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Transduction of Calcium Stress through Interaction of the Human Transcription Factor CBF with the Proximal CCAAT Regulatory Element of the *grp78*/BiP Promoter

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Mammalian GRP78/BiP is a stress-inducible 78-kDa endoplasmic reticulum (ER) protein with molecular chaperone and calcium-binding properties. The transactivation of *grp78* by the calcium ionophore A23187 provides a model system with which to study the signal transduction that allows mammalian cells to sense calcium depletion in intracellular stores and activate transcription of specific genes. Linker-scanning mutation analysis of the *grp78* promoter reveals that the single most important regulatory element is C1, which contains a CCAAT motif most proximal to the TATA sequence. The C1 element is crucial for mediating the stimulatory effects by the upstream regulatory elements under normal and stress conditions. In this report, we establish that the heteromeric CCAAT-binding factor CBF is the major component of the C1-binding factor (C1F) in human cells. A GGAGG motif flanking the CCAAT sequence also contributes to high-affinity C1F/CBF binding. We show here that the binding of C1F in vitro is sensitive to the concentration of calcium ions. At high calcium ion concentrations, the C1F-binding activity is lower because of a higher dissociation rate. This binding characteristic correlates with the induction of *grp78* transcription in response to the depletion of intracellular calcium stores. The strikingly similar behavior of C1F from nuclear extracts of control and A23187-treated cells further suggests that C1F itself does not undergo any major inherent changes after calcium depletion stress. Rather, its binding property could be modulated by the immediate calcium ionic environment in stressed and nonstressed cells. On the basis of the in vitro and in vivo site occupancies of C1F and other stress-inducible changes of upstream regulatory complexes, we present a model to explain how C1F and other upstream factors can synergistically activate *grp78* transcription in calcium-depleted cells.

Calcium is a potent intracellular signalling agent which has been implicated in the regulation of a wide variety of cellular activities (3). Despite the huge volume of studies of the effect of calcium on cell growth and differentiation, little is known about the molecular mechanism by which fluctuations of intracellular calcium stores contribute to specific induction of gene expression. One major intracellular reservoir of calcium ions is the endoplasmic reticulum (ER). Residing in the lumen of the ER are a class of proteins referred to as the glucose-regulated proteins (GRPs) (18, 23, 28). These proteins are high-capacity but low-affinity calcium-binding proteins (14). GRP78, also known as BiP, is a 78-kDa GRP known to function as a molecular chaperone in that it has the ability to bind to unfolded proteins to assist in their folding (10, 32). Recently, it has been demonstrated that calcium ions play an important role in the process of protein folding (25). Furthermore, it has been suggested that the flux of ER calcium ions, acting through calcium-binding proteins such as the GRPs, facilitates the transport of secretory proteins from the ER (37).

GRP78 is encoded by a single-copy gene in the mammalian genome (22, 40). While it is ubiquitously transcribed at a basal level, a variety of conditions that create stress in the ER enhance the transcription of the GRP78 gene (*grp78*) by 10- to 25-fold (17, 18). One of the most potent inducers of *grp78* is the calcium ionophore A23187 (35). In a typical mammalian cell, the majority of the calcium ions are sequestered in intracellular organelles, where the concentration of calcium ions could reach the millimolar range (3). In contrast, the free calcium ion concentration in the cytoplasm is extremely low and is estimated to be in the micromolar range. While A23187 is most commonly used to raise the cytoplasmic calcium ion level, it can also be used to deplete the intracellularly trapped calcium ion stores. *grp78* induction by A23187 is independent of an increase in cytoplasmic calcium ion concentration (9). Rather, it is the depletion of intracellular calcium stores which results in the transactivation of the *grp78* promoter. Thus, treatment of mammalian cells with calcium-free medium or EGTA increases transcription of *grp78* (35). Addition of A23187 to calcium-free medium further enhances the level of *grp78* induction (9). Recently, we and others demonstrated that thapsigargin (Tg), which inhibits the ER Ca^{2+}-ATPase, resulting in the draining of calcium ions from the ER store, stimulates *grp78* transcription (20, 34). Nonetheless, the slow kinetics of *grp78* induction by A23187 and Tg and its dependence on new protein synthesis (20, 34, 35) suggest that the molecular triggering mechanism is more complex than the instantaneous release of calcium ions from the trapped stores.

To understand signal transduction from the ER to the nucleus as a result of calcium ion depletion, we determined the *cis*-regulatory elements of the human and rat *grp78* promoter which respond to A23187 and Tg induction. The promoter sequences of human and rat *grp78* are remarkably conserved (40). In addition, the *grp* promoters in general exhibit a unique characteristic in that they contain a large number of CCAAT and CCAAT-like elements flanked by GC-rich motifs (5, 42). Using a combination of deletion and site-directed mutagenesis approaches, we discovered that the mammalian *grp78* promoter is functionally redundant (42). Of the multiple arrays of regulatory elements which span the *grp78* promoter, the most

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proximal CCAAT element (termed C1) to the TATA sequence, and the *grp* core, a sequence motif conserved among *grp* genes from yeast species to humans (36), are the two most crucial promoter control elements required for both basal-level expression and stress induction of *grp78* (24, 42). Recently, we have demonstrated by in vivo footprinting that during stress induction of *grp78*, including the treatment of cells with A23187, inducible changes of factor occupancy occur at the *grp* core, and a 70-kDa protein was identified as binding to the sites exhibiting a change in dimethyl sulfate (DMS) protection (21). In the case of the C1 element, both in vitro footprinting and in vivo footprinting show that the site is constitutively occupied before and after stress (1, 21). In in vivo transfection assays, a *grp78* promoter subfragment spanning positions −109 to −74 containing C1 is sufficient to confer the calcium stress response (4, 20). As the most proximal CCAAT element to the TATA motif, C1 in the native promoters serves as an essential link for the upstream regulatory factors to activate the *grp78* promoter (42).

In this report, we seek the identity of the factor (termed C1F) or factors which bind C1 and seek to understand the mechanism by which C1F mediates the *grp78* calcium stress response. Several mammalian transcriptional factors which bind to CCAAT elements have been reported. These include C/EBP, CTF/NF-1, and several CBFs. C/EBP is a heat-stable DNA-binding protein with a molecular size of 42 kDa and has a leucine zipper for dimerization (15, 16). CTF/NF-1 contains a range of isoform proteins ranging from 52 to 66 kDa resulting from alternative splicing (38). Recently, it was reported that NF-1 recognizes the sequence TGG(N6)GCCA instead of the CCAAT site (43). The nomenclature for CBF is confusing because it is used to represent several unrelated CCAAT-binding proteins. One of the better-characterized CBFs is a heteromeric protein so far found to consist of at least three components (28). Of the three components, two have been purified and characterized. The molecular sizes for the CBF components were originally reported to be around 39 to 41 kDa (12). Subsequently, the sizes of CBF-A and CBF-B were determined to be 27 and 37 kDa, respectively (27, 41). The two components can be separated by MonoQ chromatography, and maximum binding activity is restored when both components are added back to each other (12). Another CBF, referred to here as the hsp70 CBF, is a 114-kDa protein encoded by a single polypeptide shown to be able to activate the hsp70 promoter through its proximal CCAAT element (26).

Through UV cross-linking, biochemical fractionation, antibody cross-reactivity, and site specificity tests, we establish here that the multimeric CBF is a component of C1F in mammalian cells. We note that while the CCAAT motif is critical for its binding, a GGAGG sequence flanking the CCAAT motif is also needed for high-affinity binding. Fortuitously, it was discovered that CBF binding to DNA is stimulated by EDTA (12). We show here that chelation of divalent cations by EDTA or EGTA enhances C1F binding in vitro. Conversely, increases in calcium ion concentrations decrease C1F binding. The calcium ions do not affect the kinetics of formation of the C1F complex but do increase the dissociation rate of C1F binding. C1Fs prepared from stressed and nonstressed cells exhibit identical binding properties. The binding characteristic of C1F is consistent with the induction of *grp78* transcription in calcium-depleted cells, suggesting that a drop in the calcium ion concentration in the nucleus may lead to increased stability of C1F binding to the C1 site of the *grp78* promoter. This in turn may increase *grp78* transcription. Our results indicate that CBF, a general CCAAT transcription factor with binding activity sensitive to the calcium ion concentration, plays a key role in the induction of the mammalian *grp78* gene in response to calcium stress. A model integrating the depletion of the ER and nuclear calcium ion stores and the interactions of C1F with the upstream regulatory factor complexes is presented.

MATERIALS AND METHODS

Cell lines. The maintenance of the hamster K12 and NIH 3T3 cells has been described previously (4, 42).

Plasmids. The *grp78* chloramphenicol acetyltransferase (CAT) plasmids, −457CAT, LS90CAT, and LS95/98CAT, have been described previously (42). The plasmid pSV2CAT has been described previously (11). The plasmid (−109/−74)MCAT contains two copies of the synthetic *grp78* promoter subfragment subcloned in a palindromic orientation into MCAT (20). Plasmid MCAT contains the minimal mouse mammary tumor virus promoter fused to the CAT gene. The mouse α2(I) collagen promoter plasmids $pR40^{-2000}$ and $pG18^{-2000}$ (gift of B. de Crombrugghe, University of Texas) containing 2,000 bp of the collagen promoter fused to CAT have been described previously (13).

