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ORIGINAL PAPER

**Olga Pleguezuelos · Jun Zou · Charlie Cunningham
Christopher J. Secombes**

Cloning, sequencing, and analysis of expression of a second *IL-1β* gene in rainbow trout (*Oncorhynchus mykiss*)

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Abstract The full-length sequence of a second *IL-1β* gene (*IL-1β2*) in rainbow trout (*Oncorhynchus mykiss*) has been obtained. As with the first *IL-1β* gene, *IL-1β2* is organized into six exons/five introns. There are only small differences in their intron/exon sizes, with the exception of intron 3, which is 334 bp smaller in *IL-1β2*. The transcript encoded by the *IL-1β2* gene contains a 5′ untranslated region (UTR) of 121 bp, followed by a 762-bp open reading frame and a 518-bp 3′UTR. The 3′UTR contains seven instability attta motifs, typical of inflammatory genes, and a polyadenylation site 11 bp upstream of a 17-bp poly(A) tail. The predicted 254 amino acid sequence of the second *IL-1β* gene has 82% similarity to the first gene, 45% similarity to carp IL-1β, and 40% similarity to human IL-1β. Comparison of the two trout genes reveals that the *IL-1β2* gene has a deletion of 9 bases in exon 3 and an altered splicing site at the 5′ end of exon 4 giving rise to a further 9-bp deletion in the resulting cDNA. As with other nonmammalian *IL-1β* genes, no interleukin-converting enzyme (ICE) cut site has been found but the alignment of the amino acid sequence with other species shows a possible cut site between Arg^{89} and Ala^{90} that would give arise to a 165-amino acid mature peptide. Expression studies performed by RT-PCR using primers specific for the *IL-1β2* transcript revealed a clear dose-dependent induction of this gene in cultured trout leukocytes by stimulation with lipopolysaccharide.

O. Pleguezuelos · J. Zou · C.J. Secombes (✉)
Department of Zoology, University of Aberdeen,
Tillydrone Avenue, Aberdeen, AB24 2TZ, UK
E-mail: c.secombes@abdn.ac.uk
Phone: +44-1224-272872
Fax: +44-1224-272396

C. Cunningham
SARS International Center for Marine Molecular Biology,
5008 Bergen, Norway

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Introduction

Interleukin (IL)-1β is an important inflammatory mediator and belongs to the interleukin-1 family of cytokines that also include IL-1α and the IL-1 receptor antagonist (IL-1ra). In mammals, IL-1β is translated as a M_r 31,000 precursor molecule that is subsequently cleaved by the interleukin-converting enzyme (ICE) to a M_r 17,000 mature protein. Only the mature form is biologically active. There is no signal peptide in IL-1β and its mechanism of secretion is unknown (Dinarello 1997).

IL-1β is produced by a variety of cell types, including monocytes, macrophages, T and B lymphocytes, fibroblasts, and epidermal and endothelial cells (Oppenheim et al. 1986). Expression is induced by a diverse range of stimuli, including mitogens, cytokines, and microbial products (Compton et al. 1998; Di Giovine et al. 1991; Tabona et al. 1998; Wang et al. 1997). The IL-1β produced plays a key role in the inflammatory process, enhancing cell-mediated immunity by inducing the growth and proliferation of lymphocytes, connective tissue cells, and vascular endothelial cells, and by stimulating immune and inflammatory response effector cells. IL-1 acts synergistically with IL-4 to activate B cells, augmenting the humoral arm of the immune response. It also stimulates, in combination with IL-2 and interferon-γ, nonspecific immunity through the activaton of natural killer cells (Kullberg and van de Meer 1995). IL-1β as an immunoregulatory cytokine has the potential to enhance the immune response induced by a vaccine and/or to modulate the immune response leading to different effector mechanisms (Nash et al. 1993). Thus, recombinant IL-1β is currently being used as an adjuvant for vaccines in sheep (Elhay et al. 1997; Loft-

house et al. 1995), pigs (Blecha 1997), and cattle (Godson et al. 1997).

IL-1β has been cloned and sequenced recently in a variety of nonmammalian species, including chicken (Weining et al. 1998), *Xenopus* (Zou et al. 2000a), rainbow trout (Secombes et al. 1998; Zou et al. 1999a, 1999b), and carp (Fujiki et al. 1998). There is also evidence for an *IL-1β* gene in cartilaginous fish (S. Bird, personal communication), and cross-hybridization of a human *IL-1β* gene probe with the cerebral ganglion of a protochordate (*Styela plicata*) has been described (Pestarino et al. 1997). Thus, IL-1*β* is phylogenetically well-conserved.

There has been much speculation about the origins of the *IL-1* gene family, and whether *IL-1β* and *IL-1ra* arose from a common ancestor that underwent alternative splicing (Hughes 1994). In Southern blots using a trout *IL-1β* probe, a second, related gene could be detected from liver DNA digested with *Bam*HI, *Bam*HI/*Bgl*II, or *Bam*HI/*Eco*RI (Zou et al. 1999a), and studies were initiated to clone and sequence this gene. A sequence was obtained with high homology to the existing *IL-1β* gene but that was clearly distinct. The present paper describes the cloning of the full-length cDNA, analysis of the gene organization, evidence that it is a second *IL-1β* gene, and confirmation that the transcript is up-regulated by lipopolysaccharide (LPS), and thus that this gene is likely to be of relevance to trout immune responses.

Materials and methods

Polymerase chain reaction

The cloning and sequencing of the second rainbow trout *IL-1β* gene, called *IL-1β2*, began by amplifying a small region of trout genomic DNA (extracted from the liver of a single outbred fish using a Qiagen maxi prep kit) by PCR using primers IL-1*β*1-F17 and IL-1*β*1-R (see Table 1) that span intron 5 of the known *IL-1β1* gene sequence. The PCR used a 25-μl reaction volume as follows: 2.5 μl 10×NH_4 buffer, 1 μl of 10 mM dNTP mix, 0.75 μl of 50 mM $MgCl_2$, 1 μl of 10 μM reverse primer, 1 μl of 10 μM forward primer, 0.125 μl of 5 units *Taq* polymerase (Bioline)/ml, 1.5 μl of template, 17.125 μl of distilled water. The cycling protocol was: 94°C for 5 min; 28–32 cycles of 94°C for 45 s, 58–62°C for 45 s, and 72°C for 45 s; 72°C for 10 min followed by cooling to 4°C. The resulting PCR product (P1; see Fig. 1) was cloned and plasmid DNA sequenced as described below.

