Calcium-sensitive Transcriptional Activation of the Proximal CCAAT Regulatory Element of the grp78/BiP Promoter by the Human Nuclear Factor CBF/NF-Y*

(Received for publication, May 7, 1996, and in revised form, July 31, 1996)

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Transcription of the gene encoding GRP78/BiP, a calcium-binding molecular chaperone localized in the endoplasmic reticulum, is induced in mammalian cells through gradual depletion of the intracellular calcium stores. The multicentric CCAAT binding factor, CBF/NF-Y, binds to the most proximal CCAAT regulatory element (C1) of the grp78 promoter required for both basal level expression and stress response. Using an in vitro transcription system, we show through factor competition and immunodepletion that the grp78 C1-mediated enhancement of transcription requires primarily CBF. Correlating with the previous observation that CBF binding to the 78C1 site is enhanced by EGTA and EDTA, these divalent cation chelators specifically stimulate 78C1-directed transcription. In contrast, increasing amounts of calcium ions are inhibitory. These results provide evidence that CBF is functionally important in transactivating the grp78 C1 transcriptional activity, and suggest a possible mechanism by which grp78 transcription is stimulated by calcium depletion. We further discovered that in addition to binding CBF, both the 78C1 element and the CBF binding site of the α2(I) collagen promoter interact weakly with the multifunctional transcription factor YY1. Our studies show that the binding sites for CBF and YY1 are distinct for the two promoter sites, suggesting that YY1 and other interacting factors could exert differential effects on individual promoters bearing the same CBF site.

The 78-kDa glucose-regulated protein, GRP78/BiP, is a calcium-binding molecular chaperone that shares partial amino acid sequence identity with HSP70, the 70-kDa heat shock protein (1, 2). However, unlike the cytoplasmic HSP70, which is heat-inducible and is translocated into the nucleus upon stress treatment, GRP78 is a resident endoplasmic reticulum (ER) protein. The promoter of the gene for mammalian grp78 does not contain any heat shock response element; rather, this gene is highly inducible by reagents that disrupt organelle calcium homeostasis (3, 4). By monitoring the intracellular free calcium with the fluorescent indicator fura-2 while employing both intracellular and extracellular calcium buffers, and following the kinetics of grp78 induction, it was established that the induction of grp78 does not require an increase in cytoplasmic calcium; instead, it is the gradual depletion of intracellular calcium stores that activates transcription of grp78 (3, 5). In support, treatment of mammalian cells with calcium-free medium or EGTA increases transcription of grp78 (3). Addition of the calcium ionophore A23187 to calcium-free medium further enhances the level of grp78 induction (5). Thapsigargin is a specific inhibitor of the ER calcium-ATPase, resulting in draining of calcium ions from the ER store (6). It is one of the most potent inducers of the mammalian grp78 promoter (7). Thus, in contrast to other calcium influx-mediated gene activation events that occur within minutes of stimulation (8, 9), the grp78 system provides a novel example of gene activation as a result of sustained calcium depletion from intracellular stores.

The mammalian grp78 promoter is highly complex and contains multiple functionally redundant regulatory elements (4). One of the most interesting features of grp78 and other related grp promoters is the presence of a large number of CCAAT-like motifs, which by criterion of in vivo and in vitro footprinting bind with nuclear factors (10–13). The two most critical regions for grp78 stress induction have been identified through mutational analysis and their ability to confer stress inducibility to heterologous promoters (7, 11). These two regions are the conserved grp core, and the C1 element. The grp78 C1 region encompasses a 34-base pair (bp) sequence and contains the most proximal CCAAT element located about 90 bp upstream of the TATA element. Mutation of this CCAAT motif within the native promoter results in partial decrease of basal level expression; most strikingly, the stress inducibility of the grp78 promoter is greatly reduced (11). Additionally, a duplicated copy of the C1 element can act as stress-inducible element, conferring a 7–10-fold induction to a heterologous promoter (7, 14).