Transient transfections and CAT assays. Conditions for the transfections and assays have been described previously (4, 42). Essentially, the test plasmid (5 μg for the *grp78* CAT plasmids and 10 μg for the collagen CAT plasmids) was cotransfected with 5 μg of PCH110, an expression vector for β-galactosidase, by the calcium phosphate precipitation method in the presence of 3 μg of carrier HeLa genomic DNA. Twenty-four hours after transfection, fresh medium was added to the cells. For stress induction, the cells were treated with either A23187 (7 μM), Tg (300 nM), or tunicamycin (1.5 μg/ml) for 16 h prior to protein extraction. The CAT activities were measured at the linear range after normalization to the β-galactosidase activities. The developed thin-layer chromatography plates were quantified on an Ambis radioanalytic imaging system (Ambis System, San Diego, Calif.).

Preparation of nuclear extracts. The procedures for preparing HeLa nuclear extract and simplified nuclear extract from hamster K12 cells have been described previously (31, 39).

Gel retardation assays. The sequences of all synthetic oligomers used are shown in Table 1. The CTF oligomer was purchased from Promega, Madison, Wis. The oligonucleotide strands were purified on G25 gel filtration columns, and the complementary strands were annealed. The synthetic oligonucleotides were labeled by the Klenow reaction. One to 2 μg of the extract was mixed with 100 to 200 ng of poly(dI-dC) or 100 ng of salmon sperm DNA in a binding buffer consisting of 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 80 mM NaCl, and 5% glycerol. After 10 min of preincubation at room temperature, 0.5 ng of the labeled probe was added to the reaction mixture and further incubated for 15 min. The total reaction volume was 20 μl. The binding reaction mixture was then loaded on a 6% polyacrylamide gel and run at 200 V for 2 h in 1× TBE (89 mM Tris base, 88 mM boric acid, 2 mM EDTA [final pH 8]). Where noted, preincubation with poly(dI-dC) was omitted. In the case of competitions, the probe and competitor were added at the same time in the concentrations indicated. In assays examining the effect of EDTA and EGTA, regular binding buffer containing 2 mM $MgCl_2$ was used. However, EDTA was omitted from the 1× TBE used for running the polyacrylamide gel. In assays examining the effect of divalent metal ions, $MgCl_2$ was omitted from the binding buffer and EDTA was omitted from the running buffer. For these assays, $CaCl_2$ was added in the indicated concentrations to the binding reaction mixtures before the addition of nuclear extract or probe.

For the antibody shift experiments, 0.25 to 1 μl of anti-CBF antibody or preimmune serum was incubated at room temperature with the binding reaction mixture for 15 min 5 min after addition of the probe to the binding reaction mixture as described above. The reaction mixture was then loaded on a polyacrylamide gel.

To determine the on rates for C1F binding, binding buffer with or without 2 mM $CaCl_2$ was prepared. At the indicated times after addition of the probe, the reaction was stopped by immediate loading onto a 6% polyacrylamide gel. For determination of the off rates, binding buffers with or without 2 mM $CaCl_2$ were incubated with the probe for 15 min. A 50-fold molar excess of the homologous competitor was added, and at the times indicated, the reaction mixture was loaded onto the gels. All experiments were repeated independently in triplicates. The gels were dried and subjected to autoradiography. The intensity of the band corresponding to complex I was quantitated with either an LKB Ultroscan XL laser densitometer or a Hewlett-Packard Scanjet IIcx scanner with Image-Quant software from Molecular Dynamics.

UV cross-linking experiments. The bromodeoxyuridine-substituted probe was prepared as described previously (24) with the following modifications. A total of 240 ng of the C1 primer (Table 1) was reannealed with 270 ng of the sense strand of the *grp78* C1 oligonucleotide. The annealed product was radiolabeled in a Klenow reaction in which dCTP was replaced with [^{32}P]dCTP and dTTP was replaced with bromodeoxyuridine. The probe was purified by gel filtration with a G25 column. In a 40-μl binding reaction mixture, 50 μg of nuclear extract was preincubated with 5 μg of sonicated salmon sperm DNA in the binding buffer for gel mobility shift assays. After the preincubation, 10 ng of probe was added, and where indicated, a 50-fold molar excess of the competitors was added to the reaction mixture. After the incubation period, the samples were loaded on a 4%

TABLE 1. Sequences of synthetic oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Reference
mutCBF^{GA}	tcgacCGTCTCCACCAATG**ttctt**GCTGGGc gGCAGAGGTGGTTAC**aagaa**CTACCCgagct	
CBF	tcgacCGTCTCCACCAATGGGAGGGCTGGGC gGCAGAGGTGGTTACCCTCCCGACCCGagct	12
78C1	ctcgagTAGCGAGTTCACCAATCGGAGGCCTCCACGACGGG ATCGCTCAAGTGGTTAGCCTCCGGAGGTGCTGCCCagctg*	20
mut78C1	tcgacTAGCGAGTTC**gtaagctttc**GGCCTCCACGACGGGc gATCGCTCAAG**cattcgaaag**CCGGAGGTGCTGCCCgagct	20
94C1	tcgaGGACGGGAAACATGAACCCACCAATCGCGCCGCAC CCTGCCCTTTGTACTTGGGTGGTTAGCGCGGCGTGagct	5
CTF	CCTTTGGCATGCTGCCCAATATG GGAAACCGTACGACGGGTTATAC	6
78C3	tcgagGGCATGAACCAACCAGCGGCCTg cCCGTACTTGGTTGGTCGCCGGAcagct	20
mut78C3	tcgag**taagcttcaa**AACCAGCGGCCTg c**attcgaagtt**TTGGTCGCCGGAcagct	42
mut78C3^{GC}	tcgagGGCATGAACCAAC**actattaag**g cCCGTACTTGGTTG**tgataattc**cagct	
htk	aagctTGCGGCCAAATCTCCCGCCAGGTCAGC ACGCCGGTTTAGAGGGCGGTCCAGTCG	19
78 core	ctcgaGCCGCTTCGAATCGGCAGCGGCCAGCTTGGTGGCAT CGGCGAAGCTTAGCCGTCGCCGGTCGAACCACCGTAcagcg	20

[a] Boldface lowercase letters indicate mutated sequence. The CCAAT elements are boxed. Lowercase letters indicate linker sequence.
* C1 primer for UV cross-linking is underlined.

N, *N*′-bis-acrylylcystamine (BAC) acrylamide gel (24), and electrophoresis was performed at 200 V for 4 h. The gel was irradiated at 302 nm for 10 min at room temperature, followed by autoradiography for 12 h at 4°C. The bands corresponding to complex I were excised and processed, and the samples were applied to a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and electrophoresed at 35 mA for 6 h. The gel was dried and exposed for 4 days with an intensifying screen at −70°C.

MonoQ chromatography. MonoQ chromatography of the HeLa nuclear extract was performed with the SMART System (Pharmacia Biotech., Piscataway, N.J.). Two hundred micrograms of HeLa nuclear extract in a 40-μl volume was diluted with an equal volume of buffer D (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 5% glycerol) and applied to a MonoQ PC 1.6/5 column previously washed and equilibrated in buffer D supplemented with 100 mM KCl. One hundred-microliter fractions were collected at a flow rate of 50 μl/min. After about 10 column volumes of washes with buffer D at 100 mM KCl, the buffer was changed to that containing 500 mM KCl for the elution of bound proteins. Binding assays were carried out as described with 2 μl of the flowthrough fractions and 1 μl of the 0.5 M eluate in place of the nuclear extract and a lower concentration (50 ng) of poly(dI-dC). To examine the effect of high temperature on the stability of the components eluting from the column, the fractions were treated at 65°C for 5 min prior to addition to the binding reaction mixtures.

DNase I footprint analysis. The footprint analysis of a 266-bp rat *grp78* promoter subfragment spanning positions −293 to −34 with HeLa nuclear extracts was performed as described previously (1).

Methylation interference analysis. The procedure was performed as described in reference 33 with the following modifications. One microgram of the sense strand of the C1-binding site was treated with kinase, reannealed, and purified by ethanol precipitation followed by a 70% ethanol wash. The radiolabeled DNA (6.5×10^6 cpm) was DMS treated for 1 min, and 1×10^6 cpm of DMS-treated probe was mixed with 20 μg of HeLa nuclear extract in the binding reaction mixture for gel mobility shift assays. The gel was run in 1× TBE buffer and subjected to autoradiography at 4°C overnight. The bands corresponding to complex I and the free probe were excised from the gel. The DNA was electroeluted from the gel, ethanol precipitated, dried, and treated with piperidine. Equal counts of each sample were loaded on a 13% sequencing gel.

