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Short sequence report

Molecular cloning and characterisation of the flounder (*Paralichthys olivaceus*) interleukin-6 gene

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Interleukin 6 (IL-6) is a pleiotropic cytokine that plays major roles in regulating immune responses, acute phase reactions, haematopoiesis, and inflammation [1]. It is produced by many different cell types and acts on B cells [1], T cells [2], hepatocytes [3], haematopoietic progenitor cells [4], and cells of the central nervous system [5]. IL-6 is a potent inducer of the acute phase response, along with IL-1 and TNF-α [6]. Recently, the first fish IL-6 sequence has been determined in the Japanese pufferfish (*Fugu rubripes*) by exploiting the synteny found between certain regions of the human and *Fugu* genomes [7]. We have identified a partial fragment of IL-6 from a suppression subtracted cDNA library of flounder (*Paralichthys olivaceus*) peripheral blood leucocytes (PBLs) that were stimulated with lipopolysaccharide (LPS). In this paper, we report the molecular cloning and sequencing of the flounder IL-6 gene, as well as the upregulation of the expression of this gene after bacterial challenge.

PBLs were prepared by density gradient centrifugation over a Percoll gradient (1.072 g/ml) at 400 × *g* for 20 min. Following centrifugation, the PBLs were carefully removed and washed with phosphate-buffered saline (PBS). The isolated PBLs were then adjusted to 10^7 cells/ml and cultured in RPMI 1640 that contained 500 μg/ml LPS or in normal fresh medium (RPMI 1640 without LPS) at 20 °C for 1, 3, and 6 h. Following each incubation period, leucocytes were harvested, washed twice with PBS, and stored at −80 °C until used. Total RNA was isolated from the control and LPS-stimulated PBL cells with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Samples of mRNA were purified from the total RNA solution using the PolyATtract mRNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions. Two-microgram aliquots of poly(A) RNA isolated from treated and untreated cultures were used as the tester and driver, respectively. Suppression subtracted hybridisation (SSH) was performed using the PCR-Select cDNA Subtraction Kit (BD Clontech, Palo Alto, CA) according to the manufacturer's instructions. The amplified cDNA fragments were subcloned into the pGEM-T vector (Promega) for sequencing.

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Table 1
Primers used in this study

IL-6-5′-RACE	5′-TGCGCCGTCATCTTCCTGTGGAAAGT-3′
IL-6-3′-RACE	5′-CCAGCAGACAAGAGCAGCTGCTGCAA-3′
IL-6 ORF-F	5′-ATGGCCTCCAAACACAATGCCGAC-3′
IL-6 ORF-R	5′-TTATGTCATTTGGTAAGAGGGATG-3′
TNF-α-RT-F	5′-ATGGTGAAATACACAAGTGCA-3′
TNF-α-RT-R	5′-TCAAAGTGCAAAGACACCGAA-3′
IL-1β-RT-F	5′-ATGGAATCCAAGATGGAATGC-3′
IL-1β-RT-R	5′-TTAACTCTGATGATGGATGTT-3′
GAPDH-F	5′-TCCCATGTTCGTCATGGGCGTGA-3′
GAPDH-R	5′-ATTGAGCTCAGGGATGACCTTG-3′

The SSH clone LSPSL-IV-E08, which carries a 658-bp insertion, showed significant sequence homology (52%) to the C-terminal portion of the Japanese pufferfish (*Takifugu rubripes*) IL-6 gene (GenBank accession no. AJ544721). The full-length cDNA was amplified from the first-stranded cDNA of LPS-stimulated flounder PBLs using 5′-RACE and 3′-RACE using primers based on the LSPSL-IV-E08 sequence (Table 1). The primer set for IL-6-5′-RACE and the nested universal primer supplied with the kit were used for 5′-RACE, and 3′-RACE was performed with the IL-6-3′-RACE primer set and the nested universal primer. The complete cDNA of the flounder IL-6 gene was compiled by overlapping the sequences of the SSH clone and the 5′-RACE and 3′-RACE PCR products (Fig. 1). The flounder IL-6 transcript consisted of 1157 bp, which translated into a 230-amino acid (aa) open reading frame (ORF) that included a 24-aa signal peptide, an 86-bp 5′-untranslated region (5′-UTR), and a 381-bp 3′-UTR (GenBank accession no. DQ267937). The 3′-UTR region of the flounder IL-6 transcript contained a polyadenylation signal (AATAAA) and six ATTTA sequence elements. The ATTTA consensus sequence is typically found in cytokine mRNAs and mediates RNA instability [8]. Comparison of the IL-6 sequences from flounder and other species was carried out using the Genetyx program ver. 4.0 (GENETYX Co., Japan). The aa sequence identities between flounder IL-6 and its counterparts in pufferfish and human are approximately 46.3% and 22.8%, respectively. Two potential *N*-linked glycosylation sites are predicted at aa 100 and aa 175. The IL-6 aa sequences were aligned with the corresponding NCBI GenBank sequences of known IL-6 molecules using the CLUSTALW program (Fig. 2). The flounder IL-6 shows conservation of the IL-6/G-CSF signature (C-X_9-C-X_6-GL-X_2-Y-X_3-L), with the exception of the last leucine residue (Leu) at position 121. In the flounder IL-6, aa 121 shows a substitution of Leu (TTA or G) to Phe (TTT). An unrooted phylogenetic tree

