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BOB.1 of the channel catfish, *Ictalurus punctatus*: Not a transcriptional coactivator?

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Abstract

Expression of the immunoglobulin heavy chain (*IGH*) locus of the channel catfish (*Ictalurus punctatus*) is driven by the E μ 3' enhancer, whose core region contains two octamer motifs and a μ E5 site. Orthologues of the Oct1 and Oct2 transcription factors have been cloned in the channel catfish and shown to bind to the octamer motifs within the core enhancer. While catfish Oct2 is an activator of transcription, catfish Oct1 failed to drive transcription and may act as a negative regulator of *IGH* transcription. In mammals, the Oct co-activator BOB.1 (B cell Oct-binding protein1, also known as OCA-B and OBF-1) greatly enhances the transcriptional activity of Oct factors and plays an important role in the development of the immune system. An orthologue of BOB.1 has been cloned in the catfish, and its function characterized. The POU binding domain of the catfish BOB.1 was found to be 95% identical at the amino acid level with the binding domain of human BOB.1, and all the residues directly involved in binding to the Oct-DNA complex were conserved. Despite this conservation, catfish BOB.1 failed to enhance transcriptional activation mediated by endogenous or co-transfected catfish Oct2, and failed to rescue the activity of the inactive catfish Oct1. Electrophoretic mobility shift assays showed that catfish BOB.1 was capable of binding both catfish Oct1 and Oct2 when they formed a complex with the Oct motif. Analysis of recombinant chimeric catfish and human BOB.1 proteins demonstrated that the failure to drive transcription was due to the lack of a functional activation domain within the catfish BOB.1.

Keywords

Transcription factors; Gene regulation; B cells; Antibodies; Comparative immunology

1. Introduction

The Oct transcription factors are class II members of the POU (Pit1, Oct1 and Unc) domain family, which is characterized by a bipartite DNA binding domain comprised of a POU specific (POU_S) amino terminal domain and a POU homeodomain (POU_H) C-terminal domain, joined by a flexible linker region (Sturm and Herr, 1988). The Oct transcription factors bind the cis-regulatory element, the octamer motif (ATGCAAAT) or its reverse complement (Parslow et

al., 1984) to enhance the transcriptional activity of a variety of ubiquitously expressed (in the case of Oct1) and tissue restricted (in the case of Oct2) genes. Oct2 knockout mice die within several hours of birth, but possess normal numbers of B cell precursors and their production of IgM is only slightly affected. These results suggest that Oct2 is not required for the expression of immunoglobulin (*IG*) genes (Corcoran et al., 1993) and that there is a functional redundancy between Oct1 and Oct2 transcription factors in driving immunoglobulin gene expression. In order to accommodate these observations, it was postulated that a B lymphocyte restricted coactivator might also be involved in the transcriptional control of the immunoglobulin genes.

Such a B cell specific co-activator was discovered (Gstaiger et al., 1995; Luo et al., 1992; Luo and Roeder, 1995; Strubin et al., 1995) and named BOB.1 (B cell Oct-binding protein1, also known as OCA-B, Oct coactivator from B cells, or OBF-1, Oct binding factor1). The BOB.1 gene encodes a 256 amino acid residue (Gstaiger et al., 1995), 35 kDa protein that forms a ternary complex with the POU domains of either the Oct1 or Oct2 bound to its cognate motif and thereby enhances transcription driven by Oct1 and Oct2. The activation domain of BOB.1 has been mapped to the C-terminus of the protein, and most of the activity localizes to the last 90 residues, being concentrated within the last 35 residues of the protein (Krapp and Strubin, 1999). BOB.1 is expressed in two forms, one of which localizes to the nucleus (where it can act as a transcriptional coactivator) while the other is myristoylated and localizes to the plasma membrane (Yu et al., 2006) where it may interact with the signal transduction machinery of the B cell. The observed effects of BOB.1 knock-out in mice include impairment in B cell development, loss of normal germinal center formation, and failure to produce normal amounts of isotype-switched immunoglobulins (Kim et al., 1996; Schubart et al., 1996a); however, transcription and rearrangement at the *IGH* locus are not seriously impaired (Kim et al., 1996; Schubart et al., 1996a, 2001). The effects seen in BOB.1 deficient mice are likely to be attributable to impairment of signal transduction in B cells, through, for example, the non-transcriptional interaction of BOB.1 with SYK (Siegel et al., 2006) as well as through the reduced expression of Oct transcription factor target genes that include BTK (Brunner and Wirth, 2006).

The channel catfish is a well-studied model that has provided insight into the evolution of the vertebrate immune system. Catfish express only two classes of immunoglobulin IgM and IgD, and class switching by chromosomal recombination is absent (Bengtén et al., 2006; Wilson et al., 1997, 1990). A single enhancer, E μ 3', has been described that is situated immediately 3' of the *IGHM* gene. The core or minimal functional region of this enhancer consists of two variant, but fully functional, octamer motifs and a μ E5 site (Cioffi et al., 2001; Magor et al., 1997). These octamer motifs and the octamer-binding transcription factors have been shown to play important roles in driving expression of the catfish *IGH* locus. Orthologues of mammalian Oct1 and Oct2 have been cloned and characterized in the channel catfish. Catfish Oct2 is expressed as two isoforms, Oct2 α and β , both of which are transcriptionally active (Ross et al., 1998), whereas catfish Oct1 is transcriptionally inactive (Lennard et al., 2007). Human BOB.1 enhances the transcriptional activity of catfish Oct2 (Ross et al., 1999), but not of catfish Oct1 (Lennard et al., 2007). It is clear from these observations that the catfish Oct1 transcription factor has very different functional properties from its mammalian orthologue, and it is therefore of interest whether or not catfish B cells express a functional BOB.1 that can modify the transcriptional properties of the endogenous Oct1 and Oct2 factors. Here we report the results of a study to clone catfish BOB.1, characterize its function and evaluate its ability to interact with Oct transcription factors.