RESULTS

Mediation of stress response by the C1 element. The *grp78* promoter contains multiple regulatory sequence motifs which bind to protein factors according to the criteria of gel mobility shift assays, in vitro DNase I footprinting, and in vivo DMS protection (1, 21). These include sites for the transcription factors, SpI, CREB, and p70core, a 70-kDa protein which binds to the 3′ half of the core sequence (Fig. 1A). Most proximal to the TATA element is the C1 element, and 20 bp upstream is the C3 element. The functional significance of the C1 element in mediating the *grp78* calcium stress response derived from this and previous studies with A23187 and Tg as inducers is summarized in Fig. 1A. When cells were treated with A23187, the wild-type promoter containing a full array of upstream regulatory elements (−457CAT) was induced by about sixfold. Within the context of the native promoter, a 10-bp base substitution (LS90CAT) which destroys the C1 element and its flanking sequences or a 4-bp base substitution which mutates specifically the CCAAT motif (LS95/98CAT) reduced the basal level to about 30% of that of the wild-type promoter and reduced its inducibility by 60% to a level which is slightly above that of the simian virus 40 promoter (20, 42). Thus, in the *grp78* promoter, an intact C1 element is important for mediating both basal-level expression and the *grp78* calcium stress response. Furthermore, a synthetic subfragment of the *grp78* promoter spanning positions −109 to −74 containing the C1 element inserted in duplicate into the mouse mammary tumor virus minimal promoter is able to confer stress inducibility to the CAT reporter gene. As shown in Fig. 1B, cells transfected with (−109/−74)MCAT exhibited a sevenfold induction by Tg, which depletes the ER calcium store, and a fourfold induction by tunicamycin, which blocks N-linked protein glycosylation.

Sequence specificity of C1F. To characterize the factor (C1F) which binds the C1 element in mammalian cells, gel mobility shift assays were performed. Gel mobility shift assays were performed with the C1 element as the labeled probe, and the nuclear extracts were prepared from both human (HeLa) and hamster (K12) cells. Various homologous and heterologous synthetic oligomers were used as competitors, as shown in Table 1.

With the HeLa extract, two major complexes (I and II) were observed (Fig. 2A). Only complex I binds with high affinity to the C1 element, because complex II was largely eliminated by preincubation with salmon sperm DNA (compare the first two lanes). Complex I was specific for C1, because its formation

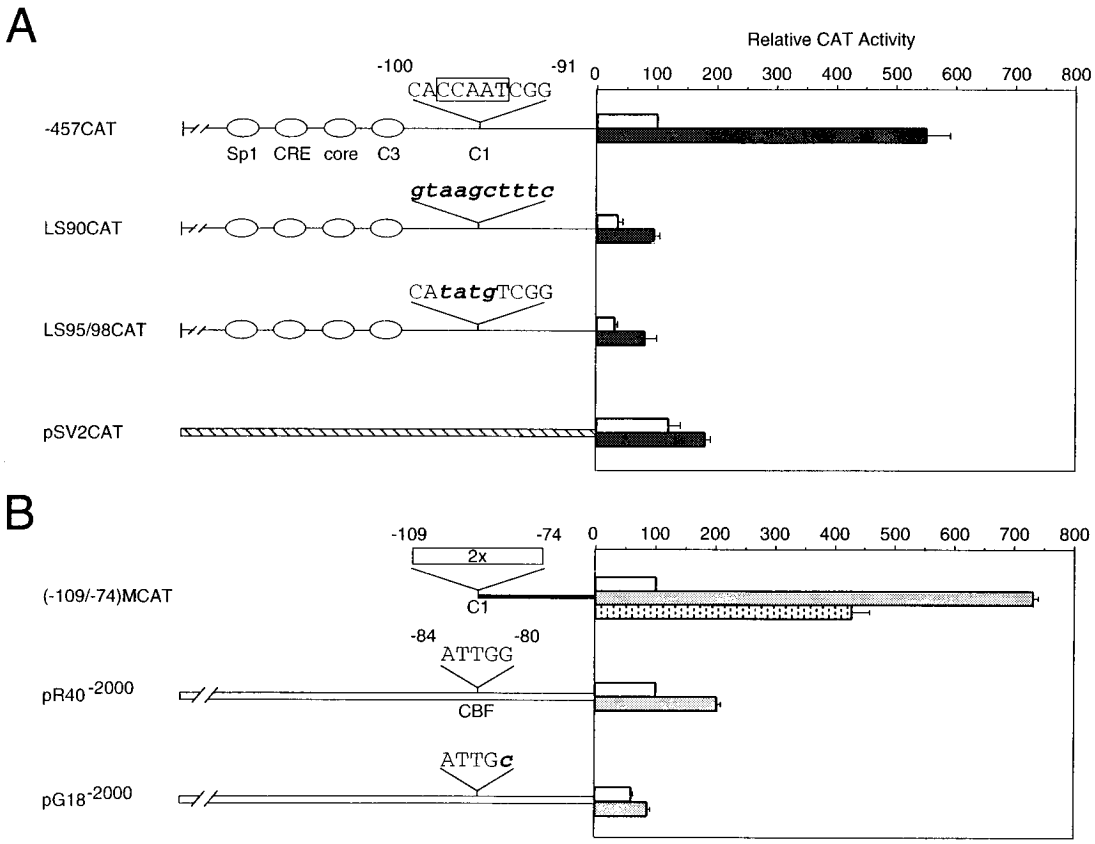

FIG. 1. Stress inducibility of promoter constructs. (A) The regulatory factor binding sites of the *grp78* promoter indicated are SpI, CRE, core, C3, and C1. The sequence of the C1 element as present in the wild-type *grp78* promoter-CAT construct (−457CAT) is compared with those of the linker-substitution mutants LS90CAT and LS95/98CAT. The basal CAT activity of K12 cells transiently transfected with −457CAT was set at 100. The relative CAT activities are summarized for cells which were not treated (open bars) or which were treated with 7 μM A23187 for 16 h (solid bars). A control with pSV2CAT is included. The standard deviations are indicated. (B) Plasmid (−109/−74)MCAT contains duplicate copies of the *grp78* promoter subfragment linked to the mouse mammary tumor virus minimal promoter. The α2(I) collagen promoter-CAT fusion genes pR40^{-2000} and pG18^{-2000} contain the wild-type CBF- and mutated CBF-binding sites, respectively. The plasmids were transiently transfected into NIH 3T3 cells. The basal CAT activities of cells transfected with (−109/−74)MCAT and pR40^{-2000} were set at 100. The relative CAT activities are summarized as follows: untreated cells, open bars; cells treated with 300 nM Tg, shaded bars; or cells treated with 1.5 mg of tunicamycin per ml, stippled bar. The standard deviations are indicated.

was severely inhibited by increasing concentrations of the homologous 78C1 oligomer. In contrast, it has little or no affinity for the mutant C1 oligomer or the CTF consensus sequence. In the case of the 78C3 oligomer and its mutant form, partial competitions were observed. In contrast, the residual complex II exhibited little difference in its binding activities when either the homologous or heterologous competitors were added. We were surprised that the CTF-binding site was a much weaker competitor than C1, because in *Drosophila melanogaster* Schneider cell line SL-2 in which the levels of CTFs were low cotransfection of the *grp78* promoter CAT constructs with CTF expression vectors resulted in its transactivation through the C1 element (42). Thus, our results indicate that, although in an insect cell background overexpression of CTF can stimulate *grp78* transcription through C1, in human cells, a factor distinct from CTF binds with high affinity to the C1 element.

To test whether this phenomenon is also observed in other mammalian cell extracts, nuclear extracts were prepared from the hamster K12 cell line, in which the majority of the previous analysis of the *grp78* promoter was performed. A highly similar result was obtained such that C1F has the highest affinity for its homologous sequence (Fig. 2B). It exhibits weak affinity for the 78C3, mutC3^{GC}, and CTF oligomers, which contain either a CCAAC or a CCAAT motif. Mutation of the CCAAT motif in mutC1 and mutC3 resulted in little or no competition. These combined results suggest that while the CCAAT element is critical in C1F binding, a CCAAT motif alone appears not to be sufficient for high-affinity C1F binding. In both nuclear extracts, a faint binding activity with competition profiles similar to those of the specific complex I was detected. Because of the greater abundance of complex I, we focused on identifying the protein components of complex I.

