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Gene structure and characterization of the murine homologue of the B cell-specific transcriptional coactivator OBF-1

Daniel B. Schubart, Patrick Sauter, Steffen Massa, Erika M. Friedl, Heidi Schwarzenbach* and Patrick Matthias+

Friedrich Miescher Institute, Maulbeerstrasse 66, PO Box 2543, 4002 Basel, Switzerland

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ABSTRACT

The B cell-specific activity of immunoglobulin (Ig) gene promoters is to a large extent mediated by the conserved octamer motif ATTTGCAT. This requires the DNA binding octamer factors Oct-1 and/or Oct-2, as well as an additional B cell-restricted non-DNA binding cofactor. We recently cloned such a coactivator specific for Oct-1 or Oct-2 from human B cells and called it OBF-1. Here we report the isolation and characterization of the murine homologue. Full-length cDNA clones as well as genomic clones were isolated and the gene structure was determined. The deduced protein sequence shows that the mouse protein has an identical length, is likewise proline rich and shows 89% overall identity to the human protein. The OBF-1 gene is expressed in a very highly B cell-specific manner and is transcribed in cells representative of all stages of B cell differentiation, including the earliest ones. We show that OBF-1 interacts in the absence of DNA with the POU domain of Oct-1 or Oct-2 and also with the general transcription factors TBP and TFIIB. Furthermore, we demonstrate that although OBF-1 efficiently activates promoter octamer sites, it does not activate enhancer octamer sites.

INTRODUCTION

The octamer motif ATTTGCAT or its reverse complement is conserved in almost all immunoglobulin (Ig) gene promoters, as well as in several of the Ig enhancers (1–3). Furthermore, this motif is also found in the promoters of several B cell-specific genes, such as the CD20 or the B29 (Igβ) genes (4,5). A number of experiments done by cell transfection (6–8) or *in vitro* transcription (9) or with transgenic mice (10) have demonstrated that the octamer motif plays a key role in mediating B cell-specific gene transcription. In particular, its role in mediating the B cell-specific activity of Ig promoters has been well established. In addition, functional conserved octamer motifs are also found in the promoters of various ubiquitous genes, such as the cell cycle-regulated histone H2B gene and some small nuclear RNA genes (11,12).

Over the last few years several transcription factors have been identified and cloned that specifically interact with the octamer site (reviewed in 13). All belong to a subfamily of the homeodomain proteins, the POU proteins (14,15). These proteins share in common a bipartite DNA binding domain, the POU domain, that is composed of a conserved POU-specific domain and of a POU homeodomain (16). Both subdomains are required for DNA binding, and efficient DNA binding results from the interaction of the individual subdomains with the major groove on opposite sides of the DNA (17). B cells contain two octamer binding proteins with identical DNA binding specificities: Oct-1 is a ubiquitously expressed protein (18) and Oct-2 is a largely lymphoid cell-restricted transcription factor (19–22). This finding, together with the observation that ectopic expression of Oct-2 could stimulate artificial B cell-specific promoters in non-B cells, led to the early notion that Oct-2 was responsible for mediating the B cell-specific function of the octamer site (21). However, several experiments challenged that model and suggested that a more complex scenario had to be considered. First, knockout of the Oct-2 gene by homologous recombination either in somatic B cells (23) or in embryonic stem (ES) cells, with subsequent generation of mice derived from these ES cells (24), showed that B cell-specific transcription from Ig promoters does not require the Oct-2 protein. Yet, Oct-2 plays a critical role for the final stage of B cell differentiation and Oct-2 –/– B cells fail to proliferate and secrete Ig in reponse to antigenic stimulation (25). Second, *in vitro* transcription experiments showed that Oct-1 and Oct-2 have an intrinsic equal capacity to stimulate transcription from an Ig promoter *in vitro* (26,27) and identified in B cells a protein fraction that was proposed to contain a specific coactivator for Oct factors (28).

Recently, using a single hybrid screen in yeast with Oct-1 as a bait, we isolated a human cDNA encoding just such a coactivator and called it OBF-1, for Oct binding factor 1 (29). The same human cDNA was also isolated independently by two others groups, either following a similar strategy or after biochemical purification of the protein, and was dubbed Bob-1 (30) or OCA-B (31). The OBF-1 protein is a novel proline-rich protein with no homology to known proteins and no obvious domains or motifs. Expression of the OBF-1 gene is restricted to B cells and we could show that OBF-1 can form a ternary complex on DNA together with

* To whom correspondence should be addressed

+Present address: Department of Oncology, St Gallen Hospital, CH-9007 St Gallen, Switzerland

either Oct-1 or Oct-2. For this, the POU domain of these factors is necessary and sufficient. In addition, by transfection assays we showed that OBF-1 specifically coactivates transcription from a natural Ig promoter, through interaction with Oct-1 or Oct-2 (29).

In order to further characterize the function and mechanism of action of this novel coactivator we have now cloned the murine homologue and determined its gene structure and RNA transcription start site. We present a detailed analysis of the highly B cell-specific expression pattern of the OBF-1 gene and show that the OBF-1 protein interacts off the DNA with the POU domain of Oct-1 or Oct-2 and also with the general transcription factors TBP and TFIIB. Furthermore, we demonstrate that OBF-1 is a coactivator mediating the activity of octamer sites from promoter, but not from enhancer, positions.

