A direct-repeat sequence of the human BiP gene is required for A23187-mediated inducibility and an inducible nuclear factor binding

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Received October 16, 1992; Revised and Accepted November 17, 1992

ABSTRACT

We have recently isolated a functional promoter encoding the human polypeptide-binding protein (BiP) gene from Burkitt's lymphoma cells by polymerase chain reaction (The EMBL Data Library accession number X59969, 1991). This promoter DNA segment (termed BIP670) was fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene and expressed in NIH3T3 cells. BIP670 retains basal and Ca\(^{2+}\) ionophore A23187-inducible activities. Using 5' deletion assay, we found three basal expression elements (BEE) in the BIP670. Removal of the distal BBE (BBE3), which is contained in a segment spanning -368/-170, caused a 50% loss of the basal activity; removal together with the middle BBE (BBE2), which is contained in a segment spanning -170/-107, resulted in a further 30% loss of the activity. Further removal of the proximal BBE (BBE1), which spans -107/-39, abolished greater than 95% of the basal expression. In addition, an A23187-inducible element (AIE) appeared to be associated with the BBE1. At least a six-fold inducibility remained as long as the BiP promoter retained the sequences -107/-39. Using an in vitro gel mobility shift assay, an A23187-inducible nuclear factor (AINF) was detected from NIH3T3 cells. DNA binding competition experiments indicate that the -107/-39 segment contains a sequence motif which interacts with this cellular factor. Further analysis showed that the two direct repeats, ranging -108/-73 and -72/-40, are the target for AINF binding. A 3-4 fold increase of the AINF binding to both repeated sequences was detected from induced cells. Similar results were also demonstrated in HeLa cells, suggesting that transcriptional control of BiP gene expression in mammalian cells is conserved. These findings also imply that the identified nuclear factor may be important in mediating transcriptional activation of the BiP gene.

INTRODUCTION

BiP (for binding protein), a 78-kDa endoplasmic reticulum (ER) protein, was originally identified in mammalian cells by its binding to immunoglobulin precursors (1-2). This ER-localized member of the HSP70 family was independently identified as a major protein that is induced by certain stresses, including Ca\(^{2+}\) perturbation, glucose starvation, and mutant proteins in the ER (3). A major physiological function of this ER protein is to assist in the assembly and transport of membrane and secretory proteins (4-5). It has been shown that the BiP is essential for yeast growth (6), and that it may also influence growth in rat cells (7). Using inhibitors of cellular metabolism, Morimoto and colleagues (8) have demonstrated the regulation of the BiP gene and have concluded that the regulation of the gene is complicated. These results suggest that although the BiP is structurally and functionally conserved in mammalian cells, the regulation of this gene expression can be dispersive.

A study has suggested that the BiP regulatory domain is highly conserved in mammalian cells (9). However, it lacks direct evidence as to what cis-acting elements of human BiP gene mediate the inducible expression of cells in response to stresses. Although the human BiP gene has been cloned from embryonic liver cells that contains 5' promoter sequences sufficient for basal and inducible transcription (10), the regulatory mechanism of the human BiP gene is mostly implicated from studies of the rat BiP homolog (reviewed in ref. 3). It is also unclear whether cellular factors interact with these potential cis-acting elements.

We have recently isolated a functional regulatory sequence of the BiP gene from human Burkitt's lymphoma cells by polymerase chain reaction (PCR) (11). In this study, we investigated the transcriptional activation of the human BiP gene and cellular factors which interact with the gene promoter in Ca\(^{2+}\) ionophore A23187-perturbed NIH3T3 cells. Using 5' deletion of the BiP gene regulatory region which had been fused to the bacterial chloramphenicol acetyltrnasferase (CAT) reporter gene, we demonstrated cis-acting elements that are essential for basal and inducible expression. In addition, using a gel mobility shift assay, we identified an inducible cellular factor that recognizes a direct-repeat sequence within the A23187-inducible element.

MATERIALS AND METHODS

Cell lines and culture conditions

NIH3T3 and HeLa S3 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Gaithersburg, MD) at 37°C in a humidified atmosphere of 5% (vol/vol) CO\(_2\)
in air according to the supplier’s specifications (American Type Culture Collection). The medium was supplemented with 10% (vol/vol) fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 μg of streptomycin and 100 units of penicillin per ml. For induction, the cells were treated with 7 μM of Ca²⁺ ionophor A23187 (Sigma Co., St Louis, MO) for 15 h.

