Functional Characterization of the Murine Homolog of the B cell-specific Coactivator BOB.1/OBF.1*

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B cell-specific transcriptional promoter activity mediated by the octamer motif requires the Oct1 or Oct2 protein and additional B cell-restricted cofactors. One such cofactor, BOB.1/OBF.1, was recently isolated from human B cells. Here, we describe the isolation and detailed characterization of the murine homolog. Full-length cDNAs and genomic clones were isolated, and the gene structure was determined. Comparison of the deduced amino acids shows 88% sequence identity between mouse and human BOB.1/OBF.1. The NH2-terminal 126 amino acids of BOB.1/OBF.1 are both essential and sufficient for interaction with the POU domains of either Oct1 or Oct2. This protein-protein interaction does not require the simultaneous binding of Oct proteins to DNA, and high resolution footprinting of the Oct-DNA interaction reveals that binding of BOB.1/OBF.1 to Oct1 or Oct2 does not alter the interaction with DNA. BOB.1/OBF.1 can efficiently activate octamer-dependent promoters in fibroblasts; however, it fails to stimulate octamer-dependent enhancer activity. Fusion of subdomains of BOB.1/OBF.1 with the GAL4 DNA binding domain reveals that both NH2- and COOH-terminal domains of BOB.1/OBF.1 contribute to full transactivation function, the COOH-terminal domain is more efficient in this transactivation assay. Consistent with the failure of full-length BOB.1/OBF.1 to stimulate octamer-dependent enhancer elements in non B cells, the GAL4 fusions likewise only stimulate from a promoter-proximal position.

The octamer motif (ATGCAAAT) or its reverse complement was originally identified as a conserved element present in virtually all immunoglobulin promoters as well as in enhancer elements of immunoglobulin genes (1, 2). In addition, it is also conserved in a variety of other genes specifically expressed in B cells (3–5). The role of the octamer motif for mediating B cell-specific transcription was most convincingly demonstrated when it was shown that a single copy of this motif confers B cell specificity onto a minimal heterologous promoter element (6). However, functional octamer motifs are also conserved in the regulatory regions of a variety of genes, which show ubiquitous expression patterns (1, 8, 9).

Several transcription factors could be identified in different cell types that specifically interacted with the octamer motif (10, 11). All mammalian cell types express the Oct1 protein (12, 13). B cells, in addition, express a second type of octamer transcription factors, the Oct2 proteins (14–18). Expression of Oct2 is largely confined to the lymphoid lineage, and there it is expressed as a family of isoforms which arise by alternative splicing from a single transcription unit (19). Oct1 and Oct2 belong to a growing family of transcription factors that all share a homologous DNA-binding domain, the POU domain (20, 21). This POU domain is a bipartite DNA-binding domain consisting of a POU-specific and a POU-homeo subdomain. Both subdomains are required for efficient DNA binding (22, 23), and recent crystallographic studies reveal that the two subdomains interact with opposite major grooves in the DNA double helix (24). In addition to Oct1 and Oct2, many other transcription factors have been identified that share a POU domain (25). Some of them, like Oct4 and Oct6, which are expressed in the germ line, also efficiently interact with the conserved octamer motif (11).

The original hypothesis that Oct2 is determining the B cell-specific functions of the octamer motifs, whereas Oct1 would be responsible for its ubiquitous activities (8, 25, 26), was questioned by a variety of observations. In vitro transcription experiments failed to reveal a significant difference between Oct1 and Oct2 proteins (27, 28). In some B cell × T cell hybrid cell lines, octamer-dependent transcriptional activity was extinguished, although Oct2 expression was maintained (29). Moreover, a thorough investigation of gene expression in somatic cell hybrids from myeloma × fibroblast cells showed a variable expression of the Oct2 gene (30, 31). Finally, stably transfected Oct2 did not activate octamer-dependent regulatory elements in NIH/3T3 fibroblasts, BW5147 T cells, or COS1 cells, whereas octamer-dependent promoter activity was evident in B cells lacking the Oct2 transcription factor (31, 32). Likewise, expression of many genes containing octamer motifs in their regulatory elements, like the immunoglobulin genes, B29/ig-β and CD20, was unaffected in B cells from mice lacking Oct2 due to a mutation introduced by homologous recombination into the endogenous oct2 gene (33, 34). These observations argued in favor of the existence of B cell-specific cofactors, which upon interaction with Oct1 or Oct2, would determine the transactivation potential of these transcription factors (31, 35). Biochemical fractionation of B cell-derived nuclear extracts revealed the presence of an activity (OCA-B = octamer coactivator from B cells) stimulating octamer-dependent immunoglobulin promoter activity (27, 36). Indeed, employing a yeast one-hybrid screen, two groups recently reported the successful isolation of cDNAs for such a B cell-specific octamer