Based on the initial sequence obtained primers (IL-1*β*2-F1, IL-1*β*2-F2, IL-1*β*2-R1, IL-1*β*2-R2; Table 1) were designed within intron 5 of the second gene, which showed the greatest sequence divergence from *IL-1β1*. These primers were used in seminested PCR with *IL-1β1* primers within exon 4 (IL-1*β*1-F8) and exon 6 (IL-1*β*1-R3) (Fig. 1), using the first-round PCR product obtained with genomic DNA as template for the second PCR reaction, and the final products (P2 and P3) were again cloned and sequenced. This process was repeated using *IL-1β2*-specific primers within intron 4 (IL-1*β*2-R3, IL-1*β*2-R4) and primer IL-1*β*1-F4 in exon 2, to obtain product P4.

Attempts to obtain the 5′ end of the gene with a primer in the 5′ untranslated region (UTR) of the *IL-1β1* gene (IL-1*β*1-F10) failed, so the cloning strategy to obtain the 5′UTR and 3′UTR (in exons 1/2 and exon 6, respectively) was switched to PCR with cDNA taking advantage of the fact that mRNA exists for *IL-1β1* that is incompletely spliced, containing intron 4 and 5 (Zou et al. 1999a). Using head kidney (HK) cDNA library as template (Hardie et al. 1998), anchored PCR was performed with vector primers and *IL-1β2* intron-specific primers. To amplify the 5′ end, nested PCR was carried out using IL-1*β*2-R4 (intron 4) with M13R, followed by a second round of PCR with IL-1*β*2-R3 (intron 4) and T3. A similar procedure was used to obtain the 3′UTR, where IL-1*β*2-F2 (intron 5) was used with primer M13F followed by PCR with IL-1*β*2-F1 (intron 5) and T7. However, in this case, touchdown PCR was used in the first round of PCR, with a protocol of: 94°C for 2 min; 5 cycles of 94°C for 1 min, 64°C for 1 min, 72°C for 3 min; 30 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 3 min; 72°C for 10 min. Both products (P5 and P6; Fig. 1) were cloned and sequenced. This new sequence allowed the design of primers to obtain the full-length cDNA for the open reading frame (ORF) of *IL-1β2* (using primers IL-1*β*2-F4 and IL-1*β*1-R3), which was cloned and sequenced. It also allowed the design of primers to obtain potential introns within the 5′UTR. For this purpose, PCR was again performed using genomic DNA, with primers IL-1*β*2-F3 located at the 5′ end of the 5′UTR of IL-1*β*2 and IL-1*β*2-R5 in the coding region of exon 2 (Fig. 1, Table 1), and the generated product (P7) was sequenced.

Table 1 Trout *IL-1β1*- and *IL-1β2*-specific oligonucleotide primers used in PCR and expression experiments

Name	Sequence (5′→3′)
First PCR to find the second *IL-1β* gene	
IL-1*β*1-F17	CCCACCCTGCAGCTGGAG
IL-1*β*1-R	ACTGGTTCATCATCAGCACGGACAT
Cloning of the *IL-1β2* gene	
IL-1*β*1-F4	CGAATTCATGGATTTGAGTCA
IL-1*β*1-F8	TCTGAGAACAAGTGC
IL-1*β*1-F10	GGATTCACAAGAACTAAGGAC
IL-1*β*1-R3	CTTAGTTGTGGCGCTGGATG
IL-1*β*2-F1	AGTGACAGTGTGTCGGCTTGTTC
IL-1*β*2-F2	CTGAGCTGAAGAATGCTGACATC
IL-1*β*2-F3	GGACTCACAACAAGGACCAA
IL-1*β*2-R1	AAACAACTCCAGCCTCAACAGAC
IL-1*β*2-R2	GTGGATAGAAATGTTACTCTGGCT
IL-1*β*2-R3	GGTCGCCAAAGAACACACTCAAAAG
IL-1*β*2-R4	CAGTCTCAGGGTGGGGTTGGTTT
IL-1*β*2-R5	GGACTCACCTTCATTAGACTGC
M13-F	TGTAAAACGACGGCCAGT
M13-R	GTTTTCCCAGTCAACGAC
Making probe for Southern blot analysis	
FS5	TTGAGTTCTGATGAATGATGCTGACTGAC
RS5	GGCTACAGGTCTTGCTTCAGTCTCA
Expression studies	
IL-1*β*2-F4	ACTACAAAACAGCCAACTACAAACC
IL-1*β*2-R8	CTCTGCTGCTGGCTTCAGT
β-Actin forward	ATCGTGGGGCGCCCCAGGCACC
β-Actin reverse	CTCCTTAATGTCACGCACGATTTC

Cloning and sequencing

PCR products were checked on an agarose gel and where a single clear band was present the products were directly ligated into the plasmid vector pCRII-TOPO (Invitrogen, UK). Otherwise, DNA was extracted from the agarose gel using a QIAquick gel extraction kit (Qiagen, UK) and then ligated. *Escherichia coli* TOP10 competent cells were then transformed with the ligation reaction according to the TOPO TA Cloning kit instructions (Invitrogen, UK) and cells incubated overnight at 37°C in LB plates containing 40 μg/μl of X-gal (Promega, Madison, Wis.) and 50 μg/ml of ampicillin (Sigma, UK). The positive clones were identified by their white colony color and checked