2. Materials and methods

2.1. Cloning and sequence determination of catfish BOB.1

The *Takifugu rubripes* genome version 3.0 at the Joint Genome Institute (JGI) was searched using the nucleotide sequence of human BOB.1 (NCBI accession number NM_006235). A region of scaffold 443 in *T. rubripes* that showed the best match to human BOB.1 was used to search the rainbow trout (*Oncorhynchus mykiss*) EST database and three sequences with homology to BOB.1 (CA384555, CA383410, and CA355180) were identified. The *Takifugu* and trout sequences were aligned with the human (Q16633), mouse (NM_011136), and chicken BOB.1 (AB052869) sequences (using Clustal V in the MegAlign suite of programs from DNA Star, Madison, WI) with a PAM 250, gap length penalty of 10 and gap penalty of 10. Degenerate primers were designed from regions of similarity in the alignment (Table 1). Gradient PCR using as target a cDNA library from the 1G8 catfish B cell line (Hikima et al., 2004) was carried out using (a) combinations of the forward and reverse primers (Table 1) and (b) 3'-RACE using the forward primers and an anchor primer (Wilson et al., 1997). The cycles used were: 95 °C for 3 min, 30 cycles of 94 °C for 30 s, 45–55 °C for 1 min, and 72 °C for 2 min, and then 72 °C for 10 min. Several bands of expected sizes were seen on a 2% agarose gel. Amplicons were cloned into the TOPO vector (PCR2.1, Invitrogen, Life Technologies, San Diego, CA) and sequenced (Biomolecular Resource Laboratory of the Medical University of South Carolina). A resulting NCBI BLAST search of two partial sequences generated with the primer sets of G-2715/G-2718 and G-2717/G-2719 gave significant *E* values (9e–20 and 1e–26) in comparison to mouse and human BOB.1. This sequence information was used to design specific primers in order to clone by PCR the full-length catfish BOB.1 sequence from the catfish B cell genomic library (see Table 1 and Fig. 1). The PCR cycles were as follows: 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, followed by 72 °C for 5 min.

Amino acid sequence alignments including the catfish BOB.1 were generated using Clustal V within MegAlign (DNASTar). The following sequences (Genbank NCBI accession number in parentheses) were used in the alignment: Human BOB.1 (Q16633), Mouse BOB.1 (NM_011136), Chicken BOB.1 (AB052869), Cow BOB.1 (NM_001075915) and Catfish BOB.1 (EF058163).

2.2. RT-PCR for BOB.1 of catfish tissues and cell lines

Total RNA was isolated using TRIzol (Invitrogen Life Technologies, San Diego, CA) from the following catfish tissues: head kidney, trunk kidney, spleen, brain, heart, gill and intestine as well as the B lymphoblastoid cell line (1G8), the T-cell line (G14D), the catfish macrophage cell line (42TA) and the catfish fibroblast cell line (G5F). Total RNA (treated with DNase I) was purified using RNeasy kits (Qiagen, Valencia, CA). Ribonuclease inhibitor (RNasin Promega, Madison, WI) was added and first strand synthesis carried out using PowerScript Reverse Transcriptase (BD Biosciences Clontech, Palo Alto, CA). PCR was carried out using primers G-2887 and G-2889 for catfish BOB.1 and G-1480 and G-1481 for actin (Table 1) with 30 cycles of 30 s at 95 °C; 30 s at 55 °C; 2 min at 72 °C.

2.3. Reporter and expression constructs

Three reporter constructs were used in the transfection studies; pO3-Δ56-CAT, pGL3/Δ56/R#2/ΔμE5, and Δ56(10-11*3)-CAT. pO3-Δ56-CAT (Ross et al., 1998) and pGL3/Δ56/R#2/ΔμE5 (Hikima et al., 2005) have been previously described. Δ56(10-11*3)-CAT possesses, in triplicate, the two octamer motifs within the core enhancer (termed #10 and #11) upstream of a minimal *c-fos* promoter and the CAT reporter gene.

The expression vectors for catfish Oct2 β (Ross et al., 1998) and Oct1 (Lennard et al., 2007) have been previously described. The construct containing the 2.2 kb human BOB.1 cDNA was a generous gift from P. Matthias (Friedrich Miescher Institute for Biomedical Research, Novartis Forschungsstiftung, Basel, Switzerland). The coding regions of human and catfish BOB.1 were amplified by PCR using the following primer sets: G-2938/G-2939 for catfish BOB.1, and G-3249/G-3250 for the human BOB.1 (Table 1), and were cloned into the HindIII and XbaI sites of the pRc/CMV expression vector (Invitrogen, Life Technologies, San Diego, CA).

A set of vectors expressing chimeric BOB.1 proteins was constructed in pRc/CMV. In order to exchange the POU binding domain (PBD) and activation domain of the catfish and human proteins, gene-fusion PCR was carried out. To clone the chimera containing the catfish PBD with the human C-terminal activation domain (CF/H), a PCR was set up with the following components: forward and reverse primers (G-3246 and G-3245, respectively) which were designed containing a 16–18 bp overlap of the human BOB.1 activation domain and 18 bp at the 3' end of the catfish PBD. Primers G-2938 and G-3250 were used to clone the entire (CF/H) chimeric sequence. To clone the chimeric BOB.1 expression vector with a human PBD and a catfish activation domain (H/CF), a PCR was set up with a forward and a reverse primer (G-3248 and G-3247, respectively) that contained 15–16 bp of the catfish activation domain and 15–16 bp at the end of the human PBD. Primers G-3249 and G-2939 were used to clone the entire (H/CF) chimeric sequence. One round of PCR was carried out for each of the chimeras using all four of the primers and two separate plasmid templates (100 ng of each template/reaction) mixed together, for the catfish BOB.1 and for the human BOB.1. For the CF/H chimera the following conditions were used: 95 °C for 5 min, 22 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 2 min, 72 °C for 5 min. For the H/CF chimera the following conditions were used: 95 °C for 5 min, 20 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 2 min, 72 °C for 5 min. The amplicons of the predicted sizes were cloned into the HindIII and XbaI of pRc/CMV parent vector, and the accuracy and integrity of the coding region was confirmed by DNA sequencing. All primer sequences can be found in Table 1.

Expression constructs for protein production by *in vitro* transcription and translation were produced using the pCITE- 4b(+) (Novagen, Cambridge, MA) expression vector. The catfish Oct2 β expression construct (Hikima et al., 2006), and the catfish Oct1 expression construct (Lennard et al., 2007) have been previously described and both contain a 6 $\times$ His tag. The catfish Oct2 α expression construct and the catfish CFEB expression construct with N-terminal S-peptide tags (S-tag) have been previously described (Hikima et al., 2004). The human BOB.1 PBD was cloned utilizing primers G-2686 and G-3074 and the BamHI and BglII restriction sites. The catfish BOB.1 PBD was cloned using primers G-3072 and G-3073 and the SacI and BglII restriction sites. Both constructs possessed an N-terminal S-tag. Template DNA for TNT reactions to produce the full-length catfish BOB.1 utilized primers G-3591 and G-3592, which contained the T7 promoter sequence and a Kozak sequence, respectively. All primer sequences can be found in Table 1 and all clones were confirmed by DNA sequencing.

