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# Cloning and characterization of the chick Oct binding factor OBF-1

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**Abstract**

We have cloned the chicken homolog of OBF-1, chOBF-1, which comprises 256 amino acids, and exhibits only 65% overall identity to the human and mouse OBF-1 proteins. Amino acid sequence alignment revealed the putative Oct-binding sequence, RPYQGVRVKEPV-KELL(K/R)RKRG, which is conserved among chicken, mouse and human. chOBF-1 protein was demonstrated to bind chicken Oct-1 protein by the in vitro immunoprecipitation experiment, and chOBF-1 was shown to functionally activate the chicken immunoglobulin (Ig) light chain promoter in the NIH 3T3 cell. Taken together, these data indicate that the Ig gene transcription machinery, including Oct-1 and OBF-1, has been highly conserved in vertebrate evolution.


*Keywords:* Chicken homolog; OBF-1/Bob1/OCA-B; Coactivator; Immunoglobulin gene promoter; Transcription factor

In many cases of eukaryotic transcriptional regulation, DNA binding transcription factors regulate target gene expression by binding to their cognate DNA elements and recruiting activators or repressors to the basal transcriptional machinery [1]. The octamer motif ATTTGCAT or its reverse complement can be found in the promoters of various ubiquitous genes, such as the histone H2B gene and small nuclear RNA [2,3] genes. The octamer motif can also be found in almost all promoters of immunoglobulin (Ig) genes [4–6] and several B cell-specific genes, such as the CD20 and the B29 (Igβ) genes [7,8]. In addition to this motif, B-cell-specific transcription of Ig genes requires two octamer binding proteins, Oct-1 and Oct-2, which have identical DNA binding specificity that can act as transcriptional activators [9,10]. Oct-1 is ubiquitously expressed [11] and Oct-2 is largely expressed in lymphoid cell [12–15]. Moreover, a B-cell-specific coactivator that binds Oct proteins was also identified. The B-cell-specific coactivator, OBF-1 (huOBF-1), was originally isolated from a human cDNA library by using a single hybrid screen in yeast with human Oct-1 as bait [16]. Two other groups also independently screened the same human cDNA, and designated it as Bob1 [17] or OCA-B [18]. Subsequently, the mouse homologue of OBF-1 (moOBF-1) was isolated and displayed 89% overall identity to the human protein [19–21]. The N-terminal part of the OBF-1 protein mediates interaction to Oct proteins and the C-terminal domain stimulates transcription [21–23]. A crystal structure analysis of a huOBF-1 N-terminal peptide revealed that a portion of 16–38 amino acids formed an ordered structure in the crystal and was responsible for binding to the Oct protein [24]. Mammal OBF-1 strongly stimulates transcription from Ig promoters [16,18,19]. The B cells that are derived from OBF-1 knockout mice with an inducible OBF-1 allele showed that the transcriptional activity of octamer-dependent promoters is dependent on OBF-1 [25]. The chicken homologue of the Oct-1 gene was isolated and displayed 96% identity to the human Oct-1 protein [26]. However, other coactivators derived from chicken have not yet been investigated.

In order to isolate the chicken homolog of the Oct binding factor OBF-1, the expressed sequence tag (EST) database by GenomeNet Database Service was searched, using BLAST (http://blast.genome.ad.jp). One of the chicken ESTs displaying sequence conservation was identified. As the clone showed similarity to N-terminal huOBF-1, to obtain the full-length cDNA, 3′ RACE was performed with a Marathon RACE kit (Clonetech) using total RNA from the chicken B-cell line DT40, and its

*Abbreviations:* Ig, immunoglobulin; EST, expressed sequence tag; β-Gal, β-galactosidase

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DNA sequence was determined by the dye terminator method (Applied Biosystems Division, Perkin-Elmer), and was then submitted to DDBJ (accession no. AB052869).

