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Common sets of nuclear factors binding to the conserved promoter sequence motif of two coordinately regulated ER protein genes, *GRP78* and *GRP94*

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ABSTRACT

The GRP78 and GRP94 are two constitutively expressed ER resident proteins that are coordinately induced in response to stress conditions. The control of their induction is at the transcriptional level. We have previously demonstrated that the *GRP78* and the *GRP94* promoters share a common regulatory domain which is highly conserved. We report here that within this 36 bp promoter region is a CG/CAAT and a GC-rich sequence motif which are important for basal level and induced expression of the gene. Gel mobility shift assays with HeLa nuclear extracts and the conserved element from *GRP78* and *GRP94* show two shared, specific protein-DNA complexes. By ultraviolet cross-linking, the sizes of the proteins labeled in the slower-migrating complex are 210-, 110-, a doublet at 90/92- and 70 kD, and in the faster-migrating complex, protein species of about 55 kD. The formation of the second complex can be inhibited by competition with the coding strand of the conserved *GRP* element in a sequence specific manner. In addition, the Ku autoantigen which is abundant in HeLa cell extracts also binds. The sizes of the nuclear factors binding to the *GRP78* and *GRP94* conserved promoter elements are strikingly similar, providing further evidence that the two genes are coordinately regulated by common *trans*-acting factors.

INTRODUCTION

The endoplasmic reticulum (ER) is the cellular organelle where lipids, proteins and complex carbohydrates destined for transport to the Golgi apparatus, the plasma membranes, the lysosomes, or to the cell exterior are synthesized and processed. Therefore, it is of interest to understand the functional components of the ER and their regulation. In mammalian cells, several proteins have been isolated, cloned and identified as proteins residing within the lumen of the ER and they include GRP78 and GRP94 (1–3). In addition to being major structural components of the ER, GRP78 and GRP94 are stress-inducible proteins sharing about 50% amino acid identity with the heat shock proteins HSP70 and HSP83 respectively (4–6).

GRP78, also known as BiP, belongs to a class of polypeptide chain binding proteins generally termed molecular chaperones (7). It can bind to nascent proteins and facilitate their transport, folding and assembly as they traverse through the ER (8,9). In physiologically stressed cells, it can also bind to under-glycosylated, malfolded proteins or proteins arrested in the ER (10–15). GRP94 is the most abundant calcium binding glycoprotein in the ER (1). Recent evidence shows that both GRP94 and GRP78 can form complexes with partially glycosylated wild-type viral glycoproteins and their mutant forms (15). Another mechanism whereby GRP94 and GRP78 can regulate ER protein transport is through their calcium binding capabilities, as it has been hypothesized that calcium flux can regulate protein flow through the ER (16). Recently, we have found that in hamster ovary (CHO) cells, inhibition of expression of GRP78 and GRP94 correlates with slower growth rates and reduced viability after calcium stress (17).

The *GRP* gene system provides a model for studying coordinate gene expression of a set of ER protein genes. Both *GRP78* and *GRP94* genes are expressed constitutively at a basal level under normal growth conditions; and under a variety of stress conditions, most notably those which block protein glycosylation or disruption of intracellular calcium stores, both genes are induced with similar kinetics (4 and reviewed in 18). We speculate that a common mechanism may be responsible for their coordinated expression, although this mechanism is not well understood.

Previously we have isolated and characterized the promoters of *GRP78* and *GRP94* from mammalian cells (3,4,19). By a combination of 5′ deletion analysis and DNase I footprint analysis we have identified promoter elements which are required for their basal and induced expression. Several evidences suggest to us that a region, referred below as the core, which spans 134 to

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170 base pairs (bp) upstream of the transcriptional initiation of the rat *GRP78* promoter, may be important for the coordinate expression of the *GRP78* and *GRP94* genes. First, the core element is highly conserved among GRP promoters from many species, including *C.elegans*, chick, rat, and human (4,20). Second, the region is footprinted in both the *GRP78* and *GRP94* promoter (4,20). Third, synthetic oligomer of the core can compete *in vitro* for nuclear factors binding to both GRP promoters. Fourth, the core sequence can reduce both the basal and induced activities of *GRP* promoters in *in vivo* competition assays (4). Fifth, stable integration and amplification of the core in CHO cells coordinately downregulates *GRP78* and *GRP94* expression (17).

In this report, we further investigate the functional domains of the conserved core and focus on identifying nuclear factors which bind specifically to the core element. We show that the core consists of a CG/CAAT and a GC-rich sequence motif with differential functions. At least two specific protein-DNA complexes are detected with nuclear extracts from HeLa cells *in vitro*. By ultraviolet (UV) cross-linking, proteins of 210-, 110-, 90/92- and 70 kD are labeled in one complex. The other complex labels protein species around 55 kD which have affinity for the coding strand of the core sequence. In addition, the Ku autoantigen (21) which is abundant in HeLa nuclear extracts also form complexes with the core. The sizes of the nuclear factors binding to the *GRP78* and *GRP94* core are strikingly similar.