MATERIALS AND METHODS

Cloning of the mouse gene

A cDNA library derived from the mouse B cell line S194 was constructed in a plasmid vector following standard procedures (32) and was screened at low stringency with the radiolabelled human OBF-1 cDNA as a probe. One full-length clone was isolated and its DNA sequence was determined by dideoxy sequencing after generation of unidirectional deletions (clone no. A55-1; accession no. U43788). This mouse cDNA was then used as a probe to screen a 129SV mouse genomic DNA library. Several overlapping phages were isolated which together span the entire OBF-1 gene, the structure of which was determined by restriction mapping, subcloning, DNA sequencing and PCR.

Primer extension assays

For primer extension the primer 5′-ACAGGATGTTGCCTTTTC-TC-3′ was 5′-end-labelled with [γ-^{32}P]ATP and gel purified. The labelled primer (120 000 c.p.m.) was hybridized to 30 µg of total RNA and cDNA was synthesized at 42°C with Superscript II reverse transcriptase. Extension products were resolved on an 8% polyacrylamide–7 M urea gel and visualized by autoradiography.

RNase protection assays

Total RNA (30 µg or 5 µg polyA$^+$) from the indicated cells or from spleen was hybridized at 50°C overnight with 150 000 c.p.m. of an antisense ^{32}P-labelled RNA probe spanning part of the first exon as well as the 5′ flanking region of the OBF-1 gene. This probe was prepared by transcription of plasmid mOBF-1/3-1 with T7 RNA polymerase, after linearization with *Ssp*I. The hybridized samples were then digested with a mixture of RNAses A and T1 and the RNase-resistant products were displayed on an 8% polyacrylamide–7 M urea gel and visualized by autoradiography.

Cell lines and RNA analysis by RT–PCR and Northern blotting

Cytoplasmic RNA (1 µg/reaction) was reverse transcribed in 20 µl volume with oligo(dT) as a primer and AMV reverse transcriptase using a kit from Promega. After heat inactivation, 10% of each cDNA reaction were used for a PCR reaction performed with the following primers (5′→3′): OBF-1, forward AGGTAGGAGGA-TGTGATGACG, reverse CAGTGCTTCTTGGCGTGACA; HPRT, forward GCTGGTGAAAAGGACCTCT, reverse CACA-GGACTAGAACACCTGC (33). The PCR reactions were performed under the following conditions: 20 cycles each at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 10 min. Subsequently, equal aliquots of the reactions were run on 2% agarose gels, blotted onto Genescreen filters and hybridized to radiolabelled OBF-1 and HPRT probes. The amplified cDNA fragments are 249 bp in length for HPRT and 644 bp in length for OBF-1.

For Northern blotting, total RNA was run on 1.1% formaldehyde–agarose gels (32), blotted to Genescreen membranes and subsequently hybridized to radiolabelled OBF-1 and β-actin probes.

For the generation of Abelson virus-transformed pre-B cell lines, single cell supensions from day 16.5 fetal livers were infected with culture supernatant from the Abl virus-producing cell line N54 (kindly provided by T. Jenuwein). Small resting B cells were purified from mice spleen by centrifugation through Percoll gradients as described (34).

Protein–protein interaction assays

The following GST expression vectors were used. pGST-OBF-1 encodes full-length OBF-1 and was made as follows: a *Bam*HI–*Xba*I fragment containing the whole human OBF-1 cDNA starting with an *Eco*RI site immediately before the ATG initiation codon was reclaimed from plasmid pEV-OBF-1/ATG and inserted into pGex2T.

pGST-hTBP encodes the full-length human TBP cDNA, pGST-hTBPn encodes the N-terminal 159 amino acids of hTBP and pGST-hTBPc encodes the C-terminal 180 amino acids of hTBP (vectors kindly provided by S. Ruppert and R. Tjian). pGST-IIB encodes the full-length human TFIIB protein (35). pGST-POU1 and pGST-POU2 have been described previously (29). Gex plasmids encoding GST fusion proteins were grown in *Escherichia coli* NB42 cells; protein expression was induced with 1 mM IPTG for 30 min at 37°C (pGST-POU1, pGST-POU2, pGST-hTBPn and pGST-hTBPc) or for 90 min at 30°C (pGST-IIB, pGST-hTBP and pGST-OBF-1). Bacteria were pelleted, resuspended in PBS containing 100 mM EDTA, 1% Triton X-100, 1 mM PMSF, 2 µg/ml aprotinin, 0.5 µg/ml leupeptin and protein extracts were prepared by sonication on ice. The lysate was cleared by centrifugation and the supernatant was adsorbed on glutathione–agarose beads. The beads were washed once with lysis buffer and three times with PBS, 100 mM EDTA. Fusion proteins were then eluted by competition with 15 mM reduced glutathione. Full length ^{35}S-labelled OBF-1 protein was prepared by coupled *in vitro* transcription/*in vitro* translation of the plasmid pBGO-OBF1/9 with the TNT system (Promega). ^{35}S-Labelled OBF-1 amino acids 1–114 was prepared by linearization of plasmid pBGO-ATG-OBF1/9-*Pvu*II with *Not*I, preparation of cRNA with T3 polymerase and subsequent *in vitro* translation in a rabbit reticulocyte lysate (Promega).

Far Western assays were done following the protocol described in Ruppert *et al.* (36). Pull-down assays were done by incubating 20 µl of GST fusion protein-loaded beads with 10 µl ^{35}S-labelled OBF-1 protein in 250 µl of a buffer containing 100 mM NaCl, 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.8, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.05% Nonidet P-40. After a 1 h nutation at room temperature, the beads were washed four times with 750 µl each of binding buffer and bound proteins were eluted by boiling and separated by SDS–PAGE.