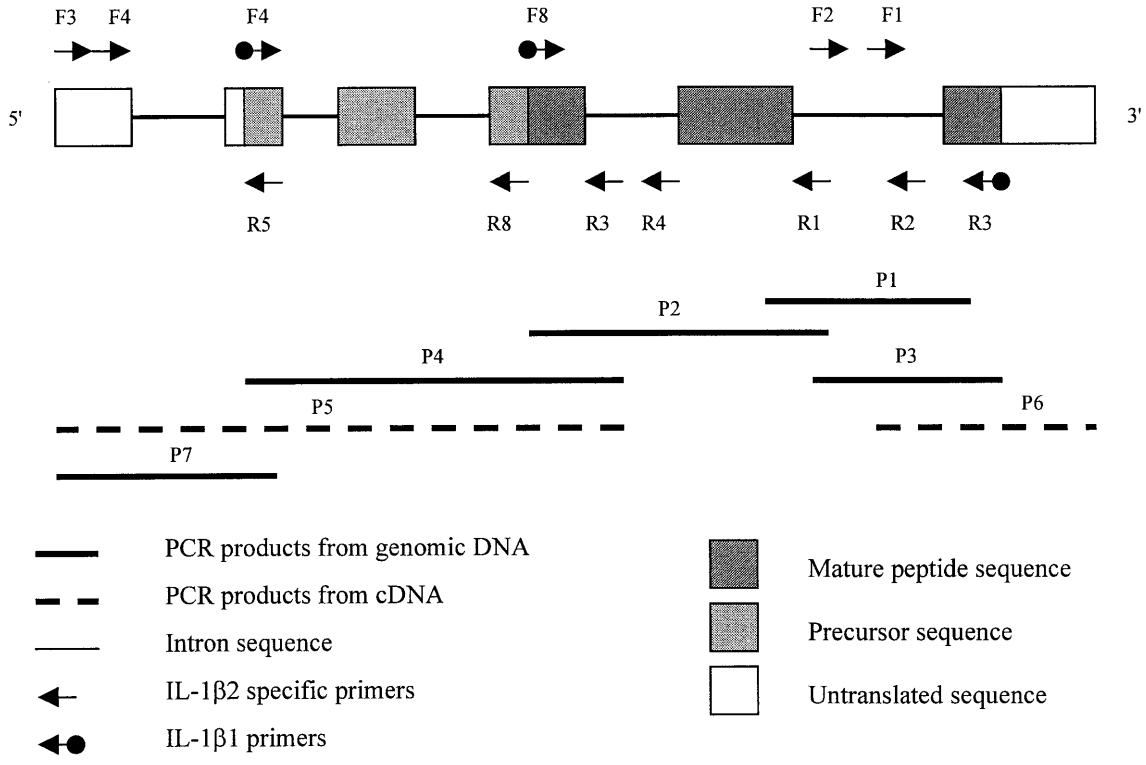

Fig. 1 Sites of primers and PCR products generated (*P1–P7*) during cloning of the trout *IL-1β2* gene. Refer to Table 1 for primer sequences. Product sizes were as follows: *P1* 713 bp, *P2* 643 bp, *P3* 651 bp, *P4* 981 bp, *P5* 625 bp, *P6* 990 bp, *P7* 384 bp

by PCR and digestion with *Eco*RI (Promega, USA) for correct-sized inserts. Selected colonies were then incubated overnight in LB medium at 37°C prior to isolation of plasmid DNA using a QIAprep Spin Miniprep kit (Qiagen, UK). The inserts were then sequenced using an ABI 377 automated sequencer (Applied Biosystems, UK) and the sequences analyzed for similarity with known sequences using the FASTA (Pearson and Lipman 1988) and BLAST (Altshul et al. 1990) suite of programs.

Direct comparison between DNA sequences was performed using the GAP program (Needleman and Wunsch 1970), within the Wisconsin Genetics Computer Group (GCG) Sequence Analysis Software Package (version 9.1, 1997) and multiple sequence alignments were generated using Clustal W (version 1.74, 1997; Thompson et al. 1994).

Southern blot

Genomic DNA, obtained as above from rainbow trout liver, was digested completely with the restriction enzymes *Bam*HI, *Pvu*II, and *Eco*RV 2 units/μg DNA (New England BioLabs, USA). The digestion products were separated in a 0.8% agarose gel and transferred to a nylon membrane. A probe to a conserved region between the *IL-1β1* and *IL-1β2* genes (probe 5, confirmed using a BLAST search) was prepared by PCR using primers FS5 and RS5 (Table 1) and spanned a region between intron 4 and exon 5 (Fig. 2). The amplified product of 120 bp was purified from an agarose gel, labelled with ^{32}P-dCTP (Ready-to-go DNA labelling beads; Amersham Pharmacia) and used for hybridization to the digested genomic DNA (20 μg per lane) at 65°C for 4 h. Following stringent washing with standard sodium citrate +0.1% SDS, the membrane was put into an X-ray cassette and film (Kodak) was exposed for 2 days. Finally, the exposed film was subjected to densitometric scanning using a UVP gel imaging system and UVP Gelworks ID advanced software.

Expression studies

HK leukocytes and HK macrophages were isolated from outbred rainbow trout using standard protocols (Secombes 1990; Zou et al. 2000b), and the cells cultured in Leibovitz L-15 medium (Gibco, Paisley, UK) at 18°C without supplemental CO_2. Different concentrations (0, 0.5, 5, 50, and 100 μg/ml) of LPS (*E. coli* serotype 0127:B8; Sigma) were used to stimulate the trout macrophage cultures. Cells (approx. 10^7) from individual fish were stimulated for 4 h prior to extraction of RNA and reverse transcription to cDNA (see below). In a further experiment, HK leukocytes suspended at 5×10^6/ml L-15 medium were plated into petri dishes (5 ml per dish) and stimulated with 5 μg/ml of LPS for 0, 1, 2, 3, and 4 h prior to RNA extraction.

RNA was extracted from the cells with RNAzol (Biogenesis, Poole, UK) and then reverse transcribed to cDNA using a Superscript II RT kit (Gibco). The cDNA was used for PCR with primers specific for either the *IL-1β1* (IL-1β1-F10 and IL-1β1-R3) or *IL-1β2* (IL-1β2-F4 and IL-1β2-R8) gene (see Fig. 1; Table 1). The PCR reaction mix consisted of 5 μl $10\times NH_4$ buffer, 1 μl of 10 mM dNTP mix, 1.5 μl of 50 mM $MgCl_2$, 2 μl of 10 μM reverse primer, 2 μl of 10 μM forward primer, 0.25 μl of 5 *unitsTaq* polymerase/ml, 1 μl of cDNA template, 37.25 μl of distilled water. The cycling program was: 94°C for 5 min; 32 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 1 min; 72°C for 10 min and cooling to 4°C. The primers gave rise to products of 860 bp for *IL-1β1* and 350 bp for *IL-1β2*. In both cases, the products were cloned, sequenced, and confirmed to be the correct form of trout *IL-1β*.