In human cells, the transcription factor that interacts with the grp78 C1 element has been identified as the multicentric CCAAT-binding factor, CBF, also known as NF-Y (15). Correlating with the increase in transcriptional activity driven by the grp78 C1 element in heterologous constructs in cells treated with thapsigargin, the binding of CBF to the C1 element in vitro is sensitive to the concentration of calcium ions (15). At low calcium ion conditions, CBF binding to the grp78 C1 site is higher because of a slower dissociation rate. The similar binding characteristics of CBF from nuclear extracts of control and stressed cells suggest that the ubiquitously expressed CBF may not undergo any major inherent changes after calcium depletion stress. Instead, its binding property may be modulated by the immediate calcium ionic environment in the stressed and non-stressed cell nuclei, resulting in a change in transcriptional activity.
These in vitro observations raise several important issues. First, is CBF functionally involved in transcription mediated by the grp78 C1 element? Second, can calcium ions affect the transcriptional activity of CBF? Third, if the ubiquitous CBF factor is a regulatory protein for the grp78 calcium stress response, why don’t other cellular promoters such as the a2(1) collagen, which contains a CBF binding site nearly identical to the grp78 C1 site, respond to calcium depletion stress as strongly as the grp78 promoter? To address these questions, we have established an in vitro transcription system that can directly transcribe specifically from the grp78 C1 element. The enhancement of transcription by the C1 element requires primarily the transcription factor CBF. Divalent metal ion chelators such as EDTA and EGTA specifically stimulate the C1-directed transcription, while increasing amounts of calcium ions are inhibitory. These results provide evidence that CBF can transactivate the grp78 C1 transcriptional activity, and that this stimulation is sensitive to calcium ions. Additionally, we show here that a second transcription factor, termed YY1, also binds grp78 C1 and the a2(1) collagen CBF sites, although this binding can only be observed under less stringent binding conditions. YY1 is an ubiquitously expressed, zinc finger DNA-binding protein. Depending on its binding sequence, YY1 can activate, repress, or initiate transcription (16, 17). YY1 can also affect gene transcription through DNA bending (18). Through site mutagenesis, we discovered that the positioning for the CBF and YY1 sites are strikingly different for the grp78 C1 and collagen promoter elements. Thus, although both elements share common factors, their interactions can be different, resulting in different regulatory responses.

MATERIALS AND METHODS

Nuclear Extract Preparation—Five liters of HeLa S-3 cells were grown to a density of approximately 3.8 \( \times \) 10\(^6\) in suspension culture and harvested. Nuclear extracts were prepared as described previously (19).

Gel Retardation Assays—Gel retardation assays were performed as described (15) with the following modifications; each reaction contained 5 \( \mu \)g of HeLa nuclear extract, 0.5 ng of \(^32\)P-labeled oligomer probe, and 200 ng of sonicated poly(dC-dC) as nonspecific competitor. For experiments with specific oligomers, the probe was mixed with the competitor prior to the addition of the reaction mixture. To test the effect of the YY1 antibody, 1 \( \mu \)l of the antibody was added to the reaction mixture containing the nuclear extract prior to the addition of the probe.

Construction of Plasmids—The construction of AdML190 and p(CAT) has been described (20, 21). The grp G-less plasmids were derived from a parental grp(1250)G-less plasmid which was constructed by inserting the region spanning –1290 to –34 of the rat grp78 promoter (22) into the SacI site of p(CAT). To generate grp(87)G-less plasmid, the grp(1250)G-less plasmid was cut with EcoRI and StuI to remove the promoter sequences upstream of –87, and the vector was religated. To generate the grp(C1)G-less plasmid, a dimerized C1 oligonucleotide was ligated into the EcoRI/StuI-cut vector fragment of the grp(1250)G-less plasmid. The sequence and orientation of the grp promoter inserts were confirmed by diodeoxyxylucleotide DNA sequencing.