Radiolabeling of the protein species in complex I of C1F. UV cross-linking was used to identify the molecular sizes of the proteins in close contact with the C1 element. For this purpose, bromodeoxyuridine was substituted for dTTP in the C1 element which was radiolabeled with [^{32}P]dCTP. This probe was mixed with either HeLa or hamster nuclear extract. Gel mobility shift assays were performed as described above, and the preparative BAC acrylamide gel was exposed to UV light. Complex I was visualized by autoradiography, excised from the gel, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) to identify the molecular sizes of the labeled proteins. In the HeLa cells, three predominant protein bands with molecular sizes of 63 kDa, a doublet of 49 and 46 kDa, and 35 kDa were detected (Fig. 3A). Similarly, the protein species with the same size were observed with the hamster extract (Fig. 3B). Thus, the protein components in close contact with C1 are

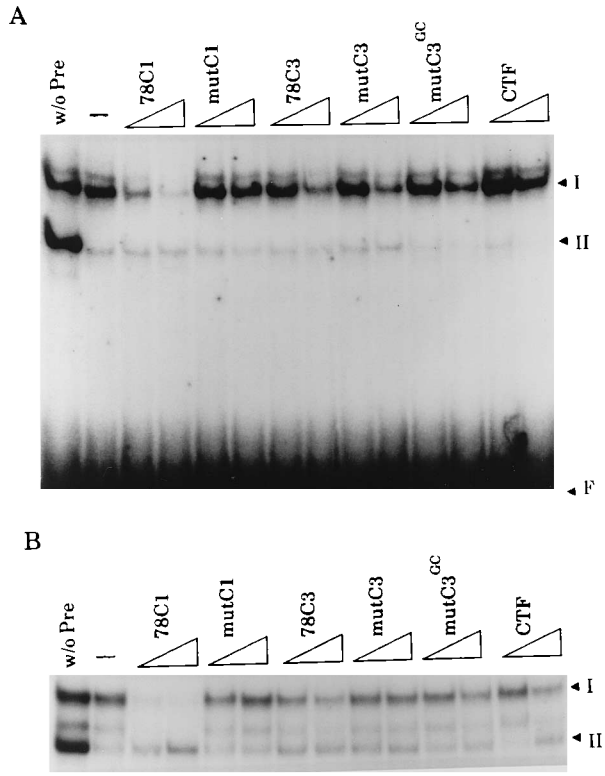

FIG. 2. Sequence specificity of the C1 complexes. Gel mobility shift assays were performed with C1 as a probe and nuclear extracts prepared from HeLa cells (A) or K12 hamster fibroblasts (B). The first lane shows reaction mixture without preincubation with 100 ng of salmon sperm DNA (w/o Pre). All other reaction mixtures were preincubated and contained either no competitor (−) or increasing amounts (10- and 50-fold) of the competitor DNA as indicated on top. The sequences of the competitor oligomers are shown in Table 1. The autoradiograms are shown. The positions of the complexes (I and II) and the free probe (F) are indicated.

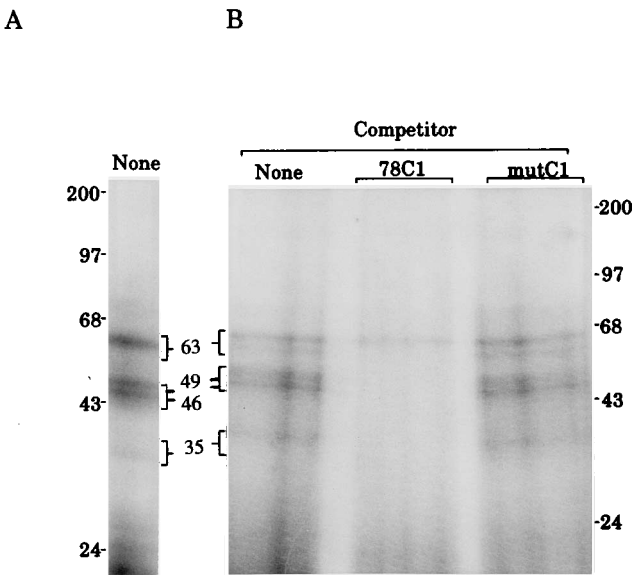

FIG. 3. UV cross-linking of C1F. Gel mobility shift assays were performed with bromodeoxyuridine substituted and radiolabeled C1 as a probe. HeLa (A) and K12 hamster fibroblast (B) nuclear extracts were used. The reaction was performed either in the absence (none) or in the presence of a 50-fold molar excess of the competitors (78C1 and mutC1) indicated. After UV crossing of the preparative gel, complex I was excised from the gel and the radiolabeled protein species were analyzed by SDS-PAGE (10% polyacrylamide). The autoradiograms are shown. The positions of the protein size markers (in kilodaltons) are indicated on the left and right. The three most prominent sets of labeled protein bands are bracketed, and their deduced sizes are indicated.

highly conserved in their molecular sizes between the human and hamster species. The 49- and 46-kDa doublet and the 35-kDa protein species are specific for the C1 element, because the labeling was eliminated when homologous 78C1 but not mutated C1 (mutC1) was used as a competitor (Fig. 3B). The specificity of the 63-kDa protein band was unclear, because its formation was only partially affected by the homologous sequence. The same was observed with the protein bands from the HeLa nuclear extract (data not shown). The size range of 30 to 40 kDa was reported for the CCAAT-binding factors CBF and C/EBP (12, 15). Furthermore, the presence of multiple proteins suggested that C1F may not consist of a single polypeptide but rather may be composed of a multicomponent protein complex, a property similar to CBF.

Chromatographic fractionation of the C1F components. The multimeric CBF consists of at least two components, one of which cannot bind to a MonoQ column but which can stimulate the bound component to bind to the CBF site with higher affinity (12). Additionally, it has been demonstrated that the MonoQ bound component is sensitive to heat treatment. To test whether C1F exhibits the same properties as CBF, we separated the HeLa extract on a MonoQ column into a flowthrough fraction and a bound fraction with a single-step elution using buffer containing 0.5 M KCl (Fig. 4A). The fractions were tested for C1F activity with gel mobility shift assays. As shown in Fig. 4B, the flowthrough fractions (Fx 1, 2, and 3) were not able to form complexes with C1; the bound fraction (Fx 13) was able to form a weak complex I and a strong complex II. However, when the flowthrough fractions 1 and 2 were added to the bound fraction, three- and fourfold increases, respectively, in complex I binding activity were observed. The increases could be even higher, because we noted that addition of either flowthrough fraction 3 or a similar buffer without protein containing 0.1 M KCl mildly inhibited the formation of complex I (Fig. 4B and data not shown). In contrast, the binding activity of the nonspecific complex II remains relatively constant. In addition, when the bound fraction was heated, it lost its binding activity even in the presence of the stimulative flowthrough fraction (Fig. 4C). Thus, C1F closely resembles CBF in its fractionation characteristics and its sensitivity to heat.

Comparison of electrophoretic mobilities of C1F and CBF. To compare more directly whether C1F is identical to CBF, we used the 78C1- and the CBF-binding sites as probes and performed gel mobility shift assays under identical conditions with both human and hamster extracts. In both cases, we observed strikingly similar complex formation (Fig. 5 [data not shown for the hamster extract]). From cross-competitions with the unlabeled 78C1- and CBF-binding sites, it was evident that both probes form a specific complex I and a nonspecific complex II. Importantly, the unlabeled CBF-binding site is as efficient a competitor as C1 and vice versa. Thus, C1F exhibits an affinity for the CBF-binding site similar to that for its own 78C1 site. On the basis of the nearly identical binding profiles of the C1F and CBF complexes, we conclude that C1F is either identical to CBF or is composed of very similar components.

Sequence identity between the CBF- and *grp78* C1-binding sites. Direct comparison between the sequences of the 78C1- and CBF-binding sites revealed that they share sequence identity (Table 1). In addition to the CCAAT motif which is

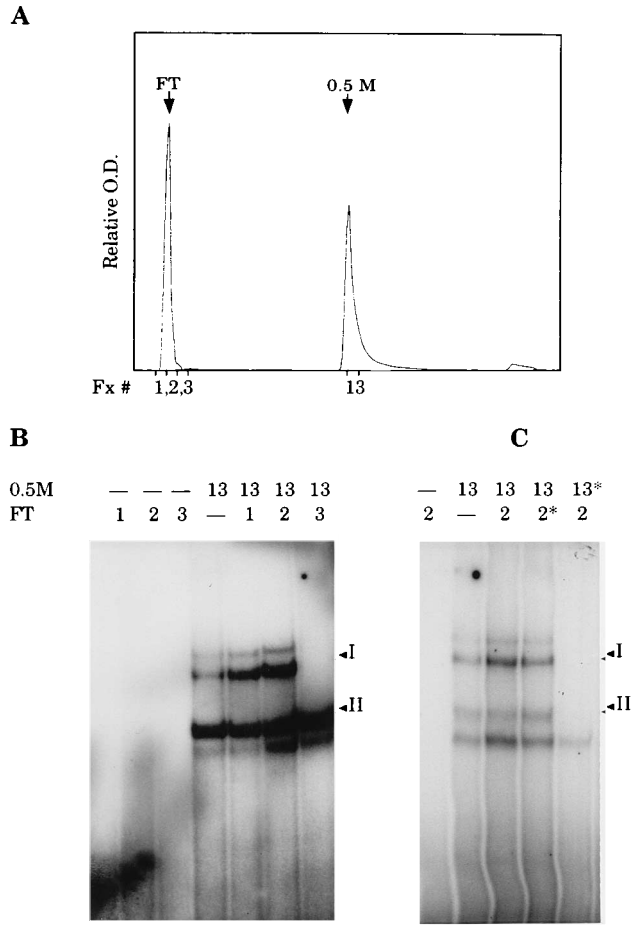

FIG. 4. MonoQ chromatography fractionation of the C1F components. (A) HeLa nuclear extract was applied to a MonoQ PC column. The unbound fractions were collected in the flowthrough (FT) fractions, and the bound fractions were collected by a single-step elution with 0.5 M KCl. The elution profile based on relative optical density (O.D.) is shown. (B) Gel mobility shift assays were performed with either the unbound (Fx 1, 2, and 3) or bound (Fx 13) fractions or their combinations as indicated. (C) Effect of preheat treatment on the binding activities. Fx 2* and 13* were heated at 65°C for 5 min prior to addition to the reaction mixture. Asterisks indicate heat-treated samples. The autoradiograms are shown. The positions of complexes I and II are indicated.

