Journal of Cellular Biochemistry 104:1708–1723 (2008)

CBF/NF-Y Controls Endoplasmic Reticulum Stress Induced Transcription Through Recruitment of Both ATF6(N) and TBP

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Abstract Previously the analysis of promoters regulated by endoplasmic reticulum (ER) stress identified a composite promoter element, ERSE that interacts with both CBF/NF-Y (CBF) and ATF6(N) transcription factors. This prompted us to investigate the underlying mechanism by which CBF, a ubiquitously binding transcription factor, specifically controls transcription activation during ER stress. The in vitro DNA binding study performed using purified recombinant proteins revealed that CBF specifically recruits ATF6(N) to ERSE DNA but it does not interact with ATF6(N) in absence of DNA binding. Inhibition of CBF binding resulted in a significant reduction of optimal transcription activation of cellular genes during ER stress. Analysis of cellular promoters by ChIP demonstrated that CBF is needed for recruitment of both ATF6(N) and TBP but not for either acetylation of histone H3-K9 or trimethylation of histone H3-K4 during ER stress. Together these study results reveal that CBF controls ER stress-inducible transcription through recruitment of both ATF6(N) and TBP but not through chromatin modifications. Our observations are in agreement with the results of recently published studies that have shown that CBF controls transcription of varieties of inducible promoters through recruitment of general transcription factors but not through acetylation of histone H4. These findings provide a paradigm of the function of CBF in inducible transcription. J. Cell. Biochem. 104: 1708–1723, 2008.

Key words: endoplasmic reticulum stress; CCAAT binding factor (CBF/NF-Y); activating transcription factor 6 (ATF6); TATA binding protein (TBP)

The endoplasmic reticulum (ER) is evolved highly specific signaling pathways, collectively termed the unfolded protein response (UPR). The UPR is activated during ER stress, which results from the expression of misfolded proteins, glucose deprivation, increase in secretory protein synthesis, perturbation in calcium homeostasis, or hypoxia. The UPR pathways contain two major parts, (1) it coordinates transcription stimulation of multiple genes that include various chaperone genes to increase the protein-folding capacity of ER, and various genes needed for ER-associated degradation, and (2) it decreases general translational initiation until normal ER function is restored [Schroder and Kaufman, 2005; van Anken and Braakman, 2005].

Previous studies have demonstrated that the ATF6α transcription factor plays an important role in transcription activation of genes during ER stress. ATF6α, a member of the bZIP family of transcription factors containing a type II transmembrane domain, is ubiquitously expressed and is localized in ER under normal conditions in mammalian cells. Under conditions of ER stress, ATF6α is cleaved by site-1 and site-2 proteases,

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Grant sponsor: National Institutes of Health; Grant number: RO1 AR46264; Grant sponsor: Living Legend Allocation for Molecular Genetics and Developmental Biology Priority Program; Grant sponsor: The University of Texas M.D. Anderson Cancer Center.

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Received 19 December 2007; Accepted 24 January 2008

DOI 10.1002/jcb.21736

which remove the transmembrane domain generating an active form of ATF6α, ATF6(N), which localizes to the nucleus [Shen et al., 2002]. ATF6(N) stimulates transcription of promoters containing the ER stress element (ERSE), which is a composite promoter element that binds both ATF6(N) and CCAAT binding factor (CBF/NF-Y) transcription factors. DNA binding analysis of a semipurified preparation of recombinant ATF6(N) and CBF/NF-Y (CBF) subunits demonstrated that ATF6(N) specifically interacts with ERSE DNA only in the presence of CBF [Yoshida et al., 2000, 2001b].

The ERSE is composed of CCAAT-(N)9-CCACG, in which CCAAT interacts with CBF and CCACG interacts with ATF6(N), and these two elements are separated by a spacing of nine nucleotides. The results of these previous studies suggest that CBF associates with ATF6(N), and the binding of CBF to ERSE facilitates recruitment of ATF6(N) to DNA in vitro [Roy and Lee, 1999; Yoshida et al., 2000, 2001b]. However, the direct association of CBF with ATF6(N) poses a possibility that CBF could then recruits ATF6(N) to many other cellular promoters containing CBF binding sites.

In this regard, multiple CBF binding sites are found not only in the promoters of various ER stress-regulated chaperone genes, but also in the promoters of various genes activated during developmental stages and cell cycle [Fang et al., 2004; Kabe et al., 2005; Hu et al., 2006]. Under ER stress condition, only the chaperone genes but not the cell cycle regulated genes are activated. At this time, however, it is not clear how the ubiquitous CBF is utilized for specific transcription induction during ER stress.

Here we analyzed DNA binding and subunit interactions of ATF6(N) and CBF in vitro using purified recombinant polypeptides. This provided a model in which CBF is needed specifically for recruitment ATF6(N) to ERSE DNA but it does not interact with ATF6(N) in absence of DNA binding. Analysis of the transcription of ER stress-regulated cellular genes showed that CBF plays a significant role in transcription activation of various genes during ER stress. Analysis of cellular promoters using chromatin immunoprecipitation demonstrated that loss of CBF binding results in inhibition of the binding of ATF6(N) and TATA-binding protein (TBP) to the cellular ER stress-regulated promoters but did not alter two other different chromatin modifications: acetylation of histone H3-K9 and trimethylation of histone H3-K4. Altogether our results demonstrate a model in which constitutive binding of CBF keeps the ER stress regulated promoters in a committed state that allows dynamic recruitment of both ATF6(N) and TBP during ER stress conditions.

MATERIALS AND METHODS

Recombinant Polypeptides

The glutathione-*S*-transferase (GST)-CBF-A and CBF-Cd polypeptides were coexpressed in *E. coli* and were purified as a GST-CBF-A/CBF-Cd complex using glutathione-agarose resin as described previously [Coustry et al., 1996]. The His-CBF-Bd and His-ATF6d polypeptides were generated in *E. coli* from cDNA constructs in the pET-23b vector (Novagen, Madison, WI). These polypeptides were all purified using Ni-NTA agarose (QIAGEN, Valencia, CA).

DNA Binding

The binding of recombinant polypeptides with DNA was analyzed using electrophoretic mobility shift assay and DNase I footprinting as previously described [Hu et al., 2002]. The ERSE DNA, which consisted of a 37-bp double-strand DNA containing sequence between −89 and −53 of the human *GRP78* promoter, was radiolabeled and then used for the electrophoretic mobility shift assay in a DNA binding buffer containing 20 mM HEPES (pH 7.9), 100 mM KCl, 10% glycerol, 1 mM DTT, 0.1% Tween-20, and 5 ng/μl of poly(dI−dC)/(dI−dC) [Yoshida et al., 2000]. For the DNase I footprinting analysis, a human *GRP78* promoter fragment containing sequences between −341 and +26 of the human *GRP78* gene was isolated by polymerase chain reaction (PCR) amplification, digested with *Kpn*I and *Xho*I, radiolabeled at the *Xho*I end using Klenow, and then used in the DNA binding reactions.

Pull-Down Assay

The association between CBF and ATF6(N) was analyzed by pull-down assay using glutathione Sepharose 4B resin (Amersham Biosciences, Piscataway, NJ) as previously described [Chattopadhyay et al., 2004]. Briefly, 150 ng of GST-CBF-A/CBF-C or GST, and 150 ng of His-CBF-B were incubated with a 20 μl of glutathione-agarose resin in the cold DNA binding

buffer for 1 h. The resin was washed to remove the unbound proteins, and was then incubated with 80 ng of ATF6(N) together with or without 1 pmol of double-strand DNA for 1 h. The resin-bound proteins were then analyzed by Western blotting using specific antibodies against each polypeptide. The polyclonal antibody against ATF6a (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to detect ATF6 protein. The cellular CBF polypeptides were detected using rabbit polyclonal antibodies against each of the CBF subunits [Hu et al., 2006].

Adenoviral and Transient Expression Vectors

Two recombinant adenoviral expression vectors, Ad-Bwt and Ad-Bmut, which express the flag-tagged wild-type CBF-B (Bwt) and the flag-tagged mutant CBF-B (Bmut), were generated and amplified as previously described [Hu et al., 2006]. Each virus was infected into the Hela tet-off cell line (BD Biosciences Clontech) at a multiplicity of infection of three plaque-forming units of virus per cell for use in the experiments in this study. In this method the recombinant Bwt or Bmut was expressed in the Hela cells in the absence but not in the presence of tetracycline. Expression of Bwt and Bmut polypeptides after the adenoviral infection was detected by Western blotting using either anti-Flag or anti-CBF-B antibody [Hu et al., 2006].