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   1  ataaaatgacagcccgtggctaagaacaggcaactccttctttctctgagagcatcaactcaacaagtgcctcccagcagcaccacATGG
                                                                                          M     1
  91  CCTCCAAACACAATGCCGACTTGTCCTCCGCAGCAATGCTGGCGGCTCTGCTGCTCTGCGCTCTCGGAGCTCCAGTCGAATACGAGCCCA
      A  S  K  H  N  A  D  L  S  S  A  A  M  L  A  A  L  L  L  C  A  L  G  A  P  V  E  Y  E  P    31
 181  CCGACAGTCCTGCAGGTGACTTTTCAGGTGAGGAGCAGGAGGTGACCCCTGACCTTTTAAGCGCCTCACCGGTGTGGGACTTGATCATCG
      T  D  S  P  A  G  D  F  S  G  E  E  Q  E  V  T  P  D  L  L  S  A  S  P  V  W  D  L  I  I    61
 271  GTGTAACCGCTCACCACCAGAAAGAGTTTGAGGATGAATTCCAACAGGAAGTGAAATATCGTTTTCTGAACCACTACAAACTCTCCTCAC
      G  V  T  A  H  H  Q  K  E  F  E  D  E  F  Q  Q  E  V  K  Y  R  F  L  N  H  Y  K  L  S  S    91
 361  TGCCAGCAGACTGCCCCAGTGCCAACTTCAGCAAGGAGGCTTGTCTCCAAcGGTTGGCTGAAGGCCTGCACACCTACATGGTTCTTTTTA
      L  P  A  D  C  P  S  A  N  F  S  K  E  A  C  L  Q  R  L  A  E  G  L  H  T  Y  M  V  L  F   121
 451  AGCAtGTGGAGAAGGAGTACCCGAGCAGCTCCATCCTCTTGCACGCCAGATATCACAGCGGGGCACTGATCGGCCTCATCAAAGAAAAGA
      K  H  V  E  K  E  Y  P  S  S  S  I  L  L  H  A  R  Y  H  S  G  A  L  I  G  L  I  K  E  K   151
 541  TGAGGAACCCTGGTCAGGTGACCGTCCCGACCAGCAGACAAGAGCAGCAGCTGCTGCAAGACATGGACAACCCCAGCACTTTCCACAGGA
      M  R  N  P  G  Q  V  T  V  P  T  S  R  Q  E  Q  Q  L  L  Q  D  M  D  N  P  S  T  F  H  R   181
 631  AGATGACGGCGCACAACATCCTGCGGCAGCTCCACAACTTCCTCCGCAATGGGAAGGTGGCAATTCGTAAAAGAGAGATGCCCAAACAGA
      K  M  T  A  H  N  I  L  R  Q  L  H  N  F  L  R  N  G  K  V  A  I  R  K  R  E  M  P  K  Q   211
 721  AGAGGAGAAAGGATGATGGAATTATTCCACCCATCCATCCCTCTTACCAAATGACATAAagatcatcttaatgaaaacatcagagatgaa
      K  R  R  K  D  D  G  I  I  P  P  I  H  P  S  Y  Q  M  T  *                                230
 811  ggagcagctcagggaaatgtctcctctcagaatcgtcacacatattgtgggtctgcagga**atttac**tttgtgctctacggcaatctactg
 901  cgcatgtgtttaatattgtggccctgagtttttaatgtttcactgttatgttactt**atttaatctatttatacttgatgaatattgtta**
 991  **ttta**tt**atttag**ttactggcgctgaaaatacagtttgtacttgatataatgatgttgaaaatgattcatttctggattgtgacaactaag
1081  gcacacatcattgttaacagt**atttatta**<u>**ataaa**</u>ctaaattattataccaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 1. cDNA and predicted amino acid sequence of the flounder IL-6. Numbering of nucleotides and amino acids are given at the left and the right margins, respectively. The putative signal peptide is underlined. The IL-6/G-CSF family signature is boxed. Two predicted *N*-linked glycosylation sites are shared. The polyadenylation signal is shown in bold and underlined. Six ATTTA sequence motifs in the 3′ untranslated region are shown in bold. The nucleotide sequence has the accession number DQ267937 in the GenBank database.