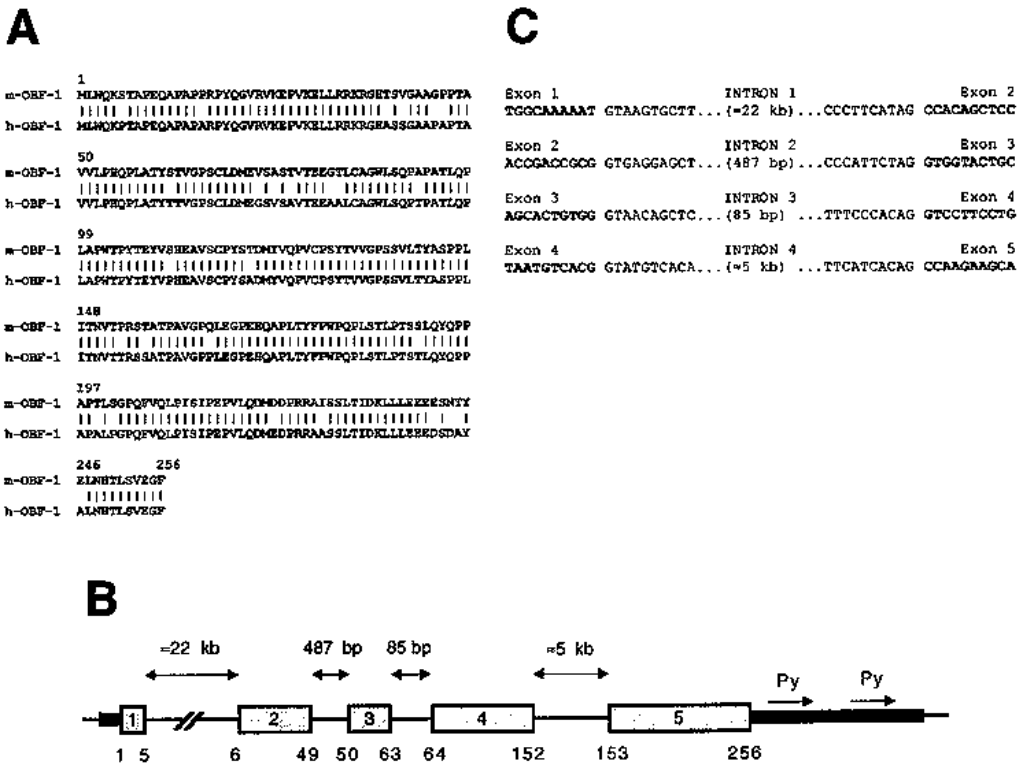

Figure 1. Deduced amino acids sequence of the mouse OBF-1 protein (cDNA sequence accession no. U43788) and corresponding gene structure. (**A**) Comparison between the mouse (top line) and the human (bottom line) OBF-1 protein sequence. Identical amino acids between the two sequences are indicated by a vertical dash. (**B**) Exon/intron structure of the OBF-1 gene (not drawn to scale). The grey rectangles represent the translated exons; the amino acids encoded by the different exons are indicated under the drawing and the intron sizes are indicated above. The thin black rectangles denote the 5′ and 3′ untranslated exonic sequences. In the 3′ untranslated exon two arrows denoted Py represent a pyrimidine-rich repeated sequence. (**C**) Nucleotide sequence of the exon/intron boundaries. The exonic sequences are indicated by upper case bold letters.

Cell transfections

HeLa cells were maintained in DMEM medium supplemented with 3% fetal calf serum and 3% newborn calf serum; Namalwa cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum and 50 µM β-MeOH. The reporter plasmids used are based on the pKappaLuc plasmids previously described (29). In pKappaLuc6xO a 300 bp fragment containing six copies of the IgH intron enhancer octamer site was inserted in place of the SV40 enhancer fragment. The OBF-1 expression vectors used were either pEV-OBF-1/9 (29) or a similar vector containing the mouse OBF-1 cDNA. The Oct-2 expression vector has been described earlier (21). B cells were transfected by the DEAE–Dextran procedure with 6 µg reporter plasmid; HeLa cells were transfected by the calcium phosphate procedure with 2–5 µg reporter plasmid and 0.5–5 µg expression vector (empty or encoding Oct-2 or OBF-1) and the total amount of DNA was brought to 20 µg with herring sperm DNA. Transfections and luciferase assays were done as previously described (37,38).

Details on all plasmid constructions are available on request.

RESULTS

Structure of the murine OBF-1 protein and gene

In order to isolate the mouse homologue of the transcriptional coactivator OBF-1, a cDNA library prepared from the mouse B cell line S194 was screened under low stringency with the human OBF-1 cDNA as a probe. The mouse cDNA isolated in such a way is 2578 bp long and contains the entire coding region as well as the 3′ untranslated region. The deduced protein sequence of the murine protein is very proline rich (~16%) and shows a high homology to the human protein: 89% identity over the entire length of the protein (Fig. 1A). Surprisingly, the conservation between the human and the mouse OBF-1 clones also extends to their 5′ untranslated leader sequence, since in the 90 nt preceding the ATG initiation codon only 7 nt are different between the two cDNAs. However, a translation of the leader sequence starting at a further upstream ATG and leading to production of a larger protein can be excluded, as the full-length mouse cDNA we isolated (accession no. U43788) starts with an in-frame TAA stop codon 96 bp upstream of the translation initiation site and no other ATG codon is found.