Two forms of ATF6(N), full length (amino acids 2–373) and a truncated (amino acids 150–373) were expressed from cDNAs cloned into p3XFLAG-CMV-14 vector (Sigma-Aldrich, St. Louis, MO), in which each polypeptide was expressed as a fusion with 3xflag epitope. Each ATF6(N) construct was transfected into Hela cells, and expression of the polypeptide was detected by Western blotting using anti-Flag antibody.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from 2×10^6 Hela cells at 48 h after Ad-Bwt or Ad-Bmut infection using an RNeasy mini kit (QIAGEN). The cells were treated with 2 mg/ml of tunicamycin for 3 and 5 h before harvesting for the RNA isolation. Ten micrograms of RNA from each sample was used for Northern blot analysis as previously described [Hu et al., 2006]. After the analysis, the signal intensity of the radioactive bands in the blot was quantified using a phosphorimager in combination with an image analysis software program ImageQuant 5.2 (Molecular Dynamics, Inc., Sunnyvale, CA). For each band, the mean intensity value and the standard deviation for three independent experiments were calculated. The *GRP78* (950–1,312 nt), *ERP72* (911–1,220 nt), and *CHOP* (161–501 nt) cDNA probes were generated by RT-PCR amplification using the total RNAs of Hela cells. The *cyclin D1* (*CCND1*) (311–731 nt) cDNA probe was obtained by PCR amplification from cDNA clone, which was purchased from ATCC (Manassas, VA). The glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) probe was isolated by *Eco*RI and *Hin*dIII digestion of pTRI-GAPDH plasmid (Ambion). The radiolabeled DNA probes were prepared using random primed DNA labeling kit (Roche).

Chromatin Immunoprecipitation Analysis

Chromatin immunoprecipitation (ChIP) was done as described previously [Hu et al., 2006]. Briefly, about 10^7 HeLa cells were incubated with formaldehyde to crosslink the chromatin DNA with bound proteins, and were then incubated with glycine to stop the crosslinking. The cells were then washed, lysed, and sonicated to produce an average chromatin DNA fragment length of 0.5–1 kb. The cell lysate containing chromatin DNAs was first precleared with protein A/G-agarose resin and then the chromatin DNAs were incubated and precipitated with each of polyclonal antibody against ATF6α (Santa Cruz Biotechnology, Inc.), TBP (Santa Cruz Biotechnology, Inc.), CBF-A [Hu et al., 2006], H3-acetylated-K9 (Upstate Technology, Greenville, SC), and H3-Tri-methylated-K4 (Upstate Technology), and with rabbit IgG as a control. The precipitated chromatin DNAs were extracted after reverse crosslinking and were used in either regular or real-time PCR reactions to detect the promoter DNA regions of specific genes. Before each immunoprecipitation, 10% of chromatin DNA was utilized to isolate DNA by reverse-crosslinking and extraction, which was subsequently used as a source for input DNA in each PCR reaction. The PCR primers for each specific promoter are listed in supplemental table (Table S1).

Real-time PCR reactions were performed using a SYBR Green approach to quantify ChIP DNAs corresponding to the promoters of *GRP78*, *ERP72*, and *CHOP* genes. Primers for these PCR reactions were designed by using Primer Express 2.0 (Applied Biosystems,

Rockville, MD). For each antibody, three independent ChIP assay were done to isolate ChIP DNAs, which were then quantified using real-time PCR, similarly as previously described [Hu et al., 2006].

RESULTS

Role of CBF in DNA Binding of ATF6(N) In Vitro

We performed DNA binding reactions using purified recombinant CBF and recombinant ATF6(N) polypeptides together with labeled ERSE DNA. Mammalian CBF consists of three subunits, CBF-A (NF-YB), CBF-B (NF-YA), and CBF-C (NF-YC), which are all needed for DNA binding [Maity and de Crombrugghe, 1998]. Initially, we used all full length recombinant CBF and recombinant ATF6(N) (ATF6(N) represents the nuclear form of ATF6 containing amino acids 2–373) subunits in the DNA binding assay. As expected from previous study, recombinant CBF formed a DNA–protein complex with ERSE DNA, but ATF6(N) did not bind by itself to ERSE DNA, rather it formed a slower mobility complex in a DNA binding reaction when it was present together with CBF; The slower mobility complex was supershifted with both anti-CBF and anti-ATF6 antibodies, indicating that the complex contained both CBF and ATF6(N) (data not shown). To better define the domains of CBF and ATF6(N) needed for formation of the ERSE–CBF–ATF6(N) complex, we used truncated CBF and ATF6(N) subunits in the DNA binding assay. The truncated CBF contains a full-length CBF-A, a truncated CBF-B (ΔCBF-B), and a truncated CBF-C (ΔCBF-C), in which each of the two truncated CBF subunits contain the DNA binding domains but not the transcription activation domains. The truncated ATF6(N) (ΔATF6) contains the DNA binding domain but not the transcription activation domain (Fig. 1A). Similar to the full-length subunits, both truncated CBF and ATF6(N) also together formed a slower mobility complex (Fig. 1B), indicating that the activation domains of CBF or ATF6(N) do not play any role in formation of the ERSE–CBF–ATF6(N) complex. The DNA binding of CBF and ATF6(N) was also examined in a DNase 1 footprinting analysis with a GRP78 promoter containing multiple CBF binding sites [McCauliffe et al., 1992; Lee, 2001]. Expectedly, footprinting of CBF was observed in several regions of the promoter, whereas no footprinting of ATF6(N) was observed when a reaction contained only ATF6(N) (Fig. 1C). Interestingly, extended footprinting as well as new hypersensitive cleavage sites were observed when a reaction contained both CBF and ATF6(N). A comparison of footprinting in the proximal *GRP78* promoter containing an ERSE showed that the addition of ATF6(N) to CBF resulted in an extended footprinting over the CCACC sequence in the ERSE (Fig. 1D). This result suggests that binding of CBF to the ERSE sequence facilitates recruitment of ATF6(N) over the CCACC sequence.

To determine the interaction between CBF and ATF6(N), we performed a pull-down assay with GST-CBF protein and two different CBF binding sites using glutathione-agarose resin (Fig. 2A,B). This showed that CBF associated with ATF6(N) in the presence of wild-type ERSE DNA, but not with ATF6(N) in the absence of DNA or in the presence of DNA containing a CBF binding site of alpha2(1) collagen promoter. This indicated that CBF did not directly interact with ATF6(N), rather the association occurred only in the presence of wild-type ERSE DNA sequence.

To examine the specificity of ATF6(N) binding, we used two different mutants of ERSE DNA with single and double nucleotide substitution mutations (Mu1 and Mu2, respectively) in the CCAAT motif (Fig. 2C). Mu2 DNA did not form a complex with either CBF alone or with CBF and ATF6(N) (Fig. 2D). In contrast, Mu1 DNA did not bind to CBF, rather it formed a much weaker complex with CBF and ATF6(N) compared to the wild-type DNA. To determine the stability of the DNA–protein complex, the DNA binding reactions were analyzed after incubating with 50-fold excess unlabeled wild-type ERSE DNA at various times (Fig. 2E). This showed that excess ERSE competed almost 90% of the CBF–DNA complex compared to almost 43% of the CBF–ATF6(N)–DNA complex, suggesting that the CBF–ATF6(N)–DNA complex was more stable than the CBF–DNA complex, that CBF and ATF6(N) stabilizes each other DNA binding.