2.4. Cell lines and DNA transfection

The catfish B lymphoblastoid cell line 1G8 and T cell line G14D were maintained as previously described (Hikima et al., 2004). Transfections were performed as previously described (Lennard et al., 2007; Cioffi et al., 2001). Reporter constructs were transfected into the cells using 2.44 pM of pGL3/ Δ 56/R#2/ Δ μ E5 and 2.63 pM of Δ 56(10-11*3)-CAT. In transfections with the pGL3/ Δ 56/R#2/ Δ μ E5 reporter construct, 1.64 pM of all expression constructs were used. In transfections with Δ 56(10-11*3)-CAT the expression constructs were transfected into the cells using 2.187 pM empty, Oct1, Oct2, and CFBOB.1 vectors. For the BOB.1 chimera transfections, 2.63 pM of the pO3- Δ 56-CAT reporter construct were co-transfected with 2.187

pM empty, CFBOB.1, HBOB.1, or the CF/H and H/CF BOB.1 chimera-expressing vectors. In all experiments 1 μ g of a *Renilla* luciferase construct with a CMV promoter (pRL/CMV; Promega) was used as the transfection control. Transfection conditions were previously described (Cioffi et al., 2001). All transfected cells were harvested 36–40 h after electroporation and assessed for expression of the reporter genes.

2.5. Reporter assays

Luciferase and CAT reporter assay were carried out as described previously (Lennard et al., 2007). In both the firefly luciferase and CAT expression assays, transfection activity was normalized to the activity of the co-transfected *Renilla* luciferase. Values were calculated as the mean $\pm$ standard errors (S.E.M.). Statistical analysis was performed using the Student's *t*-test assuming equal variance.

2.6. Electrophoretic mobility shift assays

Synthetic double-stranded oligonucleotide probes corresponding to the variant octamer motif Oct#10 found in the E μ 3' enhancer of the catfish, as well as a probe corresponding to the scrambled sequence of Oct #10 were generated from primer sets G-2110/G-2111 and G-2132/G-2133 (Table 1) respectively and used in EMSA as previously described (Hikima et al., 2006; Lennard et al., 2007). The TNT quick-coupled transcription/translation system (Promega) was used to generate *in vitro* synthesized catfish Oct1, Oct2, and BOB.1 proteins. The following binding reactions were used: first 5 μ L of buffer, 5 μ L of catfish Oct1 TNT product or 3 μ L of catfish Oct2 TNT product, 2 μ L normal rabbit serum (NRS), 1 μ L of labeled probe and water to a final volume of 20 μ L were combined together and allowed to incubate at room temperature for 30 min. Second, 10 μ L of the BOB.1 TNT products was added, mixed and allowed to incubate at room temperature for 15 min. Finally, 2 μ L of S-Tag Grade S-Protein (Novagen, Darmstadt, Germany) or NRS was added to the appropriate samples and they were allowed to incubate at room temperature for 15 min. The DNA protein complexes were then analyzed on a 4% non-denaturing polyacrylamide gel run at 100 V at 4 $^{\circ}$ C. Gels were fixed in 15% methanol, 5% acetic acid for 30 min and allowed to dry, before exposure to a Phosphorimager screen and analysis using a Typhoon Variable Mode Imager (Amersham Biosciences, Piscataway, NJ) and the ImageQuant software program.

2.7. Pull down assay

Unlabeled S-tagged recombinant proteins (human BOB.1-PBD as a positive control, catfish BOB.1-PBD, CFEB, and Oct2 α) and 35 S-methionine labeled proteins without S-tags (catfish Oct1 and full-length BOB.1) were generated by *in vitro* transcription and translation using the TNT System (Promega). The 35 S-labeled full-length catfish BOB.1 protein without an S-tag was synthesized with the PCR-generated DNA templates and T7 TNT PCR Enhancer reagents provided with the TNT system kit (Promega) and according the manufacturer's protocol. The unlabeled TNT proteins (25 μ L) were mixed with 40 μ L S-protein agarose (Novagen) and resuspended in 200 μ L of 1 $\times$ modified-binding/washing buffer [m-B/W buffer (Novagen): containing 25 mM Tris-HCl (pH 7.5), 2 mM EDTA, 250 mM NaCl]. The protein/agarose mixture was incubated with shaking at 4 $^{\circ}$ C for 3 h and the beads washed four times in 1 $\times$ m-B/W buffer by agitation and briefly centrifuged. The mixture was then combined with 35 S-labeled Oct1 TNT product (25 μ L), 1 μ g of cold annealed octamer motif and 200 μ L of 1 $\times$ m-B/W buffer. This mixture was then incubated with shaking at 4 $^{\circ}$ C for 3 h. For the assay utilizing the catfish E-proteins, the following method was used. Fifty microliters of the *in vitro* synthesized non-radioactive S-tagged catfish CFEB1 and Oct2 α were bound to 25 μ L of S-protein agarose beads for 5 h at 4 $^{\circ}$ C in 250 μ L of 1 $\times$ m-B/W buffer and then washed twice with 500 μ L of the m-B/W buffer. Fifty microliters of 35 S-labeled *in vitro* synthesized full-length BOB.1 proteins (without S-tag) mixed with (or without) 1 μ g of the octamer variant

(Lennard et al., 2007) or consensus μ E5 site (Hikima et al., 2004) DNA probes were then incubated with the agarose-bound CFEB1 and Oct2 α proteins for 5 h at 4 °C in 250 μ L of the m-B/W buffer. The mixture was then washed four times as described above. The protein/agarose mixture was then resuspended in 25 μ L of 2 $\times$ SDS-PAGE buffer and run on a 12% SDS-PAGE gel for 90 min at 100V. One 25th or 50th volumes of input (35 S-labelled catfish Oct1 or full-length BOB.1) were run as a control. The gel was allowed to dry completely and exposed to a Phosphorimager screen for 24 or 48 h before analysis using a Typhoon Variable Mode Imager (Amersham Biosciences) and the ImageQuant software program.

