonstrated that pHG2 did not cross-hybridize to *HSP70* mRNA in comparison with the translation products of pH2.3-selected mRNA (Fig. 3, lane 8). The translation products of pH2.3- and pHG2-selected mRNAs were mixed and analyzed by two-dimensional gel electrophoresis (Fig. 4). The proteins translated from pH2.3- and pHG2-selected mRNAs comigrated, respectively, with the in vivo-synthesized HSP70 and GRP78. The levels of *GRP78* mRNA were strongly induced by 2-DG and not by heat shock (Fig. 3 and 4).

To establish that pHG2 DNA hybridized to a specific mRNA induced by 2-DG, total cytoplasmic RNA was isolated from HeLa cells treated with 2-DG for 8 to 24 h and analyzed by Northern blot analysis. Between 0 and 8 h after

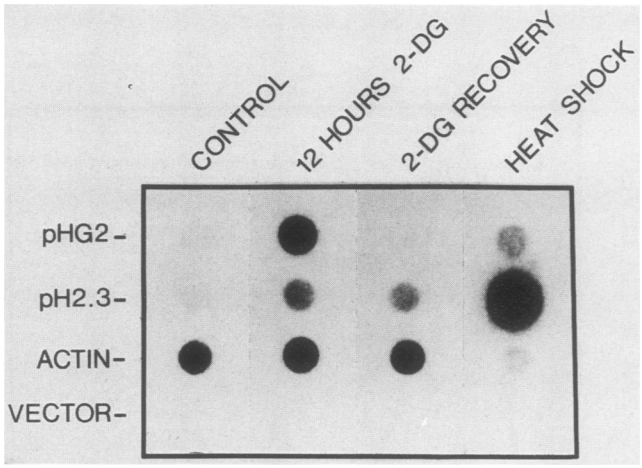


FIG. 7. In vitro transcription in nuclei of HeLa cells isolated during 2-DG treatment, recovery from 2-DG, and heat shock. Cells were treated with 2-DG for 12 h (12 hours 2-DG), treated with 2-DG for 24 h and allowed to recover for 12 h (2-DG recovery), or heat shocked at 43°C for 2 h (heat shock). Nuclei were isolated, and in vitro transcription reactions were performed. The ^{32}P-labeled RNAs were hybridized to the following filter-bound DNAs: pHG2, pH2.3, actin (pHFβA-1), and vector (pGEM1).

2-DG addition, increased levels of a 2.6-kb mRNA were detected (Fig. 5).

Expression of *GRP78* in human cells. The kinetics of induction of GRP78 protein synthesis and mRNA accumulation were measured in HeLa cells incubated with 2-DG and tunicamycin. HeLa cells were incubated with 10 mM 2-DG for 24 h, after which the 2-DG was removed and the cells were allowed to recover in normal media. At various time points, aliquots of cells were pulse-labeled with [^{35}S]methionine, and changes in the pattern of protein synthesis were detected by SDS-PAGE and autoradiography of fluorographed gels. Increased synthesis of GRP78 was detected within 4 h of 2-DG addition (Fig. 6A, lane 4), with the peak in GRP78 synthesis occurring after 8 to 12 h (Fig. 6A, lanes 5 and 6). GRP78 synthesis declined during the last 12 h of treatment (Fig. 6A, lanes 7 and 8) and returned to preinduced levels during recovery (Fig. 6A, lanes 9 to 11). We have noticed that 2-DG has variable inhibitory effects upon protein synthesis; in the experiment shown in Fig. 6, total protein synthesis, measured by trichloroacetic acid precipitation of [^{35}S]methionine-labeled proteins and scintillation counting, decreased by 60%.

Changes in the level of *GRP78* mRNA were measured by RNA dot blot analyses with total cytoplasmic RNA isolated from 2-DG-treated cells. The level of *GRP78* mRNA increased 11-fold during 12 h of treatment (Fig. 6B and C) and declined thereafter, returning to a level below that found in preinduced cells. During 2-DG treatment, the levels of actin mRNA were not affected, as determined from dot blot analysis and hybridization with an actin gene probe (12) (data not shown).

To determine whether the changes in *GRP78* mRNA levels were due to a rapid increase in the rate of *GRP78* gene transcription or due to effects on *GRP78* mRNA stability, we examined the rate of *GRP78* gene transcription in isolated nuclei. HeLa cells were treated with 2-DG for 12 h, treated with 2-DG for 24 h and allowed to recover for 12 h in fresh media, or maintained in drug-free media. In vitro transcription reactions were carried out in isolated nuclei to allow previously initiated transcripts to elongate in the presence of [^{32}P]UTP. The ^{32}P-RNA was isolated and hybridized to filter-bound DNA. As shown in Fig. 7, the *GRP78* gene is transcribed at a low basal level in untreated cells and induced 20- to 30-fold after 12 h of 2-DG treatment. The rate of GRP78 transcription during recovery from 2-DG treatment returns to a level similar to or below that found in preinduced cells. These results demonstrate that the increased expression of *GRP78* is primarily due to transcriptional activation.