Role of CBF in the Transcription of ER Stress-Regulated Cellular Genes

To determine the role of CBF in transcription activation of cellular genes during ER stress, we first inhibited CBF activity in Hela cells

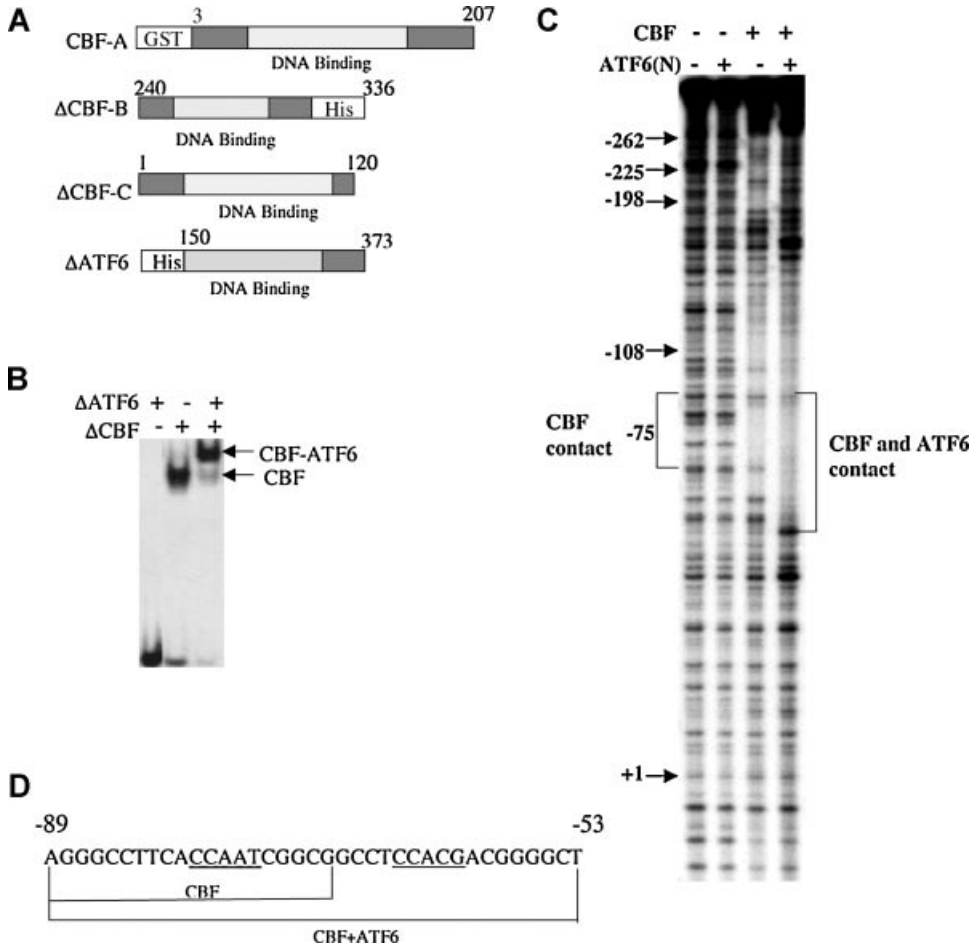

Fig. 1. DNA binding analysis of purified recombinant ATF6(N) and CBF polypeptides. **A**: Schematic diagrams of recombinant CBF and ATF6(N) subunits. A full-length CBF-A (amino acids 3–207) fused with glutathione-*S*-transferase (GST), a truncated CBF-B, ΔCBF-B, (amino acids 240–336) fused 6x-His tag, a truncated CBF-C, ΔCBF-C (amino acids 1–120), a truncated ATF6(N), ΔATF6 (amino acids 150–373) fused with 6x-His tag, were used for the DNA binding assay. The DNA binding domain in each subunit is indicated in the diagram. **B**: DNA binding of ΔATF6 and CBF, which contained GST-CBF-A, ΔCBF-B, and ΔCBF-C, with a labeled ERSE DNA. **C**: Analysis of ATF6(N) and CBF binding to the *GRP78* promoter using DNase I footprinting method. The DNA binding reactions were performed with a labeled GRP78 promoter fragment containing sequences between −341 and +26, and purified recombinant CBF subunits as described in (A), and full-length ATF6(N) (amino acids 2–336) polypeptide. The DNA binding reactions were then analyzed by DNase I footprinting method. In this analysis, ATF6(N) did not bind to the promoter DNA, but CBF alone generated footprinting in several regions in the promoter. The CBF footprinting at the −75 region was indicated by a bracket and was compared with footprinting in the presence of both CBF and ATF6(N). **D**: The DNA sequences of the footprinting regions protected by CBF, and CBF and ATF6(N).

by expressing the dominant-negative CBF-B mutant, Bmut [Hu et al., 2006]. The Bmut polypeptide and as a control, the wild-type CBF-B polypeptide (Bwt, the control) was expressed using adenovirus vector under control of a tetracycline-inducible vector, in which each polypeptide was expressed in the absence but not in the presence of tetracycline (Tc). In this method, expression of Bmut but not Bwt specifically inhibited CBF binding activity in Hela cells (Supplemental, Fig. S1).

We measured the expression of *GRP78*, *ERP72*, and *CHOP* (also called *GADD153*) genes during ER stress and under conditions of CBF binding inhibition. The *GRP78*, and *ERP72* genes that encode chaperone proteins are expressed a basal level under normal condition, and are highly activated during ER stress. Whereas the *CHOP* gene is not expressed under normal condition but is highly activated during ER stress.

The Hela cells were first infected with either Ad-Bwt or Ad-Bmut, and were cultured for 48 h in the presence or absence of Tc. The cells were treated with tunicamycin (an inhibitor of *N*-glycosylation) for 3 and 5 h to induce ER stress,

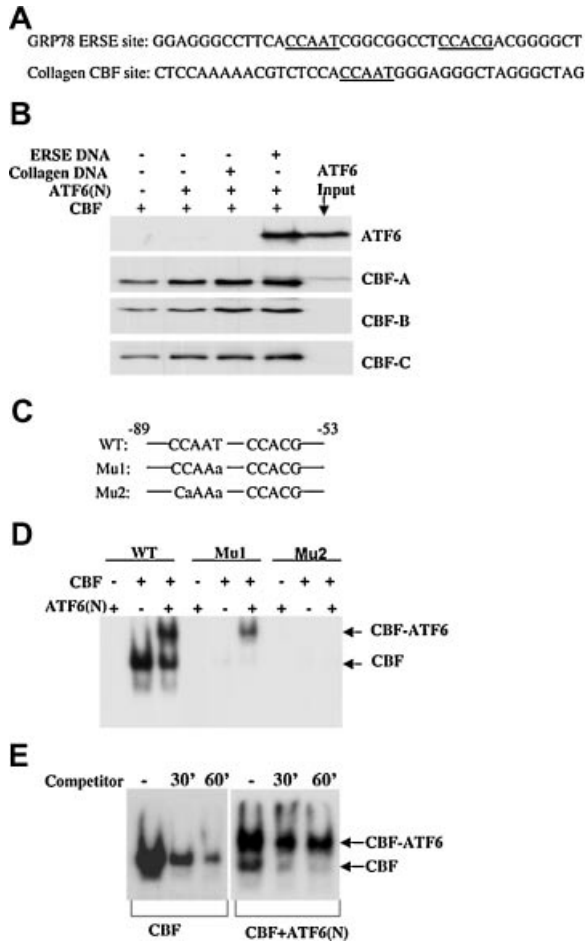

Fig. 2. Properties of CBF–ATF6(N)–DNA complex. **A**: DNA Sequences of CBF binding site of GRP78 and alpha2(1) collagen promoters. Both promoters contained CCAAT motif that binds CBF, but only GRP78 promoter contained CCACG motif that is required for ATF6(N) binding in presence of CBF. **B**: Analysis of interaction between CBF and ATF6(N) using GST-pull-down assay. The GST moiety of CBF was contributed by the CBF-A subunit as indicated in Figure 1A. After pull-down, each polypeptide was detected by Western blot using a specific antibody. **C**: Wild-type and mutant ERSE DNAs. The Mu1 and Mu2 contained single-nucleotide and double-nucleotide substitutions (respectively) in the CCAAT motif of wild-type ERSE. **D**: DNA binding of ATF6(N) and CBF with the labeled wild-type and mutant ERSE DNAs. **E**: Analysis of stability of CBF–DNA and CBF–ATF6(N)–DNA complexes. The DNA binding reactions were first incubated with excess unlabeled ERSE DNA for 30 and 60 min, and then analyzed by electrophoretic mobility shift assay. Quantification of DNA–protein complex bands by Phosphoimager showed that the unlabeled ERSE DNA competed about 90% of the CBF–DNA complex compared to 43% of the CBF–ATF6(N)–DNA complex at 60 min.

and were then used to isolate total RNAs for Northern blot analysis. Induction of *GRP78* was observed in Hela cells without expression of Bmut (Fig. 3A), and also with or without expression of Bwt (Fig. 3B). In contrast, induction of *GRP78* was significantly inhibited in cells expressing Bmut (Fig. 3A). This indicated that inhibition of CBF binding suppressed tunicamycin-induced expression of the *GRP78* gene. Similarly, inhibition of CBF binding also suppressed tunicamycin-induced expression of the *ERP72* and *CHOP* genes. Quantification of the Northern blots from three independent experiments showed that inhibition of CBF binding resulted in reduction of tunicamycin-induced expression of the *GRP78*, *ERP72*, *CHOP* genes by 3.88-, 3.44-, and 3.40-fold, respectively (Supplemental Table S2). The inhibition of CBF binding, however, resulted in much less of a change in basal expression of both *GRP78* and *ERP72* with a smaller amount of reduction by 1.5- and 1.4-fold, respectively, but did not change the expression of cyclin D1 (CCND1) and GAPDH.