PCR amplification and plasmid construction
DNA preparation, gel electrophoresis, isolation, and subcloning of plasmid DNA were carried out using accepted methods (12). The human BiP gene promoter was isolated from Namalwa genomic DNA in a DNA thermal cycler (MJ Scientific) according to the method described by Saiki et al. (13). Two primers — 5'-CCCCGGGTCACTCTGC-3' (sense strand) and 5'-TCC-TCTTGTCTCCTCCCT-3' (antisense strand) — were designed according to the human fetal liver BiP gene sequence (10). The primer pairs for PCR amplification correspond respectively to 371 and 294 base relative to the transcriptional initiation site. The expected 667-bp DNA bands were isolated from the PCR products. The sequence was the same as the reported liver sequence (10) except that an additional G was presented at +152 (Chao, C.C.-K. et al., The EMBL Data Library accession number X59969). The 5' deletion mutants (i.e., BiP-368, BiP-170, BiP-107, BiP-39 and BiP+4) were generated by restriction digestion with SmaI (indicated with closed arrowheads) by A23187 treatment in cells (Fig. 1). There was a significant induction of the CAT activity (indicated with closed arrowheads) by A23187 treatment in cells (Fig. 1).

Transcriptional activation assay
pGBiPCAT carrying a series of the BiP 5' deletion derivatives was introduced into cells by electroporation (BioRad electroporator) as previously described (15). After 72 h, cell extracts were prepared for CAT activity analysis as described before (16). The reaction was incubated at 37°C for 1 h, followed by the development on a silica thin-layer chromatography (TLC) plate (Merck, Darmstadt, Federal Republic of Germany). After autoradiography, the intensity for regions corresponding to the substrate chloramphenicol or the acetylated products was determined by scanning the X-ray film through a scanning densitometer. The reaction mixtures were then subjected to a 4% polyacrylamide gel electrophoresis under low ionic strength (6.7 mM Tris.HCl, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA) at 20°C and 15 mA. The resolved gels were dried and exposed to a Kodak XAR-5 X-ray film at -70°C with an intensifying screen. The intensity of the shifted DNA bands was determined by scanning the X-ray film through a scanning densitometer.

The DNA fragments (BiP-368, BiP-170, BiP-107, BiP-39 and BiP+4) were isolated from plasmid pBSBiP constructs by the restriction enzymes Hind III and Eco RI. BiP111, which spans -107/+4, was isolated from pBSBiP-107 by the restriction enzyme SalI. Double stranded oligonucleotides (BiP36, BiP33 and BiP24) were prepared by annealing complementary oligonucleotides synthesized using an Applied Biosystems DNA synthesizer (model 380B). A DNA probe was prepared from BiP-170. The BiP-170 was dephosphorylated with calf intestine phosphatase, labelled with [γ-³²P]ATP and kinase (3 x 10⁴ cpm/ng DNA), and then purified in spin columns using accepted methods (12). For some experiments, BiP111, BiP36 and BiP33 were also used as DNA probes. BiP111 was labeled by filling-in recessive ends with Klenow fragment of DNA polymerase I and [α-³²P]dCTP; whereas BiP36 and BiP33 were labeled with [γ-³²P]ATP and kinase.

RESULTS
The cloned BiP promoter retains basal and inducible elements
Twenty μg of a series of the pGBiPCAT (represented by BiP-368, BiP-170, BiP-107, or BiP-39) or the vector pGCAT (V, indicated on top) plasmid DNA were introduced into NIH3T3 cells, and incubated for transient expression (Fig. 1). Two μg of cell extracts from cells either uninduced ('-' lanes) or induced with A23187 ('+' lanes) were assayed in vitro for CAT activity. No CAT activity was detected in vector-transfected cells. The far left lane shows the reaction without cellular extracts (the chloramphenicol substrate is indicated with an open arrowhead). There was a significant induction of the CAT activity (indicated with closed arrowheads) by A23187 treatment in cells transfected with BiP-368, BiP-170, or BiP-107. In contrast, the CAT activity from BiP-39 transfected cells was not detected. The CAT activity in both the basal and inducible levels decreases with the length of the deletion. The relative CAT activity from three experiments was determined using scanning densitometry. After being normalized to β-gal activities (see Materials and

