Transcription Factor B Cell Lineage-specific Activator Protein Regulates the Gene for Human X-Box Binding Protein 1

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Summary
The transcription factor human X-box binding protein 1 (hXBP-1) is a basic region-leucine zipper protein implicated in the regulation of major histocompatibility complex class II gene expression as well as in exocrine gland and skeletal development. Multiple regulatory elements in the hXBP-1 promoter lie 3' to the transcription start site, including the hX2 site, whose core sequence is an AP-1-like element identical to the hXBP-1 target sequence in the HLA-DRA promoter. One complex identified by electrophoretic mobility shift assay (EMSA), complex 3, was previously shown to protect the hX2 site and more 3' bases. Sequence analysis now shows that this region contains a consensus binding site for transcription factor BSAP (B cell lineage-specific activator protein). Complex 3 and BSAP have identical cell-type specificities, as they are found only in pre-B and mature B cell lines. In EMSAs, BSAP antibody specifically recognized complex 3, and in vitro translated BSAP could bind to an hXBP promoter fragment. Cotransfections using an hXBP-1 reporter construct indicated that BSAP downregulates the hXBP-1 promoter. The highest levels of hXBP-1 mRNA were found when BSAP was not expressed, in pre-pro-B cells and in plasma cell lines. In addition, hXBP-1 and BSAP levels were inversely correlated along the early stages of B cell development. In the regulation of the hXBP-1 promoter, a strong positive transcriptional influence at the hX2 site is opposed by the downregulatory actions of BSAP.
to that of the class II DRA X2 box and is partially protected by two complexes that form on electrophoretic mobility shift assays (EMSAs) using probes of the proximal promoter. Mutation of the hX2 site caused a 90% reduction in transcriptional activity of an hXBP-1 promoter construct, establishing this site as an important regulatory region for hXBP-1 (4).

It is now shown that the hXBP-1 proximal promoter is a target for a tissue-specific and developmentally regulated DNA binding activity, complex 3, which has a distribution identical to that of the transcription factor BSAP (B cell lineage–specific activator protein) (5, 6). In EMSAs, BSAP binding sites compete for complex 3, BSAP antibody specifically recognizes complex 3, and in vitro translated BSAP binds to an hXBP-1 promoter fragment. Cotransfection experiments demonstrate that overexpression of BSAP results in downregulation of the hXBP-1 promoter by acting at the hX2 site and more 3' sequences. In the B cell lineage, hXBP-1 mRNA levels are at their highest when BSAP levels are low or absent, at the pre-Pro-B cell and plasma cell stages. Thus, both positive and negative transcriptional regulators employ the same DNA sequence element in the hXBP-1 promoter, the hX2 site.

### Materials and Methods

**Cell Lines.** All cell lines were grown in RPMI medium supplemented with 8% FCS, 100 μg/ml penicillin and streptomycin, and 10^{-4} M 2-ME.

**Plasmids.** The plasmid hX2-104/+ 128-CAT, containing hXBP-1 promoter sequences from -104 to + 128 in the vector pSVOCAT, and the plasmid LS-hXBP-CAT, containing a 13-bp hXBP-1 promoter sequences from -104 to +128 in the vector pCAT, was created by digestion of the hXBP-1 cDNA with AvaI and 10^{-4} M 2-ME.

The plasmid was found to give results equivalent to normalizing for growth hormone activity generated by cotransfected pCMV-GH (9) in pilot experiments. 40-100 μg of protein was incubated for 3 h or overnight with [^{35}S]methionine for radioactive labeling of the translated protein. The ratio of incorporated to total 14C counts spanned one intron-exon junction, and were predicted to promote the formation of BSAP-mRNA homologs of BSAP isoforms, including BSAP-1 and BSAP-2.

