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Glucose-Regulated Protein (GRP94 and GRP78) Genes Share Common Regulatory Domains and Are Coordinately Regulated by Common *trans*-Acting Factors

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We isolated the promoter of the human gene encoding the 94,000-dalton glucose-regulated protein (GRP94). The 5′-flanking region important for its expression was identified by deletion analysis. Comparison of the promoters of the genes for GRP78 and GRP94 derived from human, rat, and chicken cells revealed a common domain of 28 base pairs within the putative regulatory regions of both genes. This domain has been shown to interact with protein factors in the promoter of the gene for GRP78. Since the genes for GRP94 and GRP78 are transcriptionally regulated with similar kinetics under a variety of stress conditions, we are interested in examining the possible mechanisms for their coordinated expression. Through in vitro and in vivo competition assays, we found that the protein factors which interact with the promoter of the gene for GRP94 also have affinity for the conserved domain of the promoter of the gene for GRP78. These findings suggest that the genes for GRP94 and GRP78 are coordinately regulated through common *trans*-acting factors which recognize a common regulatory domain of glucose-regulated protein gene promoters.

Glucose-regulated proteins (GRPs) were first identified as proteins which are specifically synthesized when eucaryotic cells are depleted of glucose (38). Subsequently, it was found that a variety of reagents which block protein glycosylation or disrupt intracellular calcium stores also enhanced the syntheses of GRPs (23).

The most abundant glycoprotein in the endoplasmic reticulum (ER) is the 94-kilodalton GRP (GRP94). It is also referred to as GP100 (18), ERp99 (26), or endoplasmin (16) and was found to be substantially overexpressed in tissues or cells that are rich in ER (26). Another abundant protein in the ER is the 78-kilodalton GRP (GRP78), which has been identified as the immunoglobulin heavy-chain-binding protein (BiP; 12, 31). GRP78 can bind to immunoglobulin heavy or light chains in lymphoid cells (3, 31a) and proteins which have mutant structures (19). During stress, GRP78 is associated with many other cellular proteins (12). To understand the physiological functions and regulation of these proteins, the GRPs were purified, the amino-terminal sequences were determined (24), and their cDNA clones were isolated and sequenced (25, 30, 39, 40, 42).

By using cDNA clones encoding hamster GRP94 and GRP78 as probes, it was established that the genes for GRP94 and GRP78 are transcriptionally activated with similar kinetics in different species and tissues under stress conditions, particularly those which block glycosylation or disrupt intracellular calcium concentrations (8, 13, 23). This suggests that these two unlinked genes coding for two dissimilar ER proteins are capable of responding to some common stimuli generated by diverse physiological stress conditions. To examine the possible mechanisms by which GRPs are coordinately regulated in mammalian cells, we isolated the genes for GRP78 and GRP94 and characterized their promoters in detail. The studies on the promoter of the gene for GRP78 have already been reported (5, 27, 34, 41). Here, we report on the isolation of the promoter of the gene for human GRP94 and the identification of the region important for its expression by deletion analysis.

Previously, a cDNA and a genomic clone for chicken HSP108 have been isolated and sequenced (14, 20). The isolated gene was initially identified as one encoding a heat shock-inducible protein in chickens. However, its predicted amino acid sequence is identical to that reported for GRP94 (24, 30, 40). Direct sequence comparison between the promoters of the genes for chicken and human GRP94 indicated that a region of 130 base pairs (bp) is highly conserved. Further, a comparison of the promoter sequences of the genes for GRP94 and GRP78 isolated from human, chicken, and rat cells revealed a common domain consisting of 28 bp. This DNA domain was shown to interact with protein factors in the promoters of the genes for human and rat GRP78 and was critical for high-level expression of GRP78 (34). Since the genes for both GRP94 and GRP78 are regulated similarly at the transcriptional level, it is possible that both genes are regulated by common *trans*-acting factors binding to the common, conserved DNA domain. In support of this hypothesis, we found through in vitro and in vivo competition assays that the nuclear factors which interact with the promoter of the gene for GRP94 also have affinity for the conserved domain of the promoter of the gene for GRP78.

MATERIALS AND METHODS

Isolation of human GRP94 genes. The human fetal liver genomic library (21) was screened by using as probes a cDNA plasmid, p4A3, encoding the carboxyl half of hamster GRP94 (25, 40) and a synthetic 18-mer (5′ TTCCACAT CAACTTCATC 3′) corresponding to the noncoding strand of the amino-terminal sequence (residues 2 through 7) of human GRP94 (24; S. C. Chang and A. S. Lee, unpublished data).

For the primary screen, filters representing about 10^6 recombinant phage were prehybridized in 50% formamide–

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5× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate)–5× Denhardt solution (1× Denhardt solution is 0.02% each bovine serum albumin, polyvinylpyrrolidone, and Ficoll)–0.1% sodium dodecyl sulfate–50 mM sodium phosphate buffer (pH 6.5)–1% glycine–50 μg of denatured salmon sperm DNA per ml at 42°C for 1 h, followed by hybridization in the same buffer overnight at 42°C with a 980-bp fragment derived from *Bam*HI and *Eco*RV digestion of p4A3 (25). The DNA fragment was labeled by the hexamer method (9) to a specific activity of 10^8 cpm/μg of DNA. The filters were then washed three times in 5× Denhardt solution–3× SSC–0.1% sodium dodecyl sulfate–0.1% sodium PP_i at 50°C for 60 min each time and then twice in 1× SSC–0.1% sodium dodecyl sulfate–0.1% sodium PP_i at 50°C for 60 min each time.

The positive bacteriophage plaques isolated as described above were rescreened and, in addition, hybridized to the synthetic 18-mer end labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase. Filters were prehybridized in 6× SSC–10× Denhardt solution–50 μg of denatured salmon sperm DNA per ml–30 μg of denatured *Escherichia coli* DNA per ml at room temperature for 15 min, followed by hybridization in the same buffer with the γ-^{32}P-labeled oligomer overnight at room temperature. After hybridization, the filters were washed twice in 6× SSC at room temperature for 10 min each time and then twice at 42°C for 15 min each time.

DNA sequence analysis. The insert contained within the phage clones was isolated, restriction mapped, and sequenced by the dideoxy-chain termination method (36) with either single-stranded M13 or double-stranded pTZ18U DNA as the template (45; Genescribe-Z; description and experimental protocols, United States Biochemical Corp.). About 90% of the sequence was determined on both strands. The sequence comparison was analyzed by using the Intelligenetics Bionet SEQ: SEARCH program.

