BOB.1/OBF.1 is a lymphocyte-restricted transcriptional coactivator. It binds to the Oct1 and Oct2 transcription factors and increases their transactivation potential. Targeted gene disruption experiments revealed that BOB.1/OBF.1 is critical at different stages of B cell development. A large number of genes expressed in B cells contain octamer motifs in their regulatory regions. However, only few genes have been described so far whose expression is dependent on BOB.1/OBF.1. To understand the molecular basis of BOB.1/OBF.1 function in B cell development, we searched for BOB.1/OBF.1 target genes by expression profiling. We have identified genes both induced and repressed by BOB.1/OBF.1. Using different genetic systems, we demonstrate regulation of a selection of these genes. Identified targets included genes encoding Ahd2-like, AKR1C13, Rbp1, Sdh, Idh2, protocadherin γ, α-catenin, Ptprs, Id3, and Creg. Classification of BOB.1/OBF.1 target genes by function suggests that they affect various aspects of B cell physiology such as cellular metabolism, cell adhesion, and differentiation. To better understand the mechanism of BOB.1/OBF.1 action, we cloned the promoter of the gene encoding Ahd2-like, the gene showing the strongest regulation by BOB.1/OBF.1. This promoter indeed contains a perfect octamer motif. Furthermore, the motif was recognized by the Oct transcription factors as well as BOB.1/OBF.1. Using different genetic systems, we demonstrate regulation of a selection of these genes. Identified targets included genes encoding Ahd2-like, AKR1C13, Rbp1, Sdh, Idh2, protocadherin γ, α-catenin, Ptprs, Id3, and Creg. Classification of BOB.1/OBF.1 target genes by function suggests that they affect various aspects of B cell physiology such as cellular metabolism, cell adhesion, and differentiation. To better understand the mechanism of BOB.1/OBF.1 action, we cloned the promoter of the gene encoding Ahd2-like, the gene showing the strongest regulation by BOB.1/OBF.1. This promoter indeed contains a perfect octamer motif. Furthermore, the motif was recognized by the Oct transcription factors as well as BOB.1/OBF.1. Using different genetic systems, we demonstrate regulation of a selection of these genes. Identified targets included genes encoding Ahd2-like, AKR1C13, Rbp1, Sdh, Idh2, protocadherin γ, α-catenin, Ptprs, Id3, and Creg. Classification of BOB.1/OBF.1 target genes by function suggests that they affect various aspects of B cell physiology such as cellular metabolism, cell adhesion, and differentiation. To better understand the mechanism of BOB.1/OBF.1 action, we cloned the promoter of the gene encoding Ahd2-like, the gene showing the strongest regulation by BOB.1/OBF.1. This promoter indeed contains a perfect octamer motif. Furthermore, the motif was recognized by the Oct transcription factors as well as BOB.1/OBF.1. Using different genetic systems, we demonstrate regulation of a selection of these genes. Identified targets included genes encoding Ahd2-like, AKR1C13, Rbp1, Sdh, Idh2, protocadherin γ, α-catenin, Ptprs, Id3, and Creg. Classification of BOB.1/OBF.1 target genes by function suggests that they affect various aspects of B cell physiology such as cellular metabolism, cell adhesion, and differentiation.

BOB.1/OBF.1, also known as Bob.1, OFB-1, or OCA-B, was originally identified as a B cell-specific factor that acts as a coactivator for immunoglobulin gene transcription (1–5). Early in vitro transfection experiments had suggested a crucial role for the cell type-specific activity of immunoglobulin promoters (3, 6). BOB.1/OBF.1 shows only weak affinity for its target site, the octamer motif (7). It is primarily recruited to target promoters via interaction with one of the two transcription factors, Oct1 and Oct2. BOB.1/OBF.1 makes weak contacts with the major groove of the DNA as well as with the octamer transcription factors. This latter interaction involves the POU domains of Oct1 and Oct2. BOB.1/OBF.1 interacts with both the POU-specific domain and the POU homeodomain (8–10). It thereby acts as a molecular clamp that holds together these subdomains on DNA (11).

The octamer motif, the binding site for Oct1 and Oct2, is found in a large number of promoter sequences of genes expressed in B cells. Indeed, the octamer motif was shown to be a critical regulatory element for B cell-specific transcription (12–14). It is conserved in virtually all IgH and L chain gene promoters as well as in several Ig enhancer elements. Additionally, several other B cell-specific genes depend on functional octamer motifs for their B cell specific transcription. However, the octamer motif was also shown to be present and important for a variety of ubiquitously expressed genes, such as the histone H2B gene and most uridine-rich small nuclear RNA genes (15, 16).

The B cell-specific activation of octamer-dependent transcriptions from promoter-proximal positions critically depends on the coactivator BOB.1/OBF.1 (1, 4, 5, 17). In contrast, the activity from distal enhancer positions specifically requires the presence of Oct2 and a coactivator, apparently distinct from BOB.1/OBF.1 (2, 5, 18, 19). However, recent evidence suggests that Oct2 and BOB.1/OBF.1 can functionally interact with the 3′ IgH enhancer element (20, 21).

BOB.1/OBF.1 expression is largely restricted to B cells (22). However, expression of functional BOB.1/OBF.1 can be also induced in T lymphocytes by costimulation (23). It is expressed at all stages of B cell development, albeit at various levels. The highest expression level of BOB.1/OBF.1 was found in germinal centers and germinal center-derived B cell lymphomas (24, 25). The increased expression levels in germinal center B cells are in part due to increased transcription (24). In addition, BOB.1/OBF.1 levels are also regulated at the level of protein stability (26, 27).