In a further experiment, fish were injected intraperitoneally with a genetically attenuated (*aroA–*) strain of the Gram-negative bacterial pathogen *Aeromonas salmonicida* (Marsden et al. 1996) at a dose of 2×10^8 bacteria per 300 g fish. Two days post-injection, cells were prepared from kidney, spleen, brain, gill, and blood, RNA was isolated and used for RT-PCR as above with primers specific for the *IL-1β2* gene.

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   1  AACAAGTTGGGACTCACAACAAGGACCAAGGACTAGAAACAAGACGTTAG
  51  TACCTACTATCTACAAACTACAAAACAGCCAACTACAAACCTATTCATCC
 101  AGgtaaatgaaatgtagagtctgtttttactcacattggcaaccataaac
 151  ttactcttactgtacgactgaccatgtattatgttgttgatctgttgtgg
 201  cagtctttatttctatatagtgtttagtaaaaacgttttatttaggacta
 251  actatattgattgcctgtttacaccagatctctgaatgtgtttccagatc
 301  ttggccatctttaagcaactgactaagcatgatacaatctatgatggaca
 351  ggagttttaaaatatctgtttttatttttccagATCTAACTACAAAGACAA
 401  TATGGAATTTGAGTCAAACTGCAGTCTAATGAAGgtgagtcccaacttag
        M  E  F  E  S  N  C  S  L  M  K
 451  acatgatgataagacagacctacaatattcaggcatgacagacattcaaa
 501  tgttattctacagtaaatgttaaaacagaagaaaggaaaaaaacagacca
 551  accacaattctccatacactgatgcaacaatgttggatgaatgttgaatc
 601  gactttctctgtcctggtcagAACACCTCTGCCAGTGTAGCATGGAGCTC
                           N  T  S  A  S  V  A  W  S  S
 651  CAAACTGCCTCAGGGTCTGGATGTGGAGATATCCCATCACCCCATCACCC
        K  L  P  Q  G  L  D  V  E  I  S  H  H  P  I  T
 701  TGCGCTGTGTGGCCAACCTCATCATCGCCATGGAGAGGCTAAATGGTGGC
      L  R  C  V  A  N  L  I  I  A  M  E  R  L  N  G  G
 751  AAGGGGTTTACCCTGGGAAGAGATGAGGGCCTGTTAAATTTCTTGCTAGA
        K  G  F  T  L  G  R  D  E  G  L  L  N     F  L  L
 801  GAGCGCAGTGGAAGgtaggtcaaagatcactgggctggattagaggagcc
      E  S  A  V  E
 851  ccttcggggctagcctggccccatgtctgattgtactgtcttgcttctga
 901  tgacaatgaccataggagttgacaagacagcacaaacagaacggggtatt
 951  ttcaatgtgtaccactataataccattagctatcaccactgggctatgtt
1001  ccaatgttcacactatagaataccacttagaatgcatgcccaacacatcg
1051  cttaattctaactgctttctcctctctctctctctcctcatcaaaacata
1101  tagTGTTGGAGTTGGAGTCGGCGCGCACTGAAGCCAGCAGCAGAGCTGCT
            V  L  E  L  E  S  A  R  T  E  A  S  S  R  A  A
1151  TTCAGCAGTAAAGGAGAGTACGAGTGTAGTGTCACTGACTCTGAGAACAA
        F  S  S  K  G  E  Y  E  C  S  V  T  D  S  E  N
1201  GTGCTGGGTGCTCAACGAAGGGTCTATGGAGCTGCATGCCATAATGCTGC
      K  C  W  V  L  N  E  G  S  M  E  L  H  A  I  M  L
1251  AGGGAGGCAGCAGCTACCATAAAGgtatgttaacaaccaaggctcagtcc
        Q  G  G  S  S  Y  H  K
1301  caatttgcatttgtcttaacatagtcttaacatagtgcgttacattttgc
1351  caaagacttttgagtgtgttctttggcgaccatagatcattaaattaatg
1401  aaattacaatttgccttggggcactaagcagtggcacttcctatgaatct
1451  gaaataattggaaaggttaggtacatatgagcaatatgagattcagtgga
1501  gaggttaaaaccaaccccaccctgagactgacagataggtagcgtactta
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1551  gttcacagttgagttctgatgaatgatgctgactgactctcttctccttt
1601  cctctgtcctcagTGCATTTGAACCTGTCTACGTACATCACGCCTGTCCC
                      V  H  L  N  L  S  T  Y  I  T  P  V
1651  CAGTGAGACTAAAGCAAGACCTGTAGCCCTGGGCATCAAGGGATCCAACC
      P  S  E  T  K  A  R  P  V  A  L  G  I  K  G  S  N
1701  TCTACCTATCCTGCATCACATCAGAAGGCACGCCCACCCTGCACCTAGAG
        L  Y  L  S  C  I  T  S  E  G  T  P  T  L  H  L  E
1751  gtaaatagcactactactgctgaatatattgtcattcagtatttatagat
1801  aggataattgtctgttgaggctggagttgtttgagtcagacctggaccgc
1851  atcatgcaccacgtagagatggcaatggactgaagctgaagaatgctgac
1901  atcatcaacattgtgtctgaatcatatgtagaatgtaaaaggttgagttg
1951  cagtgtaattaaaagaaactgccagagagaccaagagagaccgaccctgg
2001  tgaaatgtgactgggaatgggatacagtgaggatgactattaattagtcc
2051  tggagagtgacagtgtgtcggcttgttccaaccaatgatgtgttgattat
2101  ataagtcattggttccaacagccacaaactcaaaccaatagccagagtaa
2151  catttctatccacccctggtgtatgacatttgacccctggtgtatgacat
2201  ttgacccctggtgtatgacatttgacctttaagtcagttttgagtctgcc
2251  attgatttgaaacccctgaccatctatctctccctctctctctctccagG
2301  AGGTGGCAGACAAGGAGCAGCTGAAGTCCATCAACCATGAGAGCGACATG
        E  V  A  D  K  E  Q  L  K  S  I  N  H  E  S  D  M
2351  GTACGCTTCCTCTTCTACAAACAGGACACCGGAGTTGACATCAGTACCCT
        V  R  F  L  F  Y  K  Q  D  T  G  V  D  I  S  T  L
2401  GGAGTCTGCCCATTACAGGAACTGGTTCATCAGCACGGCCCTACAGCAGG
        E  S  A  H  Y  R  N  W  F  I  S  T  A  L  Q  Q
2451  ACAACACCAAGATGGTGAACATGTGCCAGAGGGCAACCCTCAACCGCAAC
      D  N  T  K  M  V  N  M  C  Q  R  A  T  L  N  R  N
2501  ACCACATTCACCATCCAGCGCCACAACTAAGGTGGGTGCCGAAACGGACA
        T  T  F  T  I  Q  R  H  N  *
2551  CCACAGATCTCTCATAGAGATCCTATATAATTCAATGGTCTTTTTATGTG
2601  GTCAGGGCTGAATTGTCACTTTCTGTATGTTTAAAGAATAGGTGTATGTT
2651  TGTAAGACACAATAACTTCTTCAGGTTGTATGTATGTATGTATGTGTGTA
2701  CTACTAGAGCAATTCACCATTGTGGTGGATTGACTTGGCAAAATTTACCC
2751  AAGATGGCGCCGTTGAGTCAAATTGTTGCAAGCGCCCATAATATTTATTG
2801  ACCATATTTATTGGTGAAAGCCAAAATGAACGTATTGCTTACGGAAATTA
2851  CATTTCTACTAATCATAATATTGTTCTACTATTTATTAAGGTATTTATAT
2901  TTCAGTGATTATACTTATTTATGATTGTATTTATTTGTCAATATGTTGAT
2951  GTTGCACTTATTCATTGATGCATGCAAACATTTTGAATTGTCCTACATAT
3001  GTGCTCTTTTGAATAAATATTCAAATGG
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Fig. 2 Compiled full-length rainbow trout *IL-1β2* gene. Exons are in *uppercase* and introns in *lowercase*. The predicted translation of exon-coding regions is given. Features in *bold* include the start/stop codons and polyadenylation site. Features *underlined* include potential glycosylation sites and RNA instability motifs. The *box* indicates the region used as a probe for Southern blotting