3. Results

3.1. Identification of the catfish orthologue of BOB.1

Catfish BOB.1 cDNA (Genbank accession number, EF058163) was cloned using a PCR-based strategy that is summarized in Fig. 1. Regions of catfish BOB.1 were initially cloned from a cDNA library of the catfish B cell line (1G8) using degenerate primers G-2715/G-2718 and G-2717/G-2719 (Table 1 and Fig. 1) that were designed based on three regions of high sequence conservation in *T. rubripes* and *O. mykiss* BOB.1 orthologues. The two amplicons generated showed, upon sequencing and BLAST analysis, similarity to BOB.1 sequences of other vertebrates and allowed the design of catfish BOB.1-specific primers. These primers (Table 1 and Fig. 1) allowed the cloning of the full-length cDNA sequence of catfish BOB.1. That the correct sequence had been assembled from the overlapping PCR amplicons was confirmed by RT-PCR using primers at the 5' and 3' ends of the catfish BOB.1 (G-2887/G-2889, Fig. 1), and re-sequencing of the product. The inferred amino acid sequence of catfish BOB.1 was 11 residues shorter than that of mammalian and avian BOB.1 sequences, and could be readily aligned with the human, mouse, cow and chicken (Fig. 2). The overall sequence identity of catfish BOB.1 to the other sequences was, rather surprisingly, 43% for human, mouse, cow and chicken. The highest level of sequence conservation was observed within the POU-interacting residues (identified in Fig. 2), with only 1 difference between catfish and mammalian BOB.1 and 2 residues different between the catfish and the chicken BOB.1 sequences. Nine residues are known to be crucial for binding of the BOB.1 to the Oct transcription factors as deduced from the crystal structure (Chasman et al., 1999), and in the catfish BOB.1 sequence these residues are all conserved (Fig. 2).

3.2. Catfish BOB.1 is expressed in catfish B cells

In mammals, the expression of BOB.1 is B lymphocyte specific. In the catfish, a variety of cell lines were tested by RT-PCR for the presence of BOB.1. The catfish B cell line (1G8), T cell line (G14D), fibroblast cell line (G5F) and a catfish macrophage line (42TA) were tested for the presence of BOB.1. Detectable expression of the catfish BOB.1 transcript was found only within the B cell line (Fig. 3). A variety of tissues (brain, heart, gill, intestine, spleen, head kidney and trunk kidney) were also tested. BOB.1 transcripts were detected in all tissue types tested, with the strongest expression being seen in the spleen, head kidney and trunk kidney. This result is consistent with the presence of circulating BOB.1 expressing B cells within the non-lymphoid tissues.

3.3. Transcriptional activation potential of catfish BOB.1

The ability of the catfish BOB.1 to enhance transcriptional activation through interaction with Oct transcription factors was examined using three octamer-driven reporter constructs (Fig. 4). The capability of catfish BOB.1 coactivator to drive transcription was tested in the catfish B cell line 1G8. Transfection of BOB.1 alone was used to test its ability to interact with the endogenous Oct transcription factors expressed in the B cells, and co-transfection of BOB.1 with vectors expressing catfish Oct1 and Oct2 transcription factors was used to test its ability to interact with these factors when expressed exogenously. The results (Fig. 4) were consistent

for all three constructs: catfish BOB.1 was not capable of driving enhanced transcription with the endogenous factors present in the B cell line, nor with exogenously transfected catfish Oct2 transcription factor. Neither was catfish BOB.1 capable of conferring function on the Oct1 factor, which has previously been shown to be transcriptionally inactive (Lennard et al., 2007). Catfish BOB.1 failed to drive transcription in the catfish T cell line, which has no endogenous catfish BOB.1 (data not shown). These results raise the question of whether or not catfish BOB.1 is lacking in ability to bind to the Oct motif/octamer transcription factor complex, and whether its transcriptional activation domain is inactive.

3.4. Catfish BOB.1 binds to the complex of octamer motif/Oct transcription factor

The ability of the POU-binding domain (PBD) of catfish BOB.1 to bind to an octamer motif/Oct factor complex was tested using EMSA, with the PBD of human BOB.1 as a positive control. The experiment was designed to detect (1) a super-shift induced by the association of the S-tagged POU-binding domain (PBD) of catfish or human BOB.1 to the complex formed between the Oct transcription factor (either catfish Oct2 β or catfish Oct1) and the octamer motif, and (2) a second super shift induced by the binding of S-protein to the S-tagged PBD of catfish or human BOB.1 that had bound to the octamer/Oct factor complex. The ability of catfish Oct2 β to bind in a sequence-specific manner to the variant octamer motifs found within the catfish core enhancer E μ 3' is confirmed in lanes 1–7 of Fig. 5A. The result in lane 6 shows the control in which S-protein does not by itself bind to and affect the mobility of the octamer/Oct2 β factor complex. The ability of the catfish BOB.1 PBD to bind to the octamer/Oct2 β complex is shown by the super-shift in lane 8 of Fig. 5A, and the ability of the human BOB.1 PBD to bind this complex is shown in lane 11. The second super-shift resulting from the binding of S-protein to the octamer/Oct2 β /PBD complex upon the addition of the S-protein is shown for catfish BOB.1 PBD in lane 9, and for the human BOB.1 PBD in lane 12. That the second super-shift is not due to a non-specific effect of protein addition was shown in lanes 10 and 13, where no shift is seen upon the addition of normal rabbit serum (NRS). Additionally, Fig. 5B shows that the PBD of both human and catfish BOB.1 is capable of binding to an octamer/Oct1 complex.