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**Figure 1.** Transient CAT assay of the pGBiPCAT constructs in cells. Twenty μg of the pGCaT vector (Panel V) or a series of the pGBiPCAT constructs (indicated on top) were co-transfected with pSVβ into NIH3T3 cells. Following 57 h incubation, transfected cells were mock treated ('-' lanes) or induced with A23187 ('+' lanes) for 15 h. Ten μg of the cell extracts were used for in vitro CAT analysis. The left lane is for the reaction without cellular extracts. Symbols: open arrowhead, chloramphenicol; closed arrowhead, acetylated chloramphenicol.
Methods for details), the relative CAT activity was calculated. There is a 45% reduction of the basal CAT activity in BiP — 170 transfected cells, compared to the BiP — 368 transfected cells. A further 40% reduction was detected in BiP — 107 transfected cells. A majority of the basal levels of CAT expression was lost in the BiP — 39 transfected cells. The fold induction was calculated by taking the CAT activity of induced cells divided by that of uninduced cells. A 7— 8-fold induction was detected in BiP — 368 and BiP— 170. BiP — 107 transfected cells also exhibited a 6-fold induction, partly because the basal activities of individual constructs also decreased. In contrast, deletion down to the residue — 39, resulted in the induction being completely abolished. In any case, there is a 15— 20% variation of the CAT activities because of the uncertainties from transfection of the plasmid and the in vitro CAT assay. Thus, these results suggest that the DNA segment spanning — 107/-39 contains an A23187-inducible element (AIE).

A nuclear factor is induced by A23187 that binds to the BiP AIE

An 'A23187-inducible nuclear factor' (AINF), which binds the BiP promoter, was identified. Specificity of the AINF was investigated by the competition analyses. Results are shown in Fig. 2. An unlabeled DNA fragment BiP— 170 (lanes 3— 5), BiP— 107 (lanes 6— 8), BiP— 39 (lanes 9— 11), or BiP + 4 (lanes 12— 14) with indicated molar excess (1, 10, or 100-fold shown on top) was included in the binding reaction. It appeared that the BiP — 170 and BiP— 107 were relatively effective in inhibiting the AINF DNA binding, compared to the BiP— 39 and BiP + 4. A 10-fold molar excess of the competitors BiP — 170 and BiP— 107 revealed a slight inhibition of the DNA binding. However, with a 100-fold competitor, greater than 80% of the DNA binding was inhibited by BiP— 170 and BiP— 107, in contrast to a majority of the DNA binding that was retained in the BiP— 39 and BiP + 4 competitions.

The results of the CAT activities and the in vitro DNA-binding assays are summarized in Fig. 3. The upper part of the figure marks three basal expression elements (BEE) and an A23187-inducible element (AIE). The conserved cis -acting elements are also indicated with boxes: a closed box is for TATATAA, and five open boxes are for CCAAT. The transcriptional initiation site is marked with +1. Only the 5' residue positions are indicated to the left for each BiP fragments or CAT constructs. The uninduced or control CAT levels and the induced CAT activities are listed. The fold induction is shown in the parentheses. The basal CAT activity of BiP — 170 was reduced by 45%, compared to the BiP — 368, suggesting that a BBE is retained in the segment between residues — 368 and — 170 (i.e., — 368/-170). A 40% further decrease in the CAT activity of BiP— 107 was found, suggesting an additional BBE that is present in the region — 170/-107. Deletion of the region — 107/-39 totally abolished both the basal and the inducible expression, suggesting that an AIE as well as a BEE were contained in this segment. Three BBEs are named in numerical order as shown in the figure, with the one closest to the TATA element as BEE1. The% competition of the DNA binding by a 100-fold molar excess of the competitors is shown in the right column. A majority of the DNA binding was inhibited by the BiP— 368, BiP— 170, and BiP— 107. In contrast, only 15% inhibition of DNA binding was achieved by the BiP— 39. These results indicate that the residues spanning — 107/-39 contain a motif that is important for the binding of the AINF. This DNA segment is proposed as the target for the AINF binding.