Results

Cloning and sequencing

Analysis of the seven overlapping PCR products revealed an *IL-1β2* gene of six exons and five introns, with a size of 3028 bp (compared to 3065 bp for the trout *IL-1β1* gene; Zou et al. 1999b). Analysis of the

cDNA revealed a transcript of 1384 bp that contained a 121-bp 5′UTR, a 762-bp ORF, and a 518-bp 3′UTR including a 17-bp poly(A) tail (Fig. 2). Both the 5′UTR and 3′UTR are longer than in the *IL-1β1* transcript but the ORF is 18 bp smaller. As in trout *IL-1β1* and other known *IL-1β* genes, the 3′UTR contains mRNA instability motifs (×7 attta) and has a polyadenylation site (aataaa) 11 bp upstream from the poly(A) tail. The nucleotide identity between the two trout *IL-1β* ORFs is 85%, with the 5′UTR and 3′UTR having 65% and 74% identity, respectively. The ORF translates into a protein of 254 amino acids, containing four potential glycosylation sites, with 82% similarity to trout *IL-1β1*, 45% to carp *IL-1β*, and 40% to human *IL-1β*. As in the *IL-1β1* gene (Zou et al. 1999b) and other nonmammalian *IL-1β* genes, no ICE cut site is apparent but alignment of the sequence with other species reveals a possible cut site between Arg^{89} and Ala^{90} that would give arise to a 165-amino acid mature peptide (Fig. 3). Relatively high levels of amino acid conservation are also apparent in the regions of known secondary structure (β sheets) in the predicted mature peptide.

The sizes of the *IL-1β2* exons/introns are generally very similar to those in the *IL-1β1* gene (Table 2), with the most significant differences seen in exons 3 and 4 which are both 9 bp smaller in *IL-1β2* and intron 3 which was 334 bp smaller than in the first gene. Further analysis of the exons/introns revealed that while the deletion of 9 bp in exon 3 was likely due to a deletion event, the apparent deletion in exon 4 was due to an altered splicing site that kept the downstream sequence in-frame (Fig. 4). Analysis of the nucleotide identity within each exon/intron revealed that the highest variability between the two genes is in the intron sequences, where the lowest identity of 45% was found for intron 5. In the exons of the two genes the nucleic acids show a relatively high identity (approx. 80–90%) except for exon 1, with only 67% shared identity.

Southern blot

When the genomic DNA was fully digested with *Eco*RV and *Bam*HI restriction enzymes, a product of 836 bp was expected for the *IL-1β1* gene and of 1011 bp for *IL-1β2* gene, according to the respective restriction enzyme maps, and this is what was obtained (Fig. 5). This difference in size is due to the fact that while both genes have an *Eco*RV cut site, it is located in exon 3 in the *IL-1β2* gene and in intron 3 in the *IL-1β1* gene. In addition, the *Eco*RV/*Bam*HI digestion product includes intron 3 which, as mentioned above, is 334 bp longer for *IL-1β1*.

When the genomic DNA was digested with *Pvu*II/*Bam*HI or *Pvu*II on its own, products of 214 bp and 739 bp, respectively, were detected corresponding to the *IL-1β1* gene. Longer fragments over 3 kb belonging to the *IL-1β2* gene were obtained since the second gene does not have a *Pvu*II or *Bam*HI cut site upstream of exon 5, the region detected by probe 5. Therefore, the fragments include the *IL-1β2* gene and 5′ flanking region of the *IL-1β2* gene.

The last combination of enzymes used was *Pvu*II/*Eco*RV. In this case, a longer digestion product (1640 bp) was expected for the *IL-1β2* gene since it does not possess a *Pvu*II cut site within intron 4 as found in the *IL-1β1* gene. With the *IL-1β1* gene, a 739-bp digestion product was obtained as for *Pvu*II digestion alone.

