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Expression and antiviral activity of a β-defensin-like peptide identified in the rainbow trout (*Oncorhynchus mykiss*) EST sequences

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Abstract

The *in silico* identification of a β-defensin-like peptide sequence (omBD-1) in the rainbow trout (*Oncorhynchuss mykiss*) database of salmonid EST is reported here. We have studied the transcript expression of this β-defensin-like sequence in different organs and expressed the recombinant peptide in a fish cell line. Finally, we have demonstrated the *in vitro* antiviral activity of the recombinant trout β-defensin-like peptide against viral haemorrhagic septicaemia rhabdovirus (VHSV), one of the most devastating viruses for worldwide aquaculture. Thus, the resistance to VHSV infection of EPC cells transfected with pMCV 1.4-omBD-1 has been shown. Since EPC cells transfected with omBD-1 produced acid and heat stable antiviral activity and up regulation of Mx, a type I IFN-mediated mechanism of antiviral action is suggested. To our knowledge, this is the first report showing biological activity of a β-defensin-like peptide from any fish.

Keywords: Antimicrobial peptide; Defensin; VHSV; IFN; Mx

1. Introduction

Antimicrobial peptides (AMPs) are ancient components of the innate immune system and have been isolated from organisms spanning the phylogenetic spectrum. Defensins constitute a family of evolutionarily related vertebrate AMPs with antimicrobial, antiviral and immunomodulatory properties that have a characteristic β-sheet-rich fold and a framework of six disulphide-linked cysteines (Ganz, 2003; Selsted et al., 1985). The vertebrate defensins are classified into α-, β- and θ-defensins, which differ in the distribution of the disulphide bonds between their six conserved cysteine residues (Klotman and Chang, 2006; Selsted and Ouellette, 2005; Yang et al., 2002). Both α- and β-defensins have been identified in almost every vertebrate species searched but not θ-defensins (Pazgier et al., 2006; Tang et al., 1999). Defensin and defensin-like peptides have also been identified in invertebrates (Castro and Fontes, 2005; Garcia-Olmedo et al., 1998; Lay and Anderson, 2005; Lehrer and Ganz, 2002; Selsted and Ouellette, 2005) and plants (Castro and Fontes, 2005; Garcia-Olmedo et al., 1998; Lay and Anderson, 2005; Lehrer and Ganz, 2002; Selsted and Ouellette, 2005).

In fish, homologue sequences to β-defensins from higher vertebrates have been recently identified only in three species, zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*) and tetraodron (*Tetraodon nigroviridis*) (Zou et al., 2007). No antimicrobial, antiviral or immunomodulatory properties of these fish β-defensins-like peptides have been described so far. The presence of α-defensins in fish has not been reported yet.

Because of the economic and social impact of both bacterial and viral infections in aquaculture and since no efficient therapeutic agents against fish pathogens have yet been developed, defensin identification and characterization in commercially important fish species could be of great interest. In light of this, the identification of a β-defensin-like sequence from rainbow trout (*Oncorhynchuss mykiss*), a continental fish specie of economical importance in aquaculture is reported here. Moreover, we have studied the expression of this trout β-defensin-like sequence (omBD-1) in different trout organs, and expressed the recombinant peptide in a fish cell line. Finally, we have also assessed the biological activity of the recombinant omBD-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV),

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one of the most devastating viruses for worldwide aquaculture (Lorenzen and LaPatra, 2005; Olesen and Korsholm, 1997). All the results obtained suggest that further studies of this novel trout β-defensin-like peptide as well as its place in the fish innate immunity *in vivo* could lead to development of new strategies and agents to prevent and/or treat fish viral infections.

2. Materials and methods

2.1. Search of β-defensin-like sequences in salmonids EST databases

The individual nucleotide coding sequences of each of the 36 human β-defensins sequences (http://www.expasy.org/cgi-bin/sprot-search-de?human%20beta%20defensin) shown in Table 1 were used to screen the database of expressed sequence tags (ESTs) from salmonid (http://grasp.mbb.sfu.ca). BLAST analysis was made with the BLAST software from the EST BLAST server on the Genomic Research on Atlantic Salmon Project home page (http://grasp.mbb.sfu.ca). It retrieved one related sequence of 779 bp (GeneBank accession number, BX073898) from rainbow trout (*O. mykiss*). Analysis of the translated protein sequence was made by the translate tool from ExPASy proteomic server (http://www.expasy.org/tools/dna.html).

2.2. Sequences analysis, phylogenetics analysis and protein modelling

The existence of a signal peptide was predicted by the SignalP 3.0 program (Bendtsen et al., 2004). The molecular weight, the isoelectric point (p*I*) and the net charge of the peptide were calculated using the ProtParam tool (http://www.expasy.org/tools). Multiple sequence alignments between omBD-1 and the known β-defensin-like of several fish and human β-defensin-26 (hBD-26) sequences (Table 2) (UniProtKB/Swiss-Prot database) were generated with the Clustal W program (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1994), and identity percentages (IP) among sequences were determined with the LALING program (http://www.ch.embnet.org/software/LALIGN_form.html). A phylogenetic tree based on the deduced aminoacid sequences was performed by using the Neighbour–Joining (NJ) algorithm. Finally, omBD-1 amino acid sequence was modelled using the Swiss-PdbViewer program (Guex and Peitsch, 1997). Mouse β-defensin 8 (PDB file code: IE4R) was selected as template