The rate of GRP78 transcription is elevated at the time that *GRP78* mRNA levels peak (12 h). However, the results in Fig. 7 do not reveal the kinetics of transcriptional activation. To determine the kinetics of gene activation, we used tunicamycin, an inhibitor of N-linked glycosylation, since it has fewer pleiotropic effects on HeLa cell metabolism than does 2-DG. GRP78 synthesis and mRNA accumulation are stimulated during tunicamycin treatment (32), with kinetics similar to those seen with 2-DG.

Results of in vitro transcription in nuclei isolated from tunicamycin-treated cells assayed at 2-h intervals are shown in Fig. 8. The rate of transcription is initially low but detectable in untreated cells, remains at a low level after 2 h of tunicamycin treatment, increases 15- to 20-fold after 4 h of treatment, and remains at the fully induced level throughout 12 h of treatment. Thus, the transcriptional activation of *GRP78* is delayed, yet the gene becomes fully induced over a short period of time, well before maximal levels of *GRP78* mRNA are detected.

Complex regulation of *GRP78* and *HSP70* expression. During our studies on *GRP78* expression, we observed that inducers of *GRP78* also affected *HSP70* expression. We therefore examined the expression of the two closely related proteins in response to a wide range of metabolic inhibitors. The results are compiled in Table 1, and representative examples are shown in Fig. 9. On the basis of their effects upon *GRP78* and *HSP70* expression, we separated the inducers into three classes. Representatives of the first class induce only *GRP78* expression with no detectable effects on *HSP70*. This class includes inhibitors of glycoprotein processing such as castanospermine, an inhibitor of mammalian glucosidases I and II (33), and glucosamine, an inhibitor of complex sugar addition and tunicamycin. The second class of chemicals induces the expression of both *GRP78* and *HSP70* and includes amino acid analogs (azetidine and canavanine) and heavy metals (cadmium, copper, and zinc). The third class of chemicals induces *GRP78* expression and

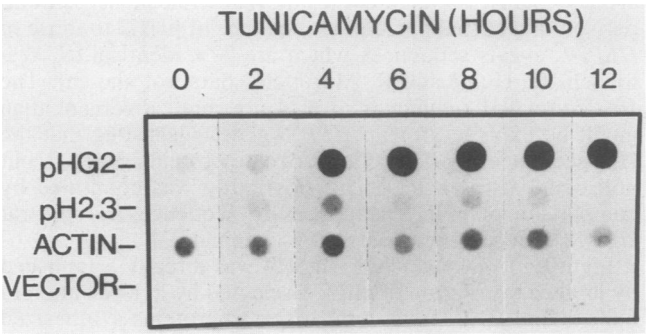


FIG. 8. In vitro transcription in nuclei isolated from tunicamycin-treated HeLa cells. Cells were harvested after 0, 2, 4, 6, 8, 10, or 12 h of incubation with tunicamycin; nuclei were prepared; and in vitro transcription reactions were performed. The ^{32}P-labeled RNAs were hybridized to the following filter-bound DNAs: pHG2, pH2.3, actin (pHFβA-1), and vector (pGEM1).

TABLE 1. Complex regulation of two members of the *HSP70* gene family

Treatment	Treatment (h)	GRP78		HSP70	
		Level of induction of mRNA	Protein synthesis[a]	Level of induction of mRNA	Protein synthesis[a]
2-DG	8–12	8–11	4–5	0.5	ND[b]
A23187	12	20	8	0.3	ND
$CaSO_4$	4		2		4
Azetidine	8	7–8	4	18	22
Castanospermine	8	2.8	2.5	NE[b]	NE
Tunicamycin	12	3	6	NE	NE
Glucosamine	8		10	NE	NE
Heat shock	2	1.2	0.7	20	10–20

[a] Cellular protein was pulse-labeled with [^{35}S]methionine at the listed times of treatment and separated by SDS-PAGE, and gels were processed by fluorography. Cytoplasmic RNA (1 and 2 μg) was analyzed by dot blot hybridization with pHG2 or pH2.3 used as gene probes. Results were quantitated by densitometry.

[b] Abbreviations: NE, no effect; ND, no detectable synthesis.

represses HSP70 synthesis and is represented by the glucose analog 2-DG and the calcium ionophore A23187. 2-DG exerts a variety of effects upon cellular metabolism, including inhibition of glycoprotein processing (39). The ionophore A23187 raises the intracellular concentration of calcium. In the following sections, we describe the effects of each class of inhibitors on GRP78 and HSP70 protein synthesis and mRNA levels in HeLa or 293 cells.

Inducers which activate *GRP78* but not *HSP70* expression. Incubation of HeLa cells with either tunicamycin or glucosamine at concentrations which inhibit glycoprotein processing induces the expression of glucose-regulated protein genes (32, 36). To determine whether *GRP78* can be induced with other inhibitors of glycosylation, we treated HeLa cells with castanospermine. After 4 to 8 h of treatment, a sixfold increase in GRP78 synthesis and a threefold increase in *GRP78* mRNA were detected (Fig. 10). During 12 h of incubation with either tunicamycin or glucosamine, the levels of GRP78 synthesis and mRNA remained elevated, whereas treatment with these inhibitors did not affect the levels of *HSP70* or actin mRNA (data not shown).