Table 2 Similarities between exons and introns of the two trout interleukin-1β genes

Region		Length	Percent nucleotide identity		Percent amino acid identity
			Exons	Introns	
Exon 1	*IL-1β1*	81	66.7		
	IL-1β2	102			
Intron 1	*IL-1β1*	288		70	
Intron 1	*IL-1β2*	280			
Exon 2	*IL-1β1*	16+33	91.8		72.7
	IL-1β2	19+33			
Intron 2	*IL-1β1*	155		50	
	IL-1β2	187			
Exon 3	*IL-1β1*	202	85.0		78.1
	IL-1β2	193			
Intron 3	*IL-1β1*	623		76	
	IL-1β2	289			
Exon 4	*IL-1β1*	180	80.7		71.9
	IL-1β2	171			
Intron 4	*IL-1β1*	236		49	
	IL-1β2	339			
Exon 5	*IL-1β1*	137	89.9		82.6
	IL-1β2	137			
Intron 5	*IL-1β1*	447		45	
	IL-1β2	549			
Exon 6	*IL-1β1*	231+466	85.7		76.3
	IL-1β2	231+518			

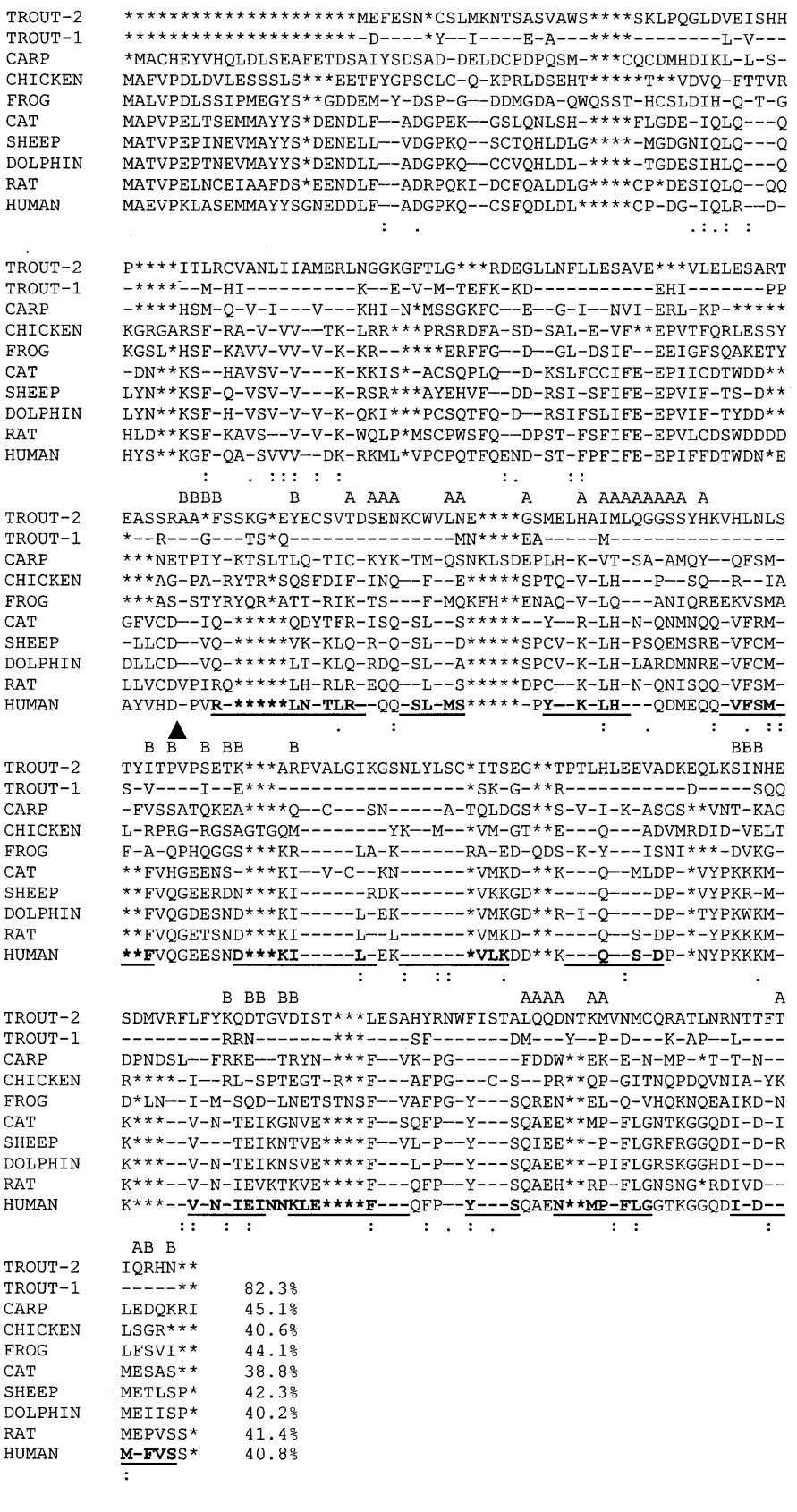

Fig. 3 Multiple alignment of the predicted translation of the trout IL-1β2 protein with other known genes. Identical (–) and similar (. and :) residues are indicated, with *asterisks* indicating gaps in the alignment. The *arrowhead* marks the beginning of the mammalian mature peptide. The twelve β sheets of the known human crystal structure are in *bold* and *underlined*, and the amino acids making contact with the two receptor binding sites (*A*, *B*) are indicated. Note the two three-amino acid deletions in the precursor region of trout IL-1β2 vs IL-1β1

```
Exon 3   V  E
IL-1β1: GTGGAAGgtagggagcaa
IL-1β2: GTGGAAGgtagg..tcaa
         V  E
Exon 4                    E  H  I  V  L  E  L
IL-1β1: tctctcttctcaccagAACATATAGTGTTGGAGTTG
IL-1β2: tctctctcctcatcaaaacatatagTGTTGGAGTTG
                          -          V  L  E  L
```

Fig. 4 Alignment of the trout *IL-1β1* and *IL-1β2* sequences at either end of intron 3. Exon sequence is in *uppercase* and intron sequence in *lowercase*. Note the change in the splice site at the 5′ end of exon 4 that results in a 9-bp (3 amino acid) deletion from the resulting mRNA (as detected using cDNA), with the new splice site retaining the sequence in frame. The mutated nucleotide is *underlined*

Expression studies

As the two trout *IL-1β* cDNA sequences share high homology, designing specific primers to distinguish their transcripts for expression analysis is difficult. The specificity of the *IL-1β2* primers was thus confirmed by sequencing five clones derived from the resulting PCR products. All five clones were identical to the *IL-1β2* sequence.