**RT-PCR was performed using B lineage cells at varying stages of differentiation (fractions A–F) isolated from murine bone marrow, as described (10). Briefly, bone marrow cells from 2–4-mo-old female BALB/cAnN mice were stained by a combination of either fluorescein isothiocyanate (FITC), PE anti-BP1, biotin anti-heat-stable antigen (bi-30F1), and allophycocyanin anti-B2 (APC-B2) for fractions A–C, or FL-S7, PE anti-IgD, biotin-anti-IgM, and APC-6B2 for fractions D–F. Texas red-avidin was used to reveal the protein reagent. Cell fractions were sorted directly into a cell lysis buffer by using flow cytometry (FACStar Plus®; Becton Dickinson Immunocytometry Systems, San Jose, CA) for further RNA preparation.**

**Reverse Transcriptase (RT)-PCR.** RT-PCR, was performed using B lineage cells at varying stages of differentiation (fractions A–F) isolated from murine bone marrow, as described (10). Briefly, bone marrow cells from 2–4-mo-old female BALB/cAnN mice were stained by a combination of either fluorescein isothiocyanate (FITC), PE anti-BP1, biotin anti-heat-stable antigen (bi-30F1), and allophycocyanin anti-B2 (APC-B2) for fractions A–C, or FL-S7, PE anti-IgD, biotin-anti-IgM, and APC-6B2 for fractions D–F. Texas red-avidin was used to reveal the reagent. Cell fractions were sorted directly into a cell lysis buffer by using flow cytometry (FACStar Plus®; Becton Dickinson Immunocytometry Systems, San Jose, CA) for further RNA preparation. PCR primers for the murine homolog of hXBP-1 were 5'-CAGAGTAGCAGCGCAGACTGC-3' and 5'-GAGCAACAGTGTCAGAGTCCG-3'.
duce a 383-bp band. The sequences of the PCR products were confirmed by the dideoxy chain termination method.

Results

EMSA Complex 3 Shows Cell Type and Developmental Specificity. To show that the entire hXBP-1 promoter had been isolated, transgenic mice were created using cosmid 20B, containing the hXBP-1 gene with 9 kb of 5' and 10.8 kb of 3' flanking sequence. Analysis of hXBP-1 mRNA levels indicated that the expression of the transgene paralleled the expression of the murine homolog of hXBP-1 in all tissues examined (data not shown), demonstrating that the transgene recreates the endogenous regulation of hXBP-1.

Further analysis of the hXBP-1 promoter has focused on the hX2 site because of its functional importance in the expression of the hXBP-1 gene. EMSAs using hXBP-1 promoter sequences +9 to +104 gave rise to four complexes, of which only complex 3 could not be competed by MHC class II X and Y box sequences. In vitro footprinting by orthophenanthroline-copper demonstrated that complex 3 protected positions +19 to +36 of the hXBP-1 5' untranslated region (4). Examination of this sequence reveals it to be a consensus binding site for transcription factor BSAP, spanning parts of the hX2 site, the CCAAT/Y box, and the interspace between them (11, 12) (Fig. 1). The hXBP-1 promoter gives an even closer match to the BSAP consensus sequence than the sea urchin histone H2B-2.2 BSAP site (Fig. 1 B), a DNA sequence known to have a high affinity for BSAP (13).

To determine if the expression of complex 3 is cell-type specific, nuclear extracts were used from a panel of cell lines. Fig. 2 A demonstrates that complex 3 appears only when Raji B cell nuclear extract is used, and not when using MHC class II-negative T cell lines HPBALL and Jurkat, MHC class II-positive T cell line HUT 78, murine thymoma cell lines EL4 and BW5147, human myeloid leukemic cell line U937, colonic epithelial cell line COLO 205, cervical epithelial cell line HeLa, or HeLa cells treated with IFN-γ to induce MHC class II expression. To further define at which stages of B cell development complex 3 is produced, nuclear extracts were made from a progenitor (Lyd-9), pre-B (70/2Z, 230-238), mature B (M12.4.1., A20), and myeloma cell lines (XAg653, J558L). Fig. 2 B shows that complex 3 appears in pre-B and mature B cells (lanes 3–6) but is absent from the progenitor cell line and myeloma cells (lanes 2, 7, and 8). This same tissue distribution has been described for transcription factor BSAP (13).