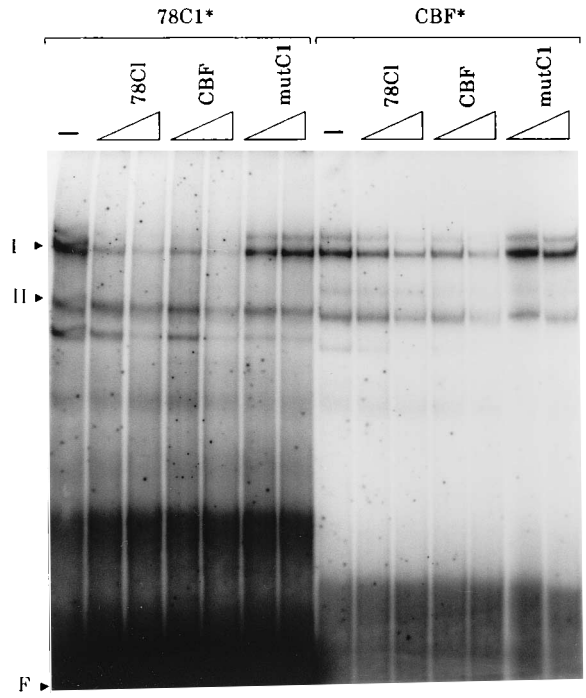

FIG. 5. Comparative gel mobility shift assays of C1F and CBF. The probes used are C1F-binding site (78C1*) and the CBF-binding site (CBF*). The labeled probes (indicated by asterisks) were mixed with HeLa nuclear extract. All of the reaction mixtures were preincubated with poly(dI-dC). Either the reaction mixtures contained no competitor (−) or increasing amounts (10- and 50-fold molar excesses) of competitors (78C1, CBF, and mutC1) were added. The sequences of the probe and competing oligomers are shown in Table 1. The autoradiograms are shown. The positions of the complexes (I and II) and the free probe (F) are indicated.

present in both oligomers, a GGAGG motif flanking the CCAAT motif is highly similar. In the case of the 78C1 sequence, the 5-bp GGAGG sequence motif represents half of a palindromic sequence. In contrast, C1 is very different from the CTF site and does not share any of the flanking sequence outside of the CCAAT motif (Table 1). Previously, we demonstrated by in vitro DNase I footprint analysis with HeLa nuclear extracts that the most-proximal CCAAT element (C1) of the *grp78* promoter was protected (1). Detailed reexamination of the footprinted region revealed that the protected region extended beyond the CCAAT motif, including that of the flanking GGAGG motif (Fig. 6A). To test directly whether the GGAGG motifs are important as protein contact points for complex I, a methylation interference assay was performed with nuclear extracts prepared from HeLa cells. The results, as shown in Fig. 6B, revealed that the GA/CT repeat sequence was partially protected, lending support to the hypothesis that it may interact with the protein components of complex I.

To test whether the GGAGG sequence is required for complex I binding, a CBF synthetic oligomer retaining the CCAAT motif but mutated at the GGAGG motif (mutCBF^{GA}) was used as a competitor (Table 1 and Fig. 7B). We also included in this series of experiments a synthetic oligomer of C1 element from a *grp94* promoter (94C1) as a competitor (Fig. 7A). The *grp94* gene is coordinately regulated with *grp78* (5), but its C1 element does not contain a GGAGG-rich motif (Table 1). We observed that the most-potent competitors are the 78C1- and CBF-binding sites, and the least-effective competitor is mutC1 without the CCAAT motif. mutCBF^{GA} and wild-type 94C1 oligomers both contain an intact CCAAT but not the GGAGG motif and thus compete less efficiently. Quantitation of the specific complex I in multiple competition experiments is summarized in Fig. 7D. A sharp decrease in complex I was observed when a 5-fold molar excess of either the CBF or 78C1 oligomer was added as competitor. At a 10-fold molar excess, the CBF and 78C1 competitors reduced the amounts of complex I to 6 and 15%, respectively, and at a 40-fold molar excess, complex I was almost totally eliminated by both oligomers. In contrast, a 40-fold molar excess of mutC1 did not affect complex I formation. Compared with CBF, mutCBF^{GA} was a less-effective competitor. At 5- and 10-fold molar excesses, mutCBF^{GA} was without effect. At 20- and 40-fold molar excesses, the amounts of complex I were reduced to 82 and 50%, respectively. Therefore, while the CCAAT motif is of primary importance, mutation of the GGAGG motif substantially reduced its ability to compete for complex I.

These competition results were confirmed further by labeling the synthetic oligomers CBF and the mutCBF^{GA} and using them as probes in gel mobility shift assays (Fig. 7C). A direct comparison of the complexes revealed that complexes I and II

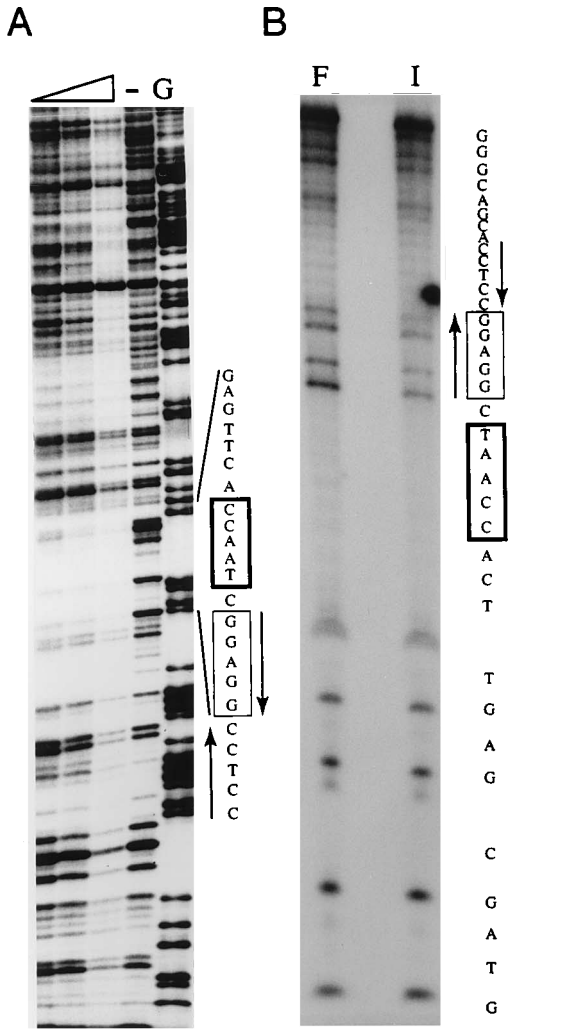

FIG. 6. Protein contact with the GGAGG sequence motif. (A) DNase I footprint analysis of the coding strand of the rat *grp78* promoter. The first three lanes indicate DNase-treated probes with increasing amounts (20, 40, and 60 μg) of HeLa nuclear extract. The fourth lane (−) was without nuclear extract, and lane G showed the Maxam-Gilbert G sequencing reaction of the labeled strand. The footprinted region around the C1 element is indicated. The CCAAT and the GGAGG motifs are boxed. The GA/CT palindromic sequence is highlighted by arrows. (B) Methylation interference assay with 78C1 as probe and HeLa nuclear extract. The coding strand of C1 was treated with kinase and reannealed with the noncoding strand prior to DMS treatment. After incubation with the HeLa nuclear extract, complex I (I) and the free probe (F) were recovered from a preparative gel, and their methylation patterns were analyzed on a sequencing gel. The autoradiograms are shown.

were formed efficiently with the CBF probe; however, when the GGAGG motif was mutated in the mutant probe, the formation of complex I but not complex II was reduced by about 60% (Fig. 7E). These combined results strongly imply that in addition to the CCAAT sequence, the GGAGG motif flanking the 3′ end of both the 78C1- and CBF-binding sites is important for high-affinity binding of C1F/CBF.

Reactivity of anti-CBF antibody with C1F. Recently, component A of CBF was purified and cloned from rats and mice (41). A rabbit polyclonal antibody generated against a peptide of CBF-A has been used with success to supershift the CBF complex formed with a CBF-binding site (27). To prove directly that CBF is a component of C1F, we used this anti-CBF antiserum preparation in gel mobility shift assays with 78C1 (Fig. 8A), CBF, and two other nonrelated synthetic oligomers (78 core and htk) as probes (Fig. 8B). In the presence of the anti-CBF antibody but not preimmune serum, complex I was eliminated and supershifted bands were observed with both the C1- and CBF-binding site probes. The specificity of the antiserum against CBF was demonstrated further by its nonreactivity against complexes formed with the unrelated probes and its nonreactivity against the nonspecific complex II (Fig. 8B). Thus, C1F is immunologically related to CBF.