In Vitro Transcriptions—The in vitro transcriptions were carried out using the G-less cassette system (21). Each 20-\( \mu \)l reaction contained 20 \( \mu \)M HEPES (pH 7.9), 75 \( \mu \)M KCl, 4 \( \mu \)M MgCl\(_2\), 12% glycerol, 2% polyethylene glycol 8000, 40 units of RNasin (Boehringer Mannheim), 150 units of RNase T1 (Ambion), 0.1 \( \mu \)g EDTA, 0.1 \( \mu \)g EGTA, 2 \( \mu \)M dithiothreitol, 1 \( \mu \)M [\(^{32}\)P]UTP, 0.3 \( \mu \)M ATP and CTP, 0.8 \( \mu \)M UTP, and 10 \( \mu \)Ci of [\(^{32}\)P]UTP (DuPont NEN, 800 Ci/mmol). The individual transcription reactions contained 75 ng of grp G-less plasmid, and 525 ng of UC18 as nonspecific DNA. After addition of 30 \( \mu \)g of HeLa cell nuclear extract, the reaction was incubated at 30 \( ^\circ \)C for 45 min. An additional master tube was incubated exactly the same conditions as described above, but containing AdML190 as template DNA. For competitions, 400-fold molar excess of competitor oligonucleotides were added. To observe the effect of cation chelators, EDTA or EGTA was added to a final concentration of 2 \( \mu \)M in the in vitro transcription reaction mixtures. To test the effect of calcium ions, CaCl\(_2\) was added to a final concentration of 2 or 5 \( \mu \)M. In case of in vitro transcriptions using immunodepleted extracts, 3 \( \mu \)l of the immunodepleted mixture was used for the in vitro transcription reactions. The in vitro transcription reactions in the AdML tubes were not subjected to the above treatments.

To terminate the reaction, a stop reaction solution containing 14 mm Tris, pH 7.5, 7 mm EDTA, 250 \( \mu \)g/ml protease K, and 0.3 \( \mu \)g/ml RNA was added, and the reactions were incubated for 10 min at 65 \( ^\circ \)C. The transcripts from the AdML tube, aliquoted into the grp reaction tubes at the end of the reaction, served as controls for the quality of the nuclear extracts used, and for loading/recovery efficiency. The in vitro transcription reactions were extracted using a mixture of phenol-chloroform:isoamyl alcohol (25:24:1), followed by chloroform extraction. The RNA was precipitated in 2.5 \( \mu \)l ammonium acetate and 2.5 volumes of 100% ethanol for 20 min at –70 \( ^\circ \)C. The RNA pellet was resuspended in 5 \( \mu \)l of formamide dye and analyzed using 4% denaturing polyacrylamide gels. The gels were dried and exposed overnight onto a PhosphorImager screen and then scanned using a Molecular Dynamics PhosphorImager. The intensity of each band was quantitated using Image Quant software from Molecular Dynamics.

CBF- or YY1-depleted Nuclear Extracts—To prepare nuclear extracts depleted of the transcription factors, 10-\( \mu \)l aliquots of HeLa nuclear extract, containing about 150 \( \mu \)g of protein, was incubated with either 2 \( \mu \)l or 5 \( \mu \)l of rabbit polyclonal antibody against CBF or YY1. The antibody against the CBF A subunit was a gift from Dr. S. Maity (University of Texas), and the YY1 antibody was purchased from Santa Cruz Biotechnology. For mock depletion, 5 \( \mu \)l of nuclear dialysis buffer was used. The reactions were incubated at 4 \( ^\circ \)C for 1 h with occasional shaking. Where required, the final volume was adjusted to 15 \( \mu \)l using nuclear dialysis buffer. At the end of the incubation period, 5 \( \mu \)l of protein A-Sepharose (pre-equilibrated in nuclear dialysis buffer) was added and incubated on ice for 1 h with occasional shaking. The reaction mixture was centrifuged, and the supernatant was tested for residual factor binding activity by electrophoretic mobility shift assays (EMSAs), and the samples showing highest depletion of the specific transcription factor were used for in vitro transcription assays.

RESULTS

Transcription Factor Binding on the grp78 C1 Element—EMSAs were used to identify transcription factors that bind to the C1 element, the sequence of which is shown in Table I. Using HeLa nuclear extracts prepared from exponentially growing cells, we consistently observed a predominant complex, previously referred to as complex I (15). By criterion of antibody supershift and chromatographic properties, complex I consisted primarily of the transcription factor CBF/NF-Y. This same complex was also observed with a CBF binding site probe derived from the a2(1) collagen promoter (15). However, in transient transfection assays, the native a2(1) collagen promoter was only weakly inducible by calcium depletion stress (15). Furthermore, the CBF site from the a2(1) collagen promoter in heterologous promoter constructs showed a much lower basal level activity than grp78 C1 element, and exhibited only a 2–3-fold induction by thapsigargin, instead of the 7–10-fold observed for grp78 C1 element (data not shown). This prompted us to examine other factors, which may interact differentially with the two CBF binding sites.