Inducers which activate both *GRP78* and *HSP70* expression. The second class of inducers included heavy metals and amino acid analogs, for example, cadmium sulfate and azetidine. After 2 to 4 h of treatment with azetidine, increased synthesis of both GRP78 and HSP70 was detected (Fig. 11A); at 4 h, these levels were near maximal levels, with only a slight increase thereafter. To determine whether the change in GRP78 and HSP70 synthesis was due to an increase in mRNA levels, total cytoplasmic RNA was isolated from azetidine-treated cells and the relative levels of *GRP78* and *HSP70* mRNA were measured by RNA dot blot analysis. As shown in Fig. 11B, the level of *HSP70* mRNA increased after 2 h of treatment and reached maximal levels by 4 h of treatment, whereas *GRP78* mRNA was only slightly induced at 2 h but also reached maximal levels by 4 h.

The cytotoxic effects of this class of chemicals may be related to their effects on primary protein structure. The effects of amino acid analogs on protein structure are most likely due to their incorporation during the synthesis of the nascent protein, resulting in deleterious effects on protein folding, stability, and, possibly, on function. Heavy metals also have pleiotropic biochemical effects, including protein denaturation.

Inducers which activate *GRP78* and repress *HSP70* expression. Members of the third class of inducers include 2-DG and the calcium ionophore A23187. During incubation with either chemical, the level of GRP78 synthesis and *GRP78* mRNA increased (Fig. 6 and 12). The ionophore A23187 induced GRP78 protein synthesis and *GRP78* mRNA levels with kinetics identical to those observed for 2-DG and similar to those for other GRP78 inducers (Fig. 12A and B). Treatment of HeLa cells with A23187 had a slight inhibitory effect upon overall protein synthesis similar to, but not as drastic as, that observed previously with 2-DG. To determine whether A23187 induction of GRP78 synthesis was due to an increase in mRNA levels, we probed duplicate RNA

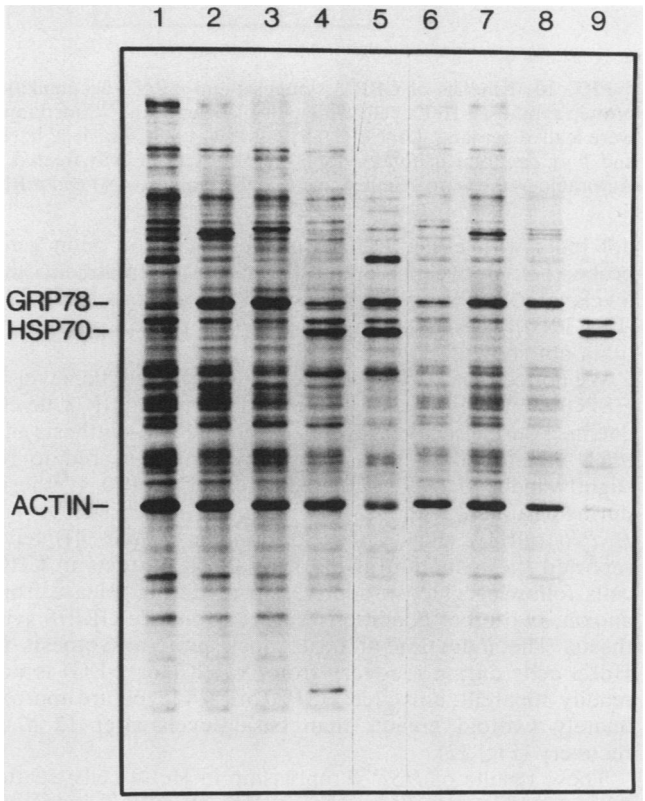


FIG. 9. Effects of various chemicals on the expression of GRP78 and HSP70 in HeLa cells. An autoradiogram of [^{35}S]methionine-labeled proteins from cells given the treatment listed in Table 1 is shown. Lanes: 1, untreated; 2, 2-DG treatment; 3, A23187 treatment; 4, cadmium sulfate treatment; 5, azetidine treatment; 6, castanospermine treatment; 7, tunicamycin treatment; 8, glucosamine treatment; 9, heat shock treatment. Equal trichloroacetic acid-precipitable counts were loaded.