Effect of divalent cation chelators and calcium ions on C1-binding activity. Previously it was noted that in binding reaction mixtures which contained 5 mM EDTA to prevent phosphatases and DNases from degrading the labeled probe, the CBF activity isolated from a murine source was unexpectedly stimulated by the inclusion of the divalent cation chelator (12). This fortuitous observation is important for the *grp78* system, because if the C1F/CBF-binding activity is sensitive to the concentration of calcium ions in the nucleus, it could provide an explanation for the transcriptional regulation of *grp78* by calcium ions and its induction when the cells were treated with EGTA (35).

To test this hypothesis, the C1F-binding activity was examined with HeLa nuclear extracts prepared from both control and A23187-treated cells in the presence and absence of EDTA and EGTA (Fig. 9). We observed that increasing amounts of EDTA or EGTA in the binding reaction mixture correlated with increased binding activity of C1F but not that of the nonspecific complex II. At 2.5 mM EDTA or EGTA, there was a two- to threefold increase in C1F-binding activity. The nuclear extracts prepared from the control and A23187-treated cells exhibited similar sensitivities and were enhanced to similar degrees with EDTA and EGTA.

Next, we determined whether an increase in calcium ion concentrations in the binding reaction mixture affects C1F-binding activity. For these experiments, increasing amounts of Ca^{2+} (2 and 10 mM) were added to the binding reaction mixture and both the control and A23187-treated extracts were tested. Furthermore, to avoid chelation of the calcium ions, EDTA was omitted from the running buffer of the gels used for the assays. We observed that increasing amounts of calcium ions specifically decreased C1F binding (Fig. 9). At 2 and 10 mM of $CaCl_2$, there were two- and threefold decreases in C1F-binding activity, respectively. This inhibitory effect was specific for the *grp78* C1F complex, because the binding activity of the nonspecific complex II was stimulated instead by the inclusion of higher concentrations of calcium ions.

High concentrations of calcium ions destabilize C1F binding to the C1 element. After establishing that chelation of divalent ions increases C1F-binding activity and that high concentrations of calcium ions decrease its binding activity, we determined whether the decrease in C1F binding in the presence of calcium ions was due to a slower formation kinetics of the complex (on rate), a faster dissociation (off rate), or a combination of both factors. The concentration of calcium ions used in these reaction mixtures was 2 mM, because at this concentration a moderate level of complex I could be formed. Also, in a typical mammalian cell, the nucleus contains high levels of sequestered calcium, such that as much as 50% of the total cell calcium (2 mM) is located in the nucleus bound to chromatin, and this calcium could be physiologically significant (3). For the on rate measurements, we monitored the kinetics of formation of the C1F complex in the presence or absence of calcium ions by using the 78C1 oligomer as a probe in gel mobility shift assays. Nuclear extracts prepared from both control and A23187 cells were examined. The results, as shown in Fig. 10A, revealed that the kinetics of C1F formation in the

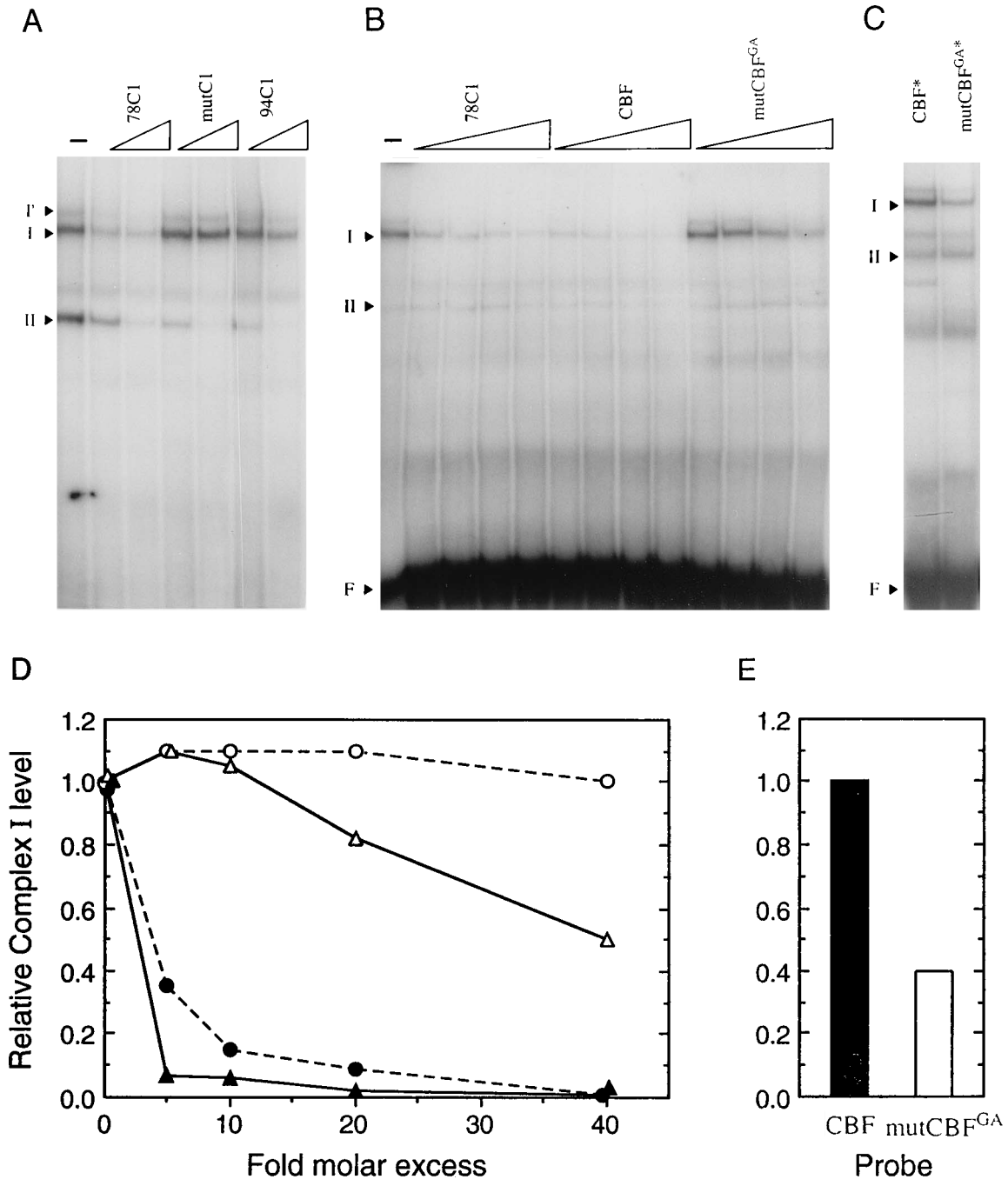

FIG. 7. Effect of GA flanking sequence mutation on C1F. (A) Gel shift mobility assay with 78C1 as probe and HeLa nuclear extract. The reaction mixtures contained either no competitor (−) or increasing amounts (10- and 50-fold molar excesses) of the competitors (78C1, mutC1, and 94C1) indicated at the top. (B) Competition with 78C1 as probe. Either no competitor (−) or increasing amounts (5-, 10-, 20-, and 40-fold molar excess) of the competitors (78C1, CBF, and mutCBF^{GA}) indicated at the top were added. (C) Gel mobility shift assay with CBF and mutCBF^{GA} as probes and HeLa nuclear extract. The sequences of the competitors and probes are shown in Table 1. The autoradiograms are shown. The positions of the complexes (I and II) and free probe (F) are indicated. (D) Summary of complex I competition by various oligomers. The complex I band intensities derived from Fig. 7B and other competition experiments were quantitated. The level of complex I without competitor in each experiment was set at 1.0. The relative complex I levels were plotted against the fold molar excess of the competitors 78C1 (●), mutC1 (○), CBF (▲), and mutCBF^{GA} (△). (E) Quantitation of the complex I band intensity shown in panel C formed by the CBF (closed bar) or mutCBF^{GA} (open bar) probe. Asterisks indicate labeled probes.

binding reaction was very rapid. Within 15 s, the maximum binding level was nearly achieved. The on rates for C1F were similar in the presence or absence of the divalent cations in the reaction buffer. Similar results were obtained for nuclear extracts prepared from control or A23187-treated cells. Thus, a difference in the rate of C1F complex formation from both control and A23187-treated cells cannot be detected in the presence of 2 mM calcium ions.