When two strong BSAP binding sites, sea urchin histone sequences H2B-2.2 and H2A-2.1 (13), were used in EMSAs with nuclear extracts from our panel of B cell lines, the pattern of binding to the histone sequences was identical to the pattern of binding of complex 3 to the hXBP-1 promoter (Fig. 2, C and D). As a control, no binding was seen using the hX2s probe, an 18-bp probe including the hX2 site of the hXBP-1 promoter, but missing the 3' half of the putative BSAP binding site (Fig. 2 E).

The specificity of the protein–DNA interactions in complex 3 was confirmed by performing EMSAs using Raji nuclear extract with varying amounts of DNA competitors. Fig. 3 shows that the hXBP-1 promoter fragment hX2-BSAP, containing a complete potential BSAP binding site, competes as well for complex 3 as the known high-affinity BSAP binding site H2B-2.2. By contrast, the hX2 fragment, which contains a 6-bp truncation at its 3' end compared to hX2-BSAP, has an incomplete BSAP recognition sequence and competes only weakly, even at 100-fold excess. Three control fragments did not compete: the mut1 fragment containing the DQ AP-1-like site in the context of the hX2 probe, the transcription factor USF binding site, and the transcription factor TFE3 binding site (not shown), even though USF and TFE3 binding sites showed sequence similarity to the hX2 site and more 3' sequences (4). These results provide strong evidence that BSAP or a closely related binding activity interacts with the

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**Figure 1.** The hXBP-1 promoter. (A) Diagram of the proximal promoter of hXBP-1 showing putative conserved sequence elements. (B) Comparisons of the hXBP-1 promoter with BSAP binding sequences. hXBP-1 sequences shown extend from +14 to +38 of the hXBP-1 promoter. BSAP binding sites are from the sea urchin histone H2B-2.2 promoter (13) and a consensus BSAP recognition sequence (11, 12).
body to BSAP supershifted complex 3 in EMSA using either the 95-bp SacI-BssHII (Fig. 4 A, lane 4) or the 27-bp hX2 probe from the hXBP-1 promoter (Fig. 4 B, lane 4). Preimmune serum (Fig. 4, A and B, lane 3) had no effect. As a control, antibody to transcription factor TFE3 did not cause a change in the EMSA complexes (data not shown).

To demonstrate direct binding of BSAP to the hXBP-1 promoter, in vitro transcription and translation were performed using the cDNA of BSAP cloned into the vector pBS. After incubation of BSAP protein with the 95-bp hXBP-1 promoter fragment, electrophoresis showed binding of BSAP, which is known to bind as a monomer (Fig. 4 C). Reticulocyte lysate without added RNA, in vitro translated transcription factors USF, TFE3, and hXBP-1, and mixtures of USF and hXBP-1 or TFE3 and hXBP-1, showed no DNA binding activity (Fig. 4 C, and data not shown). Mixtures of BSAP and hXBP-1 or BSAP and USF showed only the binding activity seen with BSAP alone (not shown). From these results, it is concluded that BSAP specifically binds to the hXBP-1 promoter.

**The Effect of BSAP on the hXBP-1 Promoter.** With EMSAs showing that antibodies to BSAP recognize a specific hXBP promoter binding activity, the functional importance of this transcription factor in regulating the hXBP promoter was addressed. Cotransfections were performed using expression vectors for BSAP together with hX2-104/+128-CAT, a construct containing all functional elements of the hXBP-1 promoter in the vector pSVOCAT (4). As shown in Fig. 5 A, transfection of BSAP downregulated hXBP-1 promoter activity in a dose-dependent manner, reaching 86.3% reduction of CAT activity upon addition of 8 μg of BSAP. Equivalent results were obtained in HeLa cells (which have no endogenous BSAP) and in M12.4.1 B lymphoma cells, and with the BSAP cDNA cloned into two different expression vectors. Squeezing was not observed, since BSAP downregulated the hXBP-1 promoter even when the lowest amounts of BSAP expression plasmid were used. Control expression plasmids had no significant effect on hX2-104/+128-CAT activity. These included antisense BSAP, the cDNA for transcription factor USF, frameshifted cDNA for hXBP-1 (delta hXBP-1), and the expression vector pSG5 without a cDNA insert (Fig. 5 A). However, mutations in the putative BSAP binding site within hX2-104/+128-CAT did affect CAT activity. A 13-bp mutation centered on the hX2 site, called LS-hXBP-CAT, allowed downregulation by cotransfected BSAP of only 22%, whereas a more drastic mutation of the entire putative BSAP binding site, hXBP-1 mutated, essentially abolished the effect of cotransfected BSAP (Fig. 5 B). These experiments demonstrate that BSAP can downregulate the hXBP-1 promoter and that a BSAP binding site in the region from +15 to +37 mediates this effect.