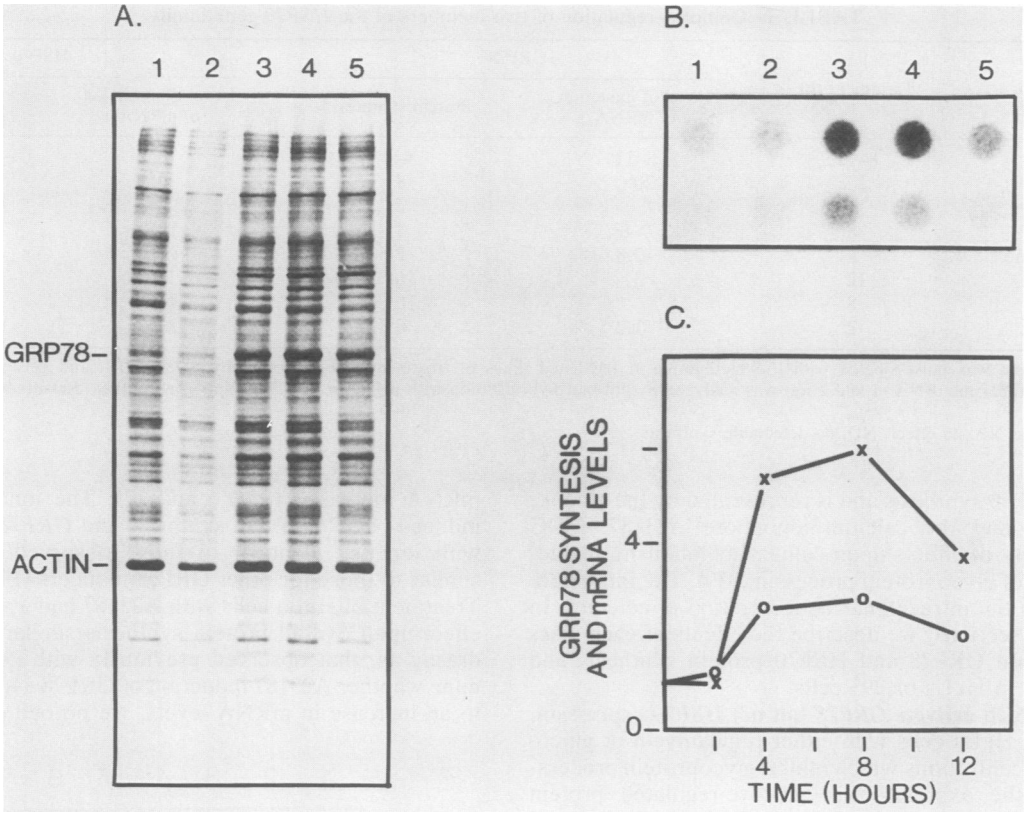


FIG. 10. Kinetics of GRP78 synthesis and mRNA accumulation in HeLa cells treated with castanospermine. (A) Induction of GRP78 protein synthesis. HeLa cells were pulse-labeled with [^{35}S]methionine after various times of treatment. Approximately equal numbers of cells were loaded per lane. Lanes: 1, 0 h; 2, 2 h; 3, 4 h; 4, 8 h; 5, 12 h. (B) Induction of *GRP78* mRNA accumulation. The filters contain 2 μg (top) and 1 μg (bottom) of total cytoplasmic RNA from cells treated for 0, 2, 4, 8, or 12 h with castanospermine (columns 1 through 5). (C) Autoradiograms were scanned and GRP78 synthesis (×) and mRNA (○) levels relative to the control are shown.

dot blots with either the plasmid pHG2 or an actin gene probe (12). Between 8 and 12 h of A23187 treatment, the levels of *GRP78* mRNA increased approximately 20-fold (Fig. 12B), whereas actin mRNA levels remain unchanged (data not shown).

We noticed that as GRP78 synthesis increased, the level of HSP70 synthesis declined. Furthermore, as GRP78 levels declined during recovery from A23187, HSP70 synthesis and mRNA returned to their basal levels or appeared to be slightly induced (Fig. 12). Reduction of HSP70 synthesis during treatment with A23187 was due to decreased levels of *HSP70* mRNA (Fig. 12C). Sciandra et al. (40, 41) have reported the induction of the heat shock proteins in CHO cells following glucose deprivation or during release from anoxia, two other conditions known to induce GRP78 synthesis. The induction of heat shock protein synthesis in HeLa cells during recovery from A23187 (or 2-DG) is not readily apparent; however, *HSP70* mRNA levels are approximately twofold greater than basal levels after 12 h of recovery (Fig. 12).

These results of *HSP70* repression in HeLa cells treated with A23187, as well as the reports of *HSP70* induction during recovery from other known inducers of *GRP78*, suggest an interesting reciprocal relationship between *GRP78* and *HSP70* expression. However, the effects on *HSP70* expression were often difficult to quantitate because *HSP70* is expressed at a low basal level in HeLa cells growing at 37°C. Therefore, we examined the effects of A23187 on the expression of *GRP78* and *HSP70* in 293 cells, cells which express high levels of *HSP70* under normal growth conditions (18, 51). The kinetics of GRP78 induction in 293 cells were identical to those observed in HeLa cells; however, the magnitude of GRP78 synthesis was reduced in this cell line (Fig. 13A). Total cytoplasmic RNA was isolated and hybridized with the cloned *GRP78* and *HSP70* gene probes to measure the relative levels of each mRNA. As the level of GRP78 mRNA increased (Fig. 13B), *HSP70* mRNA levels declined and became undetectable (Fig. 13C). During recovery from A23187, the levels of *HSP70* mRNA increased and *GRP78* mRNA levels declined. Although the results for A23187 treatment are shown, 2-DG had similar effects on *GRP78* induction and *HSP70* repression (Fig. 4, 6, and data not shown).