RT-PCR analysis with the *IL-1β2*-specific primers showed that LPS was capable of inducing the transcription of the *IL-1β2* gene in trout HK leukocytes and macrophages (Figs. 6, 7). Different concentrations of LPS were used in the first experiment with macrophages and all were capable of inducing transcription of the *IL-1β2* gene within 4 h, although there was a clear dose effect, with 0.5 μg/ml being only marginally effective while 50 μg/ml induced the highest levels of transcription (Fig. 6C), similar to the situation with the *IL-1β1* transcript (Fig. 6B). In the kinetic study using total HK leukocytes, some baseline transcripts were detected by PCR in the control cells (Fig. 7). Nevertheless, stimulation with 5 μg/ml LPS again clearly induced *IL-1β2* expression, the transcript being detectable after 2 h of incubation with LPS and increased further over the following 2 h, as seen previously with the *IL-1β1* gene (Zou et al. 2000b).

Analysis of the sites of expression of the *IL-1β2* gene after bacterial challenge revealed strong products with cDNA from kidney, spleen, gill, and blood, but only a very weak product from the brain (not shown), again similar to the findings with the *IL-1β1* gene (Zou et al. 1999a).

Discussion

The present results reveal the existence of two *IL-1β* genes in the rainbow trout genome, as confirmed by sequence and Southern blot analysis. That these two sequences do not represent alleles of the *IL-1β1* gene is further validated by the fact that a second allele of *IL-1β1* has been sequenced recently (Wang et al. 2000) and in heterozygous trout the second gene is detectable by PCR (unpublished data). A precedent does exist for the presence of two *IL-1β* genes within at least one other species, the pig *IL-1β*, where the predicted proteins from the two genes show 86% amino acid similarity (Vandenbroeck and Billiau 1997; Vandenbroeck et al. 1994). Whether in trout the existence of two genes relates to their tetraploid ancestry (Hordvik 1998) remains to be determined.

The coding regions of the two trout transcripts show 85% nucleotide identity and the two proteins show 82% amino acid similarity. This high homology between the two trout gene sequences presented some problems when designing specific primers for the *IL-1β2* gene expression studies, requiring the use of primers in the 5′UTR and precursor region of the molecule, and has, to date, precluded the production of specific probes for Northern blot analysis.

Besides the high homology between the two *IL-1β* genes, a deletion and an altered splice site were identified in the *IL-1β2* gene. The potential effects of the two deletions of three amino acids have yet to be studied, but they occur within the precursor domain of the molecule and so may not be critical for biological activity which requires cleavage of this portion in mammals. Nevertheless, the proximity of these modifications to the potential cut site could generate conformational and structural changes that might compromise the cleavage of the precursor to obtain the mature peptide. Although the IL-1*β*2 precursor, like the IL-1*β*1 precursor (Zou et al. 1999b), does not possess an ICE cut site, other enzymes may perform this function in trout. Indeed, from mammalian studies, other enzymes are known to be capable of processing the IL-1*β* precursor in the region of the authentic cut site (Granzyme A after residue 120, HIV protease after residue 94, elastase after residues 103 and 113) giving rise to a biologically active IL-1*β* mature form (Irmler et al. 1995). Precedents for deletions in the *IL-1β* transcript also exist in mammals. In the horse *IL-1β* transcript, the whole of exon 5 can be spliced out as a result of alternative splicing of the single *IL-1β* transcript (Kato et al. 1996), although the biological consequences of this are not clear. Other examples of alternative splicing in cytokines have been reported to be related to different stages of development (Kim et al. 1993) or disease states, as with an incomplete *IL-1β* splicing associated with a human skeletal muscle disease (Belec et al. 1997).

Even if the deletions in the trout *IL-1β2* gene do have little biological effect, the two molecules still differ significantly in the amino acid composition of their mature peptide region, so producing and comparing the bioactivities of the IL-1*β*1 and IL-1*β*2 recombinant proteins will be important. The crystal