Targeted gene disruption experiments revealed that BOB.1/OBF.1 is essential for B cell development, preferentially for antigen responses leading to secondary isotype production (2, 28, 29). The most prominent characteristic of BOB.1/OBF.1 knockout mice is the complete failure to form germinal centers upon immunization with thymus-dependent antigens. Although BOB.1/OBF.1-deficient mice are clearly defective in terminal B cell differentiation and cannot participate in germinal center reaction, they still proliferate normally when stimulated with lipopolysaccharide or CD40 in the presence of interleukin-4. They also induce isotype switching at a normal rate when stimulated in vitro, indicating that they are similar to normal resting B cells despite some alteration in the Ig
surface phenotype (reduced levels of IgD). Surprisingly, in mice simultaneously deficient for BOB.1/OBF.1 and Oct2, B cell development to the IgM-positive stage and immunoglobulin gene transcription were not affected (30). A more detailed analysis of BOB.1/OBF.1-deficient mice revealed that this cofactor is not only important for B cell development at late stages in secondary lymphoid organs, where the number of B cells is critically reduced. BOB.1/OBF.1 is also essential for earlier stages of B cell development in the bone marrow, namely in the transition of immature B cells to the periphery (31, 32). A BOB.1/OBF.1 protein isoform bearing an N-terminal extension is myristoylated in vivo and localized in the membrane fraction. The exact function of this BOB.1/OBF.1 isoform is not known (33).

Despite the fact that a large number of genes specifically expressed in B cells contain octamer motifs in their regulatory regions, only a small number of BOB.1/OBF.1-dependent genes has been described. The BRL1 gene is cooperatively regulated by NF-κB, BOB.1/OBF.1, and Oct2 in B cells (34). The CCR-5 gene was found to be regulated by BOB.1/OBF.1 and Oct-1 in T cells (35). In addition, BOB.1/OBF.1 activates the B cell-specific B29 and mb1 promoters (36). Similarly, only few Oct-2-dependent genes have been described, such as CD36 (37) and Crisp-3 (38). For the CD36 gene it was shown that Oct-2 regulates transcription by both BOB.1/OBF.1-dependent and -independent mechanisms (39). Recent experiments suggested that there are distinct octamer sequences that favor or disfavor recruitment of BOB.1/OBF.1. These sites were called POREs (BOB.1/OBF.1-responsive) and MOREs (nonresponsive). They may define a structural basis for BOB.1/OBF.1-regulated genes (40, 41).

To understand the B cell phenotype of the BOB.1/OBF.1-deficient mice, we searched for BOB.1/OBF.1 target genes in B cells. In this study, we used oligonucleotide microarrays to identify a large number of genes that were consistently induced or repressed by BOB.1/OBF.1. The specific changes in gene expression observed suggest new mechanisms for the biological functions of BOB.1/OBF.1 in B cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Abelson virus-transformed pre-B cell lines of BOB.1/OBF.1-deficient C57Bl/6 mice (clone MB10) (29) were established as described (42). Cells were kept in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin/streptomycin, and 50 μg/ml β-mercaptoethanol and grown at 37 °C and 10% CO₂. The generation of viruses expressing the BOB.1/OBF.1-ER fusion protein as well as the infection of BOB.1/OBF.1-deficient Abelson virus-transformed pre-B cells (MB10) with these viruses to establish a BOB.1/OBF.1-deficient cell line stably carrying the BobER gene are described (43).

NIH/3T3 and Jurkat cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal calf serum, antibiotics, and 50 μg/ml β-mercaptoethanol and grown at 37 °C and 5% CO₂. Transfections of NIH/3T3 cells were performed by electroporation (Bio-Rad) with 450 V and 250 microfarads in phosphate-buffered saline.

Pre-B cell lines were established by culturing bone marrow cells from mice of the indicated genotype under the stimulatory influence of irradiated adherent stromal cells ST2 and the cytokine interleukin-7. The culture medium used was Iscove's modified Dulbecco's medium supplemented with 2% fetal calf serum, antibiotics, and 50 μg/ml β-mercaptoethanol. Cells were grown at 37 °C and 10% CO₂ in the presence of supernatant from interleukin-7-producing cells J558 and Primatone (A. Rolink, Basel).

**Transgenic Mice**—The generation of mice, named 1.5/B1, is described elsewhere (31).

**Plasmoids**—The Ahd2 [alddehydrogenase 2-like] promoter was cloned into the pTKL2 vector containing the herpes simplex virus-thymidine kinase promoter (−105 to +52) from the pBLcat2 in front of the firefly luciferase coding region. The herpes simplex virus-thymidine kinase promoter was excised by a restriction endonuclease digest using HindIII and BglII and replaced by the Ahd2-like promoter cloned via genomic PCR using the following primers: Ahd2-B promoter, BglII (sea cga atg tgt aga ctc gc); Ahd2-like promoter HindIII (ACA AAG CTT AGA GGG AGG GC). The Oct-binding site mutant of the Ahd2-like promoter was created by in vitro mutagenesis according to the instruction manual of the QuikChange™ site-directed mutagenesis kit (Promega) using the following primers: 4× Oct mutant T2G, GST, CTA, AGC forward, TAT TTC CCG CAT TCA AAA TGC TCG CCT GGT GCA (31); 4× Oct mutant T2G, GST, CTA, AGC reverse (CAT GCA CTA AGG GTG ATA TTA GCG TCA GGA TAT G).

The wild type BOB.1/OBF.1 cDNA was cloned into the expression vector pcDNA3. For luciferase reporter assays, the octamer-dependent reporter plasmids μETI (43) and the 1× wt (5) were used as internal transfection controls. For in vitro transfection of BOB.1/OBF.1, the Pmt/IPKA-Bob expression vector was used (5).

**DNA Microarray Analysis**—Total RNA was extracted from frozen cell pellets by using Trizol® reagent (Invitrogen). RNA was purified on RNeasy Mini columns (Qiagen) for RNA cleanup and DNase treatment (RNase-Free DNase Set Protocol, Qiagen). Biotin-labeled cRNA for hybridization was prepared as described (44). 10 mg of fragmented cRNA in 200 μl of hybridization solution were hybridized onto the Affymetrix Gene Chip Multi1k array set (sub A, B), representing over 11,000 known murine genes and expressed sequence tags. Images were scanned at 3-μm resolution using a Hewlett Packard GeneArray Scanner. Data analysis was performed using Affymetrix software. Expression levels of BobER-infected and OHT-stimulated samples were compared with expression levels of empty virus-infected, OHT-treated controls. Each of the three comparisons was performed using three separately isolated sets of mRNA preparations derived from independent cellular harvests (experiments 1, 2, and 3). The genes that showed substantial regulation were selected based on the following criteria: 1) absence of genes with “absent calls” in both samples of a pairwise comparison and 2) a threshold of a 2-fold change in expression level in each of the three pairwise comparisons. This stringent threshold is based on our observation that no single gene showed a more than 2-fold change in triplicate control experiments (triplicate comparative hybridizations of BobER plus OHT samples that were separately harvested, transfected, and harvested; RNAs from BobER plus OHT experiment 1 versus experiment 2, experiment 1 versus experiment 3, and experiment 2 versus experiment 3) and effectively eliminates false positive calls due to technical and sampling variability.