Using the mouse cDNA as a probe we isolated, from a mouse genomic library, several overlapping λ phages that together cover the whole OBF-1 gene. The cDNA is split over five exons, all of which contain coding sequences (Fig. 1B). The first exon codes for only the first five amino acids of the OBF-1 protein and is then interrupted from the remainder of the gene by a very large (~22 kb) intron (Fig. 1B); all exon/intron boundaries fit the consensus sequences defined for mammalian introns and exons (Fig. 1C).

In order to know whether the cDNA we isolated was indeed full length we determined the transcription start site(s) of the OBF-1 message by primer extension and RNase protection assays (Fig. 2). The primer used extends from positions –9 to –29 with respect to

the ATG initiation codon (Fig. 2A). When RNA from S194 plasmacytoma B cells was used as template, a major extension product 88 bp in length was observed which was also observed with spleen RNA, albeit much more weakly (Fig. 2B); by contrast, no extension product was obtained with either yeast RNA or with RNA derived from BW5147 T cells (Fig. 2B). In addition, several very weak longer extension products were also seen in RNA from S194 B cells (not shown and see below). To confirm the results from the primer extension, an RNase protection assay was performed. For this, an antisense RNA probe was prepared from a genomic clone containing the region around the first exon of the gene; this probe corresponds to the OBF-1 sequence from –41 to –709 with respect to the ATG codon (Fig. 2A). When this probe was hybridized to RNA from S194 B cells, a 55 nt fragment was protected from RNase digestion (Fig. 2C). This fragment was also very weakly seen with spleen RNA and corresponds precisely to an RNA transcript initiating at the site identified by the primer extension assay presented in Figure 2B. In addition, the RNase protection assay also showed several longer but much weaker protected fragments, one of which is visible on the gel presented (Fig. 2C). Thus, both primer extension and RNase protection identified one major transcription start site for the OBF-1 gene. This start site lies ~28 bp downstream of an AT-rich sequence with the sequence 5′-TTTAAAAA-3′, which fits well the consensus for a TATA box (not shown), and the cDNA we isolated represents an RNA initiating at this start site.

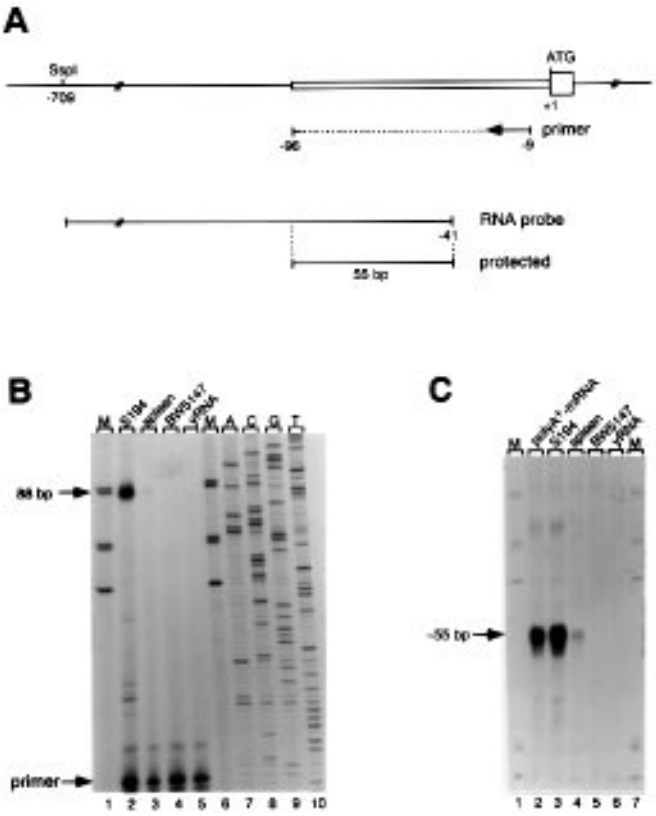

Figure 2. Analysis of the transcriptional start site by primer extension and RNase protection. (**A**) Schematic diagram of the experimental strategy used. A portion of the OBF-1 gene is shown with the first exon (thin open rectangle, untranslated leader; open square, translated sequence) as well as part of the 5′ flanking region and first intron (thin lines). At the top, the primer used for the primer extension is indicated by an arrow pointing to the left, as well as the major extension product obtained, indicated by a dotted line. The numbering is relative to the ATG translation initiation codon, with the A being position +1. At the bottom, the 668 nt long antisense RNA probe used is depicted, as well as the major protected RNA species. (**B**) Primer extension performed with the primer indicated in (A) and RNA from S194 B cells (lane 2), spleen (lane 3), BW5147 T cells (lane 4) or yeast (lane 4). Lanes 7–10 show a DNA dideoxy sequencing ladder performed with the same primer and a genomic DNA subclone as template. Lanes 1 and 6 contain end-labelled size markers; only the relevant portion of the gel is presented. (**C**) RNase protection experiment using the probe indicated in (A) and RNA from S194 B cells (lanes 2 and 3), spleen (lane 4), BW5147 T cells (lane 5) or yeast (lane 6). Lanes 1 and 7 contain end-labelled size markers. The major protected product is indicated by an arrow. A minor longer protected product is also visible in lanes 2 and 3 (see text). The full-length probe (668 nt) is not visible, as only the lower part of the gel is shown.