The decline in *HSP70* mRNA levels during treatment with A23187 or 2-DG could be due to repression of *HSP70* gene transcription or due to effects on *HSP70* mRNA transport and mRNA stability. To distinguish between these possibilities, we examined the rate of *HSP70* gene transcription in HeLa cells treated with 2-DG, in HeLa cells treated with 2-DG and allowed to recover, or in cells incubated at 43°C for 2 h. Nuclei were isolated and incubated in transcription reactions containing [^{32}P]UTP. The ^{32}P-labeled RNA was isolated and hybridized to nitrocellulose filters containing the *GRP78* gene probe (pHG2), *HSP70* gene (pH2.3), actin (pHFβA-1), and a plasmid vector (pGEM1). Surprisingly, the results (Fig. 7) show that the rate of *HSP70* gene transcription did not decrease during 2-DG treatment when *HSP70* mRNA levels declined but appears to be stimulated.

These results suggest that the block in *HSP70* expression during treatment with 2-DG or A23187 is posttranscriptionally regulated.

DISCUSSION

We have isolated human genomic sequences which encode the glucose-responsive protein, GRP78, a closely related member of the *HSP70* gene family. GRP78 is antigenically related to HSP70 and shares extensive homology at the nucleotide and predicted amino acid sequence levels. We have examined expression of both GRP78 and HSP70 genes in response to a wide range of chemicals, many of which have been previously shown to induce these genes. Our results reveal that the expression of these two genes may be independently regulated; the intracellular target of each inhibitor appears to regulate whether one or both stress genes is induced.

There are three lines of evidence to suggest that the four 70,000-Da stress proteins (GRP78, P72, HSP70, and HSP72) in mammalian cells are closely related. First, studies on the purification of HSP70 have revealed several proteins in mammalian cell extracts which have similar electrophoretic characteristics and which are copurified by ATP-agarose chromatography (49). The most acidic protein of the 70,000-Da protein family was identified as the glucose-responsive protein GRP78 (50). The constitutively expressed 72,000-Da protein in HeLa cells is very likely to correspond to an ATPase purified from bovine brain which dissociates clathrin triskelions in vitro (6, 47). Second, genes encoding a 78,000-Da glucose-responsive protein have been cloned from rat, hamster, and human genomes, and the resulting nucleotide sequences reveal homology to HSP70 (23, 30; S. Watowich, C. Hunt, and R. Morimoto, unpublished observation). The third line of evidence comes from our Western blot analysis of control or heat-shocked HeLa cell lysates with a monoclonal antibody directed against *Drosophila* HSP70. The four 70,000-Da stress proteins which share biochemical characteristics are also immunologically related.

We have observed three distinct forms of regulation for *GRP78* and *HSP70* expression: GRP78 synthesis can be induced with no effect upon HSP70, *GRP78* and *HSP70* may be coinduced, or *GRP78* may be induced and *HSP70* may be repressed. The chemicals in the last class are of interest