**Northern Blot Analysis**—Total cytoplasmic RNA was prepared using the Trizol® reagent (Invitrogen) according to the protocol of the manufacturer. After separation of RNAs on a 1% formaldehyde agarose gel, Northern blot analyses were performed as described (43).

**RT-PCR**—Total RNA was isolated using the High pure RNA isolation kit (catalog no. 1828665, Roche Applied Science) and reverse transcribed using M-MLV reverse transcriptase (Invitrogen). For the PRBs, the following primers were used: Ahd2-like forward, GCA GTC CCT GCT GAC AAG ATC; Ahd2-like reverse, TCC ATG TGG GAG GAT GTC GCT; MAPKAPK2 forward, CCT GTG ACG TTT GGT GAT GTC; MAPKAPK2 reverse, GGT GTG CAG TGG ATG CTG AGC; Pcl-1 forward, CTC CAT CAG CAT GTC ATC AGG GAT CCT; Pcl-1 reverse, GTG ACT ACC GAA CCTCCA CAT G; Shh forward, GCT CCA GGT CCT GGA TCG AGG; Shh reverse, GCC CCA GGT CCT GGA TCG AGG; TRFm forward, CCT GGG CCC GGA CCT GGA TCG; TRFm reverse, GCC CCA AAC TGT CCT AGA GGA CGG; BOB.1/OBF.1 forward, GAA CCT CTT GCC CCA CCA AGG; BOB.1/OBF.1 reverse, GAG GTT GAT ACT GCA GCC TGG AGG TG; β-actin forward, GGT CAG AAG GAC CCT TAT GTG AG; β-actin reverse, AGA GCA ACA TAG TCG GCC TTC; TGC TCC TTC; TGC TCC TCG; J558 family forward, ATG ATC CCG ATT GAC GAG GAT GAT G; J558 family reverse, ATG ATC CCG ATT GAC GAG GAT GAT G.

**EMSAs**—Preparation of whole cell extracts for EMSA and the protocol of the EMSA procedure have been described earlier (45, 46). For EMSA, the following oligonucleotides were annealed and subsequently labeled using [α-32P]dCTP in a fill in reaction: Ahd2-like BS forward, GGT CAG AAG GAC CCT TAT GTG AG; Ahd2-like BS reverse, GGA CCA GAC CCT TAT GTG AGC; Ahd2-like BS forward, GTC AAG GAC CCT TAT GTG AGC; Ahd2-like BS reverse, GGC CCA GGT CCT GGA TCG AGG; Ahd2-like BS forward, GGA CCA GAC CCT TAT GTG AGC.

1 The abbreviations used are: RT, reverse transcription; ChIP, chromatin immunoprecipitation; PLC, phospholipase C; V₅₂, immunoglobulin heavy chain variable; RNAi, RNA interference; EMSA, electrophoretic mobility shift assay; BobER, fusion protein of BOB.1/OBF.1 and estrogen receptor; OHT, hydroxy tamoxifen; MAPKAPK, mitogen-activated protein kinase-activated protein kinase.
Genes are listed in order of -fold induction in experiment 1 (BobER1). Each independent experiment compared gene expression levels between BobER-infected, OHT-stimulated samples and empty virus-infected, OHT-treated controls.

| Accession no. | BobER1 | BobER2 | BobER3 |
|---------------|--------|--------|--------|
| U96401        | 8.9    | 10.1   | 7.0    |
| AF084524      | 5.8    | 8.8    | 5.9    |
| M11024        | 3.2    | 2.3    | 2.5    |
| AB027125      | 2.5    | 2.6    | 2.6    |
| P01747        | 2.5    | 2.4    | 2.7    |
| M289498       | 2.5    | 2.0    | 2.9    |
| C77421        | 2.4    | 2.6    | 2.2    |
| U88672        | 2.1    | 4.7    | 7.9    |
| W61814        | 2.1    | 2.4    | 6.0    |
| PH09844       | 2.0    | 4.4    | 9.2    |
| U88670        | 2.0    | 3.7    | 7.4    |
| PH09784       | 2.0    | 2.3    | 2.3    |

and used for specific competition. The indicated amount of oligonucleotide was added to the EMSA reaction mixture. For supershift experiments, an anti-Oct1-antibody (serum), anti-Oct2-antibody (C-20; catalog no. sc-233; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or anti-Oct3/4-antibody (serum) were divided into 1-ml aliquots and incubated overnight on an overhead shaker with one of following antibodies: 1 mg of relevant affinity-purified polyclonal antibody against PLCγ1, cytosine-rich secretory protein 1 (Crap), EST, similar to virus-like (VL30) LTR, Immunoglobulin heavy chain variable region (J558 family) heavy chain, and Immunoglobulin heavy chain variable region (J558 family) heavy chain.

RESULTS

BOB.1/OBF.1 Targets Identified with BobER—In order to identify BOB.1/OBF.1-regulated genes, we used an Abelson virus-transformed bone marrow-derived pre-B cell line from BOB.1/OBF.1-deficient mice (43). These cells were either infected with a retroviral vector expressing an inducible BobER fusion protein, or as a control, they were infected with the parental vector (43). In the BobER-expressing cell line (MB10-K10), octamer-dependent transcription was nondetectable, unless the activity of the BobER protein was induced by treatment of these cells with tamoxifen (OHT).