MATERIALS AND METHODS

Plasmids

Plasmids pGRP78(−154)CAT and pGRP78(−130)CAT are 5′ deletion of rat *GRP78* promoter linked to the chloramphenicol acetyl transferase (CAT) gene (20). pGRP78(−169)CAT was generated from a linker-scanning mutant LS170CAT which contains an unique HindIII site at −170 to −179 (Wooden and Lee, submitted). Digestion of LS170CAT with HindIII and NdeI, followed by religation, resulted in a 5′ deletion with an end point at position −169.

To construct p78-1Xcore, a synthetic, double-stranded oligonucleotide spanning the rat core sequence (−170 to −134) was subcloned into the unique SmaI site of pUC13.

Transfection and CAT assay

The Chinese hamster fibroblast K12 cell line, maintained in DMEM containing 10% cadet calf serum, were co-transfected with 3 μg of the test plasmids and 3 μg of pCH110, which contains the β-galactosidase gene driven by the SV40 promoter (22) as previously described (23). Protein extracts were prepared from cells maintained in DMEM at 35°C or treated with 7 μM A23187 for 16 hr. For β-galactosidase assay, 10 μg of cell extract was adjusted in 0.25 M Tris, pH7.8 to a volume of 200 μl. 500 μl of solution 1 (60mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 50 mM 2-mercaptoethanol) and 100 μl of solution 2 (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 2 mg/ml o-nitrophenyl-β-D-galactopyranoside) were added to each sample, and incubated for ~30 min at 37°C until a yellow color appeared. The reaction is then stopped by adding 500 μl of Na_2CO_3. Absorbance at 420 nm were measured. The linear range of the assay is between 0.25−0.4. CAT assays were carried out as previously described (23). The CAT activities were quantitated by an AMBIS Radioanalytic Imaging System (Ambis System, San Diego, CA) and normalized against the β-galactosidase activities which served as internal control for transfection efficiencies.

Preparation of HeLa nuclear extracts

Nuclear extract was prepared from HeLa S3 (5 to 7 liters grown in suspension in DMEM containing 10% calf serum as described (24). After ammonium sulfate precipitation, the extract was dialyzed against a buffer containing 20 mM Hepes, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT and 20% glycerol. The extracts were quick frozen in liquid nitrogen and stored at −80°C. For the preparation of A23187 induced nuclear extract, the HeLa cells were treated with 2 μM of A23187 for 5 hr. prior to harvest.

Synthetic oligonucleotides

GRP78(−134/−170) and GRP94(−163/−197) contain the rat *GRP78* and *GRP94* core sequence spanning −134 to −170 and −163 to −197, respectively. For subcloning and labeling purposes, both synthetic oligomers contain an XhoI site, CTCGAG, at the 5′ terminus of the coding strand and a SalI site, GTCGAC, at the 5′ terminus of the non-coding strand. The 32 bp human *thymidine kinase* (*TK*) promoter sequence (25) spanning −83 to −57 is 5′ GGCCGGGCGCTGATTGGCCCCATGGC 3′ with GTCGAC and CTCGA attached at the 5′ termini of the coding and non-coding strand respectively. The lacZ coding sequence is 5′ TAAGCCGGGAATTCTCGACGC 3′.

Gel mobility shift assays

Radiolabeled probe containing the core sequence were prepared either from the subcloned fragment or the synthetic oligonucleotide. The 77 bp BamHI-HindIII fragment of p78−1Xcore, containing the rat *GRP78* was labeled by the Klenow enzyme in the presence of [α-^{32}P] dATP, [α-^{32}P] dCTP, dTTP and dGTP. Oligonucleotide probe containing the *GRP78* core was prepared by reannealing a 15 bp primer to the non-coding strand and extended by the Klenow enzyme with [α-^{32}P]dCTP, dATP, dTTP and dGTP. Bromodeoxyuridine probes were generated by annealing the primer to their complementary DNA strands. The reannealed DNA were extended by the Klenow enzyme in the presence of 250 μM each of dATP and dGTP, 125 μM each of dTTP and BrdUTP, and 80 μCi of [α-^{32}P] dCTP (3000 Ci/mmole). After incubation at room temperature for 30 min., dCTP was added to a final concentration of 250 μM and the mixture was incubated for 60 min.