To detect additional factor binding, the reactions were performed in the presence of lower concentrations of the nonspecific competitor poly(dC-dC) than used previously (15). Under such conditions, in addition to the CBF complex, a second major complex with a faster electrophoretic mobility was detected (Fig. 1A, lane 1). The sequence specificity of this complex was examined in EMSAs by competition with a panel of synthetic wild type and mutant C1 and CBF oligomers, and additionally, the grp78 core (Table I). As expected, the CBF complex was competed by the wild type C1 (Fig. 1A, lanes 2 and 3) and CBF oligomers (lanes 6 and 7). The 78core was only able to compete partially in high molar excess (lanes 10 and 11). In the case of the faster migrating complex, it was competed most efficiently by the 78core (lanes 10 and 11), followed by the full length field type C1 (lanes 2 and 3) and CBF (lanes 6 and 7). The 78C1 mutant, which has a 10-bp mutation in the region of the CCAAT motif (mt78C1), failed to compete for the CBF complex.
but was able to compete for the faster complex (lanes 4 and 5). Mutation of the GGAGG motif of the CBF oligomer (mtCBF GA) reduced its ability to compete for the CBF complex and eliminated its ability to compete for the faster complex (lanes 8 and 9). Thus, the faster complex binds outside the CCAAT motif of C1 and has very high affinity for the 78core.

We have recently discovered that the transcription factor, YY1, binds the grp78 core sequence.2 The efficient competition of the faster migrating complex by 78core indicated the possibility of the faster migrating complex being formed by YY1. To test this, antibody specific against YY1 or preimmune serum was added to the binding mixture prior to the addition of the probe. As shown in Fig. 1B, the formation of faster migrating complex was eliminated by the antibody against YY1 (compare lanes 1 and 3), but not by the preimmune serum (lane 2). The other complexes were not affected by the antisera. Thus, the faster migrating complex is immunologically related to YY1. The same results were obtained using the α2(I) collagen CBF site as probe (Fig. 1C).

**Positioning of the CBF and YY1 Binding Sites on 78C1**—The 78C1 and the α2(I) collagen CBF elements bind the same two factors in EMSAs, yet they exhibit different transcriptional properties. To delineate the CBF and YY1 binding sites on these two elements, we used additional mutant C1 and CBF oligomers (Table I). The results of the EMSA competition assays are shown in Fig. 2A. Confirming that the faster migrating complex consisted of YY1, a YY1 consensus site competed efficiently for this complex, but not by the CBF complex (lanes 2 and 3). The YY1 consensus site was a better competitor than 78C1.
(lanes 2–5), correlating with its relatively weak binding to 78C1. Mutation of the CCAAT motif (mt78C1) retained its ability to compete for YY1 but not CBF (lanes 6 and 7), whereas mutation of CCAC (mtC1CA) retained its ability to compete for CBF but not YY1 (lanes 8 and 9). These results show that the CBF and YY1 binding sites can be dissociated in the 78C1 element, with CBF occupying the CCAAT motif and its immediate flanking G-rich sequence, and YY1 occupying the CCAC motif and its flanking sequences toward the 3′ half of the C1 element.

In contrast, the positioning of the CBF and YY1 sites on the α2(I) collagen CBF site is different from that of grp78 C1, such that the binding sites for both transcription factors overlap. Whereas the wild type CBF site can compete for both CBF and YY1 binding (lanes 10 and 11), mutation of a 8-bp region eliminating mainly the CCAAT motif and its immediate flanking G-rich sequence (mtCBFCA) eliminated its ability to compete for both CBF and YY1 (lanes 12 and 13).

As a complementary experiment, the 78C1, mt78C1CA, CBF, and mtCBFCA oligomers were radiolabeled and used as probes in EMSAs (Fig. 2B). The mt78C1CA oligomer, while retaining 65% of CBF binding, was unable to form the YY1 complex (compare lanes 1 and 2). The mtCBFCA was unable to form both the CBF and YY1 complex (compare lanes 3 and 4). These results confirmed the competition studies shown in Fig. 2A. The binding properties of all the oligomers used are summarized in Table I.