For microarray analysis, BobER and parental vector-infected cells were induced for 12 h with OHT. We reasoned that direct BOB.1/OBF.1 targets would have increased or decreased in expression by this time, yet secondary changes in RNA levels should be low. RNA was prepared from three independent experiments and hybridized to Affymetrix DNA microarrays with the capacity to display transcript levels of some 11,000 murine genes and expressed sequence tags. Based on control experiments (see “Experimental Procedures”), we applied a threshold of a 2-fold change in expression level of all three independent experiments for identifying putative BOB.1/OBF.1 targets.

Based on these criteria, 12 genes were found to be up-regulated, and 10 were found to be down-regulated in all three experiments (listed in Table I). Of the 22 genes regulated in all three experiments, none was previously reported as BOB.1/OBF.1 target. Genes regulated by BOB.1/OBF.1 are implicated in the control of cellular metabolism (Ahd2-like, AKR1C13, Rbp1 (retinol-binding protein 1), Sdh, Idh2), cell adhesion (protocadherin γ A1, catenin α 1, Ptprs), and differentiation (Id3 (inhibitor of DNA binding 3), Creg (cellular repressor of E1A-regulated genes)). Furthermore, several members of the J558 family of immunoglobulin heavy chain variable (VH) region were found.
Several genes involved in signal transduction were found to be up-regulated (PLCγ1, TRAFamn) or repressed (MAPKAPK) in the presence of BOB.1/OBF.1 in only two of three experiments (data not shown). Regulation of these genes by BOB.1/OBF.1 was confirmed in appropriate genetic systems (see below). This observation further confirms the stringency of the threshold we have applied to identify BOB.1/OBF.1-responsive genes.

Target Verification by Northern Blot Analysis

To verify the gene expression data by an independent method, we chose several of the target genes listed in Table I for Northern blot analysis. We picked six identified targets, namely Ahd2-like, Creg, Crisp, Rbp1, thymosin β, and Id3, and studied their expression. Using RNA from OHT-treated parental vector-infected or BobER-expressing cells, we could show that the identified genes are indeed differentially expressed in these two cell populations (Fig. 1). Furthermore, the Northern blot analyses of putative target genes in these two cell populations revealed a clear concordance to the microarray screen data for all of the genes tested (see Fig. 1).

Altered Expression of BOB.1/OBF.1 Targets in BOB.1/OBF.1-deficient B Cells—The expression of these genes was investigated in primary B cells by RT-PCR. We first studied interleukin-7-dependent pre-B cell lines established from different genotypes. Fig. 2A shows the results from RT-PCRs using RNAs from pre-B cells of heterozygous (subsequently called wild type cells; there are no differences in the phenotype of wild type or heterozygous mice) or BOB.1/OBF.1-deficient genotype. In addition to Ahd2-like and Sdh as representatives for genes up- or down-regulated in all three DNA-chip experiments, we also included two genes (TRAFamn and PLCγ1) up-regulated and one gene (MAPKAPK) down-regulated in only two of three experiments. For the up-regulated genes analyzed, we observed a stronger gene-specific signal in wild type cells in comparison with BOB.1/OBF.1-deficient B cells. In contrast, in the case of the down-regulated genes, we detected a stronger gene-specific signal in BOB.1/OBF.1-deficient B cells.

In addition, we analyzed BOB.1/OBF.1-deficient cells carrying a tet-regulated BOB.1/OBF.1 transgene (Fig. 2B). Since the tet-off system was used, the presence of doxycyclin prevents the expression of the transgenic BOB.1/OBF.1 (31) and thus the expression of BOB.1/OBF.1-stimulated genes. Conversely, the expression of BOB.1/OBF.1-repressed genes is increased in the presence of doxycyclin (Dox; Fig. 2B).

Taken together, in both systems, we observed a regulation of BOB.1/OBF.1-dependent genes, which were up- or down-regulated consistent with the microarray data. This BOB.1/OBF.1 dependence could also been seen in the cases of genes regulated in only two of three DNA-chip experiments, such as TRAFamn, PLCγ1, and MAPKAPK. For these genes, we also detected a clear regulation by BOB.1/OBF.1 in both analysis systems used.

Identification of V_H Regions as BOB.1/OBF.1 Targets—In addition to the genes analyzed so far, the microarray screen also revealed 20 immunoglobulin gene variable region sequences regulated by BOB.1/OBF.1 (five sequences regulated in all three experiments and 15 sequences regulated in two of three experiments). All of these 20 immunoglobulin genes code for V_H regions of the J558L family. To test whether the expression of J558 V_H genes is dependent on BOB.1/OBF.1, we performed RT-PCR using J558 family-specific primers (47). In semiquantitative RT-PCR experiments using BOB.1/OBF.1-deficient BobER pre-B cells or control mock-infected cells and J558 family-specific primers, we observed an increase of J558 mRNA in OHT-treated BobER cells (Fig. 3), indicating that BOB.1/OBF.1 regulates the expression of V_H genes in the J558 V_H family.

Identification of the Ahd2-like Gene as Direct BOB.1/OBF.1 Target—Since BOB.1/OBF.1 is a transcriptional coactivator
that binds to promoter DNA in a complex with octamer transcription factors, we analyzed the promoters of potential BOB.1/OBF.1-regulated genes for octamer binding sites. In the case of CRBP (48) and Id3 (49), the published parts of the promoters did not contain obvious octamer binding sites. Most of the other target gene promoters have not been published. The strongest BOB.1/OBF.1-regulated gene according to the microarray analysis encodes for the Ahd2-like protein. This gene belongs to a family of aldehyde dehydrogenase proteins, comprising at least 145 members in different organisms. In humans, 16 genes coding for aldehyde dehydrogenases have been identified, including Ahd2. Ahd2 and Ahd2-like were grouped to the same subfamily on the basis of their sequence similarity. The promoter sequence of the murine Ahd2 gene was published (50). It contains one perfect (ATGCAAAT) octamer motif. Using the Celera discovery system, we identified the Ahd2-like promoter and found it to be very similar to the Ahd2 promoter close to the transcription start site. It differs markedly from the Ahd2 promoter only in the more 5' region. Importantly, the Ahd2-like promoter contains the same perfect octamer motif as the Ahd2 promoter (Fig. 4A).