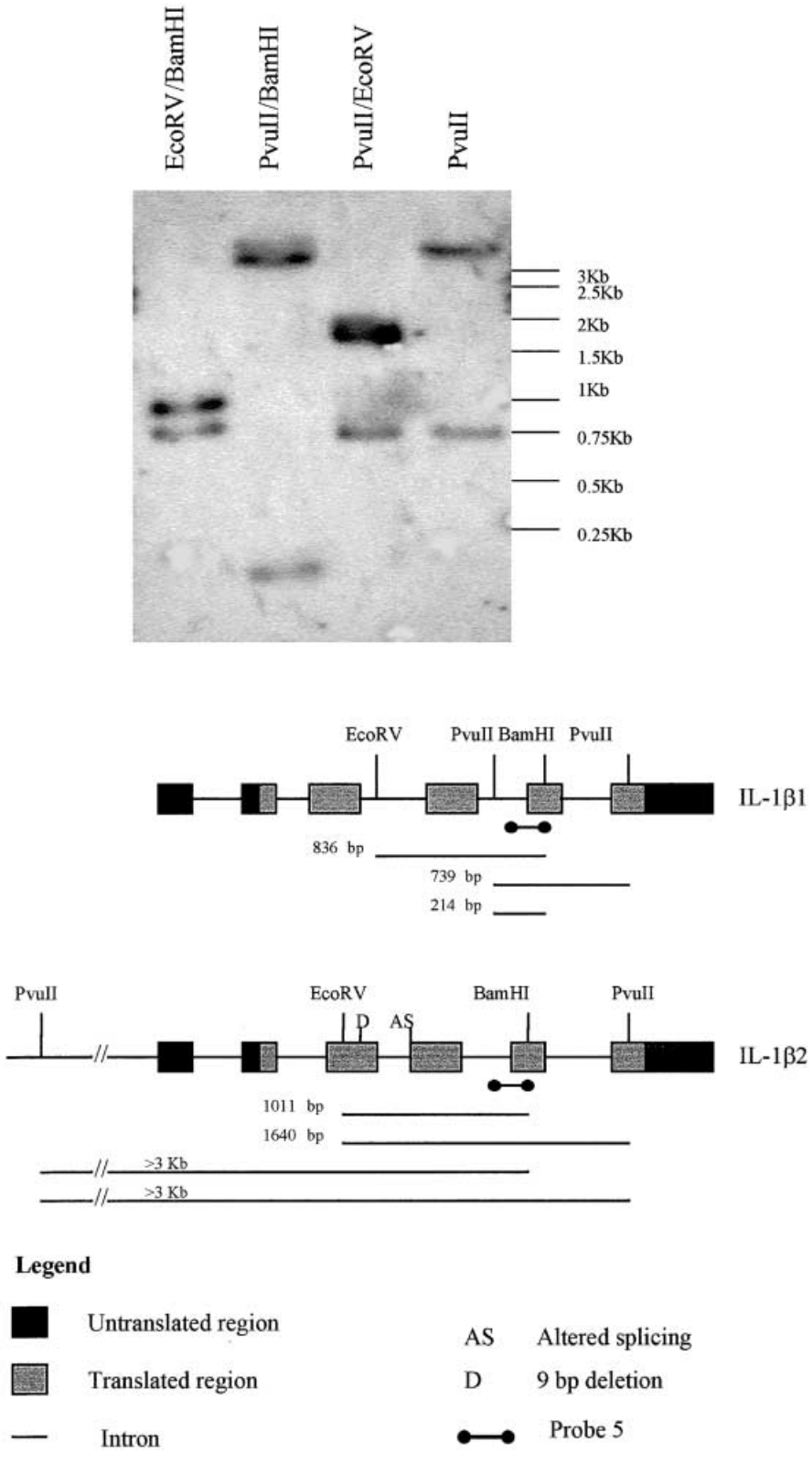

Fig. 5 Southern blot analysis of trout liver DNA completely digested with *Eco*RV, *Bam*HI, and *Pvu*II prior to electrophoresis. After blotting, the membrane was hybridized with a ^{32}P-labelled trout *IL-1β* probe encoding a nucleic acid sequence in exon 5 common to both genes. The restriction map of the two trout *IL-1β* genes below the blot shows the cut sites for each enzyme and the predicted sizes of the DNA fragments that will hybridize with the probe (*D* the site of a 9-bp deletion in *IL-1β2*, *AS* the altered splice site in *IL-1β2*, ●-● position of the probe)

structure of IL-1β complexed to its receptor has been obtained recently and allowed a precise determination of those residues required for receptor binding (Vigers et al. 1997). By alignment of the predicted trout IL-1β amino acid sequences with other known IL-1β sequences, the approximate locations of the receptor-binding residues can be defined, and these show some 70% identity. The difference of 30% could markedly influence the affinity for the receptor and hence the biological effect. Furthermore, potential differences in

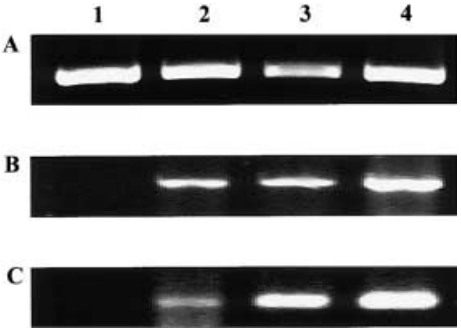

Fig. 6 RT-PCR with cDNA from trout macrophages from a single fish, stimulated for 4 h with different concentrations of lipopolysaccharide (LPS): no LPS (*1*), and 0.5 (*2*), 5 (*3*), or 50 (*4*) μg/ml of LPS. Specific primers for *β-actin* (**A**), *IL-1β1* (**B**), and *IL-1β2* (**C**) were used to amplify the products obtained. The results are representative of three performed experiments

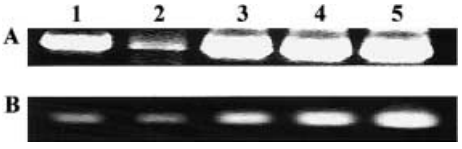

Fig. 7 RT-PCR with cDNA from trout head kidney leukocytes from a single fish, stimulated with 5 μg/ml LPS for different times: 0 (*1*), 1 (*2*), 2 (*3*), 3 (*4*), and 4 (*5*) h. Specific primers for *β-actin* (**A**) and *IL-1β2* (**B**) were used to amplify the products obtained. The results are representative of three performed experiments

the promoter could affect the expression pattern and/or cell specificity of the second gene, and warrant future study.

Both genes appear to undergo incomplete splicing of introns, whereby forms of the mRNA containing intron 4 and/or intron 5 are found (Zou et al. 1999a). Indeed, the present study took advantage of this phenomenon in the *IL-1β1* gene, and a successful strategy to obtain the 5′ and 3′ ends of the gene from cDNA was to use *IL-1β2*-specific primers designed to the relatively poorly conserved introns. The incompletely spliced forms are likely involved in the regulation of the mature transcript, as seen in certain situations in mammals (Jarrous and Kaempfer 1994).

An indication that the IL-1β2 molecule will have a role in trout immune responses comes from the RT-PCR experiments. Using specific primers for the *IL-1β2* gene in RT-PCR, the *IL-1β2* gene was demonstrated to be transcribed and up-regulated in response to LPS stimulation. The induced transcription was both dose and time dependent. At least 2 h was necessary to reach high levels of transcript and the levels continued to increase in the following hours. During this 2h period, transcription factors were likely produced that bound to regulatory elements in the *IL-1β2* promoter activating transcription (Auron and Webb 1994). That 0.5 μg/ml LPS was a suboptimal dose to induce expression of *IL-1β* is in agreement with previous Northern blot studies in trout where 0.1 μg/ml LPS was just able to induce a detectable transcript, while doses of 1.5 μg/ml LPS and above appeared optimal, using a probe that probably detected both molecular species (Zou et al. 2000b). Future expression studies are needed to understand the role of the *IL-1β2* gene relative to the *IL-1β1* gene, and to determine whether *IL-1β2* gene expression is tissue specific and if it is linked to disease states.

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