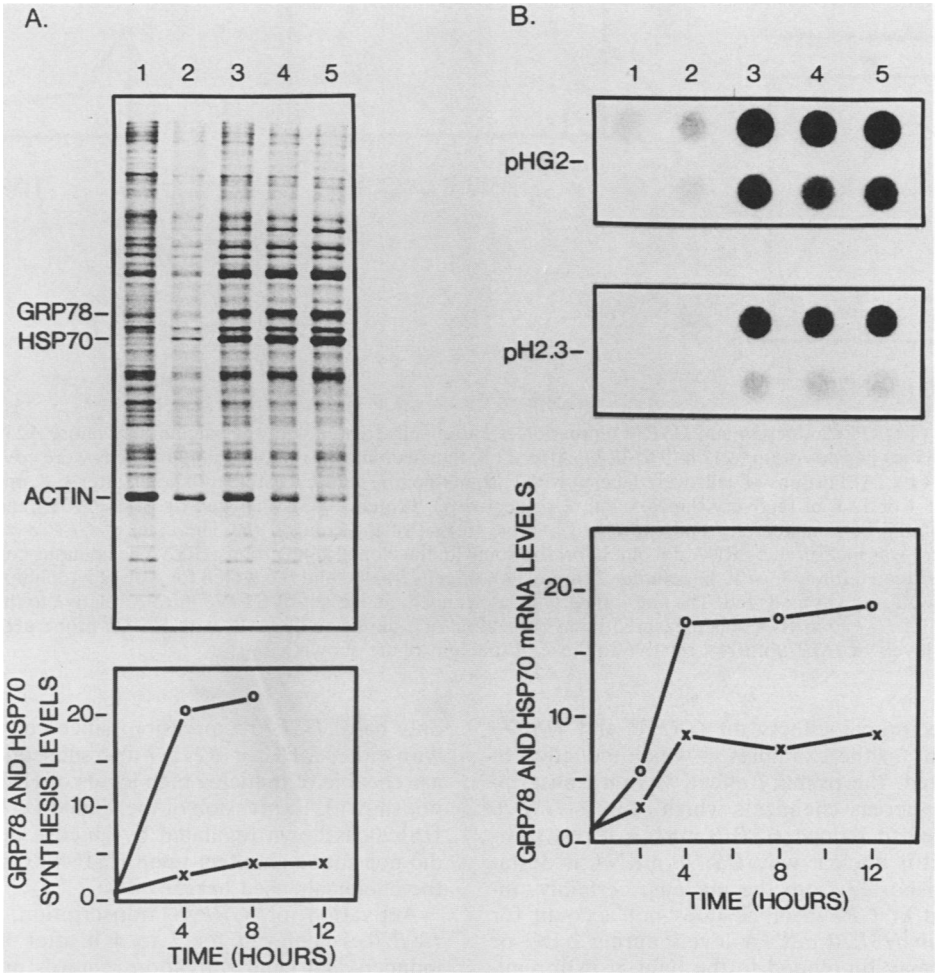


FIG. 11. Kinetics of GRP78 and HSP70 synthesis and mRNA accumulation in HeLa cells treated with azetidine. (A) Autoradiogram of HeLa cells pulse-labeled with [^{35}S]methionine during azetidine treatment and the results of quantitation are shown. Approximately equal numbers of cells were loaded per lane. Lanes: 1, 0 h; 2, 2 h; 3, 4 h; 4, 8 h; 5, 12 h. The autoradiogram was scanned, and the levels of GRP78 (×) and HSP70 (○) synthesis relative to those of the control are shown. (B) RNA dot blot analysis and quantitation. Cytoplasmic RNAs (top rows, 2 μg; bottom rows, 1 μg) isolated during treatment were immobilized on nitrocellulose and hybridized with pHG2 or pH2.3 as indicated. The autoradiogram was scanned, and levels of GRP78 (×) and HSP70 (○) mRNA relative to a control are shown.