A typical binding reaction (20 μl) contained 1 μg of HeLa nuclear extract, 1 ng of probe (2 to 4×104 cpm) and 100 ng of sonicated salmon sperm DNA as non-specific DNA competitor. The binding buffer contained 20 mM Tris hydrochloride (pH 7.5), 80 mM NaCl, 2 mM $MgCl_2$ and 5% glycerol. After 20 min room-temperature incubation, the reaction mixtures were loaded onto a 5% (40:1) polyacrylamide: bisacrylamide gel and electrophoresed in 50 mM Tris base-50 mM boric acid−1 mM EDTA. Gels were run at 150V for 1.5 hr.

Pre-incubation of salmon sperm DNA required the binding of nuclear extract with salmon sperm DNA for 3 min at room temperature prior to addition of probe. Otherwise, nuclear extract was added last. Experiments with Ku antibodies were performed by adding 1 μl of a 1:10 dilution of antiserum (generous gift from Dr. W.H. Reeves) to the binding reactions 10 min after the start

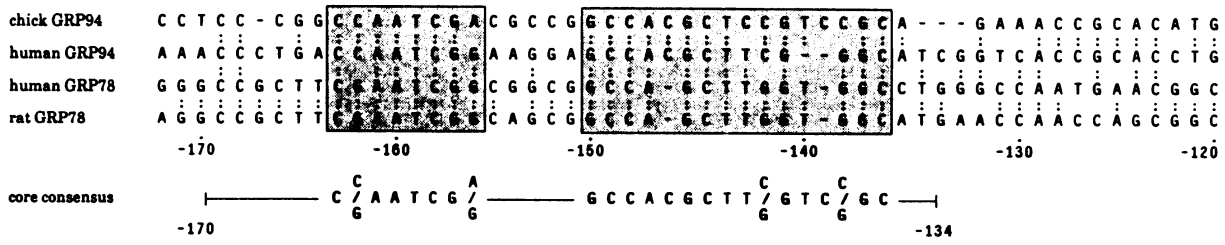

Figure 1. Core sequence of *GRP78* and *GRP94*. The core and its flanking sequence are shown for the chick *GRP94* promoter spanning from −212 to −162 (40), the human *GRP94* from −204 to −152 (4), the human *GRP78* from −135 to −83 (3) and the rat *GRP78* from −172 to −120 (20). Identical nucleotides are indicated by vertical dots. Two conserved regions among the *GRP* promoters are boxed. The numbers denote the nucleotide position of rat *GRP78* promoter. The consensus sequence of the highly conserved CG/CAAT and GC-rich motifs were shown below.

of reaction and further incubated for 20 min at room temperature. The heterologous competitor pUC was generated by digesting the plasmid pUC8 with the restriction enzymes Ava II, Hha I and HinfI. The resultant DNA represents a pool of DNA fragments, with an average size of 100 bp.

UV cross-linking

Twenty-five μg of HeLa nuclear extract was pre-incubated at room temperature for 3 min. with 2.5 μg of salmon sperm DNA (as non-specific competitor) prior to the addition of 25 ng of the BrdU substituted probe. The binding reaction was carried out at room temperature for 20 min with or without addition of the Ku antiserum or competitors. The DNA protein complexes were resolved electrophoretically on a 5% polyacrylamide gel using BAC (N, N'-bis-acrylcystamine) instead of Bis-acrylamide. The ratio of acrylamide to BAC is 40:1. After electrophoresis for 2.5 hours at 120V, the gel was placed on top of a UV transilluminator (model 3-4400, Fotodyne, New Berlin, WI), irradiated at 302 nm for 20 min at room temperature, and autoradiographed for 16 hr at 4°C. The bands corresponding to the bound complexes II and III, and free probe were excised, dissolved in 30 μl of 14.2 M β-mercaptoethanol and 100 μl of SDS sample buffer containing 0.16M Tris hydrochloride (pH 6.8), 5.8% SDS, 0.17M β-mercaptoethanol, 25% glycerol, boiled for 10 min and loaded onto a 7% SDS polyacrylamide gel. Electrophoresis was carried out at 40 mA for 4 hrs.

RESULTS

Sequence identities of the GRP core

On closer examination of the core sequence among different mammalian *GRPs*, we found that there is a CG/CAAT motif between −164 and −156 and a GC-rich motif between −150 and −137 within the *GRP78* core which may be functionally important (Fig. 1). The CCAAT sequence motif has been shown previously as one of the major cis-regulatory sites for transcriptional regulation in mammalian cells (26). GC-rich regions may also be important regulatory elements. A well-known example is the Sp1 binding site, GGGCGG (27). Among the *GRP* promoters examined, the GC-rich motif is highly conserved and consists of 81% and 71% G/C residues in *GRP78* and *GRP94* respectively. To dissect functionally the two motifs, 5′ promoter deletions at endpoint −169, −154, and −130, were constructed and fused to the chloramphenicol acetyltransferase (CAT) reporter gene.