**BSAP and hXBP-1 Levels Vary Inversely in Early B Cell Development.** The powerful downregulatory effect of BSAP on the hXBP-1 promoter predicts that hXBP-1 levels might be high in cells with low or absent BSAP expression. BSAP and hXBP-1 levels were assessed in a panel of immortalized B cell lines representing various stages of hXBP-1 promoter in a cell type-specific and developmentally regulated manner.

**BSAP Antibody Recognizes Complex 3.** Having obtained evidence that the transcription factor BSAP is involved in the formation of complex 3, a polyclonal antibody to BSAP was then used to see if it would recognize specific complexes on EMSA with B cell nuclear extract. The antibody to BSAP supershifted complex 3 in EMSA using either the 95-bp SacI-BssHII (Fig. 4 A, lane 4) or the 27-bp hX2 probe from the hXBP-1 promoter (Fig. 4 B, lane 4). Preimmune serum (Fig. 4, A and B, lane 3) had no effect. As a control, antibody to transcription factor TFE3 did not cause a change in the EMSA complexes (data not shown).
maturation, including four plasma cell lines (Fig. 6 A). As expected, BSAP mRNA was present only in pre-B and mature B cell lines, but absent from the progenitor cell line Lyd-9 and from plasma cell lines (Fig. 6 A, top). mRNA for hXBP-1 was present in all cell lines examined and was most abundant in two plasma cell lines, HOPC IF/12 and J558L (Fig. 6 A, middle).

Since the cell lines in Fig. 6 A are transformed, are of diverse origins, and have not been assigned a precise developmental maturity, RT-PCR was performed on nontransformed bone marrow-derived B cells (10) to assess BSAP and hXBP-1 levels. As shown in Fig. 6, B and C, BSAP levels are near zero in pre-Pro-B cells (fraction A, before D-J rearrangement), increase as the B cells progress through the early Pro-B (fraction B, D-J rearranged) and late Pro-B (fraction C) stages, and peak at the pre-B stage (fraction D, V-D-J rearranged). In the immature (fraction E) and mature B cell stages (fraction F), BSAP expression decreases. On the other hand, hXBP-1 levels are highest in the pre-Pro-B stage (fraction A) and decrease thereafter. There is an inverse relationship between BSAP and hXBP-1 expression in early B cell development, as seen in fractions A–D. Although hXBP-1 expression is not regulated by BSAP alone, hXBP-1 levels appear to be highest when BSAP expression is absent, such as in pre-Pro-B cells and in plasma cell lines.

Discussion

The transcription factor hXBP-1 was originally cloned from a λgt11 library by probing with a cyclic AMP response element site from the murine MHC-α gene,
known as the X2 box (1). Initial characterization revealed hXBP-1 to be a b-zip protein that could form homodimers or heterodimers with the b-zip protein Fos, but not with c-Jun. A role for hXBP-1 in the regulation of MHC class II genes was demonstrated by transfection of antisense hXBP-1 into the Raji B cell line, resulting in decreased expression of DRA and DPB genes (2).