Table 1
Human β-defensin (hBD) mature peptide sequences used to screen the salmonid EST database

hBD	UniProtKB/Swiss-Prot entry
hBD-1	P60022
hBD-2	O15263
hBD-3	P81534
hBD-4	Q8WTQ1
hBD-5	Q8NG35
hBD-6	Q8N104
hBD-7	Q8IZN7
hBD-8	Q8NET1
hBD-9	Q30KR1
hBD-10	Q30KR0
hBD-11	Q30KQ9
hBD-12	Q30KQ8
hBD-13	Q30KQ7
hBD-14	Q30KQ6
hBD-15	Q30KQ5
hBD-16	Q30KQ4
hBD-17[a]	Q30KQ3
hBD-18	Q96PH6
hBD-19	Q8N690
hBD-20	Q8N689
hBD-21	Q5J5C9
hBD-23	Q8N688
hBD-24	Q8NES8
hBD-25	Q8N687
hBD-26	Q9BYW3
hBD-27	Q9H1M4
hBD-28	Q7Z7B8
hBD-29	Q9H1M3
hBD-30	Q30KQ2
hBD-31	P59861
hBD-32	Q7Z7B7
hBD-33	Q30KQ1
hBD-34	Q4QY38
hBD-35	Q30KP9
hBD-36	Q30KP8

[a] Fragment, not complete sequence.

Table 2
β-Defensin (BD) mature peptide sequences used to compare with omBD-1

Name	Accession number	Specie	aa	Net charge	Mw (KDa)	pI
omBD-1	918595	***O**ncorhynchus* ***m**ykiss* (Trout)	41	+2	4.38	8.35
zfBD-1	AM181358	*Danio rerio* (**Z**ebra **f**ish)	43	+1	4.52	7.82
zfBD-2	AM181359	*Danio rerio* (**Z**ebra **f**ish)	43	+3	5.11	8.68
zfDB-3	AM181360	*Danio rerio* (**Z**ebra **f**ish)	43	+7	5.29	9.50
fuBD-1	BN000875	*Takifugu rubripes* (**Fu**gu)	42	+2	4.54	8.35
tnBD-1	BN000873	***T**etraodon* ***n**igroviridis* (Green spotted pufferfish)	42	+2	4.50	8.35
tnBD-2	BN000874	***T**etraodon* ***n**igroviridis* (Green spotted pufferfish)	45	+2	5.41	8.34
ogBD-1	AY129305	*Epinephelus coioides* (**O**range spotted **g**rouper)	43	+4	5.17	8.92
hBD-26	AF525928	*Homo sapiens* (**H**uman)	43	+4	4.95	8.84

Letters in bold in species names were used to identify the BD names. Fish β-defensin mature peptide sequences were obtained from Zou et al. (2007) and hBD-26 mature peptide sequence (NWYVKKCLND VGICKKKCKPEEMHVKNGWAMCGKQRDCCVP ADRRANYPVFCVQ TKTTRISTVTATTATTTLMMTT ASMSSMAPTPVSPTG) correspond to UniProtKB/Swiss-Prot entry Q9BYW3.

structure because of its higher sequence similarity among all the available β-defensin PDB files.

2.3. Fish

Rainbow trout of approximately 8–10 cm obtained from Lillogen (Leon, Spain) were maintained at the University Miguel Hernández (UMH) aquarium at 12–14 °C and fed daily with a commercial diet (Trouw, Leon, Spain). Prior to experiments, fish were acclimatised to laboratory conditions during at least 2 weeks.

2.4. Expression studies

The expression of omBD-1 in different trout tissues was analyzed by RT-PCR in 12 healthy trout by using specific primers deduced from the trout EST encoding the omBD-1 (omBD-1 forward 5′-ATGGTCACTTTGGTGCTCCTGG-3′ and reverse 5′-TTAGAAATGAGAAACACAGCACAAG-3′). Fish were sacrificed by overexposure to tricaine methanesulfonate, MS222 (Sigma) and head kidney, spleen, liver and muscle dissected for RNA extraction. The "Total RNA Isolation System" kit (Promega) was used for RNA extraction from the different rainbow trout organs following manufacturer's instructions. Isolated RNAs were resuspended in diethylpyrocarbonate (DEPC)-treated water, treated with DNase (RQ1 RNAase-Free Dnase, Promega) and stored at −80 °C until used. Two micrograms of RNA were used to obtain cDNA by using the Moloney murine leukaemia virus reverse transcriptase (M-MLV) (Invitrogen). Briefly, RNA was incubated with 1 μl of random hexamers (50 μM) (Roche) and 1 μl 10 mM deoxynucleotide triphosphate (dNTP) mix for 5 min at 65 °C. After the incubation, 4 μl of 5× first strand buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 2 μl 0.1 M dithiothreitol (DTT) were added, mixed and incubated for 2 min at 42 °C. Then, 1 μl of M-MLV reverse transcriptase was added and the mixture incubated at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min and the resulting cDNA stored at −20 °C.