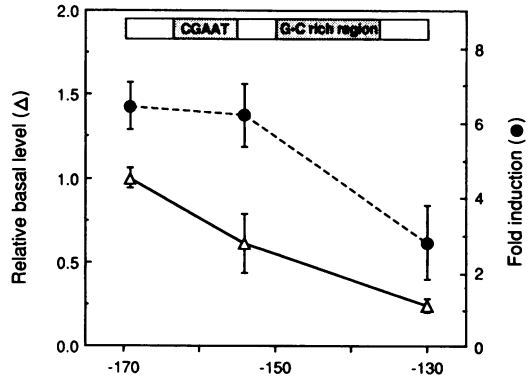

Figure 2. Functional analysis of the core sequence. Promoter activities of pGRP78 (−169) CAT, pGRP78 (−154) CAT and pGRP78 (−130) CAT after transient transfection into K12 cells. The basal level promoter activities (△) are expressed relative to the −169 CAT construct at the non-induced condition, which is set as 1. The fold induction (●) is expressed as ratio of the A23187 induced level over the basal level for each construct. The numbers and the standard deviation were calculated from three independent transfection experiments, with each condition performed in duplicate in each transfection. The CG/CAAT and GC-rich sequence motifs were aligned against the 5′ deletion endpoints.

The GRP/CAT fusion plasmids and a plasmid containing the β-galactosidase gene were co-transfected into hamster fibroblasts K12 cells which have been used previously to analyze *GRP78* expression and its regulation under a variety of stress conditions (reviewed in 18). The transfectants were either cultured under normal growth conditions or treated with 7 μM A23187, a potent inducer of the *GRP* genes as it depletes intracellular calcium and blocks protein glycosylation (8,23,28). To standardize for transfection efficiencies, the CAT activities were normalized against the β-galactosidase activities. As shown in Fig. 2, deletion from −169 to −154 results in a 40% drop of the basal promoter activity; with further deletion to −130, the residual basal promoter activity is reduced to 25% of the −169 CAT plasmid. In A23187 treated cells, deletion from −169 to −154 has no significant effect on the fold inducibility by A23187. However, deletion from −154 to −130 lowers the fold inducibility by 55%. Therefore, it appears that in GRP78 promoter the sequence between −169 and −154 containing the CG/CAAT motif is important primarily for basal level expression, whereas the region between −154 and −130 containing the GC-rich motif is important for both basal and induced expression. Similar 5′ deletions with the *GRP94* promoter revealed that elimination of

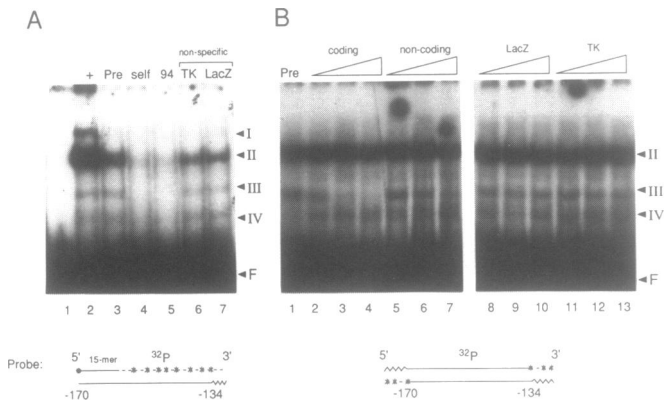

Figure 3. Interaction of nuclear factors with the *GRP78* core. Panel A: The autoradiograms of the gel mobility shift assays are shown. Lane 1 represents reaction without extract. Lane 2 represents a typical reaction with nuclear extract added at the end of the reaction (+). Lane 3 represents reaction with nuclear extract pre-incubated with salmon sperm DNA prior to addition of probe (Pre). Lanes 4 through 7 represent competition with 50 fold molar excess of various reannealed, synthetic oligomers subsequent to the pre-incubation with salmon sperm DNA as shown in lane 3. The competitors used are: self, unlabeled *GRP78* (−134/−170); 94, *GRP94* (−163/−197); TK, a 32 bp-human *TK* promoter sequence and lacZ, a 21-bp lacZ coding sequence. The complexes (I through IV) and free probe (F) are indicated. Panel B: Competition with single stranded oligonucleotides. The *GRP78* core fragment probe was used. In all the reactions, 1 μg of HeLa extract was pre-incubated with 100 ng of salmon sperm DNA prior to the addition of 1 ng of probe. The complexes formed (II, III and IV) are shown in lane 1 (Pre). Lanes 2 through 13 represent competition with increasing amounts (3, 10 and 30 fold molar excess) of various single-stranded oligomers. The competitors used are: the coding strand of *GRP78* core spanning −134 to −170 (lanes 2−4); the non-coding strand of the *GRP78* core (lanes 5−7), the coding strand of the TK oligomer (lanes 8−10) and the coding strand of the lacZ oligomer (lanes 11−13).