3.5. Catfish BOB.1 lacks a functional activation domain

The results shown in Fig. 5 demonstrate that catfish BOB.1 can bind to an octamer/Oct transcription factor complex, and suggest that the failure of BOB.1 to drive transcription may be due a lack of function in the C-terminal activation domain. To test this possibility chimeric BOB.1 proteins were designed. Reciprocal catfish/human chimeras were constructed yielding two BOB.1 proteins, one that contained the N-terminal POU binding domain (PBD) of the catfish BOB.1 fused to the activation domain of the human BOB.1 and a second that contained the PBD of the human BOB.1 protein fused to the activation domain of the catfish BOB.1 protein (Fig. 6A). These chimeric proteins were tested for their ability to enhance transcription driven by endogenous Oct transcription factors. Vectors expressing either native catfish or human BOB.1 or the chimeric BOB.1 proteins were co-transfected into the catfish B cell line 1G8 with a reporter construct driven from a trimer of consensus octamer motifs (Fig. 6B). While native human BOB.1 was capable of driving transcriptional activation significantly (approximately 25-fold enhancement over the reporter construct), the inability of the catfish BOB.1 to drive transcription was also observed. Upon exchange of the activation domains, the chimera containing the catfish BOB.1 PBD with the human BOB.1 activation domain was able to drive transcription at a significantly enhanced level similar to that of human BOB.1 alone (Fig. 6B). In contrast, the BOB.1 chimera containing the human PBD with the catfish activation domain was unable to enhance transcription. These results suggest that the activation domain of catfish BOB.1 is functionally inactive. Further evidence supporting this conclusion was seen when the ability of the BOB.1 chimera with the human activation domain to interact with exogenously transfected catfish Oct2 β was tested. This was done in catfish B cells (1G8 line)

using the reporter construct containing the core region of the catfish *IGH* enhancer (Fig. 6C). These results confirm that catfish Oct2 β is transcriptionally active but that catfish BOB.1 is inactive, when tested with or without exogenously transfected catfish Oct2 β . However, the chimeric BOB.1 with catfish PBD/human activation domain significantly enhanced transcriptional activation when compared to the activation of Oct2 β alone (Fig. 6C). Collectively, these results indicate that the failure of catfish BOB.1 to drive transcriptional activation is due to the lack of a functional activation domain.

3.6. Catfish BOB.1 physically interacts with catfish Oct transcription factors in the presence of DNA, but not with catfish E-proteins

As shown above, catfish BOB.1 is capable of interacting with both Oct transcription factors when bound to their cognate motif. A series of pull down assays were employed to further understand the nature of the interactions between the catfish Oct transcription factors, the octamer motifs, the coactivator BOB.1 and other transcription factors important for driving transcription from the E μ 3' enhancer. A pull down assay was performed in order to demonstrate, by a method additional to EMSA (Fig. 5) the binding between the catfish Oct1 and the catfish BOB.1. In the presence of the variant octamer motif found within E μ 3', catfish Oct1 was able to bind to both catfish BOB.1 (Fig. 7A, lane 3) and human BOB.1 (lane 4). The empty vector control (omission of BOB.1) is shown in lane 2. Next, pull down assays were performed to determine if catfish BOB.1 physically interacts with the catfish E-protein, CFEB1. S-tagged catfish CFEB1 and Oct2 α were produced by *in vitro* transcription and translation and their ability to interact physically with the radiolabeled full-length catfish BOB.1 protein in the presence of the variant octamer motif (Fig. 7B) or the consensus μ E5 motif (Fig. 7C) found within the core enhancer was tested by pull down assay and analysis by SDS-PAGE. The size of the full-length catfish BOB.1 is shown in the input control in lane 1. Untagged, radiolabeled BOB.1 was added to the S-protein agarose as a negative control (lane 2). In the presence of the variant octamer motif (Fig. 7B), catfish BOB.1 was capable of interacting with catfish Oct2 α (lane 6), but it did not interact with catfish Oct2 α when no DNA was present (lane 5). Catfish BOB.1 did not interact physically with CFEB1 either in the presence (lane 4) or the absence (lane 3) of the octamer motif. An interaction between catfish BOB.1 and CFEB1 was not detectable in either the presence or the absence of the μ E5 motif (Fig. 7C). In order to confirm that the S-tagged CFEB1 and Oct2 α proteins were being pulled down by their S-tag, ³⁵S-labeled S-tagged CFEB1 and Oct2 α were mixed with the S-protein beads, and the reaction confirmed (Fig. 7D). Catfish BOB.1 that was ³⁵S-labeled but not S-tagged did not, as predicted, interact with the S-protein beads (Fig. 7D). Thus, from both EMSA experiments (Fig. 5) and pull down assays (Fig. 7) it can be concluded that catfish BOB.1 is capable of interacting with Oct transcription factors only in the presence of an octamer motif and that it does not interact physically with the E-protein CFEB1, even in the presence of the cognate μ E5 motif.

4. Discussion

This study identified and subsequently characterized the function of an orthologue of the mammalian coactivator BOB.1 from the channel catfish, *Ictalurus punctatus*. Amino acid sequence alignment (Fig. 2) showed a high degree of sequence conservation, primarily within the POU binding domain. A stretch of 20 amino acids corresponding to the POU interacting residues were 95% identical in the catfish BOB.1 sequence when compared with the corresponding region in the human BOB.1 (Fig. 2). Clear conservation in the other regions of the BOB.1 sequence could also be seen. The following nine residues have been shown to interact specifically with the POU domains of the mammalian Oct1 and Oct2 transcription factors (Chasman et al., 1999): Pro18, Gln20, Gly21, Val28, Lys29, Leu31, Leu32, Lys35, Arg36. All of these residues are conserved within the catfish BOB.1 sequence (Fig. 2) and are

consistent with the identification of the catfish BOB.1 as an orthologue of the human BOB.1. A genomic analysis comparing the catfish BOB.1 to the BOB.1 gene in other teleost fish has been previously described (Lennard Richard et al., 2007). Briefly, orthologues of the human and catfish coactivator BOB.1 were identified in four out of the five fish genomes publicly available. All of the BOB.1 genes appear to be single copy (as is the case with mouse and human) with the exception of the zebrafish BOB.1, which appears to have multiple transcripts that may well be an artifact of inaccurate genome assembly (Lennard Richard et al., 2007). This analysis suggests that BOB.1 is a single copy gene in the catfish and no pseudogene is present. Additionally, no isoforms of the BOB.1 were found while cloning the catfish BOB.1 gene. When using gene-specific primers to clone BOB.1 from the B cell genomic library, only a single band could be seen. B cell specificity is a striking feature of mammalian BOB.1 (Gstaiger et al., 1995; Luo and Roeder, 1995; Massa et al., 2003; Schubart et al., 1996b; Strubin et al., 1995), and this tissue-specific expression is conserved at the level of the catfish (Fig. 3). The strong conservation of sequence (particularly within the POU interacting residues), the genomic analysis clearly identifying the catfish sequence as an orthologue (Lennard Richard et al., 2007), and the RT-PCR, which suggest that catfish BOB.1 is found only in B lymphocytes, taken collectively, indicate that the catfish molecule identified in this study is an orthologue of the mammalian coactivator BOB.1.