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cofactor from human cDNA libraries. The identified cDNAs encode the same protein which was designated BOB.1 for B cell Oct-binding protein 1 (37) or OBF.1 for Oct binding factor 1 (38). Here we describe the isolation and detailed functional characterization of the murine homolog of BOB.1/OBF.1. We show that BOB.1/OBF.1 is an efficient octamer coactivator that allows Oct1 and Oct2 to function on promoter-proximal octamer motifs. However, this factor is unable to mediate the activity of octamer motifs from distal enhancer positions.

MATERIALS AND METHODS

Cloning of the Murine BOB.1/OBF.1 Homolog—Using the published human sequence (37) the following primers were synthesized: BOB.5: 5'-GTC TCT GAG CAT GCT GCA AAA ACC C-3', BOB.3: 5'-AGC GCA GGG TAC TAA AAG CCT TCC ACA GAG AG-3'. These primers correspond to the 5'-end of the human BOB.1 coding sequence. 2 μg of poly(A)+ RNA from S194 cells were reverse-transcribed with oligo(dT) primers and superscript reverse transcriptase (Life Technologies, Inc.) following the manufacturer's instructions. 5% of the cDNA synthesized were used together with primers BOB.5 and BOB.3 in a polymerase chain reaction amplification protocol as described (19). The resulting fragments were gel-purified, digested with XhoI and BamHI and cloned into the pBluescript vector (Stratagene). This cloned fragment was sequenced and confirmed to encode the same protein which was designated BOB.1 for B cell Oct-binding protein 1 or OBF.1 for Oct binding factor 1. A second primer pair was used to determine the 3'-end of the human BOB.1/coding sequence. Two additional primers were designed, BOB.5: 5'-GGT TCC TAA AAG CCT TCC ACA GAG AG-3' and BOB.3: 5'-AGC GCA GGG TAC TAA AAG CCT TCC ACA GAG AG-3'. These primers correspond to the 3'-end of the human BOB.1 coding sequence. In addition, two μg of poly(A)+ RNA from S194 cells were reverse-transcribed and amplified by polymerase chain reaction. Gel-purification and digestion with XhoI/BamHI confirmed the translation of the complete coding region of murine BOB.1/OBF.1. The genomic map was determined by a combination of restriction enzyme mapping, subcloning, and sequencing.

Construction of BOB.1/OBF.1 Expression Plasmids—The plasmids used for the in vitro expression experiments were derived from the human sequence and had introduced an amino acid exchange in the extreme 5'-position of murine BOB.1 (position 6 in the coding sequence) is a proline in human BOB.1/OBF.1 and a serine in the murine homolog). Therefore, two new primers were synthesized that allow the precise and correct amplification of the murine coding sequence: BOB.5b: 5'-CTC TCG AGA GCC ATG CTC TGG CAA AAA T-3' (corresponding to position 446 to 465 with respect to the published human BOB.1/OBF.1 cDNA sequence, but introducing a XhoI site) and BOB.3b: 5'-GGC GAT CCT AAA AGC CCT CCA CGG A A-3' (corresponding to position 436 to 419 with respect to the published human BOB.1/OBF.1 cDNA sequence, introducing a stop codon and a BamHI site at the end).

The resulting full-length, NH2- and COOH-terminal fragments were cloned into three types of vectors: pMT/PKA (39) that allows expression as a fusion protein with a hexa-His moiety at the amino terminus; a vector containing the GAL4 DNA-binding domain (41); and a derivative of the GAL4 expression vector that had the GAL4 DNA-binding domain removed allowing expression of BOB.1/OBF.1 or the individual domains by themselves.