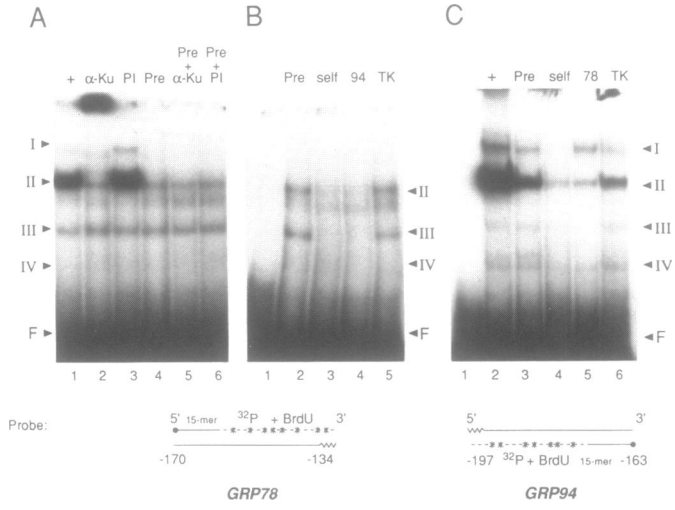

Figure 4. Interaction of nuclear factors with the BrdU substituted *GRP78* and *GRP94* probes. The autoradiograms for the gel mobility shift assays with BrdU probes are shown. In Panel A, lanes 1 through 3, the HeLa extract was added last to the reaction (+). In lanes 4 through 6, HeLa extract was pre-incubated (Pre) with salmon sperm DNA for 3 min. prior to addition of probe. The positions of the complexes (I through IV) and free DNA (F) are indicated. Lanes 1 and 4, no antiserum was added. Lanes 2 and 5, human Ku antiserum (α-Ku) was added. Lanes 3 and 6, human pre-immune serum (PI) was added. Panel B: Lane 1, *GRP78* BrdU probe alone. The protein-DNA complexes formed between the *GRP78* BrdU probe and HeLa extract after pre-incubation (Pre) with salmon sperm DNA as shown in lane 2 was competed with self, 50 fold molar excess of reannealed homologous core oligonucleotides (lane 3), *GRP94* core oligonucleotides (−197, −168) (lane 4), and the TK oligomer (lane 5). Panel C: The *GRP94* BrdU substituted probe was prepared identically to the *GRP78* probe as described in Panel A except that a 15 base primer was reannealed to the coding strand of the human *GRP94* core sequence spanning from −163 to −197 as shown. The treatments for each lane are indicated on top as described for Panels A and B. Arrows indicate DNA-protein complexes (I through IV) formed between HeLa nuclear extract and the *GRP94* probe (F).

the core sequence (between −219 and −164) resulted in a 65% drop of basal level activity and a 35% drop in A23187 inducibility (Liu and Lee, data not shown). These results, taken together, suggest that the *GRP78* and *GRP94* core function similarly *in vivo*.

Interaction with specific nuclear factors with the *GRP78* core

Gel mobility shift assays were used to detect the interaction between nuclear factors and the *GRP78* core. Using an oligonucleotide probe spanning −134 and −170, four protein complexes were formed with HeLa nuclear extracts (Fig. 3A, lanes 1 and 2). However, we noticed that the intensity of complex II is dependent on the order of addition of the reaction components. When the nuclear extract was allowed to pre-incubate with non-specific DNA prior to addition of the probe, the intensity of complex II was reduced leaving behind a residual band of the same size (Fig. 3A, lanes 2 and 3). Therefore, a majority of complex II consists of a non-specific DNA binding protein which can be removed by prior incubation of the extract with non-specific DNA. The residual complexes II and III are specific as demonstrated by competition. The formation of both complexes were inhibited by homologous competitors such as the unlabeled, reannealed *GRP78* core, and the *GRP94* core which shares high sequence identity with the *GRP78* core (Fig. 3A, lanes 4 and 5). In contrast, oligomers of similar size containing the human *thymidine kinase* (*TK*) promoter sequence or the coding sequence of the lacZ gene were not able to do so (Fig. 3A, lanes 6 and 7). It is interesting to note that the human TK sequence contains an inverted CCAAT sequence closely resembling the CP-1 binding site (29). Therefore, although the *GRP* core contains a CGAAT motif, the factor interacting with the *GRP* core is unlikely to be identical to the CCAAT binding protein, CP-1.