To examine the specificity of Bmut mediated inhibition, we analyzed tunicamycin induced gene expression after expression of Bmut together with Bwt in the same cells through coinfection of both Ad-Bmut and Ad-Bwt at a 1:1 ratio in Hela cells. DNA binding analysis of cell extracts showed that coexpression of Bmut and Bwt partially rescued DNA binding inhibition by Bmut alone (data not shown). Consistently, coexpression of Bmut and Bwt also partially rescued Bmut-mediated suppression of *GRP78* and *ERP72* gene expression in the presence of tunicamycin (Tm; Fig. 4A,B). This indicated that Bmut suppressed tunicamycin-induced gene expression through inhibition of CBF binding.

The role of CBF was also examined after knockdown of the CBF-B subunit in Hela cells using small interfering RNA (siRNA). The Hela cells were transfected with either specific CBF-B siRNA or a control siRNA, and 48 h after transfection, the cells were analyzed for CBF-B knockdown efficiency. This showed that both CBF-B mRNA and protein were reduced by almost 50% with CBF-B siRNA compared to control siRNA (Fig. 5). For analysis of tunicamycin-induced gene expression, Hela cells were transfected with siRNA, and at 48 h after transfection, the cells were treated with tunicamycin for 5 h, and then used for isolation of total RNA. Expression of *GRP78*, *ERP72*, and *CHOP* were analyzed by the quantitative RT-PCR method. This showed that knockdown of CBF-B resulted in the inhibition of tunicamy-

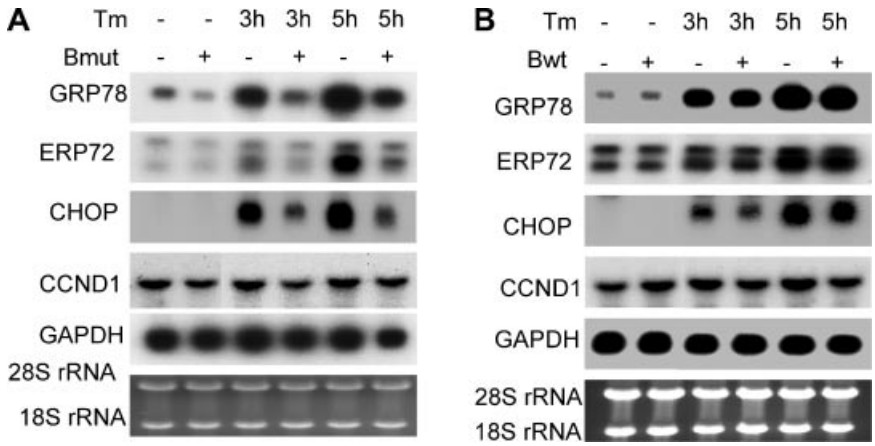

Fig. 3. Northern blot analysis of cellular gene expression with or without inhibition of CBF during ER stress. Total RNAs were isolated from Hela cells at 0, 3, and 5 h after 2 μg/ml tunicamycin (Tm) treatment, and were used for the Northern blot analysis to measure expression of each gene. The cells were first infected with Ad-Bmut (**A**) or Ad-Bwt (**B**), cultured with or without tetracycline, and were then used for Tm treatment. The Bmut or Bwt polypeptide was expressed in cells cultured without but not with tetracycline. The GAPDH bands in both (A) and (B) served as the RNA loading control. The CCND1, a cell cycle-regulated gene and showed no change by ER stress or expression of Ad-Bmut, was used here as a negative control.

cin-induced expression of *GRP78*, *ERP72*, and *CHOP* genes by 32%, 53%, and 36%, respectively. In general, the results of siRNA knockdown of CBF-B are in complete agreement with the results using the dominant-negative CBF-B. Together, these analyses indicated that CBF is needed for optimal induction of *GRP78*, *ERP72*, and *CHOP* genes during ER stress in Hela cells.

Role of CBF in Binding of ATF6(N) and TBP to the Cellular Promoters During ER Stress

To examine the effect of CBF binding to the cellular promoters of ER stress-regulated genes, we performed ChIP experiments with the anti-CBF-A antibody as described previously [Hu et al., 2006]. This showed that the cellular *GRP78* promoter was specifically

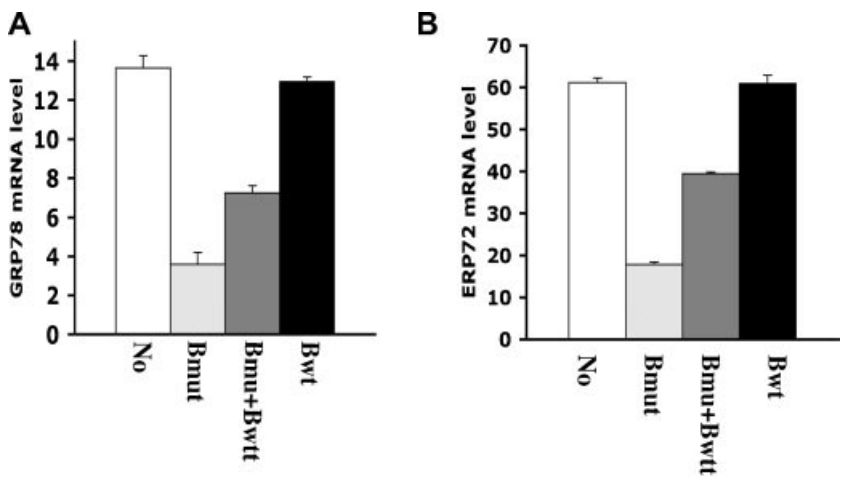

Fig. 4. Expression of Bwt rescues Bmut-mediated inhibition of GRP78 and ERP72 expression during ER stress. To rescue from Bmut-mediated inhibition, both Ad-Bmut and Ad-Bwt viruses were coinfected into cells, cultured without tetracycline, and then treated with Tm for 5 h. Total RNAs were isolated and used for the Northern blot analysis similar to the process described in Figure 3. The mRNA levels of GRP78 (**A**) and ERP72 (**B**) in each condition were quantified from three independent experiments. The error bars represent standard deviations.