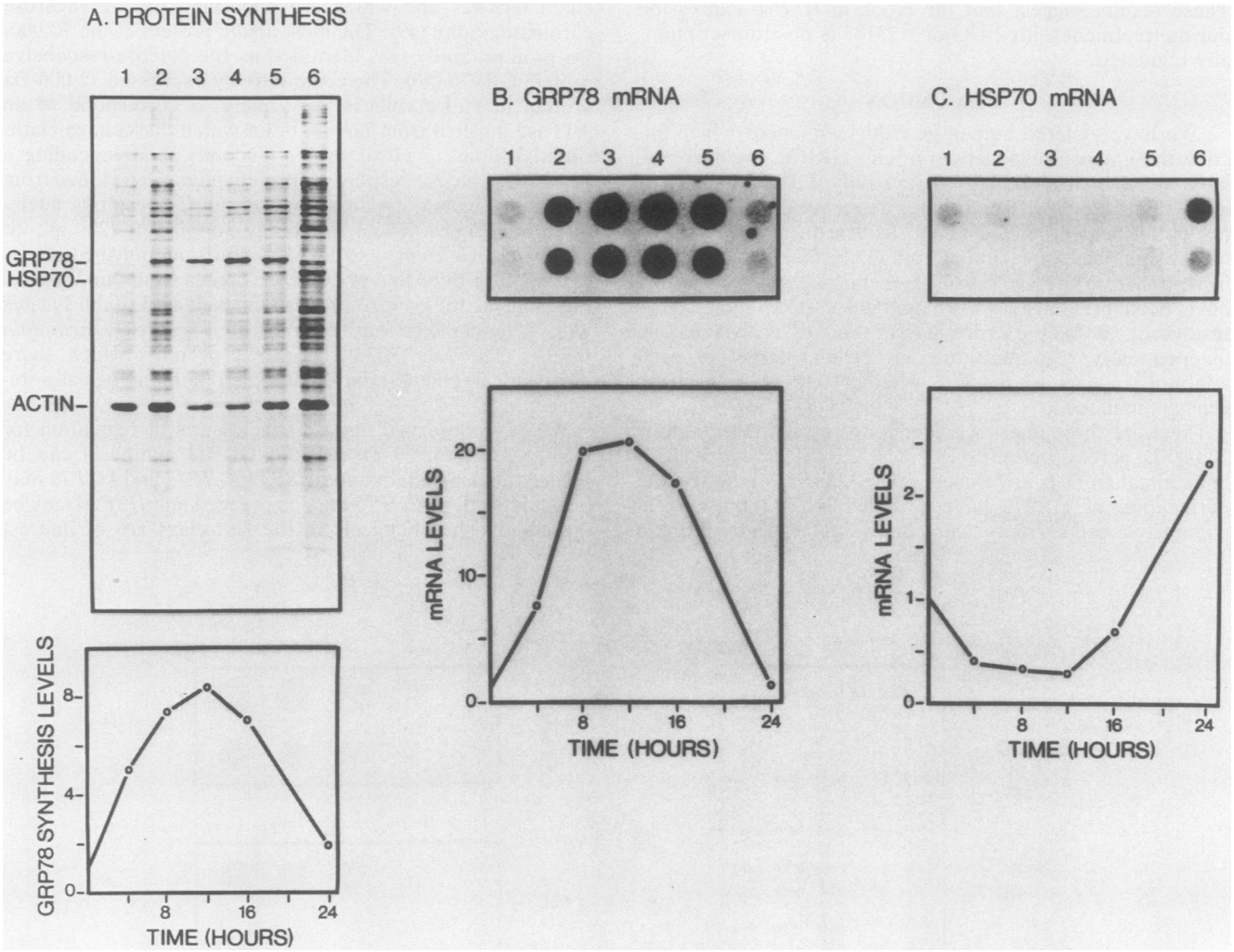


FIG. 12. Kinetics of *GRP78* induction and *HSP70* repression in HeLa cells treated with the calcium ionophore A23187. HeLa cells were incubated with A23187 for periods of up to 12 h (0 to 12 h). After 12 h, the medium was removed, and the cells were covered in fresh medium for up to 12 h (12 to 24 h). (A) Portions of cells were labeled with [^{35}S]methionine before treatment (lane 1), after 4, 8, or 12 h in A23187 (lanes 2 through 4), or after 4 or 12 h of recovery (lanes 5 and 6, respectively). Proteins were analyzed by SDS-PAGE, and the level of GRP78 synthesis was quantitated by densitometry and expressed relative to that of the control. (B) The level of *GRP78* mRNA during A23187 treatment and recovery was measured by RNA dot blot hybridization with the cloned gene probe pHG2. The columns correspond to untreated cells (column 1), cells treated for 4, 8, or 12 h (columns 2 through 4), or cells treated and recovered for 4 or 12 h (columns 5 and 6). RNA (top row, 2 μg; bottom row, 1 μg) was analyzed. The autoradiogram was scanned, and levels of *GRP78* mRNA relative to those of the control are shown. (C) The level of *HSP70* mRNA was measured by hybridization of a duplicate filter (B) with HSP70 probe pH2.3. The results were quantitated, and the levels of *HSP70* mRNA relative to those of the control are shown.

because of the reciprocal effects on *GRP78* and *HSP70* expression. In many of the examples in which induction of *GRP78* was detected, the primary effect was on transcriptional activation, whereas chemicals which repress *HSP70* expression appeared to reduce *HSP70* mRNA levels posttranscriptionally. It is unclear why *HSP70* mRNA is destabilized or not transported from the nucleus; certainly, increased expression of *GRP78* alone does not account for this. The decline in *HSP70* mRNA levels during 2-DG or A23187 treatment may be related to the tight growth-regulated expression of the human *HSP70* gene and the short half-life of *HSP70* mRNA in normally growing cells (29, 45, 52). Since the basal level of *HSP70* mRNA is due to expression at the G1/S boundary, a decrease in basal levels could reflect effects of 2-DG or A23187 on cell growth or the cell cycle. Furthermore, 2-DG or A23187 appears to affect only basal *HSP70* expression, since cells which are treated with either 2-DG or A23187 and subsequently heat shocked are capable of inducing high levels of HSP70 synthesis (data not shown). Expression of the 90,000-Da heat shock protein, HSP90, is down regulated by glucose deprivation (20); we did not notice an effect upon HSP90 expression with any of the chemicals used here.

Activation of *GRP78* transcription, in contrast with *HSP70*, is delayed for 2 to 4 h after the addition of the inducer, and gene activation requires protein synthesis (1, 37, 52). Once stimulated, *GRP78* transcription continues at the fully induced level during treatment. *GRP78* expression is strongly induced by glycosylation inhibitors (castanospermine, tunicamycin, and glucosamine [32, 36]) which affect posttranslational processing events in the endoplasmic reticulum. The primary effect of these inhibitors is to increase the