To measure the off rate of C1F, the C1F complex was allowed to form in reaction mixtures in the presence or absence

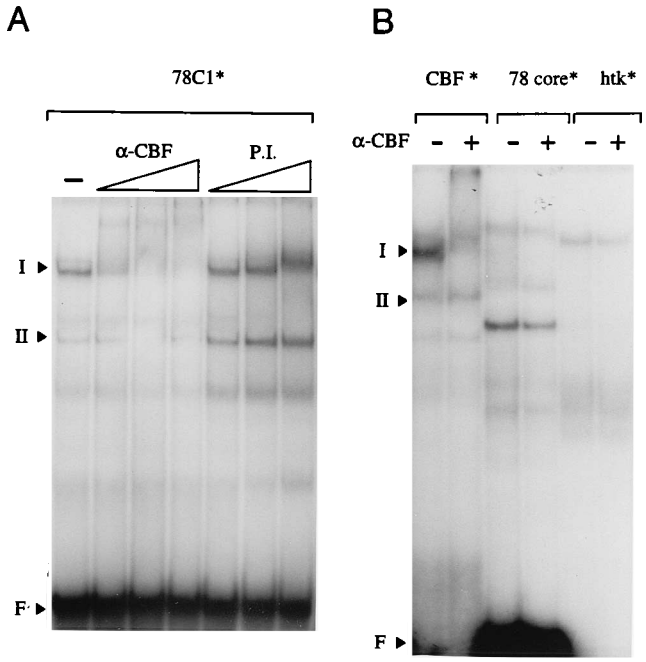

FIG. 8. Effect of anti-CBF antibody on the C1 complex. (A) Gel mobility shift assays were performed with HeLa nuclear extract and 78C1 as a probe. The reactions were carried out either in the absence (−) or presence of increasing amounts (0.25, 0.5, and 1 μl) of anti-CBF-A antibody (α-CBF) or preimmune serum (P.I.). (B) The probes used were CBF, 78 core, and htk, the sequences of which are shown in Table 1. The gel mobility shift assays were performed either in the absence (−) or in the presence (+) of 1 μl of the anti-CBF antibody. The autoradiograms are shown. The positions of the complexes (I and II) and free probe (F) are indicated. Asterisks indicate labeled probes.

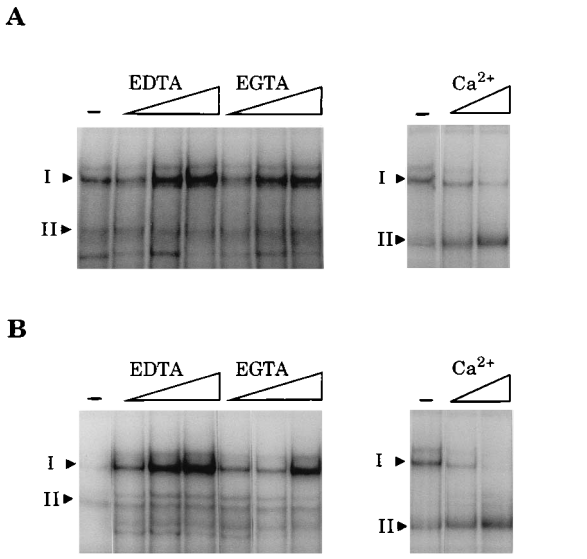

FIG. 9. Sensitivity of C1F-binding activity to divalent cations, chelators, and calcium ions. Gel mobility shift assays were performed with C1 as a probe and HeLa nuclear extracts prepared from control cells (A) and A23187-treated cells. (B). (Left panels) The reactions were carried out either in regular reaction buffer (−) or in buffers containing increasing concentrations (0.05, 0.5, and 2.5 mM) of EDTA or EGTA. (Right panels) The reactions were carried out either in buffers without $CaCl_2$ (−) or in buffers containing increasing concentrations (2 and 10 mM) of Ca^{2+}.

of 2 mM $CaCl_2$. After equilibrium of binding was reached, a 50-fold molar excess of the unlabeled homologous competitor was added to the binding reaction mixture to measure the kinetics of dissociation of the C1F complex. The results are shown in Fig. 10B. We observed that the C1F complex formed in binding reaction mixtures depleted of calcium ions was quite stable. There was an initial decrease of 15% in its binding activity after the first 15 s. This decrease was quickly stabilized such that after 5 min, 70% of the binding activity remained intact. In the presence of 2 mM calcium ions, however, the decrease was more rapid. Within 15 s, a 35% drop in binding activity was observed. By 5 min, only 40% of the binding activity remained. At higher calcium ion concentrations (10 mM), the binding activity of the C1F complex was greatly diminished and the kinetics measurements could not be performed with reliability (data not shown). Similar results were observed for nuclear extracts prepared from control and A23187-treated cells. Thus, our results indicate that the presence of high concentrations of calcium ions destabilizes C1F binding, such that they dissociate from the C1 site at a faster rate than in the absence of calcium. The strikingly similar behaviors of nuclear extracts from control and A23187-treated cells further suggest that C1F itself does not undergo any major inherent changes between stressed and nonstressed cells. Rather, its binding properties could be modulated in the nuclei of the calcium-depleted cells by the immediate calcium ionic environment.

Response of other CBF site-containing promoters to calcium stress. In in vitro assays, the synthetic 78C1 and CBF sites exhibited similar sensitivity to divalent metal ion chelators and calcium ions (unpublished results). To address the issue of whether other CBF site-containing promoters are responsive to calcium stress in vivo, we examined the inducibility of the mouse α2(I) collagen promoter by Tg treatment. This mouse collagen promoter contains an inverted CCAAT site at −80 which binds CBF (12), and the same site was used in our in vitro comparative studies with 78C1. The activities of this promoter and its mutants have been previously analyzed after transfections into NIH 3T3 cells (13). We observed that for the wild-type collagen promoter CAT construct ($pR40^{-2000}$), a twofold increase over the basal level was detected after Tg treatment (Fig. 1B). The mutant construct ($pG18^{-2000}$) is identical to the wild type, with the exception that one base was mutated at −80 to change the ATTGG motif to ATTGC. Our results indicated that the basal level of this construct was reduced to 60% of that of the wild type and that the level of Tg inducibility was 1.4-fold, a level comparable to that of the pSV2CAT control. These results suggest that the presence of a CBF-binding site alone in a cellular promoter does not result in high-level induction by calcium stress mediated by Tg. It appears that the orientation of the CBF site with respect to the other promoter elements and its interaction with other accessory factors are also important.

DISCUSSION

In mammalian cells, the stimulation of *grp78* transcription by depletion of calcium ions has been shown to involve at least two *cis*-regulatory elements of the *grp78* promoter. One is the *grp* core element, and the other is a CCAAT element most proximal to the TATA element, referred to here as the C1 element (20, 42). By combining in vivo footprinting with biochemical purifications, we have recently shown that during calcium and other forms of ER stress, changes in DMS protection occur at the conserved core region. This finding suggests that a change in the site occupancy of the core region by a transcription factor is part of the induction process (21).

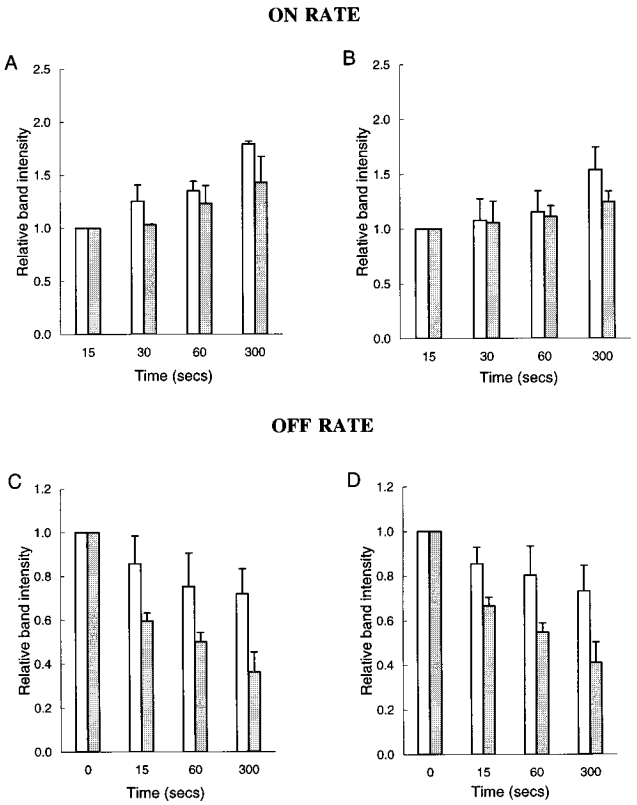

FIG. 10. Measurement of the on and off rates of C1F binding activity. Gel mobility shift assays were performed with HeLa nuclear extract prepared from control (A and C) and A23187-treated cells (B and D) and with C1 as a probe. The reactions were carried out either in buffers without $CaCl_2$ (open bars) or in buffers containing 2 mM $CaCl_2$ (shaded bars). The autoradiograms were quantitated by densitometry. The band intensities for the first time point for each sample were set at 1.0. The relative band intensities were plotted against time. The standard deviations are indicated.