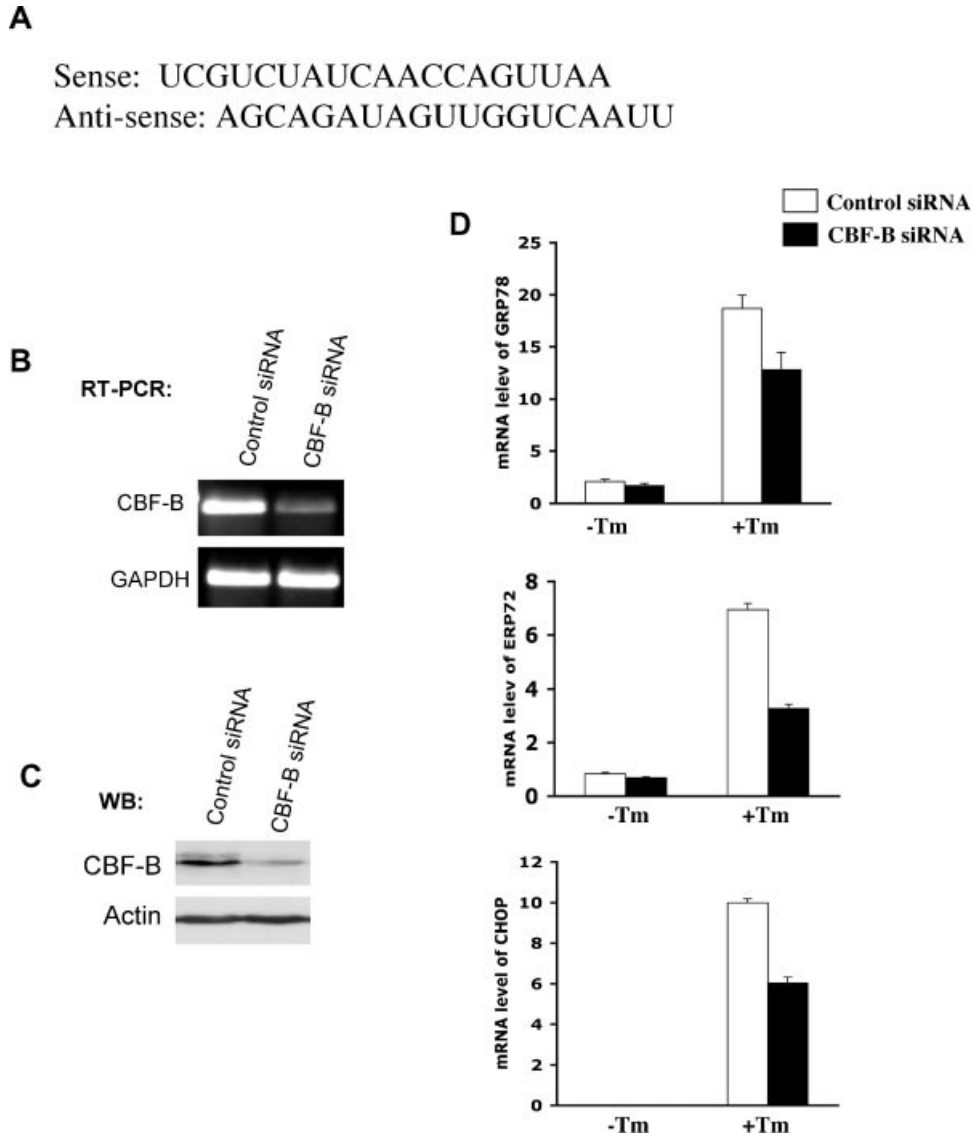

Fig. 5. Knockdown of *CBF-B* using siRNA inhibits optimal activation of *GRP78, ERP72,* and *CHOP* genes during ER stress. **A**: Sequences of siRNA of CBF-B. The siRNA sequence corresponds to a region of CBF-B mRNA between 679 and 697. The Hela cells were transfected with 60 nM siRNA using lipofectamine 2000 (Invitrogen). At 48 h after transfection, the cells were treated with or without 2 mg/ml tunicamycin (Tm) for 5 h and then collected for either total RNA isolation or protein extraction. **B**: RT-PCR analysis of *CBF-B* and *GAPDH* expression in the total RNA. **C**: Western blot analysis of protein extracts using anti-CBF-B and anti-beta-actin antibodies. **D**: Analysis of *GRP78, ERP72,* and *CHOP* expression using total RNA by real-time PCR method.

precipitated with anti-CBF-A antibody, but not with rabbit IgG under normal conditions (Supplemental, Fig. S2A). As another control, anti-CBF-A did not precipitate the downstream region of the *GRP78* gene located about 4.6 kb away from the CBF binding sites in the promoter. The *GRP78* promoter was also specifically precipitated with anti-CBF-A from cells treated with tunicamycin. Quantitative ChIP analysis showed that CBF binding to the GRP78 promoter was increased by twofold in tunicamycin treated cells compared to the untreated cells (Fig. 6A). This indicated that CBF specifically interacted with the cellular *GRP78* promoter under both normal and ER stress conditions, and that CBF binding to the promoter is increased by twofold under the ER stress conditions. As expected, expression of

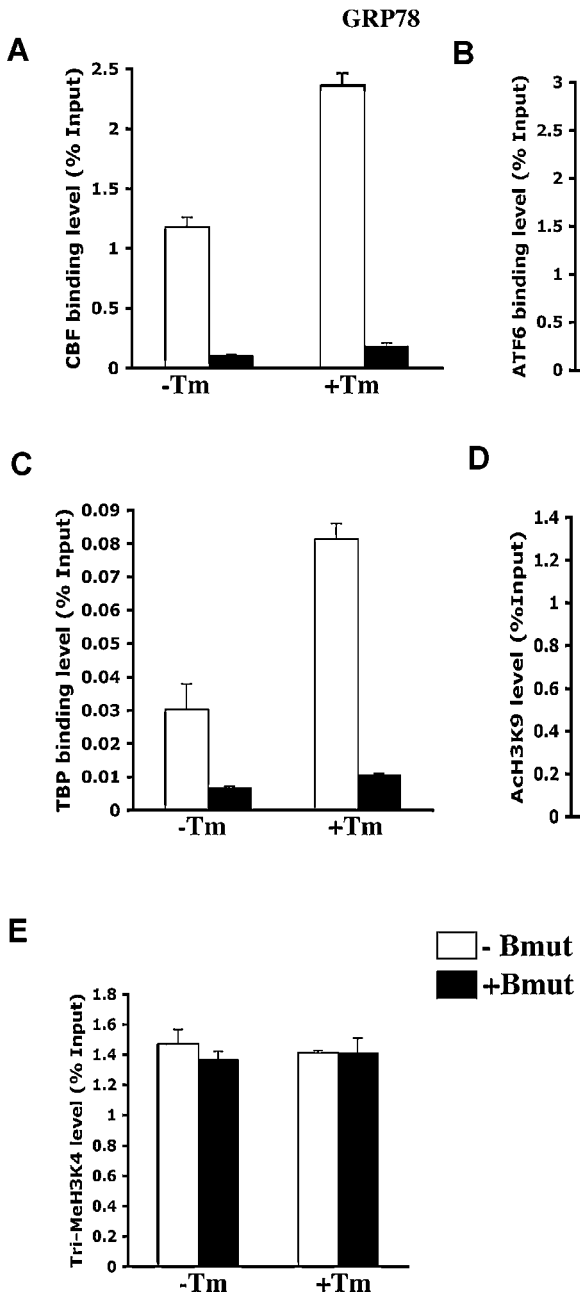

Fig. 6. Quantitative ChIP analysis of transcription factor binding and chromatin modifications in the cellular *GRP78* promoter with or without CBF inhibition during ER stress. The ChIP experiment was performed using cells, which were first infected with Ad-Bmut, cultured with (−Bmut) or without (+Bmut) tetracycline, and then treated with or without Tm for 5 h, as described in Figure 3. The antibodies against CBF-A (**A**), ATF6α (**B**), TBP (**C**), H3-acetylated-K9 (**D**), and H3-trimethylated-K4 (**E**) were used for the ChIP experiment. The amount of ChIP DNAs corresponding to the GRP78 promoter was quantified by real-time PCR with a SYBR green approach, which is expressed as a percentage of the input DNAs used in each immunoprecipitation. Data presented in the histogram is the mean for three independent experiments with standard deviations represented by error bars.

Bmut almost completely inhibited CBF binding to the promoter under both normal and ER stress conditions, indicating that Bmut was an effective inhibitor of cellular CBF for its binding to the cellular *GRP78* promoter.

ChIP analysis using anti-ATF6 antibody showed that ATF6 strongly interacted with the *GRP78* promoter in the tunicamycin treated cells, but not in the untreated cells (Supplemental, Fig. S2B and Fig. 6B). As a control, the

ChIP analysis showed that ATF6 did not interact with the cyclin B1 promoter, which interacted with CBF (Supplemental, Fig. S2A,B). As previously reported, treatment of cells with tunicamycin results in cleavage of full-length ATF6 to ATF6(N), which was translocated to the nucleus [Yoshida et al., 2000], indicating that ATF6(N) specifically interacted to the cellular *GRP78* promoter only under ER stress conditions. Inhibition of CBF binding resulted in a fivefold reduction of ATF6(N) occupancy to the promoter in tunicamycin-treated cells, indicating that CBF is needed for ATF6(N) binding to the cellular *GRP78* promoter under ER stress conditions.