To determine whether the activity of this promoter depends on BOB.1/OBF.1, we cloned the promoter DNA from genomic DNA by PCR. Additionally, the octamer motif ATGCAAAT was mutated to AgtacAAT (M). This mutation should abolish both BOB.1/OBF.1 and Oct1/Oct2 binding to DNA.

To test the binding ability of Oct proteins to the wild type and mutant promoters, we performed electrophoretic mobility shift assays (Fig. 5A). For this purpose, protein extracts from BobER pre-B cells stimulated for 24 h with lipopolysaccharide (50 μg/ml) were incubated with a labeled oligonucleotide representing the region −67 to −34 of the Ahd2-like promoter, encompassing the octamer motif. As a control, a parallel EMSA was performed with the consensus octamer motif as the AH32 promoter (Fig. 5B). In supershift experiments, we could demonstrate that the shifted complexes contain the proteins Oct1 or Oct2 (Fig. 5). Furthermore, complex formation could be efficiently inhibited by competition with the unlabeled wild type oligonucleotide. In contrast, the mutated octamer motif failed to compete for Oct1 and Oct2 binding (Fig. 5A). We then asked whether BOB.1/OBF.1 was able to form a ternary complex on the Ahd2-like promoter octamer. Therefore, in vitro translated BOB.1/OBF.1 was added to nuclear extracts from HeLa cells and tested with a consensus octamer probe and the Ahd2-like promoter. In both cases BOB.1/OBF.1 induced a supershift, which could be reversed by the addition of the BOB.1/OBF.1-specific antibody or even further supershifted by an Oct1-specific antibody. Since HeLa cells do not contain Oct2, the addition of the anti-Oct2 antibody served as a negative control (Fig. 5B).

We then wanted to investigate whether Ahd2-like promoter activity is influenced by BOB.1/OBF.1 in transfection experiments. Therefore, the wild type and mutant Ahd2-like promoters were cloned in front of a luciferase reporter gene. Two control constructs were used to compare the Ahd2-like promoter activity with known octamer-dependent promoters. The first one bears a synthetic promoter, containing a single perfect copy of the octamer motif upstream of a TATA box. The second control reporter contains an immunoglobulin κ promoter plus the intronic heavy chain enhancer. Transfection experiments in NIH/3T3 fibroblasts (no endogenous BOB.1/OBF.1) revealed that the activity of the Ahd2-like promoter could be increased by co-transfection of BOB.1/OBF.1 (Fig. 6A). Likewise, the control promoters were induced by BOB.1/OBF.1 co-transfection. Mutation of the octamer motif in the Ahd2-like promoter reduced activity by a factor of 3, indicating that this motif contributes to basal promoter activity in fibroblasts. Importantly, however, the activity of the mutant promoter was virtually unaffected by BOB.1/OBF.1.

In addition, we analyzed the activity of the Ahd2-like promoter constructs in Jurkat T cells. In this cell line, BOB.1/OBF.1 activity is inducible by treatment with phorbol 12-myristate 13-acetate and ionomycin (23). Phorbol 12-myristate 13-acetate/ionomycin treatment resulted in a large increase in the activities of the Ahd2-like promoter as well as the control promoters (Fig. 6B). This induced activity was largely decreased for the mutant promoter construct. These results indicate that the activity of the Ahd2-like promoter is influenced by the presence and activity of BOB.1/OBF.1, and this requires an intact octamer site.

To analyze the ability of Oct1, Oct2, and BOB.1/OBF.1 to bind to the Ahd2-like promoter in vivo, we performed ChIP assays. After OHT treatment of BobER cells, protein-DNA complexes were cross-linked and precipitated with antibodies specific for Oct1, Oct2, BOB.1/OBF.1, or PLCγ1. After immunoprecipitation, DNA was amplified by PCR using promoter-specific primers. A signal indicates that the analyzed proteins were bound in vivo to the promoters being amplified. The analysis of the H2B promoter, which is known to contain a functional octamer motif in the promoter, served as internal positive control. This promoter was bound by Oct1, Oct2, and BOB.1/OBF.1 in vivo. Similarly, the Ahd2-like promoter region

---

**Fig. 2.** RT-PCR analysis of putative BOB.1/OBF.1 targets. RNA was prepared from pre-B cell lines established from either BOB.1/OBF.1-heterozygous or -deficient mice (A) or BOB.1/OBF.1-deficient cells expressing BOB.1/OBF.1 in a doxycyclin (Dox)-dependent manner (1.5/B1−/−) (B). Gene-specific RT-PCR analyses were performed. The RT-PCRs for β-actin and BOB.1/OBF.1 were included as internal controls. MAPKAPK, mitogen-activated protein kinase-activated protein kinase.

**Fig. 3.** Semiquantitative RT-PCR analysis of the J558 family of \(V_\text{H} \) genes. Mock- or BobER-infected pre-B cells were treated with OHT and compared for the expression of J558 genes by semiquantitative RT-PCR analyses using J558 family-specific primers.
could be precipitated with all three specific antibodies. The specificity of the ChIP experiment was demonstrated by several controls. No signals were obtained either when we used no antibody or a PLCγ1/H9253-specific antibody or when we analyzed the Pax5 promoter, which lacks an octamer motif (Fig. 7A). As an additional control, we performed ChIP analyses on BOB.1/OBF.1-deficient pre-B cells. Whereas Oct1 and Oct2 still interacted with the H2B and Ahd2-like promoters, no BOB.1/OBF.1 binding was detectable (Fig. 7B). These data clearly show that BOB.1/OBF.1 binds specifically to the Ahd2-like promoter DNA in a complex together with either Oct1 or Oct2.

**DISCUSSION**

To explain the phenotype of BOB.1/OBF.1-deficient mice and to get more insights into the molecular function of this transcriptional coactivator, we searched for BOB.1/OBF.1 targets using oligonucleotide microarrays. In this screen, we identified several target genes regulated by BOB.1/OBF.1. The genes we identified are involved in many physiological processes of B cells: cellular metabolism, cell adhesion, and differentiation. This implies that BOB.1/OBF.1 is an important protein for B cell physiology and function. Interestingly, we did not identify other transcription factors, suggesting that BOB.1/OBF.1-regulated genes are at the end point of a cellular differentiation program.