All PCR amplification reactions were performed by using 0.5 μl dNTP mix (10 mM each), 0.125 μl Taq DNA polymerase (Roche, Barcelona, Spain), 2.5 μl Taq 10× buffer, 1 μl of each primer (20 μM) and 2.5 μl of cDNA in a final volume of 25 μl. A parallel PCR with primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Wang et al., 2004) was performed with all samples as a house-keeping gene to standardise the RT-PCR results using the conditions previously described (Tafalla et al., 2007; Wang et al., 2004). PCRs were carried out in a GeneAmp® PCR System 2700 cycler (Applied Biosystems). The amplification conditions consisted in a denaturing step of 94 °C for 5 min followed by 25–30 cycles of 94 °C 30 s, 52 °C 30 s and 72 °C 30 s followed by a final extension step of 72 °C 7 min. PCR products (8 μl) were visualised on a 1.5% agarose gel stained with ethidium bromide. A 100 bp ladder was used as a size marker.

2.5. Cloning and sequencing of omBD-1

For cloning purposes, all PCR products amplified from trout head kideny and muscle were resolved on a 1% agarose gel; bands excised from the gel, extracted by using Gene-Clean (Bio 101, La Jolla, CA, USA) and then cloned into the PCR II-Topo vector (Invitrogen, CA, USA). The sequence of the inserts was determined by Sistemas Genómicos S.L. (Valencia, Spain) using specific primers for the PCR II-Topo vector. After sequencing, one of the PCR product obtained from muscle (183 bp) was excised from the PCR II-Topo with the restriction enzymes KpnI and XhoI and subcloned into the KpnI/XhoI site of the expression vector pMCV 1.4 (Ready-Vector, Madrid, Spain) following standard procedures. The pMCV 1.4 vector contains the human cytomegalovirus immediate-early-promoter (CMV).

2.6. Cell cultures and virus

The fish cell line EPC (*epithelioma papulosum cyprinid*) (Fijan et al., 1983), purchased from the European collection of cell cultures (ECACC n°. 93120820) was used in this work. EPC cells were maintained at 28 °C in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen corporation, UK) cell culture medium containing 10% fetal calf serum (Sigma Chem. Co., St. Louis, MO, USA), 1 mM pyruvate (Gibco), 2 mM glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone.

Viral haemorrhagic septicaemia virus (VHSV 07.71) isolated in France from rainbow trout, *O. mykiss* (LeBerre et al., 1977) was propagated in EPC cells at 14 °C as previously reported (Basurco and Coll, 1989). Supernatants from VHSV 07.71 infected EPC cell monolayers were clarified by centrifugation at 1000 × *g* for 20 min and kept in aliquots at −70 °C. Clarified supernatants were used for the experiments.

2.7. Transfection assays and analysis of the expression of omBD-1 transcripts by RT-PCR in EPC transfected cells

For cell transfection, EPC cell monolayers, grown in culture flasks of 75 cm^2, were detached using trypsine (Sigma), washed, resuspended in culture medium supplemented with 10% of FCS and dispensed into 96-well plates at a concentration of 3×10^4 cells per well in a final volume of 100 μl. Next day, different concentrations (from 0.25 to 2 μg/ml) of either pMCV 1.4-omBD-1 or pMCV 1.4 complexed with 0,3 μl of FuGene 6 (Roche) were incubated for 15 min in 25 μl of RPMI-1640 containing 2 mM Cl_2Ca and then added to each well in 100 μl of culture medium with 10% of FCS (Brocal et al., 2006; Lopez et al., 2001). As an additional control, EPC cells were transfected with FuGene 6 alone following the same procedure. The plates were further incubated at 28 °C for 48 h. The expression of transcripts from the recombinant omBD-1 in transfected cells was then assessed by RT-PCR. RNA extraction from transfected cells, synthesis of cDNA and PCR amplification of the omBD-1 sequence were performed as described above (Section 2.3) but carp β-actin and their primers (Y.B. Zhang et al., 2004). PCR products (8 μl) were visualised on a 1% agarose gel stained with ethidium bromide. Samples to be compared were run in the same agarose gel. The intensity of the amplified bands was estimated using the Imagen Scion Program (www.scionorg.com). Analysis of mRNA transcription of the

recombinant omBD-1 was performed and expressed as relative to the β-actin gene transcription (expression relative to β-actin). PCR products amplified from transfected EPC cells were sequenced.

2.8. Preparation of conditioned medium (CM) from EPC cell cultures

In order to prepare conditioned medium (CM) from transfected cells, EPC cells were transfected with 1 μg/ml of pMCV 1.4-omBD-1, pMCV 1.4 or FuGene alone as indicated above (Section 2.6) for 24 h. After extensive washes with PBS, 100 μl of fresh medium supplemented with 10% FCS were added to each well. Following incubation at 28 °C for 24 h, the supernatants collected from transfected cells were clarified by centrifugation at 800 × *g* for 10 min. Clarified supernatants were then treated with 1N HCl, which reduced the pH to 2. After overnight incubation at 4 °C, the samples were heated to 50 °C for 60 min and the pH was restored to 7.6 by addition of 1N NaOH.