**In Vitro Transcription Directed by the grp78 C1 Element**—To understand the functional role of CBF and YY1 in grp78 C1-directed transcription, *in vitro* transcription reactions were set up using the G-less cassette system (21). Since CBF is a multimeric factor, *in vitro* transcription assays can circumvent the problem of reconstituting the multiple subunits of CBF *in vivo*. In addition, *in vitro* transcription systems will be amenable for testing directly the effect of calcium ions or its chelators on C1-directed transcription. In these assays, three G-less constructs were used (Fig. 3). The test constructs contained grp78 promoter sequences linked to the 380-bp G-less cassette. The construct grp−87G-less contained the minimal rat grp78 promoter spanning −87 to −34 fused to the G-less cassette. This sequence is devoid of any CCAAT elements and contains essentially the TATA element and its flanking sequence. The construct grpC1G-less is similar to grp−87G-less, except that it also contained two copies of the C1 element inserted upstream of the minimal grp78 promoter. Duplicated C1 elements in this orientation have been shown to be transcriptionally active in transfection assays (7, 15). Thus, the transcripts from the grp−87G-less construct represented basal transcription from the minimal grp78 promoter sequence, and transcripts from the grpC1G-less construct represented C1-directed transcription, utilizing the same basal promoter element. The third construct is the AdMLG-less plasmid, which contained 400 bp of the AdML viral promoter directing the transcription of a 190-bp G-less cassette. Since the AdML promoter contained similar sequence motifs (GGCC and CCACAC) to the C1 element (23), to avoid interference of the AdML promoter in *grp* promoter-directed transcription, the AdML templates were assayed in separate reactions, and served as controls for the quality of the extracts. The 190-nucleotide transcripts generated were also used as a recovery/loading control.

*In vitro* transcription of the *grp* G-less templates yielded an expected transcript size of 380 nucleotides. Presence of the duplicated C1 element resulted in a 3–4-fold increase in transcript level, as compared to the minimal promoter construct (Fig. 4, A and B, lanes 1 and 2). Addition of 400-fold molar excess of synthetic oligomers corresponding to the wild type C1 (Fig. 4A, lanes 3 and 4) or the α2(I) collagen CBF site (Fig. 4C, lanes 3 and 4) eliminated this increase. In contrast, competition by mt78C1 with a mutated CCAAT motif, which could no longer bind CBF but retained the ability to bind YY1, exhibited only a minimal effect (Fig. 4C, lanes 5 and 6). The level of the transcripts in each lane were quantitated and normalized against the AdML control. The grpC1G-less-directed transcription was compared to that of the minimal promoter construct, grp−87G-less, which was set as one in each set of experiments (Fig. 4, B and D). These results suggest that when the transcription factor CBF was titrated away by molar excess of a competent binding site, the C1-directed increase in transcription was abolished.

**Depletion of CBF Abolished C1-directed Transcription**—Another approach to test the requirement of CBF for C1-directed transcription is to deplete CBF from the HeLa nuclear extract used for *in vitro* transcription. For this purpose, the HeLa nuclear extract was either immunodepleted of CBF using CBF-A antibody (24) or mock-depleted using nuclear resuspension buffer. The depletion of CBF from the extracts were monitored by EMSAs using radiolabeled C1 as probe (Fig. 5). The position of the CBF complex was confirmed by reduction of the complex through addition of the CBF antibody to the HeLa nuclear extract (compare lanes 1 and 5). There was no major change in CBF binding in the mock-depleted extract (lane 2). With increasing amounts of the CBF-A antibody, the formation of the CBF, but not the YY1 complex, was diminished (lanes 3 and 4). Using 5 μl of the CBF antibody, the CBF complex was reduced to 25% of the mock control (lane 4), and this depleted extract was used for the *in vitro* transcription assays described below.

*In vitro* transcription assays were performed with the non-
to monitor the depletion of the YY1 complex, the position of which was confirmed by the reduction of the complex upon addition of the YY1 antibody to the nuclear extract (compare lanes 1 and 5). At 2 and 5 μl of YY1 antibody, the YY1 complex was diminished in the immunodepleted extracts, but not CBF complex (lanes 2 and 3). At 5 μl of YY1 antibody, the residual YY1 band intensity was 30% of the mock control.