Given the high level of conservation in both structure and expression pattern for the catfish BOB.1, it was predicted that its function would also be consistent with what is known for its mammalian counterparts. This expectation was supported by the ability of the catfish Oct2 to interact functionally with the human BOB.1 to enhance transcriptional activation (Ross et al., 1999), and in addition it was hypothesized that the catfish BOB.1 may rescue the transcriptional activity of the functionally inactive catfish Oct1 transcription factor (Lennard et al., 2007). Catfish BOB.1, however, was unable to enhance transcriptional activation from a variety of physiologically relevant reporter constructs (Fig. 4). Unlike the transcriptional enhancement seen when human BOB.1 was cotransfected with catfish Oct2, no increase in transcription was seen upon cotransfection of catfish Oct2 with the catfish BOB.1. Furthermore, cotransfection of catfish BOB.1 with catfish Oct1 did not confer on catfish Oct1 the capability to drive transcription (Fig. 4). To exclude the possibility that the endogenous transcription factors within the catfish B cell line may impact the ability to measure a transcriptional enhancement, transfection of the catfish BOB.1 alone or in addition to the catfish Oct transcription factors was carried out within the catfish T cell line, previously shown to lack BOB.1 (Fig. 3). The absence of endogenous BOB.1 had no influence on the transcriptional activation potential of the catfish BOB.1, and it failed to drive or enhance transcription driven by Oct factors in catfish T cells (data not shown).

The failure of catfish BOB.1 to act as a transcriptional coactivator is not the result of a failure to bind Oct transcription factors. Both EMSA and pull-down assays showed clearly that catfish BOB.1 will bind to octamer transcription factors (both catfish Oct1 and Oct2) in the presence of a cognate Oct motif (Figs. 5 and 7). It is important to note that while BOB.1 is capable of binding the Oct transcription factors, the strength of this interaction has not been determined and may indeed be weaker than the binding of mammalian BOB.1 to mammalian Oct transcription factors. However, it is necessary to question whether or not catfish BOB.1 possesses a functional transactivation domain.

Within mammalian BOB.1, only two domains have been defined; the POU binding domain (contained within the N-terminus; amino acids 1–44) and an activation domain (mapped to the remaining amino acids 44–256) (Krapp and Strubin, 1999). Since the sequence identity between catfish and human BOB.1 within the C terminus overall is only 41% (Fig. 2) the lack of a functional activation domain is a logical possibility for the failure of catfish BOB.1 to enhance transcriptional activation. To test this, vectors expressing chimeras of the catfish and

human BOB.1 coactivators were constructed and their ability to enhance transcription driven by Oct transcription factors was assessed. The results were very clear: a chimera with the catfish PBD and the human activation domain was active, whereas a chimera with the human PBD and the catfish activation domain was inactive (Fig. 6). Thus, failure of the catfish BOB.1 to enhance transcription is clearly due to an absence of a functional activation domain and not to its failure to bind the Octoctamer complex. We are left with the curious observation that the channel catfish possesses two transcription factors (Oct1 and BOB.1) that bind appropriately to their ligands but lack all transcriptional activating activity under the conditions that have been tested. This leaves open the question as to what role these two orthologues of important mammalian transcription factors may play in this species. A pull down assay to test whether a possible function of the catfish BOB.1 was the recruitment of the catfish E-proteins to the enhancer failed to demonstrate an interaction with CFEB (Fig. 7). Thus, in the case of catfish BOB.1, it is possible that it plays a (non-transcriptional) role in mediating signaling from the (B cell receptor) one of the two known roles for BOB.1 in mammalian B cells.

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Fig. 1.

Schematic for the cloning of catfish BOB.1. Block figure depicting the cloning strategy for the catfish BOB.1 (Genbank accession no., EF058163). The N-terminal POU binding domain (PBD, black box with white dots) and the C-terminal activation domain (black and white box with horizontal stripes) are denoted above. The POU interacting residues are represented by a white box and the brackets below. The dotted line indicates the partial sequence in the 5' and 3' UTR. Dashed arrows indicate the regions to which the degenerate primers were made that produced productive catfish BOB.1 sequence. The solid arrows indicate the specific primers used to verify the entire catfish BOB.1 sequence (see also Table 1).

Fig. 2.

Amino acid sequence alignment of Catfish BOB.1 and the POU interacting residues. Amino acid sequence alignment of BOB.1 proteins generated in Megalign (DNASTar). The shaded regions indicate sequence similarity between the proteins. The POU interacting residues are marked by the black brackets and a dashed box, and asterisks below the sequence denote the residues directly involved in binding. The black arrow indicates the split for the production of chimeric proteins, the POU binding domain is the N-terminal region (to the left of the arrow) and the activation domain is the C-terminal region (to the right of the arrow). Protein alignment is based on the amino acid sequences (NCBI accession number in parenthesis) of Human BOB.1 (Q16633), Mouse BOB.1 (NM_011136), Chicken BOB.1 (AB052869), Cow BOB.1 (NM_001075915) and Catfish BOB.1 (EF058163).

Fig. 3.

Catfish BOB.1 is B lymphocyte specific. Expression of catfish BOB.1 by RT-PCR in the catfish B cell line 1G8, the catfish T cell line G14D, the catfish fibroblast cell line G5F, the catfish macrophage line 42TA, spleen, head kidney, trunk kidney, brain, heart, gill, intestine. Catfish BOB.1 (upper box), actin control (lower box). Bracket indicates tissues important in immune function and an asterisk indicates the tissues expressing the BOB.1 transcript at higher levels.

Fig. 4.