2.9. Viral infectivity assays

To assay for VHSV infectivity, a previously developed immunostaining focus assay (focus forming units, f.f.u.) was used (Lorenzo et al., 1996; Mas et al., 2002, 2006; Micol et al., 2005; Perez et al., 2002). Briefly, EPC cells, grown in 96-well plates, either non-transfected, transfected or treated during 24 h with CM (diluted from 1 to 1/500) were washed extensively with PBS and then infected with VHSV (multiplicity of infection (m.o.i.) of 2×10^{-3}) in a final volume of 100 μl/well of culture medium supplemented with 2% FCS. Infected EPC cells were incubated for 24 h at 14 °C. The EPC cell monolayers were then fixed for 10 min in cold methanol and air-dried. Monoclonal antibody (MAb) 2C9 directed towards the N protein of VHSV diluted 1000-fold in dilution buffer (0.24 mM merthiolate, 5 g/l Tween 20, 50 per 1 mg of phenol red in PBS, pH 6.8) were added to the wells (100 μl/well) and incubated for 1 h at room temperature. After washing with distilled water, 100 μl of peroxidase-labelled rabbit anti-IgG mouse antibody (Ab) (Nordic, Tilburg, The Netherlands) were added per well, and incubation was continued for 30 min. After three washings by immersion in distilled water, 50 μl of 1 mg/ml per well of diaminobenzidine (DAB) (Sigma) in PBS containing H_2O_2 were added (Lorenzo et al., 1996; Sanz and Coll, 1992) and the reaction allowed to proceed until brown foci were detected with an inverted microscope (Nikon Eclipse TE2000-U, Nikon Instruments Inc., NY, USA). Once washed with water and air dried, brown foci of DAB stained cells (VHSV-infected cell foci) were counted with an inverted microscope with a 10× ocular eye grid (Lorenzo et al., 1996).

2.10. Detection of the mRNA of carp Mx1 gene in transfected EPC cells by RT-PCR

The expression of the IFN-inducible Mx1 gene of carp was analyzed by RT-PCR in EPC cells transfected with different amounts of pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6 alone as indicated in Section 2.6. As positive control of Mx expression, EPC cells, grown in 96-well plates, were treated with 30 μg/ml of polyribocytidylic acid (poly I:C) (Pharmacia, Piscataway, NJ, USA) a well-known type I IFN-inducer. After an incubation of 48 h at 28 °C cells were harvested. In all cases, after the incubation period, the medium was removed, cells dettached with Ca^{2+} and Mg^{2+}-free PBS, total RNA extracted and cDNA synthesized as indicated in Section 2.3. The primers previously designed (Y.B. Zhang et al., 2004) to amplify a 349 bp fragment in the Mx1 sequence of carp were used. The amplification conditions consisted in a denaturing step of 94 °C for 2 min followed by 30 cycles of 94 °C 1 min, 60 °C 1 min and 72 °C 1.30 min followed by a final extension step of 72 °C 5 min. PCR products (8 μl) were visualised on a 1.5% agarose gel stained with ethidium bromide. A 100 bp ladder was used as a size marker. Analysis of mRNA transcription of the carp Mx was performed and expressed as relative to the β-actin gene transcription (expression relative to β-actin). Furthermore, PCR products were sequenced.

3. Results

3.1. Sequence analysis

The search in the database of salmonid EST with one of the 36 human β-defensin nucleotide sequences used in this study (Table 1), the β-defensin-26 (hBD-26), scored a rainbow trout (*O. mykiss*) EST of 715 bp. Further analysis of the corresponding translated amino acid sequence from this EST showed that it contained a β-defensin-like peptide of 60 amino acids (Fig. 1A) that was named *O. mykiss* β-defensin 1 (omBD-1). The N terminus of omBD-1 had the features consistent with a signal peptide as defined by the SignalP 3.0 program analysis with a putative cleavage site located after position 19 (vertical arrow; Fig. 1A). After cleavage, a resulting propeptide of 41 aa (underlined; Fig. 1A), which is present in positions equivalent to those described in the β-defensins consensus sequence six conserved cysteine residues (C-$X_{4–8}$-C-$X_{3–5}$-C-$X_{9–13}$-C-$X_{4–7}$-C-C), showed 85.7% identity with the previously described β-defensin1-like sequences from other fish (zebrafish, Fugu and tetraodon) (Zou et al., 2007) and 34.9% with the hBD-26 from humans (UniProtKB/Swiss-Prot accession number Q9BYW3). As all of them, omBD-1 has a net cationic charge of +2, an isoelectric point of ∼8 and a molecular weight of 4–5 kDa (Table 2). In addition, the alignment of omBD-1 with hBD-26 and the other fish β-defensins-like (Fig. 1B) indicated the existence of other conserved amino acid residues at positions 10 (aromatic residue), 11 (serine/threonine), 17 (glycine), 20 and 21 (positively charged residues), 28 (glutamic acid) and 42 (aromatic residue). The modelling of the amino acid sequence of omBD-1 (data not shown) showed a similar peptide fold to those described for mammalian and the other fish β-defensins.

The phylogenetic tree constructed with β-defensin-like sequences from fish and hBD-26 (Fig. 2) showed that the omBD-