Expression throughout B cell differentiation

We have previously reported that, based on our analysis of RNAs from several human cell lines and organs, expression of the OBF-1 gene was B cell restricted. Here we have examined a large panel of murine cell lines representative of the various stages of B cell differentiation, as well as primary B cells and cells from other lineages. For this, we used a very sensitive reverse transcription–polymerase chain reaction (RT–PCR) assay. The PCR reactions were for 20 cycles and were followed by gel electrophoresis, blotting and hybridization with gene-specific probes. In this way the assays were essentially quantitative. As an internal control to monitor reaction efficiency and RNA integrity, primers for amplification of the ubiquitous HPRT cDNA were included in each reaction. As shown in Figure 3A, all B cells examined expressed the OBF-1 gene. OBF-1 RNA was detected in mature B cells (lanes 12–18), including in cells of the plasma stage, such as S194 or MPC11, and also in cells representing early stages of B cell differentiation, such as pro-B (lane 1) and pre-B cells (lanes 2–11). Notably, expression of the OBF-1 gene was also observed in primary IL-7-dependent pro-B cells (lanes 19–21) as well as in pre-B cell cultures established from fetal livers by transformation with the Abelson (Abl) murine leukaemia virus (lanes 22–25). Expression was also seen in primary small B cells purified from spleen by centrifugation through Percoll gradients (lanes 26–27). The expression levels were found to be roughly constant throughout B cell differentiation, an observation which was also confirmed by Northern blot analysis (not shown). Furthermore, treatment of the pre-B cell line 70Z/3 (Fig. 3A, lanes 6 and 7) or of the Abl pre-B cells (lanes 22–25) with the B cell mitogen bacterial lipopolysaccharide (LPS) did not further up-regulate OBF-1 expression, unlike what has been observed with Oct-2 (39). LPS treatment of spleen primary B cells results in their proliferation and terminal differentiation to plasma cells and showed only a weak increase in OBF-1 gene expression (lanes 26 and 27), more likely reflecting the drastic metabolic changes taking place upon mitogenic stimulation of resting B cells rather than specific induction of the OBF-1 gene. In contrast to B cells, other cells of the haematopoietic lineage, such as T cells or macrophages, did not express the OBF-1 gene, with the exception of the T cell line EL-4, in which an extremely low expression level could be detected (Fig. 3B, lane 3). In addition, this RT–PCR assay failed to detect any OBF-1 expression in RNAs from many other cells lines, such as fibroblasts, keratinocytes, melanoma, myoblastoma, neuroblastoma or teratocarcinoma cells (Fig. 3C), as well as in RNAs from mouse embryos of various stages (not shown).