The deduced amino acid sequence alignment between huOBF-1 and moOBF-1 was performed with the CLUSTALW (http://www.ddbj.nig.ac.jp/E-mail/clustalw-j.html). The alignment is shown in Fig. 1. The amino acid sequences of chOBF-1 comprises 256 amino acid residues, including a putative initiation Met, and exhibits only 65% identities in amino acid sequences to human and mouse OBF-1 proteins. In mammal, the entire N-terminal domain is well conserved and necessary for the interaction with Oct-1 and/or Oct-2 [21,22]. Interestingly, the N-terminal domain of chOBF-1 can be divided into two subdomains. The first N-terminal subdomain (1–15 amino acids) notably diverges between chicken and mammal OBF-1. The next subdomain (16–36 amino acids) is almost conserved with only one amino acid substitution (K32 of chOBF-1 corresponding to R33 of huOBF-1 and moOBF-1). A previous report from crystal structure analysis using a huOBF-1 N-terminal peptide showed that the 16–38 amino acid portion formed an ordered structure and was responsible for binding to Oct protein [24]. Thus, in the case of chOBF-1, the internal corresponding 21 amino acids (16–36 amino acids) may function as a binding domain for Oct protein.

Southern blot analysis of chicken genomic DNA with chOBF-1 as a probe is shown in Fig. 2A. Total DNA was extracted from DT40, cut with *Eco*RI, *Bam*HI and *Hin*dIII, run on 0.8% agarose gel, blotted to Hybond-N+ and hybridized to a $^{32}$P-labeled 474-bp of *Pst* I fragment as a chOBF-1 specific probe. The membrane was exposed on a Fuji-image plate and analyzed with a Bio-Image Analyzer (Bas1000). Digestion with *Bam*HI and *Eco*RI showed a single restriction fragment with a specific length in each case, whereas three fragments were detected in *Hin*dIII digestion. These results suggest that the chicken OBF-1 gene may be present as a single copy and it may have no pseudogene in the chicken genome.

In order to measure the mRNA size of chOBF-1, Northern blot analysis of total RNA from DT40 cells with chOBF-1 as a probe is shown in Fig. 2B. Total RNA was extracted from DT40, loaded on 1% agarose gel containing formaldehyde, blotted to Hybond-N+ membrane (Amersham) and hybridized with the same $^{32}$P-labeled probe used in Southern analysis. The membrane was exposed on a Fuji-image plate and analyzed with a Bio-Image Analyzer (BAS1000). A single transcript of about 3.0-kb was detected, suggesting that the 3.0-kb mRNA is a major transcript, and apparently there is no other variant transcription in DT40 cells. By contrast, chOBF-1 mRNA was not found in several non-lymphoid origin tissues, such as brain, skeletal muscle, lung, kidney and colon (data not shown). Generally, it has been estimated that the extent of genetic variation between chickens and humans is 20–25% [27]. Taking this together with the results of this study indicates that OBF-1 seems to have a relatively low degree of sequence conservation in evolution.

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chOBF-1   1  MHWQKSSASEQQPQP-RPYQGVRVKEPVKELLK 32
huOBF-1   1  MLWQKPTAPEQAPAPARPYQGVRVKEPVKELLR 33
moOBF-1   1  MLWQKSTAPEQAPAPPRPYQGVRVKEPVKELLR 33

chOBF-1  33  RKRGNVHNATAAAATTVVLPHQPLPSYSPMGQP 65
huOBF-1  34  RKRGHASSGAAPAPTAVVLPHQPLATYTTVGPS 66
moOBF-1  34  RKRGHTSVGAAGPPTAVVLPHQPLATYSTVGPS 66

chOBF-1  66  CIDVDAAAPALPGAEEGALCSSWLSQPSPSSLQ 98
huOBF-1  67  CLDMEGSYSAVT--EEAALCAGWLSQPTPATLQ 97
moOBF-1  67  CLDMEVSASTVT--EEGTLCAGWLSQPAPATLQ 97

chOBF-1  99  PLTQWTTYPDYVSHEAASCPYTADMYIQPMCPS 131
huOBF-1  98  PLAPWTPYTEYVPHEAVSCPYSADMYVQPVCPS 130
moOBF-1  98  PLAPWTPYTEYVSHEAVSCPYSTDMYVQPVCPS 130