Cell lines and culture conditions. The temperature-sensitive (ts) mutant cell line K12, derived from Chinese hamster lung fibroblast line Wg1A, has been previously described (22). It was maintained in Dulbecco modified Eagle medium (DMEM [4.5 mg of glucose per ml]) containing 10% cadet calf serum. HeLa D98 AH2 monolayer cells and HeLa S3 suspension cells were grown in DMEM containing 10% fetal bovine serum as previously described (34, 41). The induction conditions by calcium ionophore, glucose starvation, and the K12 ts mutation have been previously described (25, 28, 33).

Isolation of cytoplasmic RNA and RNA blot hybridization. Total cytoplasmic RNA was isolated from the cells as previously described (25). Ten micrograms of RNA from each sample was electrophoresed on formamide-formaldehyde agarose gels and blotted onto nitrocellulose paper (25). Hybridization was performed by using DNA fragments from either p3C5, a cDNA plasmid encoding hamster GRP78 (25, 42), or p4A3, a partial cDNA clone encoding hamster GRP94 (25, 40). The DNA fragments were labeled by the hexamer method (9) to specific activities of ≈ 10^8 cpm/μg of DNA.

Synthetic oligonucleotides. GRP94(−197/−163) was prepared by reannealing two oligomers spanning nucleotides (nt)−197 to −163 of the promoter of the gene for human GRP94 (see Fig. 1). GRP78(−170/−135) was prepared by reannealing two oligomers spanning nt −170 to −135 of the promoter of the gene for rat GRP78 (34). To facilitate subcloning and end labeling, both GRP94(−197/−163) and GRP78(−170/−135) contained an *Xho*I site, CTCGAG, at the 5′ terminus of the coding strand and a *Sal*I site, CAGCTG, at the 3′ terminus of the noncoding strand. The lacZ synthetic sequence, which contained a modified β-galactosidase coding sequence, was prepared by reannealing the 21-mers 5′ TTAGCGTCGAGAATTCCCGGC 3′ and 5′ TAAGCCGGGAATTCTCGACGC 3′.

Plasmids. Chloramphenicol acetyltransferase (CAT) gene fusion constructs pGRP94(−1170)CAT, pGRP94(−357)CAT, and pGRP94(−164)CAT contain the *Bst*EII-*Bam*HI (−1170 to +29), *Bam*HI-*Bam*HI (−357 to +29), and *Bst*EII-*Bam*HI (−164 to +29) fragments of the human GRP94 5′ region sequences, respectively, subcloned into the unique *Hin*dIII site of pSV0CAT (11). Plasmid p94Δ(−231/−43)CAT was constructed by deleting the 189-bp *Sst*II fragment spanning nt −231 to −43 from pGRP94(−357)CAT. Plasmid pGRP78(−480)CAT, containing the promoter sequence of the gene for rat GRP78 similarly fused to the gene for CAT, was previously referred to as pE43 (5).

Plasmids pGRP94(−231/−165) and pGRP94(−231/−43) are subclones of the promoter of the gene for human GRP94. Plasmid pGRP94(−231/−165) contains the *Sst*II-*Bst*EII (−231 to −165) fragment subcloned into the *Sma*I site of pTZ18U. Plasmid pGRP94(−231/−43) contains the *Sst*II-*Sst*II (−231 to −43) fragment subcloned into the *Sma*I site of pUC8. Plasmid pGRP78(−375/−88), containing the *Sma*I-*Stu*I (−375 to −88) promoter-enhancer fragment of the gene for rat GRP78 subcloned into the *Sma*I site of pUC8, was previously referred to as pUC291 (27).

Plasmid pGRP78(−170/−135) contains the synthetic promoter sequence of the gene for rat GRP78 (34) from nt −170 to −135 (with flanking *Xho*I and *Sal*I sites) cloned into the *Sma*I site of pUC8.

Transient transfection and assay for CAT activity. Transfection of DNA into K12 cells, preparation of the cell extract, measurement of the protein concentration, and assays for CAT activity have been previously described (33). Three to five micrograms of each plasmid was used for the transfection assays. Transfection for each CAT construct was repeated three to six times. Equal amounts (about 50 μg) of the cell extract were used for the CAT assays.

Preparation of HeLa nuclear extracts. Nuclear protein extract was prepared from HeLa S3 cells grown in suspension at 35°C to a density of 10^6 cells per ml in DMEM supplemented by 10% fetal bovine serum as previously described (34, 37).

Gel retardation assay. The 118-bp *Eco*RI-*Hin*dIII fragment of pGRP94(−231/−165), containing 67 bp of the promoter of the gene for human GRP94, was labeled at both ends with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase I. The binding reaction contained, in a 20-μl volume, 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 4 μg of HeLa nuclear extract, 2 μg of poly(dI-dC), and 1 ng (45,000 cpm) of the labeled fragment. Similarly, the synthetic oligomer GRP94(−197/−163) (double stranded) was labeled at both ends with [α-^{32}P]dATP by using the Klenow fragment. The binding reaction contained, in a 20-μl volume, 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 1 mM EDTA, 4% glycerol, 1.5 μg of HeLa nuclear extract, 200 ng of poly(dI-dC), and 1 ng (40,000 cpm) of the labeled oligomer. Various competitors were added to the reaction for the competition experiments. The reactions were incubated at room temperature for 20 to 25 min and electrophoresed at 120 V on 5% low-salt nondenaturing polyacrylamide gels (with an acrylamide/bisacrylamide weight ratio of 80) containing 6.7 mM Tris hydrochloride (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA for 1.5 to 2 h as previously described (10).

DNase I footprint analysis. The 118-bp *Eco*RI-*Hin*dIII fragment labeled as in the gel retardation assay was asym-

metrically digested with *Asp*718. The resultant DNA fragments were resolved on a nondenaturing polyacrylamide gel to obtain the labeled noncoding strand of the 111-bp *Asp*718-*Hin*dIII fragment. To label the coding strand, a 207-bp *Eco*RI-*Pst*I fragment containing the *Sst*II-*Sst*II (−231 to −43) sequence of the promoter of the gene for human GRP94 was isolated from pGRP94(−231/−43) and the *Eco*RI end was labeled with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase I. Binding reactions were performed as in the gel retardation assay, except that the reactions contained 0.4 to 0.7 ng of end-labeled DNA, 1 μg of poly(dI-dC), and various amounts of the nuclear extract. After 25 min of incubation at room temperature, 2 μl of 20 mM $MgCl_2$ and 20 μl of 5 mM $CaCl_2$–1 mM EDTA were added. The reaction mixtures were subjected to DNase I digestion (400 and 4 ng for samples with or without the extract, respectively) at room temperature for 60 s, and the reactions were terminated by adding 50 μl of STOP buffer containing 200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate, and 250 μg of tRNA per ml, followed by phenol-chloroform extraction and ethanol precipitation. The DNA was then suspended in formamide dye mix and electrophoresed on an 8 or 6% polyacrylamide–8 M urea sequencing gel. To localize the protected domains, the single-end-labeled DNA was subjected to the Maxam-Gilbert G sequencing reaction (29) and used as a marker.