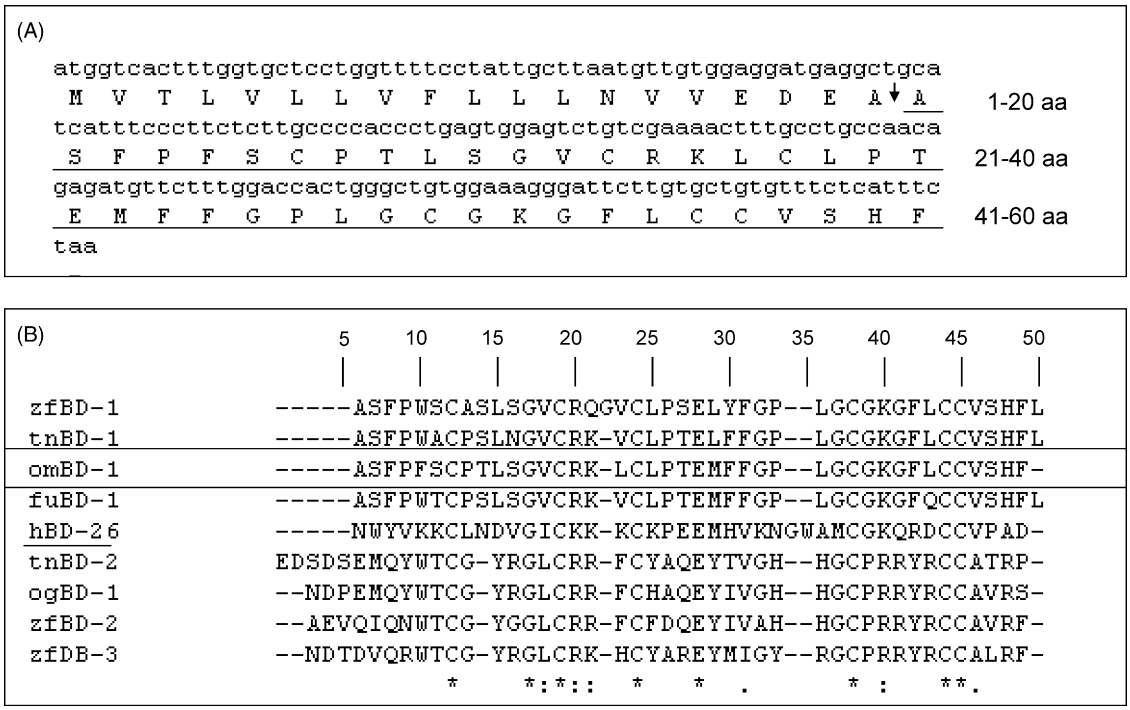

Fig. 1. Sequence (nucleotide and amino acid) of omBD-1 (A) and multiple sequence alignment of omBD-1 with β-defensin-like peptides from zebrafish, Fugu and tetraodon and hBD-26 from humans (B). (A) The nucleotide sequence is indicated in lower case letters. The deduced amino acid sequence (amino acids are numbered) is shown below the nucleotide sequence. The predicted mature peptide is underlined. The arrow shows the putative cleavage site for signal peptidase. (B) Multiple alignment was performed using the CLUSTAL W program. Fish β-defensin mature peptide sequences were obtained from Zou et al. (2007) and the hBD-26 mature peptide sequence correspond to UniProtKB/Swiss-Prot entry Q9BYW3. (*) Indicates identity, whilst (.) or (:) indicate similarity. The positions of the alignment are numbered. h, Human; om, *Oncorhynchus mykiss*; fu, *Takifugu rubripes*; tn, *Tetraodon nigroviridis*; zf, *Danio rerio*; og, orange spotted grouper; BD, β-defensin.

1 sequence branched together with tetraodon, Fugu and zebrafish BD-1 sequences, whilst tetraodon, Fugu and zebrafish BD-2 sequences and zebrafish BD-3 sequence branched in other group. By contrast, hBD-26 clusters alone showing that is more distantly related.

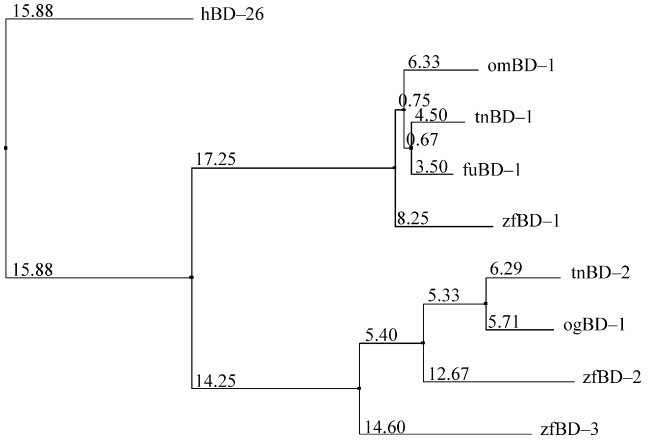

Fig. 2. Phylogenetic analysis of the mature peptide sequence of omBD-1 with known fish β-defensins-like and hBD-26 sequences. The sequences were aligned using CLUSTAL W program and the tree was generated using the Neighbour–Joining method. Fish β-defensin mature peptide sequences were chosen from Zou et al. (2007). hBD-26 mature peptide sequence correspond to UniProtKB/Swiss-Prot entry: Q9BYW3. h, Human; om, *Oncorhynchus mykiss*; fu, *Takifugu rubripes*; tn, *Tetraodon nigroviridis*; zf, *Danio rerio*; og, orange spotted grouper; BD, β-defensin.

3.2. Tissue distribution of omBD-1 mRNA revealed by RT-PCR

RT-PCR was employed by using total RNA extracted from different trout tissues (liver, head kidney, spleen and muscle) to investigate the tissue distribution of omBD-1 in healthy rainbow trout. Furthermore, the constitutive expression of omBD-1 was also assessed in the RTG-2 cell line derived from trout. In all samples analyzed a GAPDH product was amplified in parallel to confirm the good quality of the cDNA used and to serve as an internal control. Constitutive expression of omBD-1 transcripts was mostly detectable in the muscle but low levels of expression were also detected in the head kidney of all individual fingerling trout (Fig. 3). No expression was found of omBD-1 in the RTG-2 cell line (not shown).