chOBF-1 132  YTLVGPSSVLTYASQPLITNFTPRSS-TPAVVP 163
huOBF-1 131  YTVVGPSSVLTYASPPLITNVTTRSSATPAVGP 163
moOBF-1 131  YTVVGPSSVLTYASPPLITNVTPRSTATPAVGP 163

chOBF-1 164  QLEVTEQQPPLTYFPWAQPLSALPAPTLQYQPA 196
huOBF-1 164  PLEGPEHQAPLTYFPWPQPLSTLPTSTLQYQPP 196
moOBF-1 164  QLEGPEHQAPLTYFPWPQPLSTLPTSSLQYQPP 196

chOBF-1 197  SSTLPTPQFVPLPISIPEPAPQELEDARRVIGT 229
huOBF-1 197  APALPGPQFVQLPISIPEPVLQDMEDPRRAASS 229
moOBF-1 197  APTLSGPQFVQLPISIPEPVLQDMDDPRRAISS 229

chOBF-1 230  LPIEKLLLEDEDNDTYVLNHALSVEGL      256
huOBF-1 230  LTIDKLLLEEEDSDAYALNHTLSVEGF      256
moOBF-1 230  LTIDKLLLEEEESNTYELNHTLSVEGF      256
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Fig. 1. Comparison of the aligned amino acid sequences of chicken OBF-1 and mammalian OBF-1. The deduced amino acid sequences of chOBF-1, huOBF-1 and moOBG-1 are shown. Identical amino acid residues are boxed. Dashes indicate gaps inserted to maximize the alignment of the sequences.

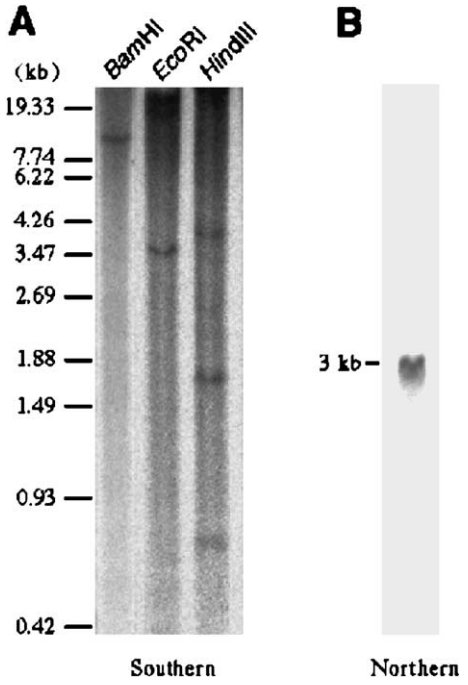


Fig. 2. Southern and Northern analysis of chOBF-1. (A) Total DNA from DT40 was digested with *Eco*RI, *Bam*HI and *Hin*dIII, run on 0.8% agarose gel, blotted to Hybond-N+ and hybridized to a $^{32}$P-labeled chOBF-1 specific probe. (B) Total RNA from DT40 was loaded on 1% agarose gel containing formaldehyde, blotted to Hybond-N+ membrane and hybridized to a $^{32}$P-labeled chOBF-1 specific probe.