RESULTS

Isolation and sequence analysis of the human GRP94 5′ region. The cDNA plasmid for hamster GRP94 and the synthetic oligomer corresponding to the amino-terminal sequence of human GRP94 were used to isolate the structural gene encoding human GRP94. A human genomic library was screened with these probes. Nine positive plaques were isolated among the 10^6 phage screened. On the basis of restriction mapping and further hybridization with the GRP94 cDNA and synthetic oligomer probes, they were found to represent two different GRP94-encoding genes. Sequence analysis indicated that one was an intronless, processed pseudogene with deletions (Chang and Lee, unpublished data). The other contained introns at the sites described for the gene for chicken HSP108 (14); this gene was further studied to establish its functionality.

To determine whether the isolated gene for human GRP94 contains a functional promoter, we identified and sequenced its 5′ region (Fig. 1). The sequences include the 5′-flanking sequence, the 5′ untranslated region (UTR), exon 1, and a portion of intron 1. The promoter of the gene for GRP94 contains several putative sites for eucaryotic transcription factors, such as Sp1 and Ap2, as well as six CCAAT sequences within 300 nt of its 5′-flanking sequence.

A comparison of the human and chicken GRP94 5′ sequences revealed several interesting features. (i) Both the human and chicken promoters possess unusual sequences, GTGAAAA and TTGATAA, respectively, around 30 nt upstream of the transcriptional initiation site. (ii) Five CCAAT sequences are conserved in both promoters with four of them in the inverted orientation, ATTGG; in addition, the human promoter has a sixth inverted CCAAT sequence. (iii) Although chickens and humans diverged from each other 300 million years ago, there is a region of high (65%) sequence conservation between nt −195 and −72, suggesting that this domain encompasses sequences important for expression of the gene for GRP94. (iv) The lengths of the 5′ UTR and the first short exon which codes for part of the leader peptide are conserved.

The genes for GRP94 and GRP78 are regulated similarly during stress. By using cDNA clones encoding hamster GRP94 and GRP78 as hybridization probes, it has been shown that the syntheses of both GRPs are greatly enhanced at the transcriptional level in mammalian cells under stress conditions, such as glucose starvation and β-mercaptoethanol and calcium ionophore A23187 treatments (13, 25, 33). Expression of the GRPs is also enhanced when the hamster fibroblast K12 ts mutant cell line, which is blocked in glycosylation at the nonpermissive temperature, is incubated at an elevated temperature (39.5°C) (23, 25). Although the kinetics of induction are similar, the magnitude of the response is always two- to fivefold higher for GRP78 than for GRP94.

Since we planned to use the HeLa cell system, which provides high yields of cellular extracts, to further study the regulation of human GRP94, we first determined whether the genes for GRP94 and GRP78 are regulated similarly in this human cell line. Cytoplasmic RNA was extracted from HeLa D98 AH2 monolayer cells subjected to either glucose starvation or calcium ionophore A23187 treatment. The levels of GRP78 and GRP94 mRNAs were monitored by RNA blot hybridization by using cDNA probes of similar specific activities and lengths and were compared with cytoplasmic RNA extracted from hamster K12 cells. Both GRP94 and GRP78 mRNA levels were induced under the same conditions (Fig. 2). Further, GRP78 hybridization signals were stronger than those of GRP94. The divergence between human and hamster coding sequences probably accounts for the stronger signals seen with the homologous hamster probe. When a human DNA probe was used for hybridization, the human RNA samples gave higher signals than did hamster RNA (Chang and Lee, data not shown).

Promoter activity and regulation of GRPs. To test whether the promoter of the gene for GRP94 isolated from the human genomic library is functional and is regulated similarly to the promoter of the gene for GRP78, a 386-bp *Bam*HI fragment (−357 to +29; Fig. 1) was subcloned into the unique *Hin*dIII site of pSV0CAT, which contains the bacterial gene for CAT (11). The resulting plasmid is referred to as pGRP94(−357)CAT.

The promoter of GRP78 has been isolated from both humans and rats. These promoter sequences are about 80% conserved within a 340-nt region upstream from the transcriptional initiation site of the gene for human GRP78 (41). The promoter of the gene for rat GRP78 has been analyzed extensively, and a plasmid, pGRP78(−480)CAT, has been constructed which contains the promoter of the gene for GRP78 fused to the gene for CAT. pGRP78(−480)CAT has been shown to be active, and it is inducible by A23187 and the K12 ts mutation, which blocks protein glycosylation (5). These plasmids and, for comparison, pSV2CAT (11), which is under the direction of the simian virus 40 early promoter-enhancer, were transfected into hamster K12 cells. After 28 h, the cells were either treated with 7 μM A23187 or shifted to the nonpermissive temperature (39.5°C). Cell extracts were prepared and assayed for CAT enzyme activity.

As shown in Fig. 3A and summarized in Fig. 3C, the 386-bp *Bam*HI fragment of the promoter of the gene for GRP94 contains the sequence required for basal-level expression and inducibility. Five- and fourfold increases in CAT activity were detected for A23187-treated cells and for K12 cells grown at 39.5°C, respectively. In contrast, with pSV2CAT, only about a 1.5-fold increase in CAT activity was detected under both induced conditions. A comparison of pGRP94(−357)CAT activity with that of pGRP78