In in vitro transcription assays, while there was a slight decrease in both basal and C1-directed stimulation of transcription in the immunodepleted extracts (Fig. 8, compare lanes 1 and 2 with lanes 3–6), the mock-depleted and YY1-immunodepleted extracts showed activities similar to those of both the grp(C1)G-less (lanes 3 and 5) and grp(−87)G-less constructs (lanes 4 and 6). These results, coupled with the lack of effect by competition with the mt78C1, which retained the YY1 binding site (Fig. 4C, lanes 5 and 6), suggests that YY1 is not a rate-limiting factor in the C1-directed transcription in vitro.

**Chelation of Divalent Cations Enhances C1-directed Transcription**—Previously, using EMSAs, it has been observed that addition of EDTA or EGTA to the reaction mixture increased the formation of the CBF complex on the C1 element (15). Measurements of the on- and off-rates of the CBF complex formation suggested that in the presence of the divalent cation chelators, the CBF complex was more stabilized. To test whether the putative increase in CBF complex formation results in increase in transcription activity, in vitro transcription reactions were set up in which EDTA or EGTA was added to a final concentration of 2 mM (Fig. 9A). Strikingly, in the presence of EDTA and EGTA, C1-directed transcription was enhanced 2- and 3-fold, respectively (lanes 3 and 4), as compared to the level without these chelators (lane 1). This stimulative effect was specific for the C1-directed transcription. The basal

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**Fig. 3.** Schematic representation of the constructs used in *in vitro* transcription. The grp(C1)G-less was constructed by inserting two copies of the 78C1 element in the orientation indicated into grp(−87)G-less, which retained the TATA sequence at −60. The grp promoter subfragments were fused to a 380-bp G-less cassette. The AdML G-less construct contained the AdML promoter sequence (−404 to +10) fused to a 190-bp G-less cassette.

**Fig. 4.** Effect of CBF competitors on 78C1-mediated transcription. A, *in vitro* transcription reactions were carried out using either grp(C1) and grp(−87)G-less plasmids templates, in the absence (lanes 1 and 2) or presence of 400-fold molar excess of the 78C1 oligomers (lanes 3 and 4). The autoradiograms are shown. The positions of the grp and the AdML transcripts are indicated. B, the intensity of the transcripts was quantitated, normalized against the AdML control, and plotted after setting the level of transcripts from the grp(−87)G-less template as 1. The number below the graph corresponds to the lanes on the autoradiograms. C, *in vitro* transcription reactions with no competitors (lanes 1 and 2), with 400-fold molar excess of the (−87)C1 collagen CBF site (lanes 3 and 4) or mt78C1 (lanes 5 and 6). D, quantitation of the relative grp-directed transcript level in each of the lanes shown in C.

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**Fig. 5.** Immunodepletion of CBF from human nuclear extract. EMSAs were performed using radiolabeled 78C1 as probe. The probe was mixed with either 4 μg of untreated HeLa nuclear extract (lane 1), mock-depleted extract (lane 2), immunodepleted extract using 2 lane 3, or 5 μl of CBF antibody (lane 4), or untreated HeLa nuclear extract with 0.5 μl of CBF antibody added (lane 5). The autoradiogram is shown. The positions of the CBF and YY1 complexes are indicated.
transcript levels, driven by the (−87)G-less construct, were slightly decreased in the presence of either EDTA or EGTA (compare lanes 1, 5, and 6). A summary of the normalized transcript levels is shown in Fig. 9B. The 2–3-fold enhancement of C1-directed transcription in divalent cation-depleted nuclear extracts correlates with the previous findings that, in mammalian cells cultured in calcium-buffered medium containing EGTA, the grp78 transcript level was elevated by about 3-fold (3).

C1-directed Transcription Is Reduced in the Presence of Calcium Ions—Previously, it has been shown that addition of calcium ions in binding reaction assays reduced the formation of the CBF complex (15). This reduction was caused by an increase in the off-rate of the complex, suggesting that in the presence of calcium ions, the binding of CBF to C1 was destabilized. The in vitro transcription described here provided a test system for the effect of calcium ions on C1-directed transcription. In such assays, addition of 2 and 5 mM calcium ions to a standard in vitro transcription mixture showed a general inhibitive effect on both C1- and (−87)G-directed transcription (Fig. 10A). However, the effect was more pronounced on the C1-directed transcription (lanes 1, 3, and 5) than the basal promoter transcription (lanes 2, 4, and 6). The normalized levels of the transcripts are shown in Fig. 10B. The results showed that addition of calcium ions, in addition to general reduction in basal promoter activity, further reduced the C1-directed transcription by about 50%. Collectively, these results are consistent with the in vitro binding properties of CBF on the C1 element, and provide evidence that that CBF-mediated transcription activation of the C1 element is sensitive to the concentration of calcium ions.