To determine whether chOBF-1 actually binds to chOct-1, in vitro immuno precipitation assay was performed with biotinylated FLAG-tagged chOct-1 and chOBF-1, using biotinylated β-galactosidase (β-Gal) protein as a control. For this, the pCITEFLchOCT-1 was constructed as follows. First, the chicken Oct-1 cDNA was amplified by PCR using DT40 and oligonucleotide primer pairs, which have eight codons of the FLAG epitope, *Bam*HI site at the 5′ end, and an *Xho*I site at the 3′ end. Next, the amplified PCR fragment was ligated between the *Bam*HI and *Xho*I sites of pCITE-4a (Novagen). To construct pCITEchOBF-1, the chicken OBF-1 cDNA was amplified by PCR using oligonucleotide primer pairs, which have a *Bam*HI site at the 5′ end and an *Xho*I site at the 3′ end, and then the amplified PCR fragment was ligated between the *Bam*HI and *Xho*I sites of pCITE-4a (Novagen). In vitro translation with Biotin–tRNA was performed as instructed by the manufacturer, and biotinylated β-Gal was also obtained using STP3 T7 Control DNA (STP3™ kit; Novagen). The immunoprecipitation experiment was performed, using 5 μl of biotinylated FLAG-tagged chOct-1 and 5 μl of biotinylated chOBF-1 protein or β-Gal, in 200 μl of the bead-binding buffer (Eastman Kodak Co.). After 60-min standing, 20 μl of the reaction mixture was removed as an input sample. The remaining mixture was added to 20 μl of anti-FLAG M2 beads (Eastman Kodak Co.) containing 10 mg/ml BSA and 4 μg of EtBr, followed by gentle rotation for 60 min at 4 °C. The affinity beads were collected by centrifugation at 12,000 rpm for 10 s, and then washed with 1 ml of the bead-binding buffer containing 0.1% PMSF three times. The beads were suspended in 30 μl of 2× SDS sample buffer and then boiled for 5 min. Aliquots (15 μl) of the resultant elutes were analyzed by 8% SDS-PAGE, and then the proteins were transferred to Hybond-P (Amersham), and detected with a Streptavidin AP LumiBlot kit (Novagen). As shown in Fig. 3, chOBF-1 was coimmunoprecipitated with FLAG-tagged chOct-1 using anti-FLAG M2 beads, but β-Gal was not. Moreover, chOBF-1 was not immunoprecipitated using the beads without FLAG-tagged chOct-1 (data not shown). Taken together, the data indicate that chOBF-1 can bind chOct-1 specifically in spite of its N-terminal divergence.

Previously, transient expression of mammal OBF-1 in non-B-cells was shown to stimulate the activity of the Ig promoter [16,18,19]. To determine whether the expression of chOBF-1 would be sufficient to stimulate transcription from an Ig promoter in non-B cells, a hybrid plasmid, pGL3–EnpchIgL, was used, containing the luciferase coding gene under the control of a 200-bp chicken Ig light chain gene promoter fragment containing one octamer motif upstream of the TATA box [28]. The pGL3–EnpchIgL was constructed as follows. The 200-bp 5′-upstream fragment with part of the first exon of the chicken IgL gene was amplified by PCR using oligonucleotide primer pairs, a *Hin*dIII site at the 5′ end and an *Nco*I site at the 3′ end; DT40 genomic DNA was adopted as a template. The amplified PCR fragment was ligated between the *Hin*dIII and *Nco*I sites of pGL3–Enhancer (Promega).

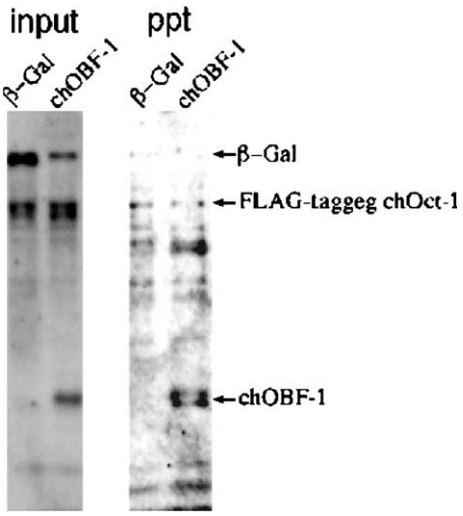


Fig. 3. In vitro interaction of chOBF-1 with chOct-1. An in vitro immunoprecipitation experiment was performed using biotinylated FLAG-tagged chOct-1 and biotinylated chOBF-1 protein or β-Gal, followed by addition of anti-FLAG M2 beads. Immunoprecipitated samples (ppt) and input samples, i.e. portions (20%) of the reaction mixtures before the immunoprecipitation (input), were separated on 8% SDS-PAGE, and then they were blotted and detected.