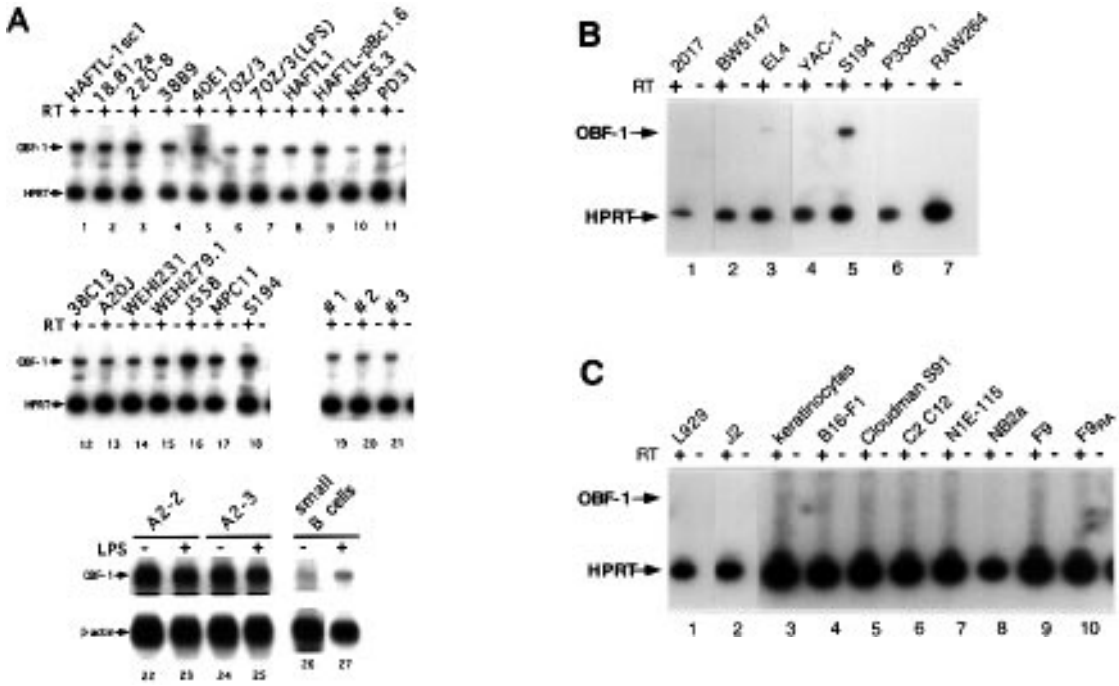

Figure 3. B cell-specific expression of the OBF-1 gene. (**A**) OBF-1 expression in B cells of all stages. RNA samples were examined by RT–PCR (lanes 1–21) or Northern blotting (lanes 22–27). The RNAs used were derived from pro-B (lanes 1 and 19–21), pre-B (lanes 2–11 and 22–25), mature B (lanes 12–15) or plasma cells (lanes 16–18). In lanes 19–21, RNAs from three fetal liver-derived IL-7-dependent pro-B cell lines were used. In lanes 22–25, RNAs from Abelson murine leukaemia virus-transformed pre-B cells were used. In lanes 26–27, RNAs from small resting B cells purified from the spleen by centrifugation over Percoll gradients were used. Where indicated, cells were treated with LPS (10 μg/ml) for 36 h before RNA extraction. (**B**) Lack of expression in other haematopoietic cell lines. The RNAs used were from pre-T cells (lane 1), T cells (lanes 2–4) or monocytes/macrophages (lanes 6 and 7). For comparison, RNA from the B cell plasmacytoma line S194 was also included in lane 5. (**C**) Lack of expression in several other non-haematopoietic cell lines. The RNAs used were from fibroblasts (lanes 1 and 2), keratinocytes (lane 3), melanoma (lanes 4 and 5), myoblastoma (lane 6), neuroblastoma (lanes 7 and 8) or teratocarcinoma cells (undifferentiated in lane 9, differentiated with retinoic acid in lane 10). Expression was measured by RT–PCR with two sets of primers amplifying a fragment of the OBF-1 cDNA or of the HPRT cDNA, respectively. The amplified fragments were run on gels, blotted and hybridized with gene-specific probes. In each case the template for the PCR reaction was a cDNA synthesis reaction that had been performed as indicated with (+) or without (–) reverse transcriptase (RT). The names of the cell lines from which the RNA had been prepared are indicated above the lanes.

Together, these data demonstrate that the OBF-1 gene is expressed in a very highly B cell-specific manner and shows no apparent regulation in the course of B cell differentiation or following B cell stimulation.

Interaction with the POU domain and two general transcription factors

We have previously shown by electrophoretic mobility shift assay (EMSA) that OBF-1 could form a ternary complex on DNA together with either Oct-1 or Oct-2, through specific interaction with their POU domains (29). We wished to determine whether this interaction could also take place in the absence of DNA. For this, we performed *in vitro* interaction assays with radiolabelled OBF-1 protein, prepared by *in vitro* translation, and several immobilized GST fusion proteins, either on filters (Fig. 4A) or by means of glutathione–agarose beads (Fig. 4B). As shown in Figure 4A and B, OBF-1 interacted efficiently with the POU domain of Oct-1 or Oct-2, but not with the GST fusion moiety, with BSA or with itself. For the interaction with the POU domain the first 114 N-terminal amino acids of OBF-1 were sufficient (Fig. 4A), in agreement with our previous data, where ternary complex formation had been scored by EMSA using a series of C-terminal deletions of OBF-1 (29). In addition, this experiment also shows that *in vitro* OBF-1 interacted efficiently with two general transcription factors, the TATA binding protein (TBP) and TFIIB. For this interaction the C-terminal evolutionarily conserved portion of TBP was necessary and sufficient and no interaction was seen with the N-terminal part of TBP (Fig. 4B, lanes 2–4). At present it is unclear whether these interactions with general transcription factors play a functional role in the transcriptional coactivation mediated by OBF-1.

OBF-1 activates promoter but not enhancer octamer sites

Previous evidence from several laboratories had demonstrated that B cells contain two kinds of coactivators for Oct factors. One that mediates the activity of octamer-dependent promoters through interaction with Oct-1 or Oct-2 and one that mediates the activity of multimerized remote (enhancer) octamer sites (23,40). This enhancer coactivator functions only in conjunction with Oct-2 and requires the Oct-2 C-terminal activation domain (37,41,42). We and others had shown that OBF-1, in cell transfections or *in vitro* transcription assays, could coactivate transcription from a promoter octamer site by interacting with Oct-1 or Oct-2 (29–31). The question whether OBF-1 might also be the cofactor permitting enhancer activation in the presence of Oct-2 had not been addressed directly. To test this we therefore compared how reporter plasmids containing or lacking an enhancer octamer site would respond to activation by OBF-1 and Oct-2. In one set of