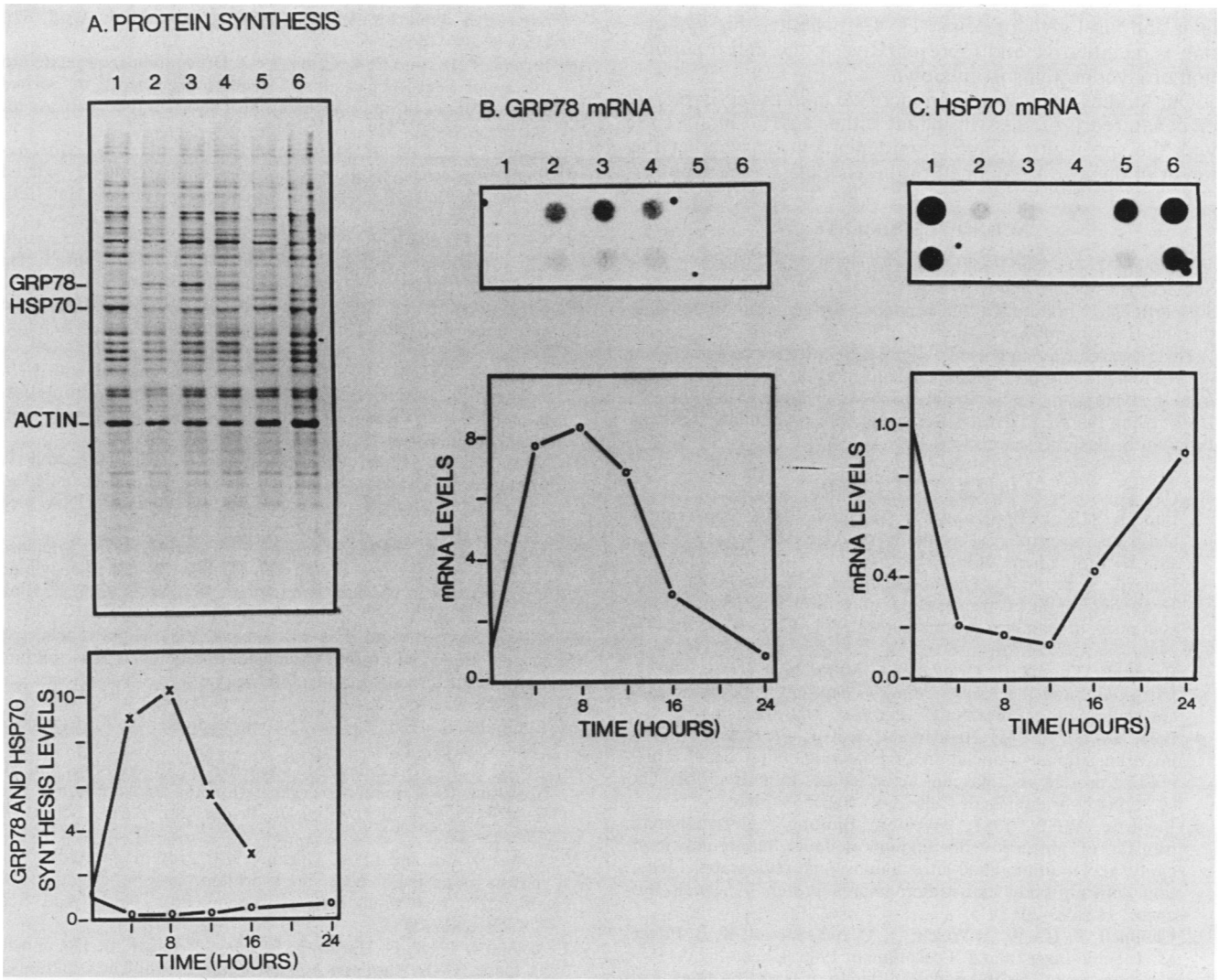


FIG. 13. Kinetics of *GRP78* induction and *HSP70* repression in 293 cells treated with the calcium ionophore A23187. Incubation of 293 cells with A23187 was done for periods of up to 12 h (0 to 12h), and cells were allowed to recover for up to 12 h (12 to 24h). (A) Analysis of [^{35}S]methionine-labeled proteins by SDS-PAGE. Cells were pulse-labeled before treatment (lane 1), at 4, 8, or 12 h of treatment (lanes 2 through 4), and at 4 or 12 h of recovery (lanes 5 and 6). The results were quantitated by densitometry, and the levels of GRP78 (×) and HSP70 synthesis (○) relative to the control are shown. (B) GRP78 mRNA levels during A23187 treatment were analyzed by RNA dot blot hybridization with gene probe pHG2. Cytoplasmic RNA (top row, 2 μg; bottom row, 1 μg) was isolated from untreated cells (column 1), cells treated for 4, 8, or 12 h (columns 2 through 4), and cells recovered for 4 or 12 h. The autoradiogram was scanned, and the levels of *GRP78* mRNA relative to the control are shown. (C) *HSP70* mRNA levels during A23187 treatment were analyzed by hybridization of a duplicate filter (B) to the HSP70 probe pH2.3. The levels of *HSP70* mRNA relative to the control are shown.

levels of improperly glycosylated proteins, possibly resulting in proteins with altered conformation. The solubility and intracellular transport of improperly glycosylated proteins appears to be affected in cells treated with glycosylation inhibitors (22). Only inhibitors which affect glycosylation events in the endoplasmic reticulum induce *GRP78* expression. For example, in cells treated with deoxymannojirimycin, an inhibitor of Golgi-localized mannosidases, no effect upon GRP78 expression was observed (data not shown).

The kinetics of GRP78 synthesis could occur in response to the accumulation of damaged proteins in the endoplasmic reticulum. Munro and Pelham (30) have suggested that an increase in improperly folded glycoproteins in the endoplasmic reticulum is a signal for *GRP78* gene activation. According to this model, there are at least two possible mechanisms for *GRP78* induction. The first possibility assumes that the endoplasmic reticulum monitors the steady-state level of improperly modified or denatured proteins. When this level is exceeded, for example during treatment with glycosylation inhibitors, the GRP78 stress gene is activated. The second possibility implies that the accumulation of damaged proteins could signal the synthesis of a factor which is necessary to stimulate the increase in GRP78 expression, hence the delay in GRP78 activation. These two mechanisms are not mutually exclusive. An obvious and interesting question which arises is how does an event which appears to be compartmentalized in the endoplasmic reticulum signal the transcriptional activation of a nuclear gene?

It has been suggested that GRP78 is similar, if not identical, to BiP (30), a protein that associates with the nonsecreted heavy chain of immunoglobulin in pre-B cells (4, 13). Others have observed that BiP associates with certain cel-

lular and viral glycoproteins (11, 42); whether this association is quantitative and representative of the entire population of glycoproteins is unknown.

Our studies suggest that an increase in improperly folded or denatured proteins will induce either *HSP70* or *GRP78*, with the specificity of induction dependent on whether the target of damage is confined to the endoplasmic reticulum.

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