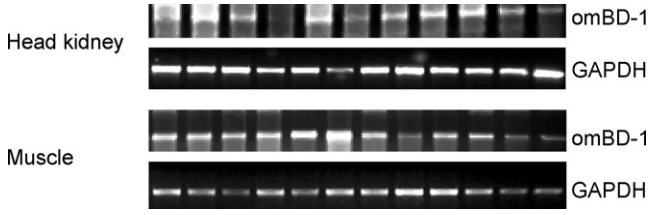

Fig. 3. RT-PCR analysis of omBD-1 mRNA expression in muscle and head kidney of healthy rainbow trout. Total RNA was extracted from these tissues from 11 fingerling rainbow trout, cDNA obtained and PCR amplifications performed as indicated in Section 2. PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide.

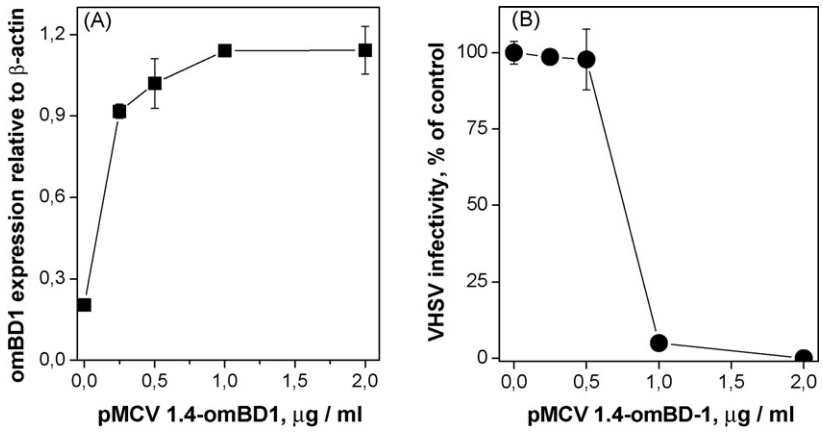

Fig. 4. Expression of omBD-1 mRNA (A) and inhibition of VHSV infectivity in EPC cells transfected with omBD-1 (B). (A) Monolayers of EPC cells were transfected with pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6 alone. After 48 h of incubation at 28 °C, total RNA was extracted and the expression of transcripts from omBD-1 was then estimated by RT-PCR. The mRNA expression relative to β-actin was calculated by the formula: intensity of omBD-1 band/intensity of the corresponding β-actin band. Data are mean ± S.D. from two experiments, each performed in triplicate. (B) EPC cells were transfected with pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6. After 24 h, transfected cell monolayers were extensively washed, infected with VHSV in cell culture media containing 2% of FCS and incubated during 24 h at 14 °C. VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. The results were expressed as the percentage of infectivity and calculated by the formula: number of VHSV-infected cell foci in cells transfected with pMCV 1.4-omBD-1/number of VHSV-infected cell foci in cells transfected with FuGene 6 × 100. Data are the mean ± S.D. from two experiments, each performed in triplicate.

3.3. Resistance of EPC cells expressing omBD-1 to VHSV infection

Since several reports showed that β-defensins possess antiviral activity against enveloped virus (Chattopadhyay et al., 2006; Hazrati et al., 2006; Klotman and Chang, 2006; Leikina et al., 2005; Sun et al., 2005), the antiviral properties of omBD-1 against a fish enveloped virus such as VHSV were studied. To carry out the antiviral activity studies of omBD-1, EPC cells were transiently transfected with different amounts of pMCV 1.4-omBD-1. RT-PCR analysis of these cells revealed that this expression vector efficiently expressed omBD-1 mRNA in EPC cells (Fig. 4A). Twenty-four hours after transfection, the EPC cells transiently expressing omBD-1 were infected with VHSV and 24 h later VHSV infectivity was determined. The results showed a 80–90% reduced VHSV infectivity in EPC cells transfected with concentrations higher than 0.50 μg/ml of pMCV 1.4-omBD-1 (Fig. 4B). The control EPC cells (EPC cells transfected with pMCV 1.4 or FuGene 6) efficiently propagated the virus since approximately 130 VHSV-infected cell foci per well were observed (data no shown). These data revealed that EPC cells expressing omBD-1 had reduced susceptibility to VHSV.

3.4. Resistance of EPC cells treated with conditioned medium from EPC cells expressing omBD-1 to VHSV infection

The inhibition of VHSV infectivity in EPC cells transfected with pMCV 1.4-omBD-1 suggested that some cellular antiviral defense mechanisms might have been induced by the expression of omBD-1 in these cells. To investigate the possibility that the expression of omBD-1 can induce secretion of soluble factors with antiviral activity, such as type I IFN, CM medium from non-transfected EPC (CM) and from EPC cells transfected with either pMCV 1.4-omBD-1 (omBD-1-CM), pMCV 1.4 (V-CM) or FuGene 6 alone (F-CM) were prepared. Fresh EPC cells were then treated with these different CM during 24 h and after extensively washing infected with VHSV in the absence of CM. As expected, the treatment of EPC cells with CM from EPC cells transfected with pMCV 1.4-omBD-1 conferred protection against VHSV infection (Fig. 5) but not to those treated with CM from non-transfected EPC cells or from EPC cells transfected with either pMCV 1.4 (not shown) or FuGene 6. Since heat and acid treatments of omBD-1-CM did not eliminate its antiviral activity against VHSV the presence of type I IFN in supernatants from EPC cells transfected with pMCV 1.4-omBD-1 is suggested.