An AINF binds to a direct repeat sequence of the BiP promoter

Although the AINF binding target was localized to a 69-nt sequence, it is not known what the exact binding site for the AINF is. Sequence analysis indicate that the 69-nt segment spanning — 107/-39 contains a direct repeat sequence (indicated with two arrowheads in Fig. 4). It is important to determine whether both halves of the sequence by itself interacts with AINF. For this purpose, double-stranded synthetic oligonucleotides (BiP36, BiP33, and BiP24) were used as DNA probes in the gel mobility shift assay. The oligonucleotides with their corresponding positions in the — 107/-39 region are indicated. The sequences of the 5'- half (— 108/-73) and the 3'- half (— 72/-40) were compared, and the homology of the sequences was aligned (shown in the bottom of the figure). The CCAAT boxes are underlined, and also indicated with boxes on the top of the figure.

These synthetic oligonucleotides were used as competitors in BiP111 (spanning — 107/+4) binding assay (Fig. 5). Ten μg of nuclear extracts were used. A 10-fold molar excess of BiP111 inhibited very little, if any, AINF binding; whereas, a 100-fold

Figure 2. Competition of in vitro DNA-binding with the BiP 5' deletion mutants. Eight μg of nuclear extracts from induced NIH3T3 cells were used for binding with 0.3 ng of BiP— 170 probe. One, 10 or 100-fold molar excess of competitors (indicated on top) is included: BiP— 170 (lanes 3— 5), BiP— 107 (lanes 6— 8), BiP— 39 (lanes 9— 11), or BiP + 4 (lanes 12— 14). Lane 1, probe alone; lane 2, without competitor. Symbols: arrowhead, bound probe; f, free probe.

Figure 3. Summary of the in vivo transcriptional activation CAT assay and the in vitro DNA-binding competition assay. Putative sequence motifs along the regulatory region of the BiP gene are indicated: BBE for basal expression element; AIE for A23187-inducible element. Conserved cis -acting elements are also indicated: closed box, TATATAA; open box, CCAAT; +1, transcriptional initiation site. The% competition of the in vitro DNA-binding (right-hand column) and the in vivo transcriptional activation CAT assay for each DNA fragments or CAT constructs (left-hand columns) are summarized. The numbers in the parentheses are the fold induction of the transcriptional activation.
Figure 4. Sequence of the BiP gene spanning -107/-39. Top: positions of the direct repeat sequence (indicated with arrows), and regions of BiP24, BiP33, and BiP36. Open bars, CCAAT elements. Bottom: alignment of the direct repeat sequences. Nucleotide positions relative to the transcriptional initiation site are indicated. The CCAAT sequences are underlined.

Figure 5. Competition of the AINF binding activity by the direct repeat sequence. Ten μg of nuclear extracts were used for binding with 0.3 ng of BiP111 probe. Ten or 100-fold molar excess of competitors are included: BiP111 (lanes 3–4), BiP24 (lanes 5–6), BiP36 (lanes 7–8), BiP33 (lanes 9–10) and f130-uv (lanes 11). Lane 1, probe alone; lane 2, without competitor. Symbols are as in Fig. 2. Molar excess of BiP111 inhibited 90% of the binding (compare lanes 3 and 4 with lane 2). In contrast, a 10- or 100-fold molar excess of BiP24 was not effective in perturbing the binding activities (lanes 5–6). Unexpectedly, a 10-fold molar excess of both BiP36 and BiP33 inhibited 80–85% of the binding activities (lanes 7 and 9). Furthermore, a 100-fold molar excess of BiP36 or BiP33 completely abolished the binding activities (lanes 8 and 10). Competition with f130-uv, a 1000 J/m² UV-irradiated DNA sequence totally unrelated to the BiP sequence (15), is also shown as a negative control (lane 11). Clearly, the BiP36 and BiP33 are equally effective in inhibiting the AINF binding to BiP111. In fact, the BiP36 and BiP33 are about 10 times more effective than BiP111 in inhibiting the AINF binding.

It is of interest to determine whether BiP36 and BiP33 are able to interact with nuclear proteins in an inducible manner. For this purpose, both sequences were used as probes for DNA binding assay (Fig. 6). Eight μg of nuclear extracts from HeLa cells without (lanes 2, 5, and 8) or with A23187 induction (lanes 3, 6, and 9) were incubated with probe BiP36, BiP33, or f130-uv. Probes alone were indicated with 'f' (lanes 1, 4, and 7). AINF binding activities were detected by both BiP36 and BiP33 probes (compare lanes 2 with 3, and lanes 5 with 6); whereas, a reduced binding activity by f130-uv was observed in induced cell extracts (compare lanes 8 and 9). The results clearly indicate that the AINF individually interacts with BiP36 and BiP33 of the direct repeat sequence.