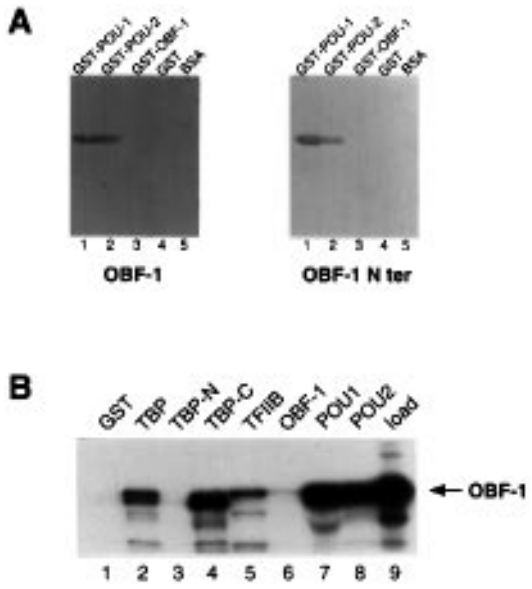

Figure 4. OBF-1 interacts with the POU domain and with the general transcription factors TBP and TFIIB. (**A**) Far Western assay. Filters onto which various recombinant proteins had been transferred by blotting after SDS–PAGE (lanes 1–5, as indicated) were incubated with ^{35}S-labelled *in vitro* translated OBF-1 protein, either full-length (left) or the N-terminal first 114 amino acids (right). Interaction was detected by autoradiography. (**B**) Pull-down assay. Various recombinant GST fusions proteins (lanes 1–8, as labelled above the photograph) were incubated with ^{35}S-labelled OBF-1 full-length protein prepared by *in vitro* translation. The protein complexes were collected by precipitation with glutathione–agarose beads and washed. Subsequently the beads/protein complexes were separated by SDS–PAGE and retention of OBF-1 (interaction) was detected by autoradiography. The following GST fusion proteins were used: lane 1, GST; lane 2, GST–TBP full-length; lane 3, GST–TBP-N (amino acids 1–159); lane 4, GST–TBP-C (amino acids 159–339); lane 5, GST–TFIIB full-length; lane 6, GST–OBF-1 full-length; lane 7, GST–Oct1 POU domain; lane 8, GST–Oct2 POU domain. Lane 9 (load), 10% of the starting material used in the binding reactions.

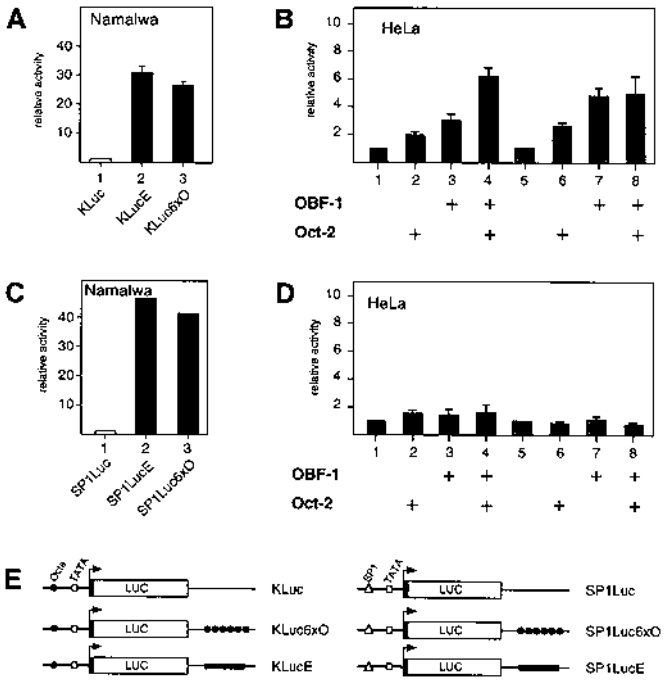

Figure 5. OBF-1 activates transcription from proximal (promoter) but not from remote (enhancer) octamer sites. (**A** and **C**) A synthetic enhancer consisting of six octamer sites (6xO) is highly active in B cells, independently of the promoter used. Namalwa B cells were transfected with the indicated reporter plasmids based either on an octamer site-containing κ light chain Ig promoter (A) or on an artificial promoter containing an SP1 binding site (C). The reporter plasmids contained no enhancer (Kluc and SP1Luc, open bars), an SV40 enhancer (KLucE and SP1LucE, solid bars) or an enhancer octamer site (KLuc6xO and SP1Luc6xO, grey bars). Two days after transfection cell extracts were prepared and luciferase activity was measured with equal protein amounts. The activity obtained with the reporter lacking an enhancer was arbitrarily set to 1. (**B** and **D**) Co-transfection of OBF-1 and Oct-2 does not activate enhancer octamer sites. HeLa cells were co-transfected with the reporter plasmids KLucE (B, solid bars), KLuc6xO (B, grey bars), SP1LucE (D, solid bars) or SP1Luc6xO (D, grey bars) and either an empty expression vector (lanes 1 and 5) or an expression vector encoding OBF-1 or Oct-2 (lanes 2–4 and 6–8). Cell extracts were prepared 2 days after transfection and equal protein amounts were used for the luciferase assays. The activity of the reporter co-transfected with the empty expression vector was arbitrarily set to 1. In non-B cells, the reporters containing the 6xO enhancer have an activity similar to that of corresponding reporters with no enhancer. The data presented represent the average of at least three independent experiments and the standard deviations are indicated. (**E**) Schematic structure of the reporter plasmids used. The κ light chain gene promoter which drives expression of the luciferase gene is indicated by a thick black line; the position of the TATA box (TATA, open square) and of the octamer site (Octa, solid circle) are indicated. KLuc contains no enhancer; KLuc6xO contains an artificial enhancer consisting of six copies of an IgH enhancer-derived octamer site downstream of the luciferase gene (solid circles); KLucE contains the SV40 enhancer (solid rectangle). Similar constructs containing an SP1-dependent promoter are depicted at the bottom (SP1Luc, SP1Luc6xO and SP1LucE). The SP1 binding site in the promoter is indicated by an open triangle.

experiments (Fig. 5A and B) we used plasmids with the luciferase gene under the control of an Ig κ light chain gene promoter. This promoter contains an octamer site and we have previously shown that it is well activated by OBF-1 (29). When transfected into B cells, the reporter with the synthetic enhancer octamer site(KLuc6xO) was, as expected (43,44), 25- to 30-fold more active than the reporter with no enhancer (Fig. 5A). By contrast, when transfected into HeLa cells (or other non-B cells; not shown) the KLuc6xO reporter was not better activated by OBF-1 and Oct-2 than a reporter lacking the 6xO enhancer (Fig. 5B). This complete inactivity of the enhancer octamer site in response to transactivation by Oct-2 and OBF-1 was observed over a range of effector and reporter plasmid concentrations in the transfection assays and did not reflect insufficient expression of the proteins.