3.5. Transfection of EPC cells with omBD-1 induces expression of the Mx1 gene

If the antiviral activity against VHSV observed in EPC transfected with pMCV 1.4-omDB was mediated by type I IFN, the up regulation of IFN-stimulated genes (ISGs) should be detectable in these cells. To investigate this possibility, EPC cells were transfected with pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6 or incubated with poly I:C (a well-known type I IFN-inducer) and the expression of the IFN-induced gene Mx1 was then assessed by RT-PCR. The IFN-inducible Mx1 gene of carp was selected as a marker for IFN responses because direct induction of this gene by carp IFN has been demonstrated (Y.B. Zhang et al., 2004). Carp Mx1 gene was up-regulated in both poly I:C treated (not shown) and omBD-1 transfected EPC cells (Fig. 6) but not in EPC cells transfected only with pMCV 1.4 or FuGene 6. The level of expression of carp Mx1 transcripts was proportional up to eight-fold to the amount of pMCV 1.4-omBD-1 transfected showing a plateau-like at DNA concentrations ≥0.5 μg/ml (Fig. 6B).

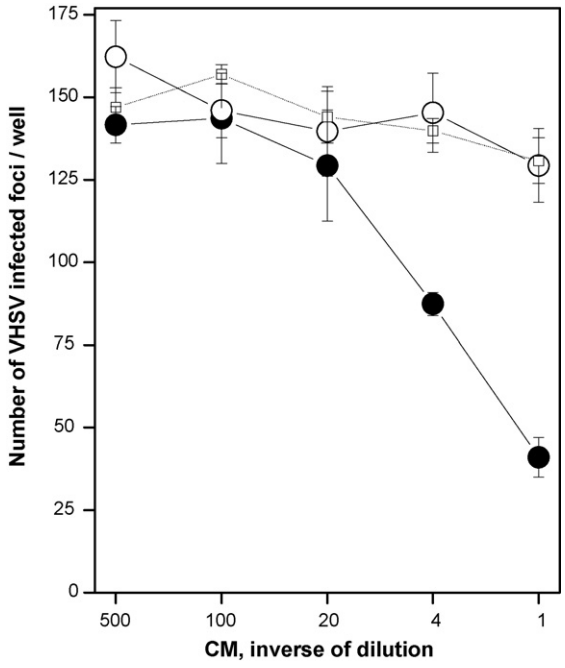

Fig. 5. Inhibition of VHSV infectivity in EPC cells treated with conditioned medium prepared from cells transfected with pMCV 1.4-omBD-1. Fresh EPC cells were incubated during 24 h with CM prepared from EPC cells non-transfected (CM) or transfected with pMCV 1.4-omBD-1 (omBD-1-CM), pMCV 1.4 (V-CM) or FuGene 6 (F-CM). Treated EPC cells were then infected with VHSV in cell culture medium containing 2% of FCS and incubated during 24 h at 14 °C. VHSV infectivity was estimated by counting the number of foci of VHSV-infected cells by the immunostaining focus assay described in Section 2. The results were expressed as the number of VHSV infected cell foci per well. Data are the mean ± S.D. from two experiments, each performed in triplicate. EPC cells treated prior to the infection with CM (□), F-CM (◯) or omBD-1-CM (●).

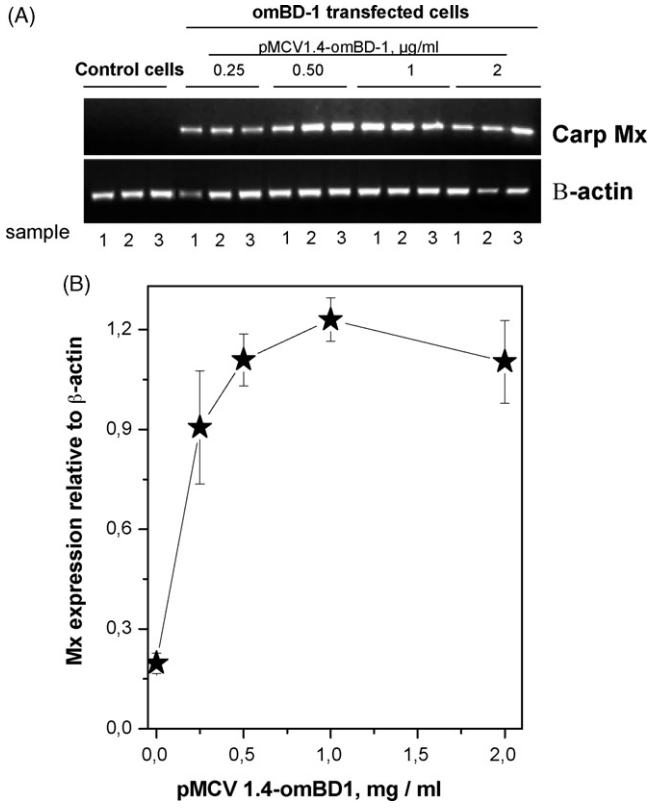

Fig. 6. Expression of carp Mx1 transcripts in EPC cells transfected with pMCV 1.4-omBD-1. Monolayers of EPC cells were transfected with of pMCV 1.4-omBD-1, pMCV 1.4 or FuGene 6 alone. After 48 h of incubation at 28 °C, total RNA was extracted and the expression of transcripts from the IFN-inducible carp Mx1 gene was then estimated by RT-PCR. The mRNA expression relative to β-actin was calculated by the formula: intensity of Mx1 band/intensity of the corresponding β-actin band. (A) Photography of an agarose gel of Mx1 and β-actin band mRNA amplified by RT-PCR. The gel is representative of two experiments. (B) Data are the mean ± S.D. from two experiments, each performed in triplicate.