To our surprise, we identified approximately the same number of activated genes compared with repressed genes. This was unexpected, since BOB.1/OBF.1 was typically described as a transcriptional coactivator. At this stage, we do not know

---

**FIG. 4.** A, sequence of the Ahd2-like promoter. The octamer motif is indicated, as well as the transcription start site. B, comparison of the consensus PORE sequence and the octamer motifs within the Ahd2-like and Igκ promoters.

**FIG. 5.** The Ahd2-like promoter octamer is recognized by Oct transcription factors and by BOB.1/OBF.1. A, lipopolysaccharide-stimulated B cells were analyzed for octamer binding using the consensus octamer site, the octamer motif within the Ahd2-like promoter, or the octamer mutation of the Ahd2-like promoter. For supershift experiments, anti-Oct1 or anti-Oct2 antibodies were used as indicated. For competition, unlabeled oligonucleotides in x-fold excess were used as indicated. B, to demonstrate ternary complexes containing BOB.1/OBF.1, EMSA experiments with HeLa nuclear extracts were performed. Unprogrammed reticulocyte lysate or in vitro translated BOB.1/OBF.1 protein was included. Complexes were analyzed by adding specific antibodies for Oct1, Oct2, or BOB.1/OBF.1 as indicated.
that affects E1A function indirectly (52).

A follow-up study suggested that Creg is a secreted glycoprotein expressed as a product contributing to the transcriptional control of several cell types, including hematopoietic cells (54). E2R had been shown to be important for the regulated transcription of many genes whose products contribute to cell cycle control and DNA replication (53) and to differentiation of several cell types, including hematopoietic cells (54–56). However, follow-up studies suggested that Creg is a secreted glycoprotein that affects E1A function indirectly (52).

The promoter sequences of two of the identified targets expressed by BOB.1/OBF.1 are published: the promoters of cellular Rbp (48) and Id3 (49). Both promoters contain no obvious Oct, E1A, or E2F binding motifs. These observations favor a model of indirect repression by BOB.1/OBF.1.

According to our microarray study, the gene most strongly induced by BOB.1/OBF.1 was the Ahd2-like gene. The promoter sequence of this gene has not yet been published. Aldehyde dehydrogenases belong to a superfamily of proteins that are implicated in the formation of retinoic acid from retinol and retinal (57, 58). The promoter sequence of the Ahd2-like protein, Ahd2, contains one consensus octamer motif. We found that the Ahd2-like promoter is very similar to the Ahd2 promoter near the transcription start site and contains the same consensus octamer motif. Mutational analysis revealed that octamer transcription factors Oct1 and Oct2 are able to bind to this site. In supershift experiments using in vitro translated BOB.1/OBF.1 and HeLa cells, a specific complex formation of BOB.1/OBF.1 together with Oct1 to the octamer site within the Ahd2-like promoter was detected. Moreover, chromatin immunoprecipitation assays demonstrate that Oct1 and Oct2 bind together with BOB.1/OBF.1 to the Ahd2-like promoter region. We conclude that Ahd2-like is a direct target of BOB.1/OBF.1.

Recently, we have shown that the POU domain transcription factors Oct1 and Oct2 recruit BOB.1/OBF.1 and thereby activate transcription when they form dimers on so-called PORE sequences (the palindromic Oct factor recognition element) (40, 41). The Ahd2-like promoter does not show such a characteristic PORE sequence and most likely represents a classical monomeric Oct-binding site (Fig. 4B).

As already mentioned, aldehyde dehydrogenases play an important role in oxidizing retinol and retinal. The already discussed Rbp protein is a cytoplasmic retinol-binding protein that is thought to play an important role in providing tissues with vitamin A for regulated retinoic acid-responsive gene expression, in retinol storage and metabolism (59). Thus, two BOB.1/OBF.1-responsive gene products, Rbp and Ahd2-like, are involved in the regulation of retinol fate in the cell. Retinoic acid (RA) has been implicated in a wide array of physiological processes. The requirement of RA for normal embryogenesis and tissue maintenance has previously been shown (60). Additionally, there is considerable evidence for a function of RA during hematopoiesis. Mouse bone marrow hematopoiesis is affected by a lack of retinoids, resulting in myeloid cell expansion (61). It has also been shown that RA regulates primitive (yolk sac) hematopoiesis (62). Several in vitro studies have analyzed the effect of RA on hematopoietic progenitors and differentiation and, in some cases, indicate a positive role of clonal proliferation of progenitors (63). RA appears to elicit a complex response of cell proliferation and/or commitment to a more differentiated cell type, depending on the differentiation stage of the cell receiving the signal (64, 65). Moreover, several studies demonstrate an important role of retinol for growth of activated B lymphocytes (66–68). Therefore, it seems to be possible that the developmental defects observed in BOB1.1/OBF1-deficient B cells are caused, at least in part, by a perturbed Ahd2-like and Rbp-dependent retinol metabolism.

Immunoglobulin promoters were originally thought to be the prime targets of BOB.1/OBF.1. However, analysis of the BOB.1/OBF1-deficient animals showed virtually normal levels of Ig heavy and light chain transcription. However, a recent report showed that at least some Ig Vκ gene families are regulated by BOB.1/OBF1. The authors analyzed the octamer motif in these promoters and suggested that they resemble PORE motifs (69). The PORE/MORE hypothesis suggests that the Ig heavy chain promoter octamer motif (the heptamer/octamer element) represents a typical MORE element. MOREs

![Figure 6](https://example.com/f6.png)