Recently, several published studies have demonstrated that CBF binding to various promoters is needed for the recruitment of the TBP as well as other general transcription factors during inducible transcription [Fang et al., 2004; Kabe et al., 2005; Hu et al., 2006]. Previous studies have also implicated a role of CBF in chromatin modifications [Li et al., 1998]. Thus we performed the ChIP analyses using anti-TBP, anti-acetylated histone H3-K9, and anti-trimethylated histone H3-K4 antibodies. This showed that TBP binding to the GRP78 promoter was increased by 2.5-fold in tunicamycin treated cells compared to the untreated cells (Fig. 6C). Interestingly, the inhibition of CBF binding significantly reduced TBP binding to the GRP78 promoter in both tunicamycin-treated and untreated cells. However, neither tunicamycin treatment, or inhibition of CBF binding, changed the level of cellular TBP, as measured by Western blotting, and also did not change TBP binding to the promoter of the proliferating cell nuclear antigen, *PCNA*, gene, which is expressed in proliferating cells but not regulated by ER stress (data not shown). This indicated that TBP binding to the *GRP78* promoter was specifically increased in tunicamycin treated cells. In contrast, the level of two chromatin modifications, acetylation of H3-K9 and trimethylation of H3-K4, in the GRP78 promoter region was not significantly changed in tunicamycin treated cells compared to the untreated cells (Fig. 6D,E). Furthermore, the inhibition of CBF binding also resulted in no significant changes in the level of these two chromatin modifications in the *GRP78* promoter.

A similar set of ChIP experiments were also done for the *CHOP* promoter. This showed that tunicamycin treatment induced ATF6(N) binding and increased TBP binding to both promoters, and that inhibition of CBF binding significantly reduced both ATF6(N) and TBP binding to the promoters, similar to our observations in the *GRP78* promoter (Fig. 7A–C). Interestingly, the tunicamycin treatment increased acetylation of H3-K9 in the *CHOP* promoter, but not in the *GRP78* promoter (Fig. 7D). Inhibition of CBF binding, however, resulted in no significant change the increased level of acetylation. This suggested that the ER stress conditions could change the status of H3-K9 acetylation in some but not all ER stress-regulated promoters, and that the status of the H3-K9 acetylation is not dependent on CBF binding to the promoters. Altogether, this study indicated that CBF is needed for binding of both ATF6(N) and TBP, but not for chromatin modifications during transcription activation under ER stress condition.

The binding of ATF6(N) and TBP was also measured after treatment of cells with two other ER stress-inducing agents, thapsigargin (Tg) and dithiothreitol (DTT). Treatment of cells with either of these agents induced ATF6(N) binding and increased TBP binding to the promoters, which however, were significantly reduced after the inhibition of CBF binding, similar to what has been observed after the tunicamycin treatment (Fig. 8; data not shown for ATF6(N) binding). Thus various ER stress conditions stimulated binding of both ATF6(N) and TBP, which is dependent on CBF binding to the promoters in the ER stress-regulated genes.

ATF6(N) Stimulates TBP Binding to the Cellular Promoters

Because the ER stress stimulated recruitment of both ATF6(N) and TBP to the promoters, we then examined whether overexpression of ATF6(N) can stimulate TBP binding to the promoters. Previously, it was demonstrated that the ER stress also activates two other transcription factors, XBP-1(S) and ATF4, which were mediated by IRE1 kinase/endonuclease and PERK kinase, respectively [Schroder and Kaufman, 2005; van Anken and Braakman, 2005]. Previous studies also reported that XBP-1(S) could also bind to ERSE DNA in a CBF-dependent manner, similar to ATF6(N) [Yoshida et al., 2001a]. We have also observed that ER stress conditions also stimulated the binding of XBP-1(S) to both *GRP78* and *ERP72* promoters

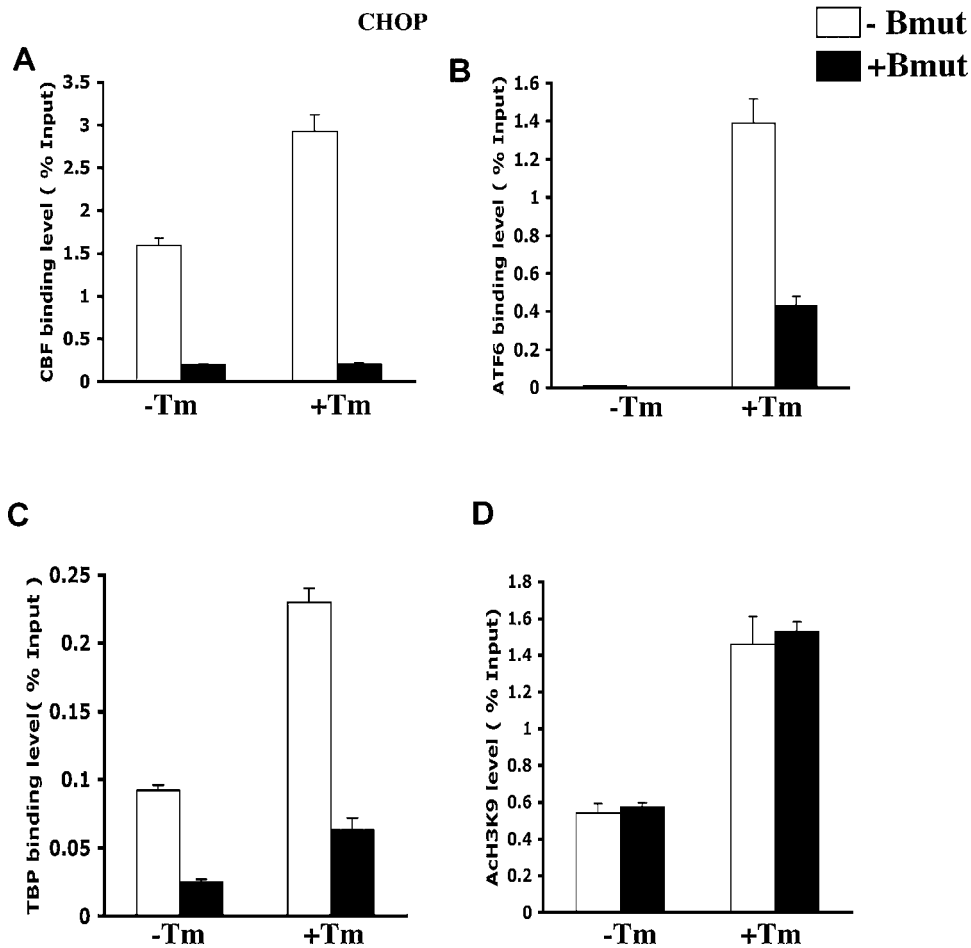

Fig. 7. Quantitative ChIP analysis of transcription factor binding and chromatin modification in the cellular *CHOP* promoter with or without CBF inhibition during ER stress. The ChIP experiments and the quantification of ChIP DNAs were performed same as for Figure 5. The ChIP DNAs precipitated with antibodies against CBF-A (**A**), ATF6α (**B**), TBP (**C**), and H3-acetylated-K9 (**D**) were used to quantify DNAs corresponding to the CHOP promoter.

(data not shown). Thus, in order to determine the specific effect of ATF6(N) binding to the promoter, ATF6(N) was expressed in Hela cells. The full length and a truncated ATF6(N) (ΔATF6) containing DNA binding but lacking transcription activation domain were expressed as fusion with flag epitope tag (Fig. 9A). This showed that expression of the full-length ATF6(N) provided a strong transcriptional stimulation of the *GRP78*, *ERP72*, and *CHOP* genes, which, however, was significantly reduced after the inhibition of CBF binding (Fig. 9B). Expression of the truncated ATF6(N), however, did not stimulate expression of the *GRP78*, *ERP72*, and *CHOP* genes (Fig. 9C; data for *ERP72* and *CHOP* genes are not shown). The expression of ATF6(N) however, did not activate XBP-1(S), which was measured by RT-PCR (Supplemental, Fig. S3). As a control, activation of XBP-1(S) was observed in Hela cells after treatment with tunicamycin. The RT-PCR product corresponding to XBP-1(S) mRNA is 26 nucleotides shorter than that of XBP-1(U) mRNA expressed without ER stress. This showed that overexpression of the full-length ATF6(N) specifically stimulated transcription of the *ERP72*, *GRP78*, and *CHOP* genes.