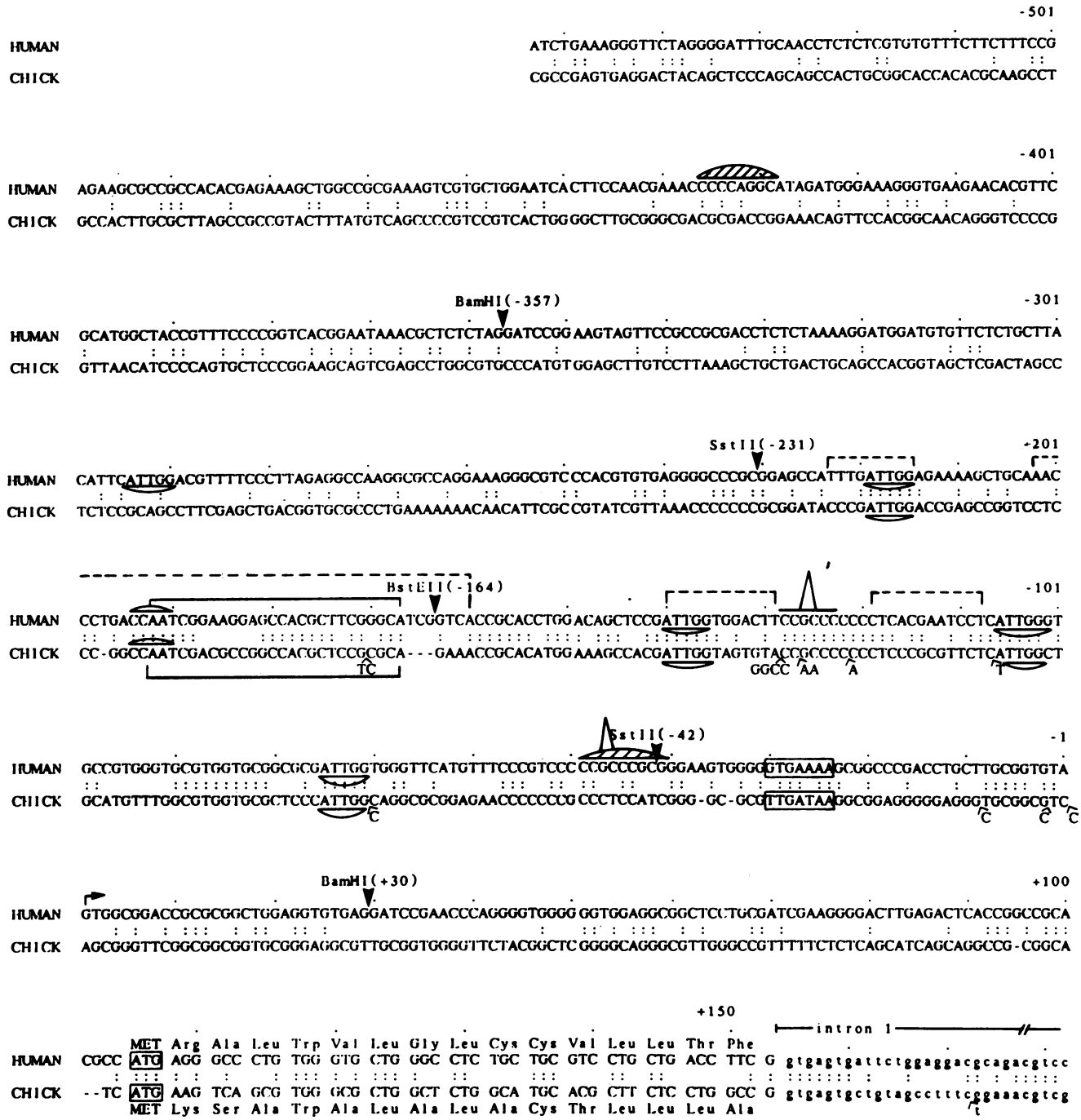

FIG. 1. Sequence of the 5′ region of the gene for human GRP94 and comparison with that of the gene for chicken HSP108 (GRP94). Nucleotides are numbered with the transcriptional initiation site (↱) of the human gene for GRP94 at +1. Downstream bases are numbered positively, and upstream bases are numbered negatively. The restriction sites used for subcloning and for constructing GRP94-CAT fusion genes are noted. Symbols: ⌒, CCAAT; ⌣, CCAAT inverted; △, putative Sp1-binding site; ⩘, Ap2-binding site. The Goldberg-Hogness sequences and the translational start codon, ATG, are boxed. Exon 1 of the GRP94 protein sequence is translated. Exon-intron junction 1 is indicated, and the intron sequence is in lowercase. Identical nucleotides between the sequences of the genes for human and chicken GRP94 are indicated by vertical dots. The domains shared with the genes for GRP78 are bracketed with solid lines. The footprinted regions are bracketed with dashed lines.

(−480)CAT demonstrated that, although the CAT fusion genes for GRP94 and GRP78 are similarly regulated, the CAT activity under the direction of the promoter of the gene for GRP78 is about four- to fivefold higher than that of GRP94. These observations are consistent with previous transcriptional measurements showing that the gene for GRP78 is expressed at a higher level than that for GRP94 (13, 25, 28, 33).

Localization of the regulatory region important for high-level expression of the gene for GRP94. To localize the

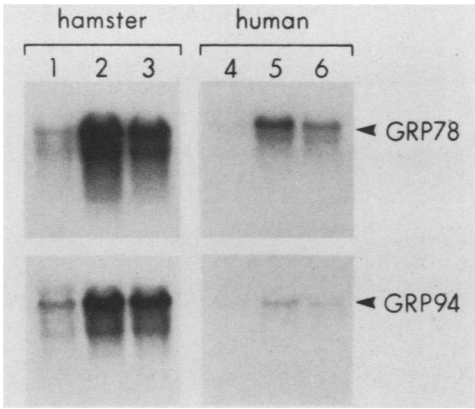

FIG. 2. Transcript levels of GRPs under induced conditions. Total cytoplasmic RNA was extracted from the hamster fibroblast K12 and human HeLa cell lines, which were maintained at 35°C in DMEM (lanes 1 and 4), treated with 7 μM A23187 at 35°C (lanes 2 and 5), shifted to the nonpermissive temperature (39.5°C; lane 3), or grown in glucose-free medium at 35°C (lane 6). Except for lane 5, for which the cells were treated for 5 h, all cells were treated for 16 h. RNA (10 μg) from each sample was used for Northern (RNA) blot analysis. The filters were hybridized with hexamer-labeled probes from cDNA encoding either hamster GRP78 (p3C5) or hamster GRP94 (p4A3). The autoradiograms are shown.

regulatory region in the promoter of the gene for GRP94 that is important for expression, three other CAT fusion plasmids were constructed. pGRP94(−1170)CAT and pGRP94 (−164)CAT contain 1,170 and 164 bp of the GRP94 5′-flanking sequence, respectively (Fig. 3C); and p94Δ(−231/−43)CAT contains an internal deletion in the promoter region spanning nt −231 to −43. Their promoter activities were tested (Fig. 3B), and the results are summarized in Fig. 3C. For the basal activity, we observed a slight decrease (less than twofold) when the 5′-flanking sequence was reduced from 1,170 to 357 bp and a large decrease (about sevenfold) when the sequence was reduced to 164 bp. The basal level was further reduced (by twofold) when the 5′ region was deleted between −231 and −43. Both pGRP94(−1170)CAT and pGRP94(−357)CAT are highly inducible by A23187 and the K12 ts mutation, while partial A23187 and K12 ts inducibility were observed with pGRP94(−164)CAT. The internal deletion mutation p94Δ(−231/−43)CAT, even though it still contains the TATA element and 126 nt of the upstream sequence spanning nt −357 to −232, had minimal basal-level promoter activity and had lost inducibility. These combined results indicate that an important domain required for basal expression of the gene for human GRP94 is located between nt −357 and −164, while the region important for inducibility