**Figure 6.** The Ahd2-like promoter is activated by BOB.1/OBF.1. Analysis of the BOB.1/OBF.1 dependence of the Ahd2-like promoter and its mutation in transient transfection experiments using NIH 3T3 fibroblasts (A) or Jurkat T cells (B), either left uninduced (−) or treated for 16 h with phorbol 12-myristate 13-acetate/ionomycin (+ PMA/Iono). As internal controls, octamer-dependent reporter constructs were included in this experiment: 1× wt (a synthetic promoter containing one consensus octamer site) and μ ET1 (a reporter construct driven by a natural Ig promoter in combination with a strong intronic enhancer). The value of the 1× wt promoter vector was set to 1 in A. In B, the values of all promoter activities without induction were set to 1. The -fold induction was determined.
are a second type of palindromic octamer elements, which do not efficiently recruit BOB.1/OFB.1. The microarray screen revealed 20 immunoglobulin gene sequences, which were upregulated more than 2-fold by BOB.1/OFB.1. All of these immunoglobulin genes code for V\textsubscript{H} regions of the J558 family. Although this result is at odds with the above mentioned PORE/MORE model, it is supported by earlier observations (32), which showed an almost 2-fold reduction of V\textsubscript{H} usage of the J558 family in hybridomas derived from BOB.1/OFB.1-deficient mice. The murine V\textsubscript{H} genes can be grouped into 15 families based on their sequence similarities. It was shown that the octamer motifs of the different V\textsubscript{H} gene family promoters contribute differently to overall promoter activity (70). The reason for the different relevance of the octamer motif within distinct V\textsubscript{H} gene promoters is not exactly known. Our results indicate that BOB.1/OFB.1 might only be involved in the regulation of one family (the J558 family) of V\textsubscript{H} genes. This would be similar to the situation observed for Ig \kappa light chain genes, where also only a subset of promoters critically depends on BOB.1/OFB.1 (69). In any case, both our data on Ahd2-like and IgH promoters do not easily agree with the PORE model (Fig. 4B). Since all PORE data are derived from \textit{in vitro} experiments or ectopic transfections into non-B cells, it is possible that there is a stronger interaction of Oct proteins with BOB.1/OFB.1 on MORE sequences in B cells \textit{in vivo}.

Acknowledgments—We thank Alexey Ushaymov for help with ChIP analyses and Sabine Fenik for excellent technical assistance.

REFERENCES

1. Gataiger, M., Knoopf, L., Georgiev, O., Schaffner, W., and Hovens, C. M. (1995) Nature 373, 360–362
2. Schubart, D. B., Rolink, A., Kosco-Vilbous, M. H., Botteri, F., and Matthias, P. (1996) Nature 380, 538–542
3. Luo, Y., Fujii, H., Gerster, T., and Roeder, R. G. (1992) Cell 71, 231–241
4. Luo, Y., and Roeder, R. G. (1995) Mol. Cell Biol. 15, 4115–4124
5. Pfisterer, P., Ziegler, S., Hess, J., and Wirth, T. (1995) J. Biol. Chem. 270, 29870–29880
6. Pierani, A., Huguy, A., Fujii, H., and Roeder, R. G. (1990) Mol. Cell Biol. 10, 6204–6215
7. Cepke, K. L., Chasan, D. L., and Sharp, P. A. (1996) Genes Dev. 10, 2079–2088
8. Chasan, D., Cepke, K., Sharp, P. A., and Pabo, C. O. (1999) Genes Dev. 13, 2650–2657
9. Gataiger, M., Georgiev, O., and Schaffner, W. (1996) Trends Genet. 12, 393–394
10. Babb, R., Cleary, M. A., and Herr, W. (1997) Mol. Cell Biol. 17, 7295–7305
11. Sauter, P., and Matthias, P. (1998) Mol. Cell Biol. 18, 7897–7909
12. Wirth, T., Staudt, L., and Baltimore, D. (1987) Nature 329, 174–178
13. Staudt, L., and Lenardo, M. J. (1991) Annu. Rev. Immunol. 9, 373–398
14. Libermann, T. A., and Baltimore, D. (1991) in The Hormonal Control of Gene Transcription (Cohen, P., and Foulkes, J. G., eds) pp. 385–407, Elsevier-Biomedical, Amsterdam
15. Schaffner, W. (1989) Trends Genet. 5, 37–39
16. Verrijzer, C. P., and Van der Vliet, P. C. (1993) Biochim. Biophys. Acta 1173, 1–21
17. Strahm, M., Newell, J. W., and Matthias, P. (1995) Cell 80, 497–506
18. Pfisterer, P., Annweiler, A., Ullmer, C., Corcoran, L., and Wirth, T. (1994) EMBO J. 13, 1654–1663
19. Annweiler, A., Muller-Immenpluck, M., and Wirth, T. (1996) Mol. Cell Biol. 16, 3107–3116
20. Tang, H., and Sharp, P. A. (1999) Immunity 11, 517–526
21. Stevens, S., Wang, L., and Roeder, R. G. (2000) J. Immunol. 164, 6372–6379
22. Schubart, D. B., Sauter, P., Massa, S., Friedl, E. M., Schwarzenbach, H., and Matthias, P. (1996) Nucleic Acids Res. 24, 1913–1920
23. Ziegling, S., Deckmann, A., Pfisterer, P., Angel, P., and Wirth, T. (1997) Science 277, 221–225
24. Qin, X.-F., Reichlin, A., Luo, Y., Roeder, R. G., and Nussenzweig, M. C. (1998) EMBO J. 17, 5066–5075
25. Gehner, A., Muller, K., Hess, J., Pfeffer, K., Muller-Hermelink, K. H., and Wirth, T. (2000) Am. J. Pathol. 156, 501–507
26. Boehm, J., He, Y., Greiner, A., Staudt, L., and Wirth, T. (2001) EMBO J. 20, 4153–4162
27. Tiedt, R., Bartholdy, B. A., Matthias, G., and Corcoran, L. (1997) Mol. Cell Biol. 21, 1531–1539
28. Schubart, D. B., Rolink, A., Schubart, D., and Matthias, P. (2000) J. Immunol. 164, 18–22
29. Yu, X., Wang, L., Luo, Y., and Roeder, R. G. (2001) Immunity 14, 157–167
30. Yeh, I., and Lim, E., Kaisers, B., Beaudard, G., Claudio, E., Siebenlist, U., Forster, R., and Lipp, M. (1998) J. Biol. Chem. 273, 28831–28836
31. Morozzi, M., and Morozzi, H. (2001) J. Biol. Chem. 276, 8683–8642
32. Malone, C. S., and Wall, R. (2002) J. Immunol. 168, 3369–3375
33. König, H., Pfisterer, P., Corcoran, L., and Wirth, T. (1995) Genes Dev. 9, 1598–1607
34. Pfisterer, P., König, H., Hess, J., Lipowsky, G., Haendler, B., Schleunig, W.-D., and Wirth, T. (1996) Mol. Cell Biol. 16, 6160–6168
35. Shere, P., Dietrich, W., and Corcoran, L. M. (2002) Nucleic Acids Res. 30, 1767–1773
36. Tomlin, A., Remenyi, A., Lordi, S., Friend, G., Wilmans, M., and Scholer, H. R. (2000) Cell 103, 853–864
37. Remenyi, A., Tomlin, A., Pohl, E., Lins, K., Philippens, A., Reinbold, R., Scholer, H. R., and Wilmans, M. (2001) Mol. Cell 8, 569–580
38. Hahne, M., Weng, K., Hahne, M., and Wirth, T. (1997) J. Exp. Med. 139, 1391–1395
39. Laumen, H., Nielsen, P. J., and Wirth, T. (2000) Eur. J. Immunol. 30, 458–469
40. Delem, K., Hemmann, U., Erk, Chena, M., Hauflë, E., Fruhlick, M., Stielow, J., Niestroj, C., Daiber, C., Enken, B., Guilliard, B., Lauretice, I., Muller, E., Pascale, E., Sauter, G., Pantic, M., Martens, U. M., Wenz, C., Lindner, R., Krait, N., Retig, W., and Schnapp, A. (2001) EMBO J. 20, 6958–6968
41. Lernbecker, T., Muller, U., and Wirth, T. (1995) Nature 326, 767–770
42. Wirth, T., and Baltimore, D. (1988) EMBO J. 7, 1109–1113
43. Wehbi, C. F., Deu, S., Buchan, K. L., Hessa, R., Smithson, G., and Smith, E. A. (1997) Mol. Immunol. 34, 743–750
44. Smith, W. C., Nakahari, H., Leroy, P., Rees, J., and Chambon, P. (1991) EMBO J. 10, 2223–2230
45. Yeh, K., and Lim, B. W. (2000) Gene (Amst.) 254, 163–171
46. Bond, S. L., and Singh, S. M. (1994) Biochim. Biophys. Acta 1207, 155–159
47. Stevens, S., Ong, J., Kim, U., Eckhardt, L. A., and Roeder, R. G. (2000) Nucleic Acids Res. 28, 4011–4018.
BOB.1/OBF.1 Target Genes in B Cells