Catfish BOB.1 fails to enhance transcription. Transcriptional activation of catfish BOB.1 in the catfish B cell line 1G8. For all diagrams; Octamer number depicted within the light grey boxes. Octamer motifs (light grey boxes), deleted μ E5 motif (dark grey rectangle with strikethrough), promoters (dark grey arrows), luciferase gene (white box), CAT gene (diagonally stripped box). (A) *Top*: schematic diagram of the octamers only reporter construct with a deleted μ E5 motif (pGL3/ Δ 56/R#2/ $\Delta\mu$ E5). *Bottom*: transcriptional activation from the reporter construct (pGL3/ Δ 56/R#2/ $\Delta\mu$ E5) by reporter construct alone, pRc/CMV, catfish Oct2 β (pRc/CMV/Oct2 β), catfish Oct1 (pRc/CMV/Oct1), catfish BOB.1 (pRc/CMV/CFB0B.1), or by the cotransfection of catfish Oct2 β (pRc/CMV/Oct2 β) and catfish BOB.1 (pRc/CMV/

CFBOB.1), or catfish Oct1 (pRc/CMV/Oct1) and catfish BOB.1 (pRc/CMV/CFBOB.1). (B) *Top*: schematic diagram of the octamer trimer (pO3- Δ 56-CAT). *Bottom*: transcriptional activation from the octamer trimer (pO3- Δ 56-CAT) reporter construct by reporter construct alone, pRc/CMV, catfish Oct2 β (pRc/CMV/Oct2 β), catfish Oct1 (pRc/CMV/Oct1), catfish BOB.1 (pRc/CMV/CFBOB.1), or by the cotransfection of catfish Oct2 β (pRc/CMV/Oct2 β) and catfish BOB.1 (pRc/CMV/CFBOB.1), or catfish Oct1 (pRc/CMV/Oct1) and catfish BOB.1 (pRc/CMV/CFBOB.1). (C) *Top*: schematic diagram of the replicated octamer sixmer (Δ 56 (10-11*3)-CAT). *Bottom*: transcriptional activation from the replicated octamer sixmer (Δ 56 (10-11*3)-CAT) reporter construct by reporter construct alone, pRc/CMV, catfish Oct2 β (pRc/CMV/Oct2 β), catfish Oct1 (pRc/CMV/Oct1), catfish BOB.1 (pRc/CMV/CFBOB.1), or by the cotransfection of catfish Oct2 β (pRc/CMV/Oct2 β) and catfish BOB.1 (pRc/CMV/CFBOB.1), or catfish Oct1 (pRc/CMV/Oct1) and catfish BOB.1 (pRc/CMV/CFBOB.1). Values presented by the mean $\pm$ standard error for six replicate experiments (A and C) and nine replicate experiments (B). Statistical significance was calculated by a Student's *t*-test, assuming equal variances. Single asterisks above solid brackets indicate a *p*-value of <0.05, double asterisks above solid brackets indicate a *p*-value of <0.01.

Fig. 5. Binding of BOB.1 proteins to the Oct-octamer complex. *In vitro* binding of the catfish BOB.1 POU binding domain (CFPBD) or the human BOB.1 POU binding domain (HPBD) to catfish Oct1 or Oct2β bound to the variant octamer motif found within the core enhancer of Eμ3'.

(A) Binding of the BOB.1 PBDs to the catfish Oct2β. Free probe (lane 1), TNT lysate (lane 2), catfish Oct2β (lane 3), catfish Oct2β plus 100X cold competitor (lane 4), catfish Oct2β plus 100X scrambled competitor (lane 5), catfish Oct2β plus S-protein (lane 6), catfish Oct2β plus NRS (lane 7), catfish Oct2β plus CFPBD (lane 8), catfish Oct2β plus CFPBD plus S-protein (lane 9), catfish Oct2β plus CFPBD plus NRS (lane 10), catfish Oct2β plus HPBD (lane 11), catfish Oct2β plus HPBD plus S-protein (lane 12), catfish Oct2β plus HPBD plus NRS (lane

13). Black and white lines indicate the central point of the shifted region. Black arrows indicate non-specific binding, free probe. The shifted bands are labeled to the right of the figure. (B) Binding of the BOB.1 PBDs to the catfish Oct1. Catfish Oct1 (lane 1), catfish Oct1 plus 100X cold competitor (lane 2), catfish Oct1 plus 100X scrambled competitor (lane 3), catfish Oct1 plus S-protein (lane 4), catfish Oct1 plus NRS (lane 5), catfish Oct1 plus CFPBD (lane 6), catfish Oct1 plus CFPBD plus S-protein (lane 7), catfish Oct1 plus CFPBD plus NRS (lane 8), catfish Oct1 plus HPBD (lane 9), catfish Oct1 plus HPBD plus S-protein (lane 10), catfish Oct1 plus HPBD plus NRS (lane 11), empty (lane 12), catfish Oct1 alone (lane 13). Black and white lines indicate the central point of the shifted region. The shifted bands are labeled to the right of the figure.

Fig. 6. Transcriptional activity of the chimeric BOB.1 proteins. (A) Schematic of the experimental design for the production of BOB.1 chimeric proteins. Upper portion shows the original BOB.1 proteins, the human BOB.1 POU binding domain (PBD, white box with black dots) and the human BOB.1 activation domain (white box with vertical black stripes), and the catfish BOB.1 PBD (black box with white dots) and the catfish BOB.1 activation domain (white box with horizontal black stripes). The lower portion shows the resulting chimeric proteins, the H/CF chimera has the human BOB.1 PBD and the catfish BOB.1 activation domain, while the CF/H chimera has the catfish PBD and the human activation domain. (B) Transcriptional activity of the chimeric BOB.1 proteins from the reporter construct (pO3-Δ56-CAT) by the reporter construct alone, pRc/CMV alone, pRc/CMV/CFBOB.1, pRc/CMV/HBOB.1, pRc/CMV/CF/H chimera, pRc/CMV/H/CF chimera. (C) Transcriptional activity of the chimeric BOB.1 proteins from the (pGL3/Δ56/R#2/ΔμE5) reporter construct by the reporter construct alone, pRc/CMV alone, pRc/CMV/Oct2β, pRc/CMV/CFBOB.1, pRc/CMV/CF/H chimera, or the cotransfection of pRc/CMV/Oct2β and pRc/CMV/CFBOB.1, or the cotransfection of pRc/CMV/Oct2β and pRc/CMV/CF/H chimera. Values are displayed as the mean ± standard error for six replicate experiments. Statistical significance was calculated by a Student's *t*-test,

assuming equal variances. Single asterisks above solid brackets indicate a p value of <0.05 , double asterisks above solid brackets indicate a p value of <0.01 .

Fig. 7.