Primer Extension Analysis—The conditions for the primer extension assays were as described (42). Sequencing of the primer used are as follows: P1: 5'-TGA AGC AGA CAG TTT GGC 3' (corresponding to position 2 to 47 with respect to the AUG codon); P2: 5'-CAC GCT TTC TTA GTA GAT 3' (corresponding to position 107 to 90 with respect to the AUG codon).

Conditions for EMSA and Supershift Experiments—Nuclear extracts from the indicated cells were prepared as described (43). For EMSA experiments, 1-4 μg of nuclear extract was incubated in a 20-μl reaction with 30,000 to 40,000 cpn (0.5-1 ng of DNA fragment), 0.5 μg of poly(dI-dC) in a buffer containing 50 mM KCl, 20 mM Hepes, pH 7.7, 1 mM EDTA, 0.25 mg/ml bovine serum albumin, and 4% Ficoll 400. 2 μl of in vitro translated BOB.1/OBF.1 protein (or the respective subdomains) generated with the hexa-His moiety or 2 μl of the untagged translation-TNT lysates (Promega) were added where indicated. All reactions were incubated for 10 min at room temperature prior to loading on a prerun 4% polyacrylamide gel (19), run in 0.35 X TBE.
Characterization of Murine BOB.1/OBF.1

A

FIG. 1.

B

C

Exon I  Intron 1  Exon II
ATGCGCTGCGAAAT  gtaagtgctt(... = 17kb)  ...cccctctatag  CCACAGCTCCA....

Exon II  Intron 2  Exon III

Exon III  Intron 3  Exon IV

Exon IV  Intron 4  Exon V

Exon V  Intron 5

.....CTAATTGGCACT  gttatggtcaca. ...4kb ...ttctgcacag  CCACAGCTCCA....

Fig. 1.
by a large 17-kilobase pair intron. Exons 2, 3, and 4 are small exons of 131, 43, and 266 base pairs separated by introns of 492 and 82 base pairs, respectively. Exon 5 is just over 2000 base pairs long and encompasses the 3′ end of the coding sequence as well as the complete 3′-noncoding sequences. The 3′-noncoding sequence contains multiple simple nucleotide repeats, and these repeats show some mouse strain polymorphism as they differ between the cDNAs isolated from S194 cells (BALB/c mouse strain) and the genomic cosmid clone derived from a 129 mouse strain library (data not shown). With the exception of the splice acceptor site of exon 3, which is divergent from the consensus sequence, all other exon-intron boundaries are in excellent agreement with consensus sequences known for mammalian exons and introns (Fig. 1C). At the 3′ end of the mRNA a consensus poly(A) addition signal is conserved (Fig. 1A).

Inspection of the 5′ leader sequence of BOB.1/OBF.1 of the longest cDNA isolate showed that there is no in frame stop codon present in this sequence. We therefore determined the 5′ end of the murine BOB.1/OBF.1 RNA by primer extension analysis to test whether there might be a longer upstream reading frame. Two primers were utilized for this analysis, one extending from -30 to -47 with respect to the AUG initiation codon (P1), the second one was localized in exon 2 at a position extending from +107 to +90, again with respect to the AUG codon (P2) (Fig. 2A). Primer extension analysis was performed on two different murine B cell RNAs, S194 and WEHI 231, representing plasmacytoma and mature B cell lines, respec-

Fig. 2. Localization of the start site of transcription by primer extension analysis. A, schematic representation of the primer extension strategy. On top, a scheme of the 5′ end of the cDNA is shown. The thin line represents the 5′ leader sequence present in the longest cDNA isolate; the coding sequence is indicated as a thick line. Numbers above indicate the individual exons, and arrowheads mark the exon/intron boundaries. Below, the position of the two primers (P1 and P2) is given. Primer extension products are shown as dotted lines and their length as judged from B, is shown.