The murine homolog of hXBP-1 is expressed ubiquitously in adult mice, with the highest levels of mRNA found in lung, liver, spleen, and testis (3; Reimold, A. M., and L. H. Glimcher, unpublished observations). During murine fetal development, hXBP-1 mRNA reaches high levels in chondroblasts and osteoblasts, in exocrine glands such as the pancreas and salivary glands, in brown fat, and in whisker follicles (3). In developing bone, the rise and fall of hXBP-1 mRNA levels parallels that of alkaline phosphatase and tissue inhibitor of metalloproteinase, suggesting that common factors or stimuli regulate the transcription or mRNA stability of these three genes. Nevertheless, the role of hXBP-1 in developing fetal tissues has not been fully elucidated.

In studying the hXBP-1 promoter, a previous report described the presence of several conserved sequence elements in the 5' untranslated region of the gene (4). The hX2 site had a core sequence identical in seven of seven base pairs to the X2 site of the DRA promoter. In addition, a CCAAT/Y box was located 15 bp 3' of the hX2 site, producing a grouping in the hXBP-1 promoter similar to the canonical X and Y boxes of MHC class II promoters. EMSA of the proximal hXBP-1 promoter using Raji B cell nuclear extracts revealed four complexes, of which only complex 3 could not be competed for by MHC class II X or Y box sequences. Copper footprinting had shown that complex 3 spanned a 17-bp region extending 3' from the center of the hX2 site (4), an area that overlaps all but two bases of the 19-bp consensus BSAP site. It has now been established that complex 3 contains the transcription factor BSAP. The first piece of evidence for this was the tissue distribution of complex 3, which was found only in pre-B and mature B cell lines, but not in progenitor or plasma cell lines (Fig. 4), using the high-affinity histone H2A-2.1 and H2B-2.2 BSAP binding site probes (13) as comparisons to an hXBP-1 promoter probe in EMSA. This distribution of expression for complex 3 is identical to that described for BSAP (13). The presence of complex 3 was not correlated with expression of MHC class II molecules, since complex 3 was absent in the class II-positive T cell line HUT 78 and in HeLa cells induced to express class II by treatment with IFN-γ. The specificity of complex 3 for a DNA probe containing a potential BSAP binding site was demonstrated by EMSA using competitor DNA or mutated BSAP sites. Probes with intact BSAP sites (histones H2B-2.2 and H2A.2.1, and hX2-BSAP from the hXBP-1 promoter) competed for complex 3, whereas probes without intact BSAP sites (hX2, hX2s, hX2 mut1, μE3 site, USF site) competed weakly or not at all.

Direct evidence that BSAP or an antigenically related protein forms complex 3 was provided by use of polyclonal antiserum to BSAP. BSAP antibody specifically shifted complex 3 in EMSA, whether using the 95-bp or 27-bp hX2 probe from the hXBP-1 promoter. In addition, in vitro translated BSAP protein bound to the 95-bp probe, whereas other in vitro translated transcription factors hXBP-1, USF, and TFE3 showed no binding, even when given the opportunity to form homodimers or heterodimers. A probe from the hXBP-1 promoter containing the hX2 site and sequences 3' to it had been used to screen a λgt11 expression library and resulted in the isolation of a clone for TFE3 (Ponath, P. D., J. L. Strominger, and L. H. Glimcher, unpublished observations). Potential binding
sites for TFE3 and USF can indeed be identified within and 3′ to the hX2 site in the hXBP-1 promoter. Our inability to demonstrate binding of in vitro translated TFE3, USF, or hXBP-1 proteins to this region may indicate a requirement for different binding conditions that we were unable to achieve, or the presence of potential binding sites that are not occupied by these factors under more physiologic conditions.

Targeted disruption of BSAP in the mouse germline has demonstrated postnatal growth retardation, abnormal morphogenesis of the midbrain and cerebellum, and arrest of B cell differentiation at an early precursor stage in BSAP-deficient mice (14). From this phenotype, it appears likely that BSAP regulates genes important for the growth of the skeletal system, of which hXBP-1 is one candidate. Most known BSAP binding sites are located upstream of genes specific to the B cell lineage, such as CD19, VpreB and A5, and the S region of immunoglobulin genes (15–19). Although the hXBP-1 gene has now also been shown to contain a BSAP binding site, hXBP-1 has no known role in the processes of B cell differentiation, switch recombination, or immunoglobulin gene expression.