DISCUSSION

To understand the stress induction mechanism of the mammalian grp78 promoter, we have previously identified several cis-acting regulatory elements (12, 13). Of these, a number of CCAAT and CCAAT-like motifs were found to be important in the expression of this gene (7, 11). The most proximal CCAAT motif, referred to as the C1 element, has the unique property that it is required for full basal level activity as well as for mediating the stimulative effects of the upstream regulatory elements. While a single copy of the C1 element is insufficient for stress induction (11), a duplicated copy of C1 linked to a heterologous promoter exhibits as strong a stress response as
in vivo genomic footprinting (13). The 5'-CGAAT motif of grp core also has weak but detectable affinity for CBF/NF-Y (25). Thus, these new observations reveal that the two important regulatory regions of the grp78 promoter, C1 and core, share common factors, but with different affinities.

Unlike the grp78 core binding site, the YY1 binding site on the grp78 C1 element is a weaker binding site and does not contain significant sequence similarity to the YY1 consensus sequence. In contrast, CBF binds very strongly to the C1 element and only weakly to the CGAAT motif in the grp core. Here we describe an in vitro transcription system to test the relative contribution of each factor in C1-mediated transcription. Through competition with CBF binding sites and immunodepletion of the CBF-A subunit required for the CBF complex to bind DNA (24), we provide evidence that CBF is a rate-limiting factor, such that either titration or depletion of the CBF from the human nuclear extract abolished the increase in transcription mediated by the grp C1 element. In contrast, competition by the mutant C1 oligomer (which lacks the CBF binding site but retains the YY1 site) or immunodepletion of YY1 has only minimal effect on C1-directed transcription. Nonetheless, YY1 is a multifunctional transcription factor, which can affect gene expression through many different mechanisms (17). YY1 has also been shown to be associated with the nuclear matrix, which can affect chromosomal topology (26). It is possible that in the in vitro systems described here in which duplicate copies of C1 are fused to a heterologous promoter to optimize the effect of C1, YY1 is not a rate-limiting factor. The relative contribution of CBF and YY1 and other grp78 promoter binding factors awaits a comprehensive analysis of the multiple effects of these factors on each of the repetitive control elements on the grp78 promoter.

With the discovery that CBF/NF-Y plays major role in C1-mediated transcription, it is intriguing that why other promoters bearing a highly similar CBF binding site, such as the α2(I) collagen CBF site, is not strongly responsive to calcium depletion stress (15). Duplicate copies of the collagen CBF site in an orientation identical to the grp78 C1 in identical heterologous promoter constructs yielded low basal level and mild stress response. Nonetheless, synthetic oligomers of both sites bind CBF and YY1 in EMSAs. Here we dissected the binding sites of these two factors on the grp78 C1 and collagen CBF sites. A closer examination of the α2(I) collagen CBF binding site showed that overlapping the CCAAT site, in the non-coding strand, bears a sequence CCTCCAT, which resembles the consensus YY1 site CGGCCAT (Table I). It has also been observed that, in cases where YY1 acts as a repressor, the YY1 site is very close to, or overlaps with, the activator site (17). Mutation of this sequence eliminated both CBF and YY1 binding on the collagen site, clearly demonstrating that the two factors bind to closely overlapping, if not identical, sites. Thus, either direct

**Fig. 9. Effect of EDTA or EGTA on 78C1-mediated transcription.** A, in vitro transcription reactions were carried out in the absence (lanes 1 and 2) or presence of 2 mM EDTA (lanes 3 and 5) or 2 mM EGTA (lanes 4 and 6), using either grp(C1) or grp (−87)G-less plasmids as templates. B, the relative grp transcript level from each lane was plotted, after setting the level of (−87) in each set of the reactions as 1.