B, primer extension experiment using primer P1 (lanes 1, 3, and 5) or primer P2 (lanes 2, 4, and 6) and either tRNA (lanes 1 and 2), WEHI231 RNA (lanes 3 and 4), or RNA from S194 cells (lanes 5 and 6). Extension products for P1 and P2 are indicated by arrows. The arrow with a question mark indicates a longer extension product specifically obtained with primer P2 (see text). The asterisk marks a prematurely terminated primer extension product seen in the reactions with P2. M, size marker (labeled pBR 322 DNA digested with MspI). C, comigration of P1 primer extension products with a sequencing ladder derived from sequencing the genomic clone with primer P1. Sequencing reactions (A, C, G, T) were loaded on the left half of the figure, the extension products for WEHI231, S194, and, as a negative control, tRNA, are shown. The arrowhead indicates the position of the P1-specific extension products.

Fig. 1. Sequence and organization of the murine BOB.1/OBF.1 gene. A, composite nucleotide and predicted peptide sequence of murine BOB.1/OBF.1. The complete nucleotide sequence of the longest cDNA isolate is shown. The first 15 nucleotides were derived from the genomic sequence and the primer extension data shown in Fig. 2. The complete peptide sequence of the murine clone is shown. Only those amino acids that differ in the human sequence are shown underneath the murine sequence. Arrows indicate the exon/intron boundaries. B, restriction map of the genomic locus. The five exons are shown as black boxes; their sizes are not drawn to scale. C, nucleotide sequences of the exon/intron boundaries. Exon sequences are shown as capital letters and intron sequences as lowercase letters. Sizes of the different introns are indicated.
tively. The size of the extension product with primer P1 was 65 nucleotides for both cell lines, primer P2 gave rise to an extension product of 205 nucleotides (Fig. 2B). An additional extension product of roughly 350 nucleotides was observed with primer P2 (marked by a question mark in Fig. 2B). As no corresponding product could be seen with P1, the significance of this extension product remains elusive. These analyses localize the start site of transcription at a position about 15–20 nucleotides upstream of the end of the longest cDNA isolate. To identify the starting nucleotide of the RNA sequence, the primer extension reaction with the P1 primer was run on a sequence gel together with the sequence of the genomic region using the same primer (Fig. 2C), and the deduced sequence was included in Fig. 1A. Consistent with the presence of a single major initiation site, inspection of the 5’ upstream putative promoter region revealed a sequence element fitting known TATA consensus motifs (TTTTAAAAA) at a position –22 to –29 relative to the transcriptional initiation site (data not shown).

A hallmark of the human BOB.1/OBF.1 protein is its interaction with the Oct1 and Oct2 transcription factors, which is thought to be a prerequisite for the transactivation of octamer-dependent promoters. Likewise, in vitro translated murine BOB.1/OBF.1 protein interacted with both Oct1 and Oct2 and resulted in supershifts which were detectable in an EMSA experiment (Fig. 3A). The identity of the individual complexes was confirmed using antibodies specific for Oct1, Oct2, or the BOB.1/OBF.1 protein (Fig. 3B). Inspection of the intensities of the various complexes suggests that Oct1 and Oct2 interact with BOB.1/OBF.1 with similar affinities. In an effort to functionally dissect the BOB.1/OBF.1 protein, we expressed the NH2- and COOH-terminal half of murine BOB.1/OBF.1 individually and tested the interaction with Oct proteins in an EMSA experiment. Only the NH2-terminal domain was able to interact with Oct proteins, resulting in an indicative supershift (Fig. 3C). In contrast, no supershift could be detected with the isolated COOH-terminal domain of BOB.1/OBF.1.

So far, all experiments analyzing the interaction of BOB.1/OBF.1 and Oct1/Oct2 had been performed as EMSA supershifts (37, 38) and above). In these experiments, the POU domain of the octamer proteins had been identified as the domain sufficient for interaction; however, interaction of BOB.1/OBF.1 with other domains of the octamer proteins could not be ruled out. We have therefore utilized a coprecipitation assay to study the interaction of various Oct2 domains with BOB.1/OBF.1. Unlabeled in vitro translated BOB.1/OBF.1 was generated as fusion protein with an NH2-terminal hexa-His moiety. Individual domains of Oct2 fused to the GAL4 DNA binding domain were in vitro translated as labeled proteins, mixed with BOB.1/OBF.1, and precipitated with nickel-nitrilotriacetic acid-agarose. Only the POU domain of Oct2, but not the NH2- and COOH-terminal domains, nor the GAL4 DNA binding domain alone, was coprecipitated efficiently together with BOB.1/OBF.1 (Fig. 4A).