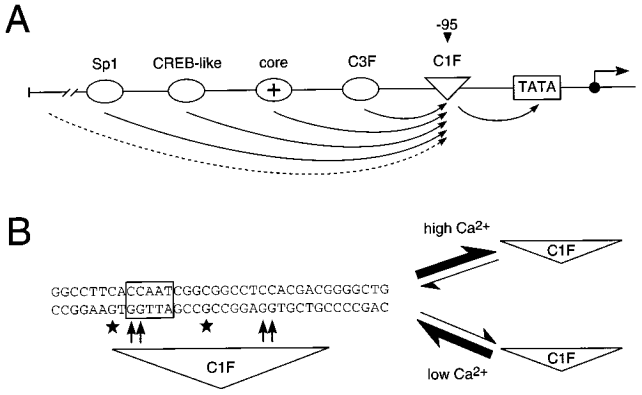

FIG. 11. Model for calcium stress induction of the *grp78* promoter through C1 and the upstream regulatory elements. (A) Schematic drawing of the coordination of the responses of the upstream regulatory units (SpI, CREB-like, core, and C3F) through the C1F unit to the TATA element, resulting in transcription initiation (┌→). The + indicates stress-inducible site changes for the core binding factor. (B) The sequence of the human C1 element is indicated with the CCAAT motif boxed. The arrows and stars point to bases protected or hypermethylated, respectively, in in vivo DMS protection (21). In the presence of a high calcium ion concentration, C1F exhibits a higher off rate, and in the presence of a low calcium ion concentration, the binding of C1F exhibits a slower off rate and is more stable.

Furthermore, we identified a 70-kDa nuclear factor from HeLa cells as the DNA-binding component of the complex, which specifically interacts with the core region necessary for calcium stress induction (21).

In this study, we focus on the transcription factor that occupies the C1 element in HeLa cells and the mechanism by which this factor can enhance *grp78* transcription when calcium is depleted from the cell. Within the *grp78* promoter containing a full array of regulatory elements (Fig. 11A), the C1 element is shown, by linker-scanning analysis, to be the single most important element, because its mutation has the most drastic effect. Thus, the upstream regulatory elements including the conserved *grp* core are not able to effectively activate *grp78* transcription if the C1 element is mutated, suggesting that the C1 complex serves as a crucial link between the TATA complex and its more distal regulatory complexes. Furthermore, a 35-bp subfragment of the *grp78* promoter containing the C1 element is able to confer stress inducibility to a heterologous promoter. Its response to calcium depletion stress is greater than its response to glycosylation block stress.

To try to identify the CCAAT-binding transcription factor which can stimulate the *grp78* promoter through the C1 element, we observed that neither hsp70 CBF nor C/EBP was effective in cotransfection studies (42). Here, we discovered that while in the *Drosophila* Schneider cell system expression of mammalian CTF can stimulate *grp78* transcription through the C1 motif (42), in HeLa cells, the CCAAT factor which binds to the C1 element of the *grp78* promoter has little or no affinity for a CTF-binding site. Rather, by the criteria of its molecular size, biochemical fractionation, heat stability, sequence specificity, and immunocross-reactivity, we show that C1F characterized in HeLa and hamster nuclear extracts is in fact composed of the widely studied CCAAT-binding factor CBF. Our combined studies indicate that in heterologous host systems such as *Drosophila* Schneider cell line SL-2, overexpressed CTF could stimulate *grp78* transcription. Nonetheless, in mammalian cells, the actual factor that interacts and regulates the C1 element is likely to be the multimeric CBF.

CBF consists of at least two components, factors A and B, both of which are required for high-level efficiency in DNA binding (12). CBF is evolutionarily conserved with the *Saccharomyces cerevisiae* HAP2 and HAP3 gene products, a multisubunit heterologous complex recognizing CCAAT-containing transcription elements (7, 29). CBF has been shown to interact with the α2(I) collagen, albumin, and major histocompatibility complex II promoters (29). Here, we show that CBF is also the primary factor which occupies the C1 regulatory element of the mammalian *grp78* promoter. Close examination of 78C1 with the CBF-binding site derived from the α2(I) collagen promoter reveals that immediately 3′ to the CCAAT motif is a GGAGG motif shared between the two sequences. In the *grp78* promoter, this sequence is extended into a 10-bp palindromic sequence which is protected by nuclear extracts in both DNase I protection and methylation interference assays. Mutation of the GGAGG motif weakens the ability of the sequence to bind the C1 complex and to compete for C1F/CBF binding. The high specificity of CBF binding to the C1 site could be stabilized by accessory factors interacting with the GGAGG sequence. It is interesting that, although the mouse α2(I) collagen promoter also contains a CBF site flanked by a GGAGG motif and the synthetic 78C1- and the collagen CBF-binding sites exhibit almost identical behaviors in vitro, the *grp78* promoter is highly inducible by calcium stress mediated by Tg, whereas the mouse α2(I) collagen promoter is only weakly inducible, if at all. This could be due to the existence of mul-

tiple CCAAT elements in the *grp78* promoter (42) compared with the single CBF-binding site with an inverted CCAAT motif on the collagen promoter (13). Also, promoter-specific associating factors may account for the stimulus-specific responses of the different promoters containing apparently the same CCAAT sequences.

Having established that a general CCAAT factor CBF is recruited to bind on C1 on the *grp78* promoter, how does it contribute to its basal-level expression and induced transcription under calcium stress conditions? First, the CBF-binding site is needed for high-basal-level transcription of the collagen promoter (13); thus, the interaction of CBF with the C1 element can contribute to *grp78* basal-level expression. Second, our finding that the binding of CBF/C1F to C1 is sensitive to divalent cations such as Ca^{2+} may be the key explanation for the contribution of C1 toward the higher transcriptional rate of the *grp78* promoter under calcium stress conditions. From in vitro and in vivo footprinting of the human *grp78* promoter in stressed and nonstressed cells, it is shown that binding sites for a number of enhancing transcription factors, including those of Sp1 and CREB, are constitutively occupied (Fig. 11A [1, 21]). The same finding is observed for the multiple CCAAT-binding sites, including C1 and C3. In agreement with the in vitro studies presented here, the C1 CCAAT motif and its immediate flanking sequences are constitutively protected from DMS, suggesting that those G residues are protected by factors in vivo in both stressed and nonstressed cell nuclei (Fig. 11B). Thus, under normal culture conditions in which the nuclear calcium ion concentration is high, we envision some CBF occupancy on the C1 site, as suggested by constitutive DMS protection, contributing to basal promoter activity. However, CBF binding on the site is of low stability, because the dissociation of the factor is more rapid in the presence of high calcium ion concentrations.

In the event of calcium depletion, we hypothesize that calcium ions could be depleted from the intracellular stores simultaneously, because sequestered calcium concentrations in the organelles are much higher than the free cytoplasmic calcium ion concentration. In the cases of the ER and the nucleus, recent evidence suggests that the biochemical properties of the nuclear Ca^{2+}-ATPase are similar to those of the ER (2). In fact, the calcium pump in the nuclear envelope corresponds to that of the ER. If this is the case, depletion of ER calcium by Tg, which inhibits the ER Ca^{2+}-ATPase, could also result in the draining of calcium ions from the nucleus. In such an event, ER calcium ion efflux could lead to malfolded protein formation and changes in the need for more calcium-binding or chaperone proteins, requiring more of GRP78 synthesis. While signals could be generated from the ER to reach into the nucleus through a network of kinase- and phosphatase-mediated steps (4, 8, 30, 34), a faster and complementing response could be achieved through sensitivity of transcription factor binding to the *grp78* promoter control elements to the level of calcium ions in the nucleus. Because CBF/C1F binding to C1F is stabilized in calcium-depleted conditions in vitro, it is possible that similar stabilization of CBF binding could be achieved in vivo. Thus, the C1 factor can be visualized as a crucial coactivating complex occupying the C1 site without which the upstream regulatory complexes cannot interact effectively with the TATA complex for transcription initiation. In the absence of C1F, as in the case of the linker-scanning mutations LS90CAT and LS95/98 CAT, the upstream regulatory complexes could not act optimally, leading to moderate reduction in basal level expression and much-impaired stress inducibility. When the local nuclear calcium ion concentration which could affect C1F binding is low, the C1F protein-DNA complex is more stabilized through a slower dissociation rate. Although the magnitude of this increase in CBF binding is only two- to threefold as measured by in vitro assays and may not be detectable by in vivo footprinting, the increase could couple with other stress-inducible changes of the upstream regulatory complexes, such as the change in the site occupancy of the *grp* core factor, and could act synergistically to increase *grp78* transcription initiation (Fig. 11). It should be emphasized that our interpretations are based primarily on the results from in vitro experiments. Although calcium depletion stress is the most potent inducer for the C1 element, it remains to be determined whether calcium regulates C1 directly in vivo. Nonetheless, this hypothesis predicts that divalent cations such as calcium ions could affect the stability of CBF binding to its site and could affect transcription and is one mechanism by which calcium-responsive genes such as *grp78* enhance their transcription during calcium stress. While the direct test of this hypothesis awaits purification of the multiple components of CBF and successful reconstitution of the multimeric CBF activity in mammalian cell systems, the discovery of CBF as a major transcription factor occupying a key regulatory CCAAT element of the *grp78* promoter is an important step toward understanding the mechanism of *grp78* transcriptional control in calcium-depleted cells.

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