**Fig. 10. Effect of calcium ion on 78C1-mediated transcription.** A, in vitro transcription reactions were carried out in the absence (lanes 1 and 2) or presence of 2 mM (lanes 3 and 4) or 5 mM CaCl₂ (lanes 5 and 6). The autoradiogram is shown. B, the relative grp transcript level from each lane was plotted, after setting the level of (−87) in each set of the reactions as 1.

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binding site competition or structural/conformational changes conferred by YY1 or other interacting factors may account for the lower basal activity of the collagen CBF element and its inability to respond to stress induction.

In the case of the grp78 C1 element, the CBF binding site centers around the CCAAT motif and can be clearly dissociated from the downstream core YY1 binding site. Nonetheless, the peripheral contact points for the two factors could overlap partially. Previously, we reported that the binding of CBF to the grp78 C1 site is calcium-sensitive in EMSAs (15). Here we extend these in vitro observations to address the critical issue of whether the C1-directed transcription mediated by CBF is sensitive to calcium. Our results show that in vitro transcription, addition of EDTA or EGTA enhances grp78 C1-directed transcription, whereas addition of calcium ions represses it. Is there any relationship between these in vitro findings and the in vitro observations that treatment of cells with EGTA, A23187, or thapsigargin, activates grp78 transcription? Unlike other calcium-mediated gene activation processes (30), which control the ATP-mediated nuclear loading does not alter the mechanism of gradual depletion of intracellular calcium stores (5, 7).

Further, since clamping of cytosolic calcium by BAPTA loading does not alter grp78 induction after calcium depletion (5), the mechanism communicating the ER state to the nucleus cannot be due to changes in cytosolic Ca2+ concentration. Interestingly, accumulating evidence suggests that the intranuclear level of free Ca2+ is regulated independently of the cytosolic level (27–29) and may contribute to the specific control of gene transcription. The nucleus contains an ER-type Ca2+-ATPase activity (30), which controls the ATP-mediated nuclear uptake. Thapsigargin severely inhibits the ATP-stimulated Ca2+ accumulation in the nucleus (27) at concentrations (250–300 nM) that also activate grp78 transcription. In other studies, inhibition of Ca2+-ATPase evoked Ca2+ release from Ca2+-loaded nuclei (31, 32). Thus, the simultaneous depletion of intracellular calcium stores could be a coupling factor in the induction of grp78. However, it should be emphasized that the physiological conditions of the nucleus, in particular, the compartmentation of bound and free calcium, is highly complex and not well understood. Although the total Ca2+ concentration in the nucleus can reach millimolar range, the free Ca2+ is much lower (33). A range of Ca2+-binding proteins exist in the nucleus to sequester calcium ions, and they can modulate transcription factor activity in a calcium-dependent manner (33). Therefore, the in vitro results must be interpreted with caution as to the physical mechanism for grp78 transcriptional activation. Alternatively, the coupling mechanism that mediates the state of ER calcium depletion to the CBF/C1 complex may be calcium-independent and involve, for example, the genistein-sensitive pathway (14), or the recently described calcium influx factor (35, 36). The calcium influx factors are small, relatively polar molecules produced in a time-dependent manner following thapsigargin treatment of Jurkat cells. In addition to mediating the signal for “capacitative” entry of extracellular calcium, they may serve other signaling functions. For the genistein-sensitive pathway, phosphorylation of critical components of the regulatory machinery may be involved in mediating the grp response.

In this study, we established through in vitro transcription that CBF/NF-Y plays an important role in C1-mediated transcription. However, the ubiquitous CBF/NF-Y transcription factor is unlikely to act alone in mediating the grp78 calcium depletion stress. CBF is composed of multiple subunits and contains multiple activation and protein interaction domains (37). Our studies raise the hypothesis that in the grp promoter system, CBF/NF-Y resides in C1 and perhaps also in other upstream CCAAT motifs, and synergistically interacts with other associating transcription factors. Through its sensitivity to calcium ions, or other signaling pathways, its transcription activity could be modulated by calcium homeostasis in the mammalian cell.

Acknowledgments—We thank Drs. Edward Little and Axel Schonthal for helpful discussions and technical advice. We thank Dr. S. Maity for the gift of the CBF antibody.

Note Added in Proof—Recently, GRP94, an ER localized calcium-binding molecular chaperone coordinately regulated with GRP78, is shown to be associated with a calcium-sensitive kinase activity (34).

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