The POU domain is a multifunctional domain important both for specific DNA binding as well as for protein-protein interactions (39, 45). We wanted to determine whether DNA binding of the POU domain was a prerequisite for interaction with BOB.1/OBF.1. We therefore mixed labeled full-length BOB.1/OBF.1 or the individual NH2- and COOH-terminal domains with nuclear extracts from HeLa cells containing ectopically expressed Oct2 protein. We then performed coprecipitation experiments with antibodies specific for Oct2 in the presence and absence of ethidium bromide. As ethidium bromide intercalates into DNA and thereby interferes with protein-DNA interactions, this method has been established as a tool to discriminate between bona fide protein-protein interactions versus assembly of proteins on the same piece of DNA (46). Full-length BOB.1/OBF.1 as well as the NH2-terminal domain efficiently coprecipitated with the anti Oct2 antibody regardless of the presence or absence of ethidium bromide (Fig. 4B). In contrast, the COOH-terminal domain was not recovered in these coprecipitation experiments regardless of the conditions. This result is in line with the failure of the COOH-terminal domain to interact with Oct proteins in the EMSA experiments (Fig. 3B) and suggests that this domain does not make stable contact with the Oct proteins. No BOB.1/OBF.1 proteins were coprecipitated when Oct2 protein was missing from the nuclear extracts confirming the specificity of the assay (Fig. 4B).

The fact that Oct2 binding to DNA was not required for interaction with BOB.1/OBF.1 did not exclude the possibility that the interaction with BOB.1/OBF.1 would affect the Oct-DNA interaction. To analyze this possibility, we performed high-resolution in-gel chemical footprinting analyses of the binary Oct-DNA complex as well as the ternary BOB.1/OBF.1-Oct-DNA complex. Identical protection patterns were observed for both complexes, regardless of whether Oct1 or Oct2 containing complexes were analyzed (Fig. 5, A and B). Using slightly modified conditions for EMSA experiments, we had previously identified a B cell-specific complex migrating slower than the Oct1 complex, but containing the Oct1 protein (31). This complex resembled the ternary BOB.1/OBF.1-Oct1 complex in several respects, such as migration behavior and the fact that no extra contacts were detectable by copper-orthophenanthroline footprinting (31). We therefore investigated whether this complex contained the BOB.1/OBF.1 protein. Whereas the preimmune serum did not affect this ternary complex, the antibody raised against the recombinant BOB.1/OBF.1 protein completely abolished this EMSA complex, suggesting that indeed this complex represents the endogenous BOB.1/OBF.1-Oct1 complex in B cells (Fig. 5C). We do not know presently why this endogenous complex is more difficult to detect in EMSA experiments as compared with the one containing the recombinant BOB.1/OBF.1 protein.

We had previously proposed that B cells contain two types of octamer coregulators (35). One that can interact with either Oct1 or Oct2 to mediate octamer-dependent promoter activity, and a second one that specifically interacts with the carboxyl terminus of Oct2 and is involved in mediating octamer-dependent enhancer activity in B cells. The observation that BOB.1/OBF.1 interacts with both Oct1 and Oct2 suggested that it would be the first type of transcriptional coregulator, namely a specific promoter cofactor. To test this hypothesis, a BOB.1/OBF.1 expression vector was cotransfected with an octamer-dependent promoter reporter into NIH/3T3 fibroblasts. In the absence of cotransfected BOB.1/OBF.1, the wild type octamer reporter showed the same activity as a mutant version, bearing point mutations in the octamer motifs (Fig. 6A). BOB.1/OBF.1 stimulated the wild type reporter construct to a level of activity comparable with the activity of this reporter in B cells (Fig. 6A and Ref. 28). Activation depended on the integrity of the octamer motifs because the mutant reporter was not stimulated by BOB.1/OBF.1 cotransfection. When the NH2- and COOH-terminal halves of BOB.1/OBF.1 were tested individually, only the NH2-terminal domain gave a low, but reproducible, activation of the wild type octamer containing reporter (Fig. 6B). In contrast, the COOH-terminal fragment failed to show any stimulation, consistent with our previous observation that this domain does not interact specifically with the octamer transcription factors.