Catfish BOB.1 physically interacts with catfish Oct2α in the presence of DNA, but not with catfish E-protein. Pull down assay by affinity chromatography on S-protein agarose. (A) Binding of catfish Oct1 to the PBD of catfish BOB.1 and human BOB.1. Lane 1, input (1/25 volume) of ³⁵S-methionine labeled catfish Oct1; lane 2, empty pCITE vector plus catfish Oct1; lane 3, catfish PBD (CFPBD) plus catfish Oct1; lane 4, human PBD (HPBD) plus catfish Oct1. Arrows denote free probe and Oct1 (as labeled). (B and C) *In vitro* synthesized ³⁵S-labeled catfish BOB.1 was mixed with *in vitro* synthesized non-labeled S-tagged catfish Oct2α (O2) or CFEB1 (C1) pre-bound to S-protein agarose beads in the presence of the variant octamer motif (B) or μE5 motif (C). Lane 1 shows the input (1/50 volume) of ³⁵S-labeled BOB.1. Lane 2 was assayed without S-tagged protein as a negative control. In lanes 3–6 the addition of Oct2α (O2) or CFEB1 (C1) is indicated by (+). (D) Reaction of S-tagged proteins with S-protein agarose as demonstrated in SDS-PAGE pull-down experiments. ³⁵S-labeled S-tagged proteins (CFEB1-S or Oct2α-S) or non-tagged BOB.1 were reacted with S-protein agarose beads. The reaction with the S-protein is confirmed in the lanes labeled S-tag PD. One-tenth volumes of the input ³⁵S-labeled proteins were analyzed in the input lanes.

Table 1

Synthetic oligonucleotides.

Primer #	Description	Length	Sequence ^a
<i>Degenerate primers used to clone catfish BOB.1</i>			
G-2715	Degenerate primer near POU binding domain	20	F: 5'-CCV TAC CAR GGN GTB CGH GT-3'
G-2716	Degenerate primer within POU binding domain	20	F: 5'-CCM GTS AAR GAR YTR YTG AR-3'
G-2717	Degenerate primer within second conserved region	21	F: 5'-CAG MCY VTG TGY CCC AGY TAC-3'
G-2718	Reverse primer of G-2717	21	R: 5'-GTA RCT GGG RCA CAB RGK CTG-3'
G-2719	Degenerate primer within third conserved region	21	R: 5'-GWR AGG GGY TGD GSC CAS GGG-3'
<i>Specific primers used to clone catfish BOB.1</i>			
G-2804	Specific primer within 5' region of CFBOB.1	21	F: 5'-ACA GCC AAA ACA TTG CCT CC-3'
G-2805	Specific primer within 5' region of CFBOB.1	21	F: 5'-TTG GAG GCA ATG TTT TGG CTG-3'
G-2806	Specific primer within 3' region of CFBOB.1	17	R: 5'-GGG GAG GGT GGA GAT GC-3'
G-2807	Specific primer within 3' region of CFBOB.1	24	F: 5'-AGA GCA TCT CCA CCC TCC CCT CCC-3'
G-2887	Specific primer within 5' UTR	22	F: 5'-GAG GCG AAT TAG CGT GTT AGT G-3'
G-2888	Specific primer within 3' UTR	21	F: 5'-CCT TTG CTG TTC TAA GCA CTG-3'
G-2889	Specific primer within 3' UTR	21	R: 5'-CAG TGC TTA GAA CAG CAA AGG-3'
<i>Primers used in vector cloning</i>			
G-2938	Catfish BOB.1 in pRc/CMV	39	F: 5'-TAT AAG CTT GGG GCC ACC ATG CAC ATG GGA AAA TCA CTG-3'
G-2939		33	R: 5'-GCT CTA GAG CCT AAA TGT CTT GTG TAA ACA GTG-3'
G-2686	Human BOB.1 POU binding domain into pCITE	35	F: 5'-TTT GGA TCC GAT GCT CTG GCA AAA ACC CAC AGC TC-3'
G-3074		32	R: 5'-TTT AGA TCT GGT GCT GCC CCA CTG CTG GCG TG-3'
G-3072	Catfish BOB.1 POU binding domain in pCITE	30	F: 5'-TTG AGC TCG ATG CAC ATG GGA AAA TCA CTG-3'
G-3073		31	R: 5'-TTT AGA TCT TGT TTT GGC TGT ATT TCC TCT C-3'
G-3591	Catfish BOB.1 into pCITE	56	F: 5'-GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GCA CAT GGG AAA ATC ACT GT-3'
G-3592		24	R: 5' TTA AAT GTC TTG TGT AAA CAG TGA 3'
<i>Primers used to clone BOB.1 chimeras</i>			
G-3245	Internal primers to clone CF/Hu chimera	34	R: 5'-CCG CCG TAG GTG CAG G/TG TTT TGG CTG TAT TTC C-3'
G-3246		34	F: 5'-GGA AAT ACA GCC AAA ACA/CCT GCA CCT ACG GCG G-3'
G-3249	Primers to clone HuBOB.1 into pRc/CMV	33	F: 5'-TTT AAG CTT GGC ACC ATG CTC TGG CAA AAA CCC-3'
G-3250		33	R: 5'-TTT TCT AGA CTA CTA AAA GCC TTC CAC AGA GAG-3'

Primer #	Description	Length	Sequence ^a
G-3247	Internal primers to clone Hu/CF chimera	31	R: 5'-CTG CAG TTG GAG GCA A/TG CTG CCC CAC TGC T-3'
G-3248		31	F: 5'-AGC AGT GGG GCA GCA/TTG CCT CCA ACT GCA G-3'
<i>Oligonucleotides for EMSA (octamer motifs are underlined)</i>			
G-2110	Catfish Eμ3' Oct#10	28	F: 5'-GCA AAA CAC TGC <u>ATG TAA AT</u> AGT CTA AT-3'
G-2111		28	R: 5'-CAT TAT TAG ACT <u>ATT TAC AT</u> GCA GTG TT-3'
<i>Scrambled oligonucleotides for EMSA</i>			
G-2132	Catfish Eμ3' Oct#10	28	F: 5'-GCA AAA CAC TGC <u>TAT ATG AA</u> AGT CTA AT-3'
G-2133		28	R: 5'-CAT TAT TAG ACT <u>TTC ATA TA</u> GCA GTG TT-3'
<i>Primers used in RT-PCR</i>			
G-2887	Specific primer within 5' UTR	22	F: 5'-GAG GCG AAT TAG CGT GTT AGT G-3'
G-2889	Specific primer within 3' UTR	21	R: 5'-CAG TGC TTA GAA CAG CAA AGG-3'
G-1480	Primer to catfish Actin	22	F: 5'-CCC ACA CTG TGC CCA TCT ATG A-3'
G-1481	Primer to catfish Actin	21	R: 5'-GAC AGG GAG CCC AGG ATT GAG-3'

^aF: forward; R: reverse.