By downregulating the hXBP-1 gene, BSAP may indirectly influence MHC class II levels in B cells. BSAP mRNA levels peak at the pre-B cell stage and remain substantial in mature B cells (Fig. 6), preceding the expression of MHC class II, which is at very low levels in pre-B cell lines and peaks in mature B cell lines (20). However, the promoters of the MHC class II genes do not contain high-affinity BSAP binding sites (13), suggesting that BSAP does not directly influence class II transcription. Nevertheless, antisense hXBP-1 can downmodulate the levels of MHC class II DRA and DPB expression in the mature B cell line Raji (2), and BSAP might have a similar effect by decreasing hXBP-1 levels, indirectly reducing class II levels. Such a mechanism provides only a partial explanation of MHC class II regulation, since neither BSAP nor hXBP-1 levels show a strict correlation with class II expression.
There is a striking inverse relationship of hXBP-1 and BSAP mRNA levels in the earliest stages of B cell development, with the pre-Pro-B stage (fraction A in Fig. 6 C) showing very low BSAP and high hXBP-1 levels, followed by a steady rise in BSAP but a concomitant drop in hXBP-1 levels thereafter, through the pre-B cell stage (fraction D). These findings suggest that BSAP is a powerful downregulator of the hXBP-1 gene in the B cell lineage, since in those circumstances where BSAP is absent (pre-Pro-B and plasma cell stages), hXBP-1 levels are at their highest. However, there are likely to be other important regulators of hXBP-1 mRNA levels, since BSAP and hXBP-1 levels do not vary inversely in all cases (e.g., Fig. 6 C, fractions E and F). A further downregulator of hXBP-1 expression has been identified as hXBP-1 itself, which in cotransfection experiments represses its own promoter by ~40%, acting at the hX2 site (our unpublished observations). Elimination of hXBP-1 expression by targeted disruption of its gene is expected to provide more definitive information on the role of hXBP-1 in B cell development.

BSAP acts as a positive or negative regulator of gene transcription, depending on its target of action. In this study, BSAP negatively regulated the activity of an hXBP-1 promoter fragment from sequences -104 to +128. In addition, BSAP was recently shown to repress the mouse immunoglobulin heavy chain 3'α enhancer, allowing increased activity of the enhancer in terminally differentiated plasma cells, where BSAP is absent (21, 22). On the other hand, BSAP is required for induction by lipopolysaccharide and IL-4 of ε germline transcripts (23), and the BSAP sites from 5' of Sy2a and from the CD19 promoter function as positive promoter elements in vitro (15, 16). Multiple other BSAP binding sites, within and 5' of immunoglobulin switch regions, in blk and in VpreB and A5 genes, have not yet been shown to have positive or negative regulatory activity (24).

Individual DNA sequence elements have been shown to mediate either positive or negative transcriptional regulation, depending on the combination of bound transcription factors and their levels (25). For example, the composite glucocorticoid response element, pGRE, showed enhanced transcription when bound by glucocorticoid receptor plus c-Jun homodimers, but transcription was repressed upon binding of glucocorticoid receptor plus c-Jun/c-Fos heterodimers (26). Similarly, one can postulate that hXBP-1, BSAP, and an unknown positively acting transcription factor combine in various ratios at the hX2 site, which might behave as a composite response element. In this model, hXBP-1 binds at the hX2 site, BSAP binds at hX2 and to more 3' sequences, and the putative positive factor makes further protein–protein or protein–DNA contacts to ensure a positive transcriptional response. However, when BSAP or hXBP-1 are overexpressed in transfection experiments, they may alter the balance of transcription factors or displace the positively acting factor, resulting in repression of transcription. In this model, although a transcription factor such as hXBP-1 is ubiquitously expressed, it may achieve cell-type specificity by interacting with a unique combination of DNA sequences and transcription factors (25).

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