In a second set of experiments we used similar reporter plasmids under the control of a promoter containing a binding site for the ubiquitous transcription factor SP1. When tested in the context of this plasmid, the synthetic enhancer octamer site was, as expected, highly active in B cells (Fig. 5C). However, in HeLa cells the synthetic enhancer was again not activated by OBF-1 and Oct-2 (compare SP1Luc6xO with SP1LucE in Fig. 5D). Thus, independently of the promoter present in the reporter plasmid, it appears that OBF-1 and Oct-2 are not sufficient to activate a synthetic enhancer octamer in non-B cells. Similar findings have been reported very recently by Pfisterer *et al.* (45).

DISCUSSION

In this report we have presented the cloning and analysis of the murine homologue of the B cell-specific coactivator OBF-1. This factor is also known as Bob-1 (30) or OCA-B (31). We have shown

that expression of OBF-1 was found almost exclusively in B cells and that cells of related lineages, such as T cells or monocytes/macrophages, did not express this gene. One exception was the T cell line EL-4, a line that also expresses high levels of the Oct-2 RNA and protein (46). Interestingly, B cells of all stages expressed roughly equivalent levels of OBF-1 RNA, including very early cells such as primary pro-B cells, which are in the process of rearranging the D and J segments of their Ig genes (47). This finding suggests that OBF-1 may already play a role at an early stage of B cell development.

We have shown that OBF-1 can interact *in vitro* in the absence of DNA with the POU domain of Oct-1 or Oct-2 (Fig. 4). Thus, OBF-1–Oct complexes can form both on DNA (29–31) and off DNA. There is no evidence that OBF-1 itself binds to DNA or that it stabilizes the Oct–DNA interaction (29,31). Therefore, the mechanism of coactivation is unlikely to be a modulation of DNA binding by Oct factors.

We have found that OBF-1 interacts *in vitro* with the general transcription factor TBP. This interaction takes place with the C-terminal evolutionarily conserved domain of TBP, which has been shown to interact with several transcription factors (48,49). It is interesting to note that the POU domains of Oct-1 and Oct-2 also interact *in vitro* with TBP (50). Thus, one could envisage that the OBF-1–POU domain complex (as an OBF-1–Oct complex) might interact better with TBP than its individual components and this might be part of the coactivation mechanism. However, this hypothesis remains to be tested experimentally. In addition, OBF-1 was found to also interact with TFIIB, the second major target for upstream factors (51,52).

Experiments by Gstaiger *et al*. showed that OBF-1/Bob-1 activates transcription by itself when directly tethered to DNA by the Gal4 DBD (30). This indicates that OBF-1 contains a transcription activation domain, and our own analysis confirms this observation (data not shown). However, coactivation seems to also require the activation domains of Oct factors, since *in vitro* transcription experiments by Luo and Roeder (31) showed that OBF-1/OCA-B did not activate transcription when indirectly tethered to the DNA through interaction with just the POU domain, as if some interplay with the activation domain(s) was necessary (31). Therefore, one might envisage a scenario whereby OBF-1 activates transcription both by bringing its own activation domain and by perhaps helping Oct factors to present their own activation domains. The targeting of TBP and TFIIB by OBF-1 may be yet another aspect of the coactivation mechanism. It is clear, however, that a lot more work will have to be done to precisely define the mechanism of transcriptional activation by this cell-specific coactivator.

Previous experiments have shown that in B cells there are two pathways of gene activation through octamer sites (23,40): (i) activation from a promoter octamer site requires Oct-1 or Oct-2 and a cell-restricted coactivator (28); (ii) activation from a remote enhancer octamer site requires Oct-2 and a cell-restricted coactivator (44). Several lines of evidence suggested that the coactivators for promoter and for enhancer activation were different. First, they have different requirements with respect to Oct factors: the enhancer cofactor requires exclusively Oct-2 and in particular its C-terminal activation domain (40,41). This was further demonstrated by the use of Gal4 fusion proteins. There it was shown that a protein containing the Oct-2 C-terminal activation domain fused to the Gal4 DNA binding domain mediated transcription activation from remote Gal4 binding sites in B cells, but not in fibroblasts (37,42). Furthermore, in these experiments co-transfection of an OBF-1 expression vector had no influence on the inactivity of the Gal fusion proteins in fibroblasts, in agreement with the finding that OBF-1 interacts biochemically with the POU domain of Oct-2 (29,31). The data presented here directly show that OBF-1, while efficiently activating promoter octamer sites, does not activate a remote enhancer octamer site and this independently of the promoter present in the reporter plasmid. Similar observations were also made recently by another group (45). Therefore, the enhancer cofactor synergizing with the C-terminal activation domain of Oct-2 remains to be molecularly identified and cloned.

Apart from the various mechanistic aspects of coactivation, another key question concerning OBF-1 is the regulation of its expression and most importantly its physiological role in transcription in B cells, in particular in Ig gene transcription. This will be assessed by the generation of a mouse strain deficient for the OBF-1 gene and these experiments are currently in progress in our laboratory.

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