A slightly different strategy had to be used in order to investigate whether BOB.1/OBF.1 would also stimulate transcrip-
tion from a distal enhancer position, because we had previously shown that this activity is strictly Oct2-dependent (31, 47). We therefore analyzed BOB.1/OBF.1 activity in NIH/3T3 cells that were stably transfected with an Oct2 expression vector (31). These stably transfected fibroblasts express amounts of the Oct2 protein similar to typical B cell lines (31). We first tested the stable transfectants in a BOB.1/OBF.1 cotransfection experiment with the octamer-dependent promoter reporters described before. Interestingly, the stimulation observed by BOB.1/OBF.1 cotransfection was comparable in the parental NIH/3T3 cells and the Oct2-positive NIH/3T3 cells (Fig. 6C).

This result suggests that Oct1-BOB.1/OBF.1 complexes have similar transactivation potential as Oct2-BOB.1/OBF.1 complexes. To test activation from an enhancer position, reporter
constructs bearing a multimerized octamer motif-containing fragment from the murine heavy chain enhancer at a position 3′ of the luciferase gene driven by an upstream chicken lysozyme promoter was used (31, 47). This reporter has been previously used to detect octamer-dependent enhancer activity in B cells. Interestingly, no stimulation of this reporter could be observed by cotransfection of full length BOB.1/OBF.1 (Fig. 6C). This result suggests that BOB.1/OBF.1 is in fact a promoter-specific cofactor and unable to mediate the B cell-specific octamer enhancer activities. This inability of BOB.1/OBF.1 to stimulate the enhancer reporter was not due to the fact that no functional ternary Oct-BOB.1/OBF.1 complexes can be formed on the multimerized heavy chain enhancer fragment used. When the same multimerized fragment was moved into the proximity of the TATA box, BOB.1/OBF.1 was again capable of activating this element (Fig. 6C).

The above interaction domain mapping experiments and cotransfection experiments had revealed that the NH2-terminal domain of BOB.1/OBF.1 interacts with the Oct transcription factors and contains a residual transactivation function, yet transactivation by the full-length BOB.1/OBF.1 clone was significantly more prominent. In order to map the potential transactivation functions within the BOB.1/OBF.1 coactivator, we generated fusion proteins with the GAL4 DNA-binding domain and either full-length BOB.1/OBF.1 or the NH2- or COOH-terminal domain of BOB.1/OBF.1 separately (Fig. 7C). These
clones were cotransfected with a reporter bearing multimerized binding sites for the GAL4 transcription factors in a promoter proximal position (41). All three fusion proteins efficiently stimulated this reporter. The COOH-terminal domain of BOB.1/OBF.1 showed about 3-fold higher activity than the NH$_2$ terminus, however, (Fig. 7A). This result suggests that the predominant transactivation function of the BOB.1/OBF.1 protein resides in the COOH-terminal 130 amino acids. Given the above described inability of BOB.1/OBF.1 to stimulate octamer-dependent enhancer elements together with Oct2 in fibroblasts, we wanted to determine whether the GAL4 fusion proteins would be able to stimulate transcription from a distance. To this end, we utilized a reporter bearing the GAL4 binding sites in a distal enhancer position and cotransfected this reporter with the GAL4 fusion protein expression vectors. Fig. 7B shows that neither of the GAL4 fusions is capable of stimulating this reporter, suggesting that failure to stimulate from a distance is an intrinsic property of the transactivation domains of the BOB.1/OBF.1 protein.

**DISCUSSION**

One of the hitherto unique features of B cell-specific transactivation mediated by the octamer transcription factors is their requirement for the presence of additional B cell-specific coactivators. Here, we describe the molecular analysis of one such coactivator that allows Oct1 and Oct2 to activate transcription from a promoter-proximal position in B cells.

We had previously shown that B cells contain two distinct types of coactivators. One responsible for the B cell-specific activity of octamer-containing promoters and a second type that confers activity on octamer regulatory elements from distal enhancer positions (31, 35, 47). The results presented here unequivocally identify BOB.1/OBF.1 as a specific coactivator from promoter-proximal positions. This conclusion is supported by the following lines of evidence.