52. Veal, E., Eisenstein, M., Tseng, Z. H., and Gill, G. (1998) *Mol. Cell Biol.* **18**, 5032–5041
53. DeGregori, J. (2002) *Biochim. Biophys. Acta* **1592**, 131–150
54. Yamasaki, L. (1999) *Biochim. Biophys. Acta* **1423**, M9–M15
55. Lissy, N. A., Davis, P. K., Irwin, M., Kaelin, W. G., and Dowdy, S. F. (2000) *Nature* **407**, 642–645
56. Rempel, R. E., Saenz-Robles, M. T., Storms, R., Morham, S., Ishida, S., Engel, A., Jakoi, L., Melhem, M. F., Pipas, J. M., Smith, C., and Nevins, J. R. (2000) *Mol. Cell* **6**, 293–306
57. Duester, G. (1996) *Biochemistry* **35**, 12221–12227
58. Duester, G. (2000) *Eur. J. Biochem.* **267**, 4315–4324
59. Quadro, L., Blaner, W. S., Salchow, D. J., Vogel, S., Piantedosi, R., Gouras, P., Freeman, S., Cosma, M. P., Colantuoni, V., and Gottesman, M. E. (1999) *EMBO J.* **18**, 4633–4644
60. Zile, M. H. (2001) *J. Nutr.* **131**, 705–708
61. Kuwata, T., Wang, I. M., Tamura, T., Ponnampерuma, R. M., Levine, R., Holmes, K. L., Morse, H. C., De Luca, L. M., and Ozato, K. (2000) *Blood* **95**, 3349–3356
62. Ghatpande, S., Ghatpande, A., Sher, J., Zile, M. H., and Evans, T. (2002) *Blood* **99**, 2379–2386
63. Deuer, D., and Koeffler, H. P. (1982) *J. Clin. Invest.* **69**, 1039–1041
64. Purton, L. E., Bernstein, I. D., and Collins, S. J. (1999) *Blood* **94**, 483–495
65. Purton, L. E., Bernstein, I. D., and Collins, S. J. (2000) *Blood* **95**, 470–477
66. Buck, J., Ritter, G., Dannecker, L., Kutta, V., Cohen, S. L., Chait, B. T., and Hammerling, U. (1990) *J. Exp. Med.* **171**, 1613–1624
67. Buck, J., Derguini, F., Levi, E., Nakanishi, K., and Hammerling, U. (1991) *Science* **254**, 1654–1656
68. Buck, J., Grun, F., Derguini, F., Chen, Y., Kimura, S., Noy, N., and Hammerling, U. (1993) *J. Exp. Med.* **173**, 675–680
69. Casellas, R., Jankovic, M., Meyer, G., Gazumyan, A., Luo, Y., Roeder, R., and Nussenzweig, M. (2002) *Cell* **110**, 575–585
70. Buchanan, K. L., Smith, E. A., Dou, S., Corcoran, L. M., and Webb, C. F. (1997) *J. Immunol.* **158**, 1247–1254
Expression of the Aldehyde Dehydrogenase 2-like Gene Is Controlled by BOB.1/OFB.1 in B Lymphocytes

Cornelia Brunner, Helmut Laumen, Peter J. Nielsen, Norbert Kraut and Thomas Wirth

J. Biol. Chem. 2003, 278:45231-45239.
doi: 10.1074/jbc.M302539200 originally published online August 28, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302539200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 69 references, 34 of which can be accessed free at http://www.jbc.org/content/278/46/45231.full.html#ref-list-1