The ChIP analysis using anti-Flag antibody was used to determine binding of ATF6(N) polypeptides to cellular promoters. This showed that both the full length and the truncated ATF6(N) were bound to *GRP78* promoter; the full-length protein has higher level of binding than that of the truncated protein (Fig. 9D). Interestingly, the ChIP analysis using anti-TBP antibody showed that binding either the full

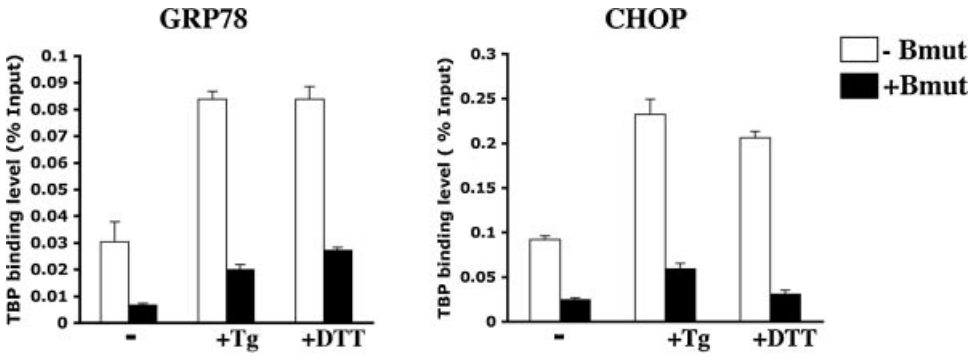

Fig. 8. Analysis of TBP binding to the *GRP78* promoter during various ER stress conditions. The ChIP experiment was performed using the antibody against TBP similar to the process described in Figure 5 except the cells were treated with 5 μM thapsigargin (Tg) and 800 nM dithiothreitol (DTT) for 5 h.

length or the truncated ATF6(N) stimulated TBP binding to *GRP78* promoter. This indicated that binding of ATF6(N) increased level TBP binding to *GRP78* promoter even in absence of transcription activation.

Our study also showed that CBF is also required for TBP binding under normal condition in which almost no ATF6(N) binding was detected. This suggests that CBF controls binding of ATF6(N) and TBP independently. Our previous study showed that CBF does not physically associate with TBP in nuclear extracts of Hela cells, suggesting that CBF indirectly regulates TBP binding to the promoters [Hu et al., 2006]. To determine whether the CBF–ATF6–DNA complex could associate with TBP, we performed a pull-down assay using biotinylated-ERSE DNA incubated with HeLa cell nuclear extracts and recombinant ATF6(N). This showed that the ERSE DNA precipitated with cellular CBF and recombinant ATF6(N), but not with cellular TBP (data not shown), indicating that the CBF–ATF6(N)–ERSE DNA complex does not physically associate with TBP. This suggested that the CBF and ATF6(N) indirectly regulate TBP binding to the promoters under ER stress condition.

DISCUSSION

The in vitro DNA binding study using purified recombinant proteins confirmed the previously published observation that ATF6(N) does not bind to ERSE DNA by itself but requires CBF in order to form a specific DNA–protein complex [Yoshida et al., 2000, 2001b]. This study also provides new information that CBF does not interact with ATF6(N) in the absence of DNA binding but rather recruits ATF6(N) to ERSE DNA containing both CBF and ATF6(N) contact sites. This suggests an important notion that although CBF binds to many mammalian promoters, it is able to recruit ATF6(N) only to promoters containing a ERSE, thus allowing transcription activation of specific genes under ER stress condition.

The gene expression analysis demonstrated that the inhibition of CBF binding either by expression of a dominant-negative CBF mutant or by knockdown of a CBF subunit results in a significant reduction of tunicamycin-induced transcription, indicating that CBF plays an important role in optimal transcription activation during ER stress. In this experiment, we selected to study three ER stress-regulated genes, *GRP78*, *ERP72*, and *CHOP*, in which both *GRP78* and *ERP72*, but not *CHOP*, expressed basal levels without ER stress. The inhibition of CBF, however, did not significantly change the basal mRNA levels of *GRP78* and *ERP72* genes, indicating that CBF plays no role or a minimal role in basal expression of these genes in non-stressed cells. In this regard, a previous study demonstrated that expression of ER stress-regulated genes is regulated by mitogenic signals in non-stressed cells [Brewer et al., 1997].

The ChIP analysis demonstrated that a constitutive level of CBF binding was observed in the cellular promoters of *GRP78*, *ERP72*, and *CHOP* genes without ER stress, indicating that CBF was constitutively bound to the promoters irrespective of their basal expression under normal conditions. This also showed that the dominant-negative mutant robustly inhibits