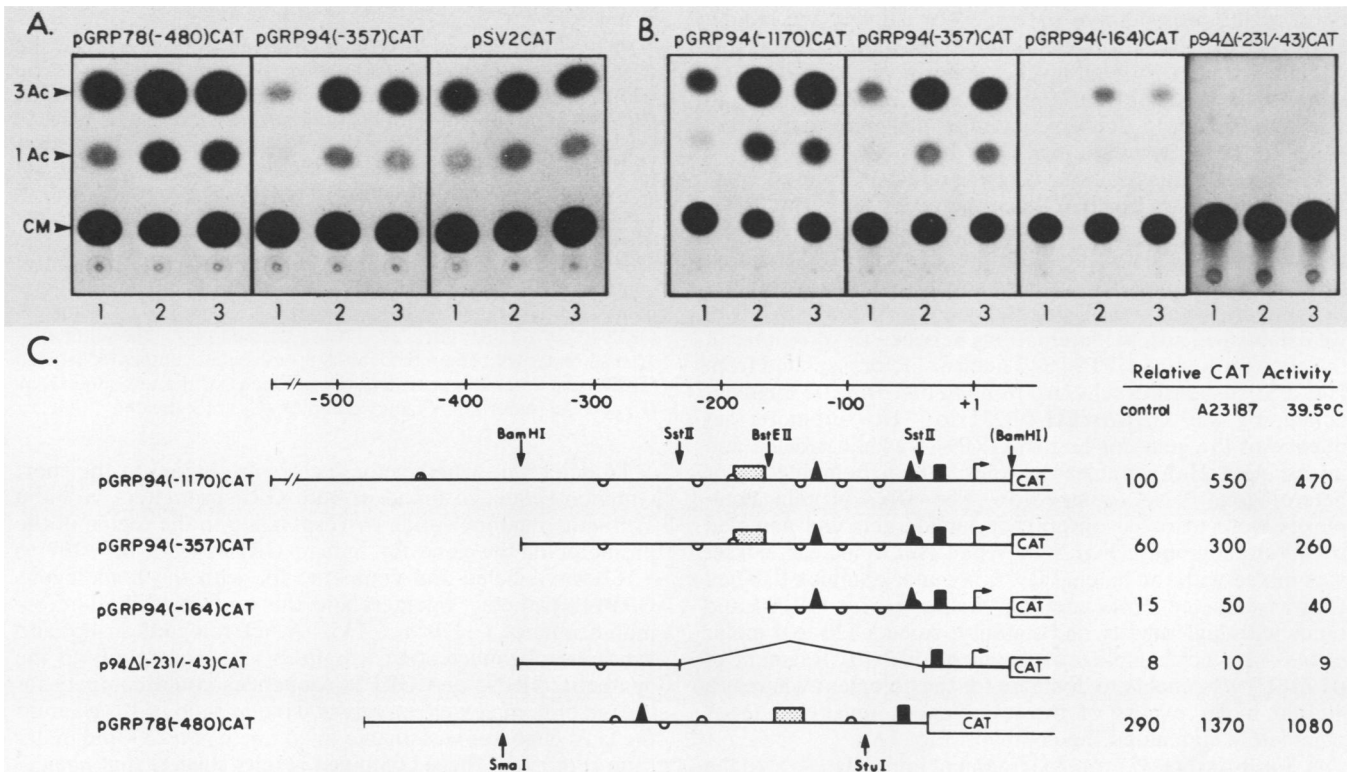

FIG. 3. Promoter activities of GRP-CAT fusion constructs. (A) K12 cells were transfected with pGRP94(−357)CAT, pGRP78(−480)CAT, or pSV2CAT. After 28 h, the cells were maintained at 35°C (lanes 1), treated with 7 μM A23187 at 35°C (lanes 2), or shifted to 39.5°C (lanes 3). After 16 h, protein extracts were prepared from the transfectants and 12 μg from each sample was assayed for CAT activity. The autoradiograms are shown. The positions of chloramphenicol (CM) and its acetylated forms (3Ac and 1Ac) are indicated. (B) K12 cells were transfected with CAT fusion genes containing various lengths (1,170, 357, or 164 bp) of the promoter of the gene for human GRP94 or an internal deletion mutation, p94Δ(−231/−43)CAT. The conditions of treatment were as described above. Equal amounts of cell protein extract from the samples were used for CAT assay analysis. The autoradiograms are shown. (C) The features of GRP promoters contained within the CAT fusion genes are schematically presented. Symbols: ■, Goldberg-Hogness sequence; ⌓, CCAAT; ⌔, CCAAT inverted; ▲, putative Sp1-binding site; ◠, putative Ap2-binding site; ▨, common domain between the promoters of the genes for GRP94 and GRP78; ↱, transcriptional initiation site. The *Sma*I-*Stu*I (−375 to −88) enhancer fragment of the promoter of the gene for rat GRP78 is indicated. The relative promoter activities of the five GRP-CAT fusion constructs are summarized.

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Chick GRP94    -202   AATCGACGCCGGCCACGCTCCGTCCGCA   -175
Human GRP94    -193   AATCGGAAGGAGCCACGCTTCG--GGCA   -168
Human GRP78    -135 GGGCCGCTTCGAATCGGCGGCGGCCA-GCTTGGT-GGCC   - 99
  Rat GRP78    -172 AGGCCGCTTCGAATCGGCAGCGGCCA-GCTTGGT-GGCA   -136
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FIG. 4. Common sequences among the GRP promoters. Sequences shared between the promoters of genes for GRP94 and GRP78 isolated from human, chicken, and rat tissues are shown. The location of each sequence is numbered on the basis of its own transcriptional initiation site. The arrows indicate an inverted repeat found in the genes for both human and rat GRP78 (33).

resides in part within this region and in part between nt −164 and −43.

Common sequence between promoters of genes for GRPs. To identify the putative common regulatory domains of the promoters of the genes for GRP94 and GRP78, the promoter of the gene for human GRP94 was compared with that of the gene for chicken GRP94 and the genes for human and rat GRP78. The promoter sequences of the genes for GRP94 and GRP78 are dissimilar except for a short domain located from nt −193 to −168 (Fig. 4). This highly similar region lies within the *Sam*I-*Stu*I (−375 to −88) fragment (Fig. 3C) implicated for high-level expression of the gene for GRP78 by deletion analysis and in vivo competition (5, 27). Further, this domain lies within the region which is highly conserved between the promoters of the genes for chicken and human GRP94 (−195 to −72). Deletion analyses indicated that this domain is crucial for high-level expression and partial inducibility of the gene for GRP94. This domain has been shown to bind specifically to nuclear factors in the promoters of the genes for both rat and human GRP78 (34).