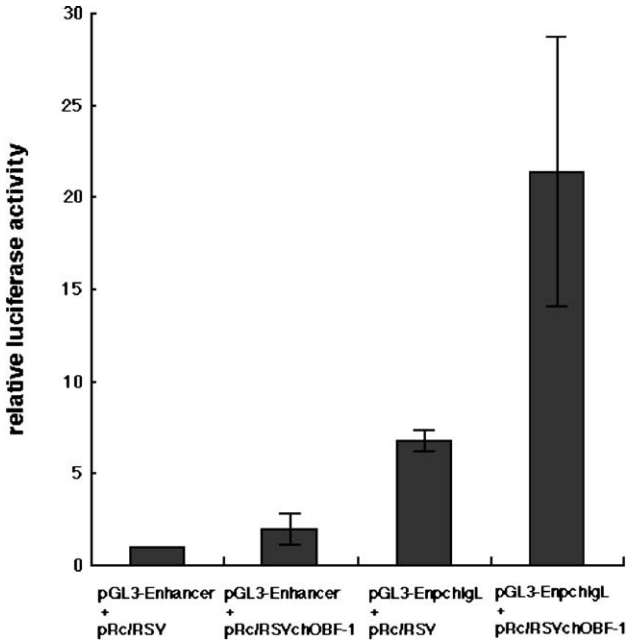


Fig. 4. chOBF-1 activates the promoter activity of the chicken Ig light chain gene. NIH 3T3 cells were cotransfected with the reporter plasmid (pGL3–Enhancer or pGL3–EnpchIgL) and the expression vector (pRc/RSV or pRc/RSVchOBF-1). One day later, transfection cell extracts were prepared and luciferase activity was measured with equal protein amounts. The activity of the pGL3–Enhancer plus the pRc/RSV was arbitrarily set to 1. The averages of three experiments are presented.

The chOBF-1 expression vector pRc/RSVchOBF-1 was constructed as follows. First, the chOBF-1 cDNA was amplified by PCR using oligonucleotide primer pairs, a *Hin*dIII site at the 5′ end, and an *Xba*I site at the 3′ end; then the amplified PCR fragment was ligated between the *Hin*dIII and *Xba*I sites of pRc/RSV (Invitrogen). NIH 3T3 cells were maintained in DMEM medium supplemented with 5% fetal calf serum. Luciferase assay was run with a Promega kit as recommended by the manufacturer. NIH 3T3 cells were cotransfected with 0.5-μg reporter plasmid pGL3–Enhancer (no promoter) or pGL3–EnpchIgL and 0.5-μg expression vector pRc/RSV (vector alone) or pRc/RSVchOBF-1 (chOBF-1 expression vector). One day later, cell extracts were prepared and luciferase activity was measured with a Promega kit as recommended by the manufacturer using equal protein amounts. As shown in Fig. 4, this reporter plasmid supports basal transcription probably mediated by endogenous Oct-1 on its promoter element (pGL3–EnpchIgL plus pRc/RSV) compared with the no-promoter reporter plasmid (pGL3–Enhancer plus pRc/RSV or pGL3–Enhancer plus pRc/RSVchOBF-1). This basal activity was stimulated about threefold when the chOBF-1 expression vector was cotransfected (pGL3–EnpchIgL plus pRc/RSVchOBF-1). These results clearly indicate that chOBF-1 can stimulate the activity of the Ig light chain gene promoter.

With these results as a background, the cloning and characterization of chicken OBF1 follows. Overall, there is only 65% amino acid sequence identity between chicken and mammal OBF-1, although its partner factor Oct-1 has been reported to show 96% amino acid sequence identity between chicken and human [26]. Amino acid sequence alignment revealed that the putative Oct-binding sequence, RPYQGVRVKEPVKELL(K/R)RKRG, is conserved among chicken, mouse and human. In addition, chOBF-1 was shown to bind with chOCT-1 by an in vitro immunoprecipitation experiment and also to activate Ig light chain gene promoter in transient expression assay. Thus, the Oct-1/2 and OBF-1 system was in fact demonstrated to function in chicken Ig gene expression and this would be conserved in evolution. Further experiments are now in progress to elucidate the fuctions of chOBF-1. A detailed comparison of mammalian OBF-1 with chOBF-1 will enable us to further understand the molecular functions of OBF-1.

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