Complex I is non-specific as it disappeared after pre-incubation treatment with either salmon sperm DNA or poly(dI·dC) (Fig. 3A and data not shown). The specificity of complex IV cannot be established since its formation was not inhibited by both homologous and heterologous competitors. Therefore, we focus on the identity of the proteins involved in the specific complexes II and III, which are shared between the *GRP* cores.

Competition of complex III by single stranded oligonucleotides

Complex III was first detected in the gel mobility shift assays using synthetic oligonucleotides as probes. To test whether complex III was only formed with synthetic probes, the gel mobility shift assays were repeated with a *GRP78* fragment probe isolated from a plasmid containing the *GRP78* core sequence. To eliminate the non-specific DNA binding complexes, the reactions were performed with the pre-incubation conditions with salmon sperm DNA. We observed that complex III can be formed with either fragment (Fig. 3B, lane 1) or the synthetic probes (Fig. 3A, lane 3). However, the relative band intensities of complex III were consistently higher using the oligonucleotide

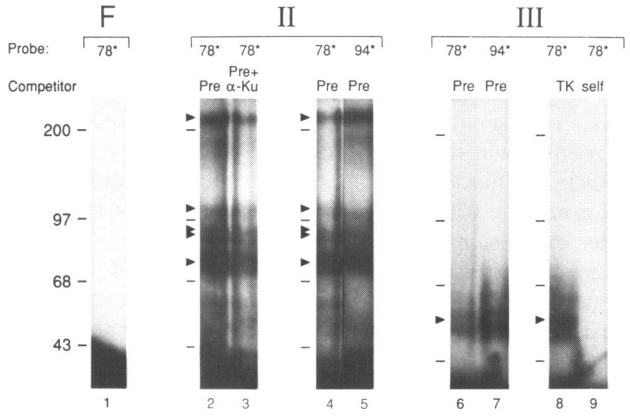

Figure 5. Identification of proteins interacting with the *GRP* core. The autoradiograms for the UV cross-linking experiment are shown. The BrdU probe used for each lane (either *GRP78* or *GRP94* core, 78* and 94* respectively) was indicated on top. Lane 1, UV cross-linked profile of free 78* probe. Lane 2, proteins labeled from 78* complex II. Lane 3, same as 2 except that 1 μl of undiluted Ku antibodies was added to the reaction mixture. Lane 4, proteins labeled from 78* complex II. Lane 5, proteins labeled from 94* complex II. Lane 6, protein labeled from 78* complex III. Lane 7, protein labeled from 94* complex III. Lanes 8 and 9, same as 6 except that a 50 fold molar excess of a heterologous competitor (TK) or a homologous competitor *GRP78* (−134/−170) (self) was added respectively. The labeled protein bands are indicated by arrows. The gel electrophoretic mobilities of the protein molecular size markers (in kD) are shown.

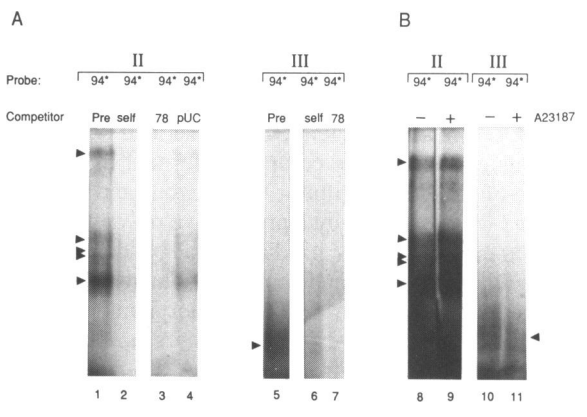

Figure 6. Nuclear factors binding to the *GRP94* core. The autoradiograms for the UV cross-linking experiments as described in the legend of Figure 5 are shown. The BrdU probe used for all the lanes was the *GRP94* core (94*). Panel A: Competition of complexes II and III with 50 fold molar excess of a homologous competitor *GRP94*(−163/−197) (lanes 2 and 6); *GRP78*(−134/−170) (lanes 3 and 7) or a heterologous competitor pUC (lane 4). No competitor was added in lanes 1 and 5. Panel B: The *GRP94* probe was mixed with either non-induced (−) (lanes 8 and 10) or A23187 induced (+) (lanes 9 and 11) HeLa nuclear extracts. The labeled protein bands for complexes II and III as described in Figure 5 are indicated by arrows.

probes. This prompted us to examine whether complex III has affinity for single-stranded DNA, which may be more abundant in the reannealed synthetic probes. To test this, the complexes were subjected to competition by single-stranded oligonucleotides containing the coding (lanes 2 to 4) and non-coding (lanes 5 to 7) of the *GRP78* core, and two other heterologous, TK (lanes 8 to 10) and lacZ (lanes 11 to 13) single-stranded sequences. The formation of complex III was most efficiently inhibited by molar excess of the coding strand of the *GRP78* core, while complex II and complex IV were resistant to competition by all single-stranded DNA tested. These results led us to conclude that complex III has the unique property that its components have affinities for the coding strand of the *GRP78* core.