Promoters of the genes for GRP94 and GRP78 can compete for nuclear factors in vitro. Since the genes for both GRP94 and GRP78 are regulated similarly, it is possible that the regulation of the genes for both proteins involves common *trans*-acting factors. We further investigated this hypothesis by competition assays. Using the gel retardation assay (10), we established specific interactions between the promoter of the gene for human GRP94 and nuclear factors isolated from HeLa S3 suspension cells. A 118-bp *Eco*RI-*Hin*dIII fragment containing the *Sst*II-*Bst*EII (−231 to −165) promoter sequence of the gene for human GRP94 was end labeled and mixed with HeLa nuclear extract with a homologous or heterologous DNA competitor. The DNA-protein complexes were resolved on polyacrylamide gels and detected by autoradiography (Fig. 5A). When HeLa nuclear extract was mixed with the labeled DNA, a major complex (labeled C) was detected. This complex is specific for GRP94 and binds with high affinity and stability, since a 120-fold molar excess of a nonhomologous fragment (a *Pvu*II fragment of pTZ18U) was unable to compete for the complex, whereas a 40-fold molar excess of the homologous unlabeled DNA completely eliminated the complex (Fig. 5A).

A *Sma*I-*Stu*I (−375 to −88) fragment immediately 5′ to the TATA element of the gene for rat GRP78 (27, 34; as indicated in Fig. 3C) has been identified as an enhancer. In cotransfection assays, it reduced the activity of the promoter of the gene for GRP78, suggesting that it can compete for cellular factors interacting with the promoter of the gene for GRP78 under induced conditions (27). When the GRP78 *Sma*I-*Stu*I (−375 to −88) fragment was used as a competitor, it was capable of competing for the complex at a 40-fold molar ratio of unlabeled GRP78 DNA/labeled GRP94 DNA (Fig. 5B).

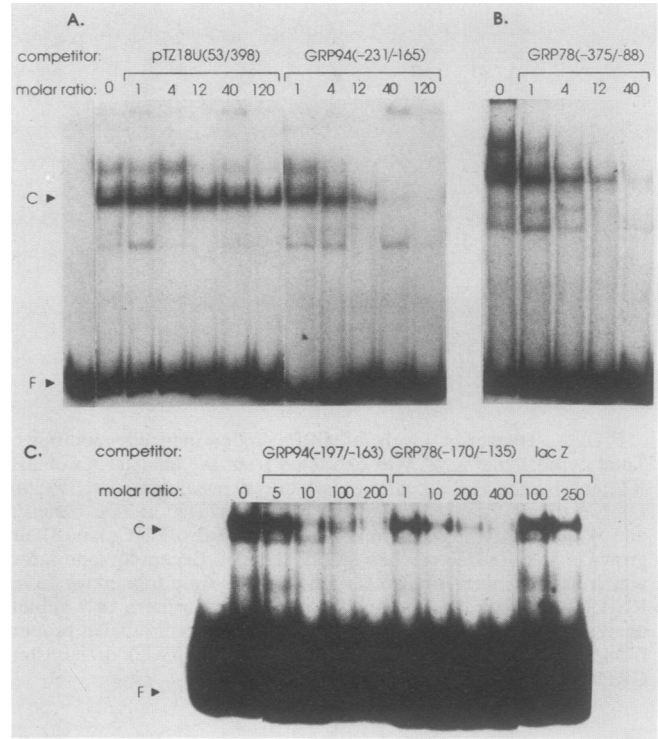

FIG. 5. Competition for cellular factor in vitro. For panels A and B, the labeled 118-bp *Eco*RI-*Hin*dIII fragment containing 67 bp (*Sst*II-*Bst*EII [−231 to −165]) of the promoter of the gene for human GRP94 was mixed with 4 μg of HeLa nuclear extract. For panel C, the labeled oligomer GRP94(−197/−163) was mixed with 1.5 μg of HeLa nuclear extract. The exceptions were lanes 1 in panels A and C, to which only the labeled probes were added. Various amounts of unlabeled competitor were added together with 1 ng of the probe. The following competitors were used: A, a *Pvu*II fragment (53 to 398) of pTZ18U and the unlabeled 118-bp *Eco*RI-*Hin*dIII fragment containing GRP94(−231/−165); B, the 298-bp *Eco*RI-*Bam*HI fragment containing GRP78(−375/−88); C, synthetic oligomers GRP94(−197/−163), GRP78(−170/−135), and lacZ. The autoradiograms are shown. The molar ratios of unlabeled competitor/labeled GRP94 promoter fragment and the positions of the unbound DNA (F) and the major DNA-factor complex (C) are indicated.

To determine whether the factors are bound to the short common sequence found in both GRP promoters, a 46-bp synthetic oligonucleotide corresponding to the region of the promoter of the gene for human GRP94 from nt −197 to −163 was labeled and competed for with the homologous GRP94 synthetic oligomers and the synthetic GRP78 common sequence (−170 to −135). A heterologous 21-bp lacZ synthetic oligonucleotide was used as a control. Both the synthetic GRP94 and GRP78 sequences could compete for the major complex effectively at a molar ratio of 10, whereas the lacZ oligomer was unable to compete at a 250-fold molar ratio (Fig. 5C). These combined results suggest that nuclear factors which specifically bind to the promoter of the gene for GRP94 also have affinity for the GRP78 conserved domain. These in vitro competition results support the hypothesis that GRP94 and GRP78 are coordinately regulated through common *trans*-acting factors.

Protein-binding sites within the promoter of the human GRP94 gene. With the gel retardation assays, we detected a major complex formed between HeLa nuclear extract and the *Sst*II-*Bst*EII (−231 to −165) fragment of the gene for human GRP94. To define more precisely the binding sites,

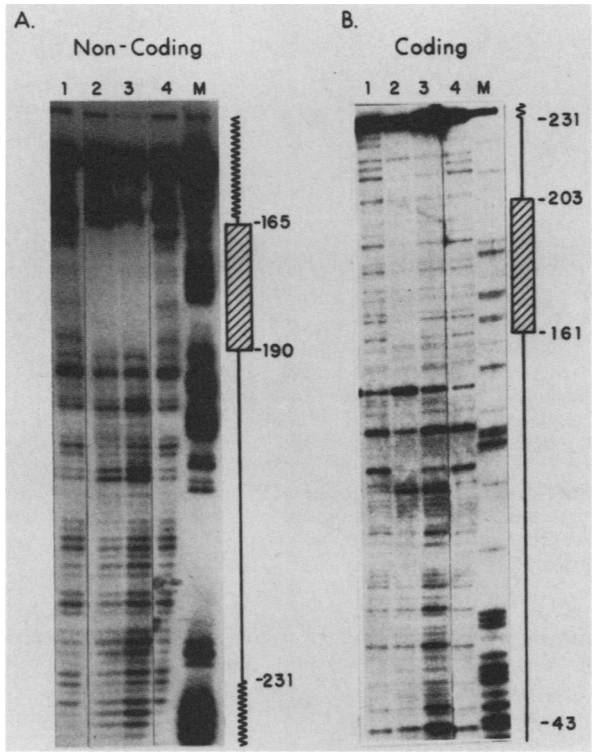

FIG. 6. Footprint analysis of the promoter of the gene for human GRP94. The DNA probe was incubated with HeLa nuclear extract and then digested with DNase I. The autoradiograms are shown. (A) Noncoding strand footprint. Lanes: 1 and 4, DNase-treated probes without protein extract; 2 and 3, DNase-treated probes with 63 and 42 μg of protein extract, respectively. The box (−190 to −165) represents the footprinted region. (B) Coding strand footprint. Lanes: 1 and 4, DNase-treated probes without protein extract; 2 and 3, DNase-treated probes containing 72 μg of protein extract without (lane 2) or with (lane 3) a 55-fold molar excess of the synthetic rat GRP78 promoter sequence (−170 to −135). The major footprinted region (−203 to −161) is boxed. Lanes M contained the Maxam-Gilbert G sequencing reaction (29) of the labeled strand.