1) Whereas cotransfection of BOB.1/OBF.1 into NIH/3T3 fibroblasts was sufficient to fully activate octamer-dependent promoter elements, it did not result in enhancer activation, regardless of the presence or absence of Oct2.

2) Fusion proteins of the GAL4 DNA binding domain with full-length BOB.1/OBF.1 or individual domains of the coactivator efficiently stimulated reporters containing GAL4 binding sites in a promoter-proximal position but failed to activate when the binding sites were present in distal enhancer positions.

3) BOB.1/OBF.1 promiscuously interacts with both Oct1 and Oct2, whereas the putative enhancer cofactor specifically requires Oct2 for a functional interaction (31, 35, 47). Furthermore, our previous experiments suggested that Oct1, if anything, reduced octamer-dependent enhancer activity (31, 47).

4) Finally, we had previously identified the COOH-terminal transactivation domain of Oct2 as a prerequisite for stimulating octamer-dependent enhancer activity in B cells. This result suggested that the enhancer cofactor might specifically interact with this domain rather than the POU domain. In our protein-protein interaction analysis presented here, we failed to detect any evidence for specific interaction between BOB.1/OBF.1 and the COOH-terminal domain of Oct2. In summary, these results suggest that BOB.1/OBF.1 is the one of the B cell-specific coactivator(s) responsible for the B cell-specific function of octamer-containing promoters. Furthermore, they suggest that additional, distinct cofactors exist in B cells that are required for
mediating the octamer-specific functions from distal enhancer positions.

At present, the molecular mechanism responsible for the observed coactivation by BOB.1/OBF.1 is largely obscure. When transactivation properties of the NH₂- and COOH-terminal transactivation domains of the Oct2 transcription factor were measured in GAL4 fusion experiments, significant activity of the individual domains could be scored, which were not significantly lower than the transactivation observed for GAL4 fusion proteins containing full-length BOB.1/OBF.1 or individual domains thereof (41, 48). A possible explanation for this apparent paradox could be that due to an intramolecular masking process, the transactivation domains of Oct1 and Oct2 might not be accessible for interaction with general transcription factors, most likely the transcription factor IID (TFIID) complex (49). The main function of the coactivator then would be to unfold and unmask these transactivation domains due to its physical interaction with the Oct1 and Oct2 transcription factors. Intramolecular masking of the transactivation domain has been suggested for the MyoD transcription factor (50). A specific conformational change induced by binding of MyoD to DNA is hypothesized to be responsible for the release of the masked transactivation domain (50, 51). From our results it is unclear whether a similar masking/unmasking process is responsible for the activation of the octamer transcription factors in B cells. Clearly, a more complex situation than for MyoD has to be envisaged, as an additional cofactor, namely BOB.1/OBF.1, is required for the activation to take place. Interestingly, involvement of additional cofactors could also not be ruled out in the MyoD activation process (51). Although our results demonstrate that Oct2 and BOB.1/OBF.1 can interact off DNA, these experiments do not exclude a role for DNA-binding in the activation process. In that respect it is of interest to note that in our previous GAL4 fusion experiments with