The Ku autoantigen as the non-specific component of complex II

As a first step towards the characterization of the protein factor(s) which bind to the *GRP* core, we used the BrdU cross-linking method previously described for the identification of size of the Drosophila heat shock factor (30). To test whether the BrdU substituted *GRP78* probe prepared formed identical complexes as the non-substituted probe, the gel mobility shift assays were performed. As shown in Fig. 4A, three complexes (I through III) were observed (lane 1). The formation of complex IV was variable with this probe. With pre-incubation with salmon sperm DNA, complex I was eliminated and complex II was substantially reduced (lane 4).

The pre-incubation protocol has suggested to us that complex II, using either BrdU substituted or non-substituted probes, consists of a major component which has general affinities for DNA and is present in high abundance in nuclear extracts prepared from HeLa cells. One such candidate is the p70 (Ku) autoantigen, a phosphoprotein with a molecular size of 70 kD which mediates binding of the Ku (p70/p80) complex to DNA (21). Using antiserum against the Ku autoantigen, we were able to supershift the majority of complex II, leaving behind complex I, a residual complex II and complex III (Fig. 4A, lane 2), reproducing the effect of pre-incubation with salmon sperm DNA (lane 4). After pre-incubation, the residual complex II was not affected by the Ku antiserum (lane 5), confirming the results of the competition assays (Fig. 3A) that the components of the residual complex are rid of the non-specific protein Ku. As a control, pre-immune serum has no effect on the formation of any of the complexes (lanes 3 and 6).

Similar gel mobility shift patterns of the *GRP* cores

Since the residual complex II and III formed with the *GRP78* could be competed with the *GRP94* core, it is possible that the same factors bind to both *GRP* sequences and we wished to determine the sizes of proteins interacting with both core sequences. For these purposes, radiolabeled, BrdU-substituted *GRP94* core was also prepared. Prior to the UV cross-linking experiments, the specificity of the complexes formed with the BrdU-substituted probes was established by competition with homologous and heterologous competitors as described for the non-substituted probe in Fig. 3A. The results are shown in Figs. 4B and C. While confirming that BrdU substitution does not alter the binding pattern for the *GRP78* core as compared to the non-substituted probe and the specificity of the complexes was retained, it also shows that the DNA-protein complex patterns using the *GRP78* and *GRP94* probes shared some similarities and differences. In both cases, specific complexes II and III were observed and they exhibited similar electrophoretic mobilities. In contrast to the *GRP78* probe, complex I formed with the *GRP94* probe was still present after the pre-incubation step with salmon sperm DNA (Fig. 4C, lane 3). The formation of this complex was inhibited by the *GRP94* sequence (lane 4), but not by *GRP78* or other heterologous competitors (lanes 5 and 6). The non-specific complex IV was observed in these assays.

Identification of protein factors by UV cross-linking

For these studies, we focus on the proteins forming the specific complexes II and III with both *GRP78* and *GRP94* probes. The labeled, double-stranded BrdU probes were mixed with HeLa nuclear extracts in a 25 fold scale-up as described in the analytical experiments of Fig. 4. The complexes formed in the presence or absence of either antiserum or homologous and heterologous competitors were resolved by a preparative polyacrylamide gel. After *in situ* exposure to UV irradiation, the bands corresponding to the free DNA probe (F) and complexes II and III were excised from the gel, re-loaded on a SDS-polyacrylamide gel and the protein bands were resolved by electrophoresis. The results are shown in Fig. 5. As a control, free DNA did not produce any labeled protein band (lane 1). Using the *GRP78* probe, for complex II, five protein bands were labeled by this method. They had molecular sizes of about 210, 110, 90/92 doublet and 70 kilodaltons (kD) (lane 2). Densitometry revealed that incubation with the Ku antiserum did not significantly reduce any specific protein band, confirming the previous observation (Fig. 4A, lane 5) that the components of residual complex II are primarily rid of the non-specific protein Ku. Comparisons of the protein bands labeled in complex II using the *GRP78* and *GRP94* probe revealed near identical protein band patterns (lanes 4 and 5). Complex III consisted of protein species around 55 kD with both *GRP94* and *GRP78* probes (lanes 6 and 7). This protein band was competed by the unlabeled, homologous oligomer (lane 9) but not by a heterologous oligomer (lane 8).