the noncoding strand of the same fragment was end labeled, incubated with HeLa nuclear extract, and then digested with DNase I. A region spanning from nt −165 to −190 was protected from DNase I digestion (Fig. 6A). This domain resides within the region required for high-basal-level expression and partial inducibility of the gene for GRP94 (Fig. 3) and is shared between the genes for GRP94 and GRP78 (Fig. 4).

A bigger domain spanning nt −161 to −203 was protected when a 207-bp *Eco*RI-*Pst*I fragment containing the *Sst*II-*Sst*II (−231 to −43) sequence of the promoter of the gene for GRP94 was end labeled at the coding strand and used for footprinting analysis (Fig. 6B). Some other minor footprinted regions were also seen. The footprinted regions are indicated in Fig. 1. Addition of the unlabeled conserved domain of the gene for GRP78 to the binding reaction reduced the protection (Fig. 6B). This confirms the finding by the gel retardation assay that nuclear factors which bind to the promoter of the gene for GRP94 also have affinity for the conserved domain of the gene for GRP78.

Promoters of the genes for GRP94 and GRP78 can compete for cellular factors in vivo. To correlate the in vitro competition results with biological functions in vivo, we tested whether the promoter of the gene for GRP78 could also compete for the factors interacting with the promoter of the gene for GRP94 in vivo. Various amounts of pGRP78(−375/−88), which contains the promoter fragment of the gene for GRP78 subcloned into plasmid pUC8, were cotransfected with test plasmid pGRP94(−357)CAT into K12 cells. Previously, pGRP78(−375/−88) has been shown to compete for cellular factors binding to the promoter of the gene for GRP78 (27). To maintain a constant amount of exogenous DNA in each transfection, pUC8 was added to adjust the total plasmid DNA concentration. Addition of increasing amounts of the competitor plasmid diminished the CAT activity driven by the promoter of the gene for GRP94 under both induced and noninduced conditions, suggesting that the promoter fragment of the gene for GRP78 can also compete for cellular factors which interact with the promoter of the gene for GRP94 (Fig. 7). This provides the first in vivo

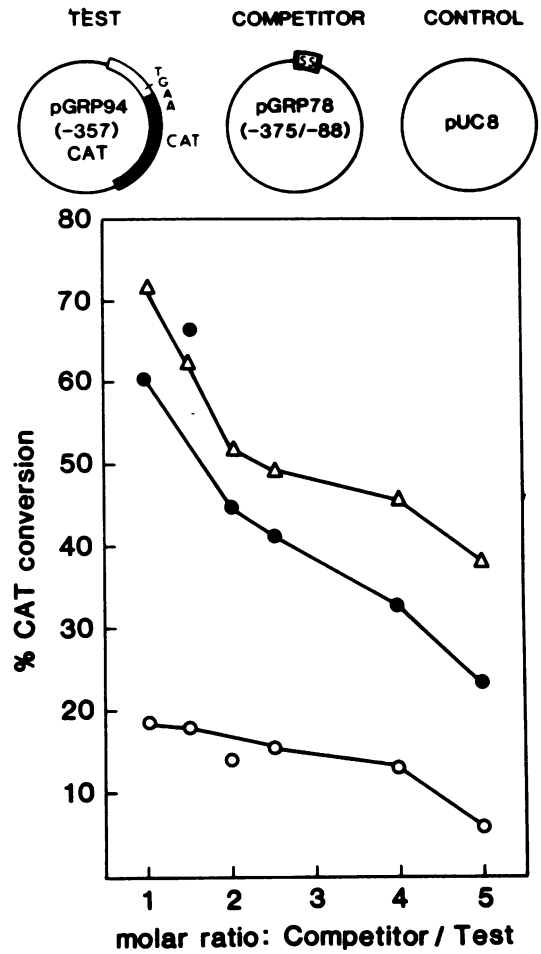

FIG. 7. Competition for cellular factors in vivo by the *Sma*I-*Stu*I (−375 to −88) fragment of the gene for rat GRP78. K12 cells were cotransfected with 5 μg of test plasmid pGRP94(−357)CAT and increasing amounts of competitor plasmid pGRP78(−375/−88). To maintain a constant amount of DNA for each transfection, pUC8 was added to adjust the total plasmid DNA to 20 μg. Protein extracts were prepared from the transfected cells maintained in DMEM at 35°C (○), treated with 7 μM A23187 (△), or shifted to 39.5°C (●). The CAT activities were determined by using 60 μg of protein from each cell extract. The CAT activity, expressed as percent conversion of [^{14}C]chloramphenicol to its acetylated forms, was plotted against the molar ratio of the competitor to the test plasmid.