DISCUSSION

In this study, we have demonstrated three cis-acting elements on a cloned human BiP gene promoter that are important for the basal expression in NIH3T3 cells. We have also identified an element spanning -107/-39 which is important for A23187-mediated inducible expression of the gene. A23187-inducible cellular factors, which interact with the regulatory sequence of the gene, were detected from NIH3T3 cells. The AINF binding target was further investigated and localized to a direct repeat sequence motif spanning -107/-39, the same region was important for transactivating the promoter activity. These results suggest that the AINF may be important for transcriptional activation of the BiP gene. The repeat sequences (i.e., BiP36 and BiP33) interact with similar AINF protein patterns when resolved on a SDS-PAGE (C.C.-K.C., unpublished data), suggesting that the number of the repeats may be important in mediating the gene activity. Since there are two CCAAT motifs located in the AIE-contained domain, the identified AINF may be the CCAAT binding nuclear factor such as CTF/NF1. Studies of the rat BiP homolog using an in vitro DNA binding by other investigators have suggested that the basal and induced expression of the BiP gene requires a CG/CAAT and a GC-rich sequence motifs (22). These motifs are conserved in mammalian cells and correspond to the human BiP gene spanning residues -135/-83. The human GC-rich motif overlaps with the 5' part of the putative AIE-containing motif (i.e., BiP36) suggested in this report. It is possible that this region is critical for inducible expression of the gene. Since the proximal CCAAT motif is located at the residue -61, which is out of the CG/CAAT and GC-rich motifs, the AINF detected by BiP36 or BiP33 is probably not the CTF/NF1 proteins (also see below).
It is conceivable that the AIE is located at the 5' part of the -107/-39 segment. More recently, Wooden et al. (23) have shown a proximal region containing a CCAAT motif of the rat BiP promoter as an essential mediator for its induction by A23187 and other stresses. Factors (including the AINF) binding to the upstream regulatory elements may then, through the interaction with the proximal CCAAT motif, activate the transcriptional machinery at the TATA element for transcriptional initiation. Therefore, the results shown in this study, together with others, may formulate a model for BiP transcriptional activation to which the proposed AIE-containing motif, the putative AINF, and C-TF/NF1 are essential. Furthermore, interaction of the human BiP gene promoter with human (11) and mouse (this study) cellular factors, leading to transcriptional activation of the promoter, may suggest that the transcriptional regulation of the BiP gene is controlled by a common mechanism in mammalian cells.

Since a 100-fold CTF/NF1 binding sequence did not significantly affect the AINF binding to BiP36 or BiP33 (C.-K.C., unpublished data), the CTF/NF1 is unlikely not the AINF. In addition, BiP24 does not affect the AINF binding, suggesting that a novel motif for interaction with the AINF is located downstream to the CCAAT elements of each repeats. However, footprinting analysis (data not shown) indicated protection by nuclear proteins not only at this sequence, but also at its spanning region. The footprinting patterns is essentially the same as that reported previously (9) except we detected an additional protection at the direct-repeat sequence. This is the first evidence showing that an AINF directly interacts with 5' sequence of the human BiP gene (i.e., BiP36 and BiP33). It should be noted that the molecular basis of the transcriptional activation of the human BiP gene documented so far is inferred from rat studies (24). In any case, our data strongly suggest that additional cis- and trans-acting factors may also participate in mediating activation of the gene. Furthermore, the abundance of the AINF binding activity in mouse is several fold lower than in human cells. By contrast, the efficiency of transcriptional activation of the human BiP promoter is similar in both cell systems. This raises a possibility that mouse, or particularly NIH3T3, cellular factors are more effective in transactivating the human BiP sequence. Alternatively or coordinately, mechanisms other than the cellular factor-DNA binding detected in vitro are also involved in mediating the control of the transcriptional activation of the BiP gene. Here, we suggest that interaction of the AINF and the direct repeat sequence is partly attributed to the inducible expression of the human BiP gene.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Olivier Brison for the pGCAT plasmid, and colleagues for helpful discussion. They also thank N.-K. Sun and W.-C. Yam for excellent technical assistance. This research was supported in part by grants from Chang Gung Medical College (CMRP138), the National Institute of Health, R.O.C. (DOH83-HR-C09) and the National Science Council, R.O.C. (NSC80-0412-B182-26 to C.-K.C. and NSC80-0203-B001-14 to S.L.-C.).

REFERENCES