Using the *GRP94* probe, competition experiments were performed for complexes II and III (Fig. 6A). In the case of complex II, all five labeled protein bands (lane 1) were competed by either the homologous, unlabeled *GRP94* oligomer (lane 2), or the *GRP78* oligomer (lane 3). In contrast, a heterologous competitor (pUC) was much less effective (lane 4). Similarly, the labeled protein bands of complex III were competed either by the *GRP94* or *GRP78* oligomer (lanes 5 to 7).

We further compared the proteins labeled using HeLa extracts from A23187 induced and non-induced cells. As shown in Fig. 6B, using the *GRP94* probe, near identical protein bands were labeled for complexes II (lanes 8 and 9) and III (lanes 10 and 11).

DISCUSSION

GRP78 and GRP94 are both ER resident proteins which are expressed constitutively and are induced coordinately to high levels when the cells are subjected to a variety of stress conditions (reviewed in 17). The regulation has been demonstrated to be at the transcriptional level. Sequence comparison of *GRP* promoter sequences from different species revealed a region of 36 bp of high sequence identity which we termed the *GRP* core. We show here that in the *GRP78* core can be subdivided into 2 sequence motifs with different contributions to the basal and induced promoter activities. Nonetheless, the *GRP* promoters contain highly redundant sequence elements (31) and its regulation is mediated through a complex interaction of multiple elements. Thus, while the core is important as revealed by 5′ deletions, the *GRP78* promoter still retains partial basal level and stress inducibility when it is deleted. This is due to the presence of other similar GC-rich motifs downstream, as revealed by sequence comparison and linker-scanning mutagenesis (Wooden and Lee, submitted). Internal deletion analysis also shows that the *GRP* core is functionally redundant. In fact, the core sequence is duplicated at 230 bp upstream of the transcriptional initiation site (31). Duplications of regulatory control elements have been observed in viral (27) and cellular promoters (32). Since the *GRP* genes are present as single copy genes (3,4,33,34) and *GRP78* has been shown to be important for cell viability (9,17), we speculate that functional duplication of important control elements such as the core within the *GRP* promoter would insure the gene's proper expression and regulation.

Since the core sequences of *GRP78* and *GRP94* are similar, one mechanism for the coordinate regulation of the two promoters is that the same set of nuclear factors interacts with both core elements. Alternatively, different sets of proteins interacts with the two core elements but they are controlled by a common *trans*-activator. Our results lend support to the first model and identify several proteins which interact specifically with the GRP cores and one protein which interacts non-specifically with the core.

What are the nature of these proteins? Five protein bands with sizes around 210, 110, 90/92, 70 and 55 kD were detected by the UV cross-linking method. The 70 kD protein is identical to the size of Ku, although under our experimental conditions, the majority of Ku would have been competed out with the pre-incubation step and the use of the Ku antiserum. While we could not rule out the possibility that the 70 kD band was due to labeling of residual Ku, we favor an alternative explanation that another protein, also 70 kD in size, was involved in the specific complex formation with the *GRP* core. The 55 kD protein species are interesting in that there appears to be a cluster of 3 protein bands and can bind to the coding strand of the *GRP78* core in a sequence-specific manner. Single-stranded DNA binding proteins have been implicated in DNA replication, recombination and repair in prokaryotes. Recently, the estrogen receptor has been shown to bind selectively the coding strand of an estrogen element, strongly suggesting that single-stranded DNA binding proteins can function in transcriptional regulation (35). During transcription, the *GRP78* core can conceivably exist in single-stranded form and bind to this protein preferentially. However, whether such binding occurs *in vivo* remains to be determined.

The non-specific protein has been identified as Ku, an abundant HeLa nuclear factor which binds to ends of double stranded DNA fragments *in vitro* (36) and regions of open chromatin *in vivo*. While its binding specificity appears to be random, Ku has recently shown to be a substrate for a DNA-activated protein kinase (37). It would be interesting to determine whether Ku plays any role in the transcription *in vivo*.

The 110, 90/92 doublet, 70 and the 55 kD protein species are uniquely labeled by the crosslinking method. How specific are these proteins to the *GRP* core? First, this method has been used to identify successfully transcriptional factors binding specifically to their perspective interactive sites (30,38,39). Second, competitions using homologous and heterologous DNA fragments show that all the crosslinked bands were competed by the *GRP78* and *GRP94* core fragments but not with others. Third, using DNA affinity chromatography, several protein bands using DNA affinity chromatography, several protein bands resembling the size of the UV labeled proteins were observed in partially purified protein fractions (our unpublished results). However, further attempts to demonstrate the binding specificity of the individual proteins were unsuccessful since after denaturing gel electrophoresis and blot transfer, their binding activities were lost, suggesting that the proper protein confirmation may be important for their binding. However, information derived from the present

study will facilitate their isolation and characterization, which will greatly enhance our knowledge on the coordinated regulation of the *GRP* genes.

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