4. Discussion

In the present study, we describe the identification of a β-defensin-like sequence in the present EST sequences from trout and demonstrated their *in vivo* expression in different tissues of rainbow trout. In addition, we have cloned the β-defensin-like peptide into an eukaryotic expression vector and shown that fish cells transfected with omBD-1 were protected against infection with VHSV, a fish rhabdovirus. Although homologue sequences to β-defensins from higher vertebrates have been previously described for three non-salmonid species (zebrafish, Fugu and tetraodon) (Zou et al., 2007), their possible biological activities have not yet been described.

Based on the homology comparison and phylogenetic analysis, the multiple defensin-like genes discovered recently in zebrafish, pufferfish and tetraodon have been classified into two subgroups, the BD-1 group and another group containing BD-2 and 3 (Zou et al., 2007). A phylogenetic tree (Fig. 2) constructed with omBD-1 and zebrafish, Fugu and tetraodon β-defensin-like peptides as well as hBD-26 peptides included omBD-1 in the BD-1 group of fish β-defensins, close to fugu and tetraodon BD-1 but in a different branch than zebrafish BD-1. On the contrary, fish β-defensins-like and hBD-26 grouped in a distant branch in this tree.

The omBD-1 expression analyzed by RT-PCR was constitutive in muscle and head kidney (Fig. 3). In contrast, zebrafish β-defensin-like homologues were constitutively expressed in every organ analyzed (Zou et al., 2007). The differential tissue expression of omBD-1 could suggest a tissue-specific regulation of β-defensin in fish such as it has been described for β-defensins from other vertebrates (Pazgier et al., 2006). In mammals, where defensins are more diversified than in other animal groups, β-defensins are predominantly expressed in epithelial cell tissues (Ganz, 2003; Klotman and Chang, 2006; Yang et al., 2004) whilst α-defensins are expressed in immune cells. Since there is no evidence of fish α-defensins existence so far, it might be not surprising that fish β-defensins could be expressed in a broader range of tissues/organs including immune system related organs such as the head kidney. However, the present ESTs might not include yet every trout possible expressed sequence. On the other hand, the lack of omBD-1 expression in the trout liver may be related to the presence of other antimicrobial peptides produced there, such as hepcidins (Douglas et al., 2003) and LEAP-2 (Y.A. Zhang et al., 2004), similarly to what was previously proposed to explain the absence

of expression of fish cathelicidins in fish liver (Chang et al., 2006).

Although the antiviral activity of β-defensins is well known long time ago, the importance of β-defensins against viruses has only recently coming to light. Thus, it has been reported against several viruses, including HIV-1 (Quinones-Mateu et al., 2003; Sun et al., 2005), adenovirus (Klotman and Chang, 2006), influenza virus (Leikina et al., 2005), parainfluenza virus 3 (PIV-3) (Grubor et al., 2004), respiratory syncytial virus (RSV) (Meyerholz et al., 2007), vaccinia virus (VV) (Howell et al., 2007) herpes simplex virus (Hazrati et al., 2006) and Chandipura virus (Chattopadhyay et al., 2006).

To investigate whether the omBD-1 also exhibited antiviral activity, omBD-1 was transiently expressed in EPC cells (a carp derived cell line) under the control of the cytomegalovirus immediate-early (CMV) promoter and then the transfected EPC cells challenged with the viral haemorrhagic septicaemia rhabdovirus (VHSV), one of the most devastating viruses for worldwide aquaculture. The *in vitro* expression system CMV promoter/EPC cells was chosen because: (i) the CMV promoter-enhancer has been found previously to work very efficiently in fish cells (Anderson et al., 1996; Hansen et al., 1991; Trobridge et al., 1997) and (ii) EPC cells are highly susceptible to VHSV (can yield up to 10^9 ffu/ml; Marroqui et al., 2007; Mas et al., 2004) and can be readily transfected (Castric et al., 1992; Lopez et al., 2001; Moav et al., 1992; Rocha et al., 2004) showing higher transfection rates than trout cell lines. In fact, one of the most important limitations of fish cell lines is their relatively low transfection efficiencies compared to those of mammalian cell lines (Altmann et al., 2003). As an example, the RTG-2 cell line, a fibroblastic cell line originated from rainbow trout and theoretically suitable to perform the present study, has transfection efficiencies ranging from 5 to 7% (Tafalla et al., 2007). The results of the transfection–infection assays demonstrated that omBD-1 was expressed in EPC cells (Fig. 4A) and induced an antiviral activity against VHSV (Fig. 4B). Since EPC cells transfected with omBD-1 produced acid and heat-stable antiviral activity (Fig. 5) and also showed up regulation of carp Mx1 gene (Fig. 6), a type I IFN-related antiviral response could be operating in the fish cells transfected with omBD-1. However, further studies are needed to clarify the mechanism underlying the antiviral activity of omBD-1.

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