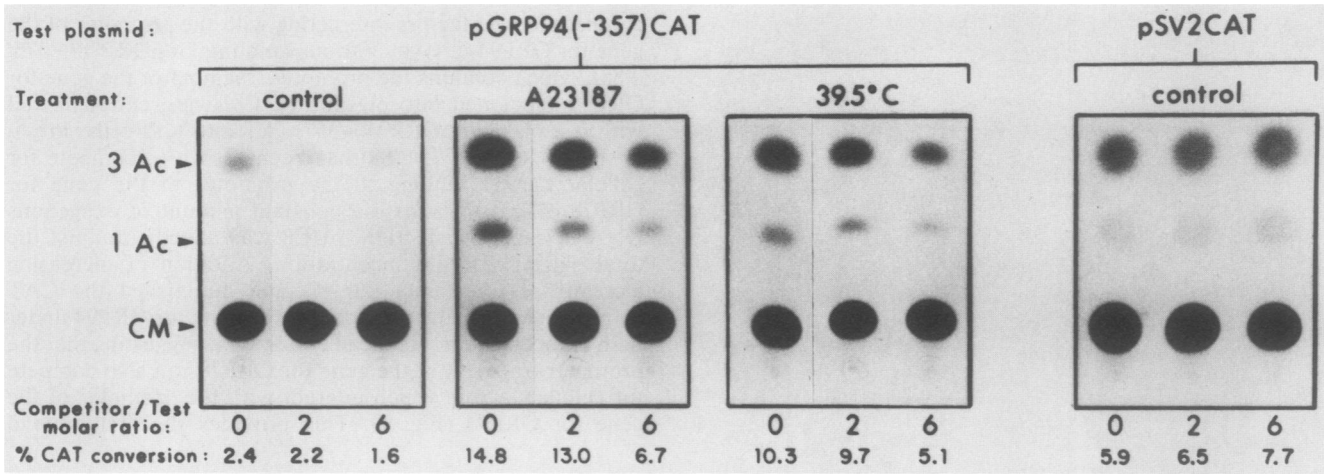

FIG. 8. In vivo competition by GRP78 common domain (−170 to −135). The test plasmids used were pGRP94(−357)CAT and pSV2CAT. The competitor plasmid used was pGRP78(−170/−135). The competition was performed as described in the legend to Fig. 7, except that 3 μg of the test plasmid was used for transfection and 120 μg of protein extract was used for each CAT assay. The positions of chloramphenicol (CM) and its acetylated forms (3 Ac and 1 Ac) are indicated to the left.

evidence that common *trans*-acting factors are important for coordinated expression of the genes for GRP94 and GRP78.

To test further whether the common factors recognize the common GRP regulatory domain, competition experiments were performed with the synthetic GRP78 sequence (−170 to −135) subcloned into pUC8. This 36-nt region effectively competed for cellular factors involved in both basal-level and induced expression of the gene for GRP94 by A23187 and the K12 ts mutation (Fig. 8). At a competitor/test plasmid molar ratio of 6 to 1, the CAT activity of the test GRP94 CAT plasmid was reduced by half. In contrast, the promoter of pSV2CAT was not affected in parallel competition experiments (Fig. 8). These combined results indicate that the factors necessary for expression of the gene for GRP94 recognize the common GRP domain. Since this domain is unique to the GRP genes and is devoid of binding sites for general transcription factors, such as Sp1, Ap2, or CAAT, it is likely that novel nuclear factors specific for the GRP genes are involved.

DISCUSSION

The GRP genes encode a set of ER proteins whose syntheses are greatly enhanced under conditions which specifically affect cellular glycosylation (5, 23) or protein conformation in the ER (13, 19). It was established that the genes for both GRP94 and GRP78 are constitutively expressed at low levels in eucaryotic cells and are regulated with similar kinetics at the transcriptional level under a variety of stress conditions (23). Thus, the GRP genes present a useful model system for studying coordinated gene regulation in mammalian cells.

Although the structure and function of GRP78 as a binding protein in the lumen of the ER is established (3, 12), the exact structure and biological function of GRP94 is unknown. GRP94 is a major glycosylated protein in the ER. However, it is controversial whether it is a transmembrane or soluble protein located in the ER lumen (17, 30). Previously, we have hypothesized that acidic amino termini of GRP94 and GRP78 may provide binding sites for calcium ions (24). Sequence analysis subsequently revealed highly acidic residues at the carboxyl termini as well (30, 31, 42). Calcium-binding studies showed that GRP94 contains low-affinity but high-capacity calcium-binding sites (15). Interestingly, GRP94 shares about 50% amino acid identity with cytoplasmic *Saccharomyces cerevisiae* HSP90 and *Drosophila melanogaster* HSP83 (30, 40). Thus, GRP94, HSP90, and HSP83 may have evolved from the same protein family. It has been shown that HSP90 possesses highly negatively charged amino acid domains. It is possible that these domains and the helical conformation of HSP90 can mimic a DNA-binding domain in that HSP90 was shown to complex with steroid receptors and maintain them in an inactive form (4, 35). We and others have observed that GRP94 has a native molecular size of 192 kilodaltons, suggesting that the protein either forms a dimer or is covalently linked with another protein (24). By analogy to HSP90, GRP94 may be capable of binding to membrane and surface receptors which are cycled through the ER, stabilizing them until they are properly folded or fully assembled.

GRP94 mRNA was reported to be induced by progesterone or estrogen in chicken oviduct tissue in a tissue-specific manner (1). The consensus sequences required for steroid inducibility were found in the chicken promoter (14). However, within 550 nt upstream of the transcriptional initiation site of the gene for human GRP94, we did not detect sequences similar to either the steroid-responsive or heat shock consensus elements. GRP94 is not inducible by heat or progesterone in wild-type hamster fibroblasts such as Wg1A. However, in the T47D human breast cancer cell line treated with 5 to 20 nM progesterone for 24 h, the GRP94 mRNA level was elevated by 2.5- to 3-fold (Chang and Lee, unpublished data).

The promoter of the gene for GRP94 possesses an uncommon sequence, GTGAAAA, upstream of its cap site. While it remains to be determined whether this sequence functions as the ATA sequence for the gene for GRP94, atypical ATA sequences in which a G residue interrupts the A-T-rich oligonucleotides are also found in the genes for chicken GRP94 (TTGATAA; 14), β-globin (GATAAAA; 6), and type 5 actin (ATAGAAA; 2). Such T-to-G, T-to-A transversions and A-to-G transitions reduce the levels of transcription both in vitro and in vivo (7, 43, 46). Perhaps the uncommon GTGAAAA sequence of the gene for GRP94 partially accounts for the lower promoter activity of the gene for GRP94

compared with that of the gene for GRP78. Assuming that the GTGAAAA sequence is not generated by a cloning aberration, we speculate that it interacts with a new class of promoter factors different from those recently isolated which recognize the typical TATA sequences (32).

A sequence comparison of the human GRP94 5′ flanking region with that of chicken HSP108 revealed a highly conserved domain between nt −195 and −72, implying that this region contains sequences important for the expression and regulation of the gene for GRP94. The crucial functional domain for high-basal-level expression and partial induction of the gene for GRP94 was located between nt −357 and −164 (Fig. 3). Interestingly, a comparison of the common domains of the genes for GRP94 and GRP78 revealed that the gene for GRP94 contained only half of the palindromic sequence observed in the gene for GRP78 (Fig. 4). If palindromic domains are more favorable for cooperative interaction of regulatory factors (44), this may account for the lower basal-level activity of the gene for GRP94 relative to that of the gene for GRP78.

Both in vivo and in vitro competition assays showed that the factors which bound to the promoter of the gene for GRP94 also had affinity for the *SmaI-StuI* (−375 to −88) fragment and/or the conserved domain of the promoter of the gene for GRP78 (Fig. 4). Further, the sequences common to the genes for both GRP94 and GRP78 were protected from DNase I digestion (Fig. 6; 34). These combined results demonstrated that the genes for GRP94 and GRP78 share common positive transcription factors. Identification of the minimal DNA sequence involved in GRP expression and purification of the *trans*-acting factors will further our understanding of the mechanism for coordinated control of the GRP genes.

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