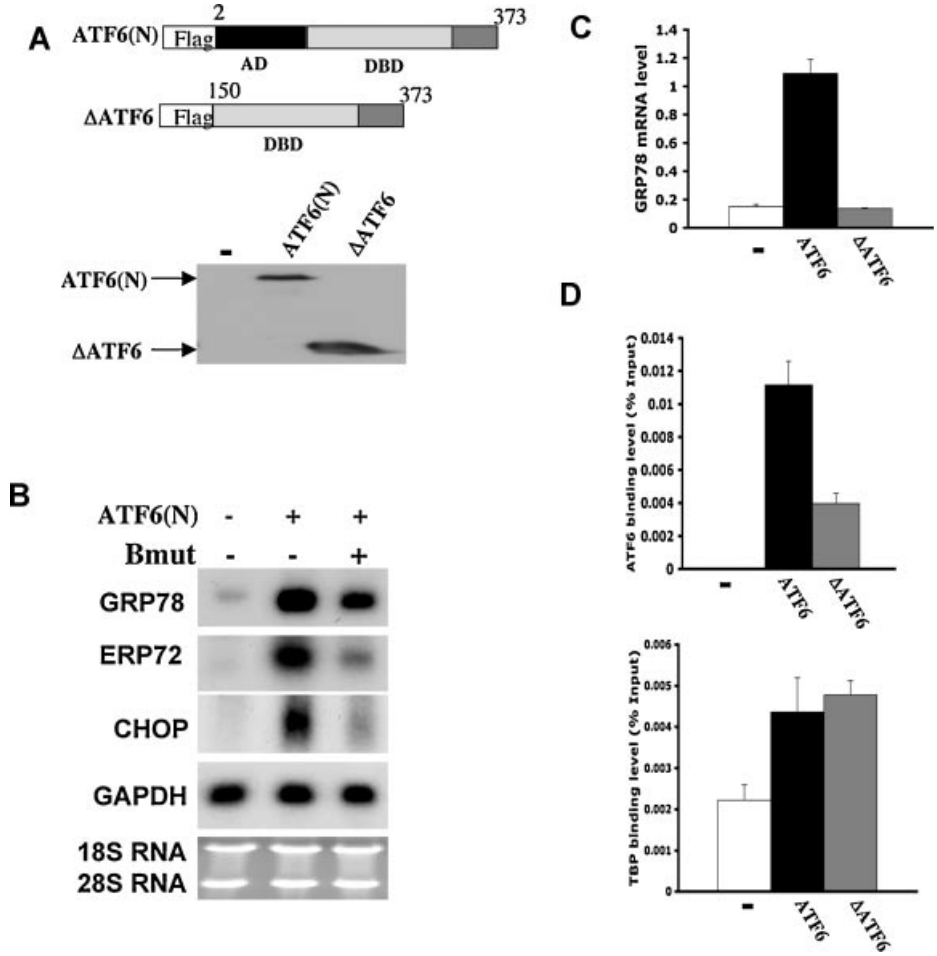

Fig. 9. Analysis of transcription activation and TBP binding after transient expression of full length and a truncated ATF6(N). **A**: Top—Schematic diagram of full-length ATF6(N) (amino acids 2–373) containing both DNA binding (DB) and transcription activation (AD) domains, and a truncated ATF6(N), ΔATF6, (amino acids 150–373) containing only DNA binding domain. Each polypeptide was expressed as fusion with 3x-Flag tag. Bottom—Each polypeptides was expressed in Hela cells after transient transfection of expression construct DNA, and was detected by Western blot analysis using anti-flag antibody. **B**: Effect of CBF inhibition in ATF6(N) dependent activation of cellular genes. The cells were first infected with Ad-Bmut, cultured with or without tetracycline, and then transfected with ATF6(N) expression construct. Total RNAs isolated from the cells at 48 h after the transfection were used for the Northern blot analysis to measure the expression of the *GRP78*, *ERP72*, and *CHOP* genes. The expression of the *GAPDH* gene was measured as a control. **C**: Analysis of *GRP78* expression by real-time PCR method. Total RNAs isolated from the cells at 48 h after transient transfection of either ATF6(N) or ΔATF6 expression construct, and then were used for real-time PCR. **D**: Role of ATF6(N) in binding of TBP to the cellular GRP78 promoter. The cells were transfected with ATF6(N) or ΔATF6 expression construct, and at 48 h after the transfection the cells were used for quantitative ChIP experiment using anti-Flag or anti-TBP antibody to measure binding of ATF6 or TBP to the GRP78 promoter similarly as described in Figure 5.

CBF binding to the cellular promoters under both normal and ER stress conditions. In this regard, the knockdown of CBF-B by siRNA also reduced CBF binding to the cellular promoters, which, however, displayed much less inhibition than that of the dominant-negative mutant. The ER stress conditions in the presence of tunicamycin increased CBF binding by about twofold in both the cellular promoters, which were also inhibited robustly by the dominant-negative mutant. Thus we believe that the robust inhibition of CBF binding by the dominant-negative mutant provided a better experimental system to determine function of CBF binding in the cellular promoter activity during ER stress.

The ChIP analysis for ATF6(N) and TBP binding demonstrated that whereas a constitutive level of TBP binding to the cellular promoters was observed, almost no ATF6(N) binding to any of the promoters was observed under normal conditions. Interestingly, the ER

stress conditions strongly stimulated ATF6(N) binding, and also significantly increased TBP binding to both the cellular promoters. The ChIP analysis for chromatin modifications showed that the ER stress conditions did not change the level of histone H3-K9-acetylation or K4-tri-methylation in *GRP78* promoter, but it increased the K9-acetylation in CHOP promoter. The inhibition of CBF strongly reduced binding of both ATF6(N) and TBP to both the promoters. In contrast, it did not change the level of the chromatin modifications in the promoters. The increase of H3-K9-acetylation in the chromatin of the *CHOP* promoter was also not changed by the inhibition of CBF binding. Altogether, this part of the study indicates that CBF is required for binding of both ATF6(N) and TBP but not for chromatin modifications during transcription activation under ER stress condition.

The increase of TBP binding to the cellular promoters was also observed in the presence of Tg and DTT, the two other ER stress inducers, indicating that an increase of TBP binding to the promoters is common during transcription activation under ER stress conditions. Importantly, our data indicate that binding of full length or a truncated ATF6(N), which was expressed in cells exogenously, also stimulated binding of TBP. The full length but not the truncated ATF6(N) activated transcription of ER stress genes. This suggests that the increase of TBP binding by ATF6(N) is independent of transcription activation.

Because CBF is required for ATF6(N) binding during ER stress, and also for TBP binding under both normal and ER stress conditions, the formation of the CBF–ATF6(N) promoter complex possibly results in an increase of TBP binding. Because ER stress also increased a modest but reproducible level of CBF binding, it is possible that binding ATF6(N) could stabilize CBF binding to the promoter similar to what we observed in our in vitro binding study. It is also possible that stabilization of CBF binding by ATF6(N) could also increase binding of TBP to the promoters.

The CBF binding sites are present in many kinds of promoters regulated by different inducible conditions. Although analyses of different promoters for more than a decade have demonstrated requirement of CBF binding site(s) in both basal and inducible transcription, function of CBF in the transcription of cellular promoters in vivo was not revealed until recently. We previously analyzed CBF function in vivo by inactivation of CBF by either expressing a dominant-negative CBF mutant in mouse fibroblasts, or by conditional inactivation of the *CBF-B* gene in mouse embryonic fibroblasts. This showed that CBF is essential for cell growth [Hu and Maity, 2000; Bhattacharya et al., 2003]. The gene expression analysis of unsynchronized mouse fibroblasts revealed that the inactivation of CBF did not, however, alter expression of many genes, which is in contrast to the observation that more than 30% mammalian promoters contain CBF binding sites [Hu and Maity, 2000]. Rather, analysis of specific cell cycle-regulated genes in synchronized fibroblasts demonstrated that CBF is needed for cell cycle-regulated transcription of cellular genes specifically at G2/M phase [Hu and Maity, 2000; Hu et al., 2006]. These studies first indicated that CBF plays an important role in transcription activation of several genes at G2/M phase, but not in basal expression of these genes at G1 or S phase of cell cycle, suggesting that CBF only regulates transcription induction at specific cell cycle stage.

A recent study analyzed CBF function after knockdown of the *CBF-B* (*NF-YA*) gene in osteoclasts. This demonstrated that CBF is needed for vitamin D3-inducible transcription of the osteoclast differentiation factor (*ODF*) gene [Kabe et al., 2005]. Interestingly, inhibition of CBF binding in the cellular *ODF* promoter resulted in a loss of recruitment of general transcription factors, such as TBP and RNA polymerase II, but did not alter recruitment of p300 or acetylation of histone H4 in the presence of vitamin D3. In agreement with this observation, our recent study also demonstrated that the activation domain of CBF-B is needed for recruitment of TBP to the promoters of the *cyclin B1* and *aurora A* genes, and plays a role in activation of these two genes at G2/M phase of the cell cycle [Hu et al., 2006]. Similarly, the CBF binding to a human gamma-globin promoter is also required for recruitment of TBP and RNA polymerase II to the promoter in adult erythroblasts but not in embryonic erythroid cells [Fang et al., 2004]. Taken together these studies provide some evidences that CBF controls inducible or developmentally specific transcription of various genes through recruitment of TBP and other general transcription factors.

Although CBF controls recruitment of TBP to the cellular promoters, it does not appear to play a major role in basal expression of the genes. Our present study provides clear evidence that although both CBF and TBP were recruited to the cellular *CHOP* promoter without ER stress, there no basal expression of *CHOP* was observed in the absence of ER stress. Our observation is consistent with a previous publication that also showed constitutive binding of CBF and general transcription factors to the cellular *CHOP* promoter in the absence of basal expression [Barsyte-Lovejoy et al., 2004].

In summary, our study led to a model by which CBF controls transcription during ER stress (Fig. S4). In this model constitutive binding of CBF to the ER stress regulated promoters keeps the promoters in a preset condition in which CBF plays a role in recruiting TBP and possibly other general transcription factors but not in maintaining active chromatin modification. Upon ER stress, CBF recruits the specific transcription factor ATF6(N), which then stimulates further recruitment of TBP and general transcription factors to activate transcription. In this system, ATF6(N) strengthens CBF-dependent recruitment of general transcription factors, but it does not change acetylation of histone H3-K9. As mentioned earlier, since CBF also controls recruitment of general transcription factors to various promoters induced by different conditions, this implies that CBF controls the inducible transcription of a specific promoter through interaction with specific factor (or factors) depending on the promoter sequence, which then stimulates crosstalk between CBF and general transcription factors to induce transcription.

ACKNOWLEDGMENTS

We thank Dr. Joseph W. Brewer, University of South Alabama, for providing ATF6(N) construct. We also thank Vickie J. Williams for editing this manuscript. This work was supported partly by National Institutes of Health Grants RO1 AR46264 (to S. N. M.), a Living Legend Allocation for Molecular Genetics and Developmental Biology Priority Program, and an Institutional Research Grant from The University of Texas M. D. Anderson Cancer Center (to S. N. M.). The DNA sequencing facility at The University of Texas M. D. Anderson Cancer Center is supported by a National Cancer Institute Grant CA 16672.

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