**FIG. 6.** Cotransfection of BOB.1/OBF.1 activates octamer-dependent promoters, but not enhancers in NIH/3T3 fibroblasts. A, BOB.1/OBF.1 cotransfection activates octamer-containing promoters. Cotransfection with the indicated reporter plasmids and either the parental expression vector (pSV) or a full-length BOB.1/OBF.1 expression vector. The activity of the reporter plasmid containing four copies of the mutant octamer motif upstream of the TATA-box was arbitrarily set to 1. B, cotransfection of the wild type octamer reporter with expression vectors for full-length BOB.1/OBF.1, the NH₂-terminal, or the COOH-terminal domain of BOB.1/OBF.1 as indicated. The activity of the reporter cotransfected with the parental expression vector (pSV) was set to 1. C, cotransfection of the indicated reporter plasmids into NIH/3T3 cells stably expressing Oct2. An expression vector for full-length BOB.1/OBF.1 (or the parental expression vector) was cotransfected where indicated. D, schematic outline of the various expression and reporter plasmids used in the cotransfection experiments described in this figure. The SV40 based expression vector was derived from pSG5 (Stratagene). The different reporter plasmids have been described previously (28, 31, 47). Briefly, 4 × wt.TATA contains four copies of a synthetic octamer motif upstream of the HSV-thymidine kinase TATA box (T). The octamer motifs contain a single point mutation in 4 × mut.TATA. CL(ED) contains the chicken lysozyme promoter and six copies of a 50-base pair fragment derived from the murine heavy chain intronic enhancer element comprising the E4 and Oct motifs. The octamer motif contains several point mutations in the CL(ED) construct. The same hexameric wild type and mutant enhancer multimers were cloned upstream of the minimal HSV-TATA region in (ED)/(ED)TATA.
mediated transactivation. A recent investigation of Oct1 binding component in this system, it was unclear how specific octamer protein, HCF (54–58). However, as Oct1 is the only DNA-binding protein, VP16, as well as an additional cellular protein, ATGARAT consensus is responsible for efficient coactivation of several viral promoters after infection of cells with these viruses. The Oct1 protein is a critical component for the activation of Oct1 and Oct2 proteins is involved. The Oct1 protein is a critical component for the activation of several viral promoters after infection of cells with herpes simplex virus. There, a complex between the viral VP16 protein and Oct1 on specific promoter motifs containing a TAATGARAT consensus is responsible for efficient coactivation (52, 53). The functional complex formed on the DNA actually is composed of Oct1, VP16, as well as an additional cellular protein, HCF (54–58). However, as Oct1 is the only DNA-binding component in this system, it was unclear how specific octamer motifs (the GARAT-containing motifs) were selected for VP16-mediated transactivation. A recent investigation of Oct1 binding to different octamer motifs by high resolution chemical footprinting suggested that the Oct1 POU domain adopts a specific conformation when binding to GARAT-containing motifs as compared with two other octamer motifs. It was suggested that this specific conformation would then be recognized by the viral coactivator (59). However, the additional binding of the coactivator to Oct1 did not affect the POU domain contacts to DNA as measured by chemical footprinting. In agreement with these findings, we also failed to detect any alterations of Oct1 or Oct2 DNA contact upon binding of the BOB.1/OBF.1 cofactor. This does not exclude the possibility, however, that, upon interaction with the cofactor, the overall conformation of the Oct1 and Oct2 proteins is changed.

Could different conformation of Oct proteins on different octamer binding sites be responsible for the differences observed with respect to promoter and enhancer activation by BOB.1/OBF.1? This interpretation is highly unlikely for the following two reasons. First, supershifted EMSA complexes containing Oct1 or Oct2 plus the BOB.1/OBF.1 coactivator could be observed on the various binding sites used for the promoter and the enhancer reporter constructs (compare, for example, Figs. 3 and 5). Furthermore, we have shown that the very same elements that failed to function at a distance, were efficiently activated by BOB.1/OBF.1 when placed in a promoter-proximal position (Fig. 6D). These results together with the evidence discussed above argue for an independent distinct enhancer coactivator.

The observation that BOB.1/OBF.1 only contacts the POU domain of the octamer transcription factors further supports the dual function of the POU domain in these proteins. In addition to being responsible for specific DNA-binding, its role in orchestrating protein-protein interaction is becoming more and more apparent over the last years. In addition to viral proteins such as the described VP16 herpes simplex virus coactivator or the adenovirus E1A protein (30), interaction with cellular proteins has also been shown to be mediated by the POU domain of Oct1 and Oct2. We could previously show that the POU domains of Oct1 and Oct2 specifically interact with TBP, the TATA-binding protein component of transcription factor IID (45). More recently, using the POU domain as probe in a protein-protein interaction screening protocol, we were able to isolate HMG2, an abundant non-histone nucleoprotein, as an interacting partner protein (39). In contrast to the interaction with BOB.1/OBF.1 which has been described to be specific for Oct1 and Oct2 (37, 38), HMG2-POU domain interactions are more promiscuous as the Oct6 POU domain was also shown to interact with HMG2 (39).

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