Immunogenetics (2006) 58: 422–432
DOI 10.1007/s00251-006-0106-5

ORIGINAL PAPER

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# The granulocyte colony-stimulating factors (CSF3s) of fish and chicken

Received: 1 November 2005 / Accepted: 15 February 2006 / Published online: 28 April 2006


**Abstract** Granulocyte colony-stimulating factor (CSF3) is a glycoprotein cytokine, which influences the hematopoiesis of the phagocytic neutrophils and its precursors and was used extensively in cancer therapy and for the treatment of neutropenia in mammals. However, CSF3 is yet to be identified in nonmammalian species mainly because of its rapid mutation. Here, we report the first CSF3 genes from three teleost fishes: Japanese flounder (*Paralichthys olivaceus*), fugu (*Takifugu rubripes*), and green-spotted pufferfish (*Tetraodon nigroviridis*) and present evidence that the chicken (*Gallus gallus*) myelomonocytic growth factor is in fact the chicken CSF3 orthologue. We support this by showing significant conservation of the CSF3 genes' structure, domains, regulatory motifs, and synteny across species and by phylogenetic analysis. CSF3 orthologues are indeed evolving rapidly and appears to be undergoing purifying selection in mammals but positive selection in fish and chicken. Furthermore, the paralogous fugu and pufferfish CSF3-1s and CSF3-2s are shown to be the ancestral and duplicate genes, respectively. Finally, we demonstrate that the Japanese flounder CSF3 gene is at least involved in immunity based on its basal expression in immune-related tissues and its upregulation in kidney and peripheral blood leukocytes after in vitro stimulation with lipopolysaccharide and a combination of concanavalin A/phorbol myristate acetate.

**Keywords** Granulocyte colony-stimulating factor · Japanese flounder · Fugu · Green-spotted pufferfish · Chicken

**Electronic Supplementary Material** Supplementary material is available for this article at (http://dx.doi.org/10.1007/s00251-006-0106-5)

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## Introduction

Colony-stimulating factors play a central role in mediating the development of pluripotent hematopoietic stem cells of the myeloid lineage (for review, see Barreda et al. 2004). Granulocyte colony-stimulating factor (CSF3), which is a member of this cytokine group, mediates the proliferation, survival, terminal maturation, and functional activation of mammalian neutrophils and its precursors during inflammation or steady-state in a lineage-specific manner (for review, see Basu et al. 2002). It is one of the few cytokines used successfully as recombinant therapeutics in mammals (Vilček and Feldmann 2004; Welte et al. 1996). However, CSF3 and the other colony-stimulating factors are yet to be identified and characterized in lower vertebrates. This is in spite of its clinical importance and the availability of genome resources in fugu *Takifugu rubripes* (Christoffels et al. 2004), green spotted pufferfish *Tetraodon nigroviridis* (Jaillon et al. 2004), zebrafish *Danio rerio* (National Center for Biotechnology Information 2005), and chicken *Gallus gallus* (International Chicken Genome Sequencing Consortium 2004). Such failure is believed to be due to the gene's rapid evolution rather than deletion from the genome, which make it difficult for current homology-based predictions to detect them (Jaillon et al. 2004; Aparicio et al. 2002). Previously, it was reported that the chicken CSF3 could not be detected in the chicken genome albeit its putative locus showed conserved synteny with mammalian CSF3s (Kaiser et al. 2005). It is interesting to note that a chicken myelomonocytic growth factor (cMGF) was reported to have intriguingly significant sequence similarity with human CSF3 and interleukin 6 (IL6) (Leutz et al. 1989) but was suggested to be unique based on DNA cross hybridization, proliferation, and antibody assay (McGruder et al. 1996; Kogut et al. 1997; Oshibe et al. 1999; and for review, see Siatskas and Boyd 2000).

Here, with the use of cloning, exhaustive use of available genome resources through automatic and manual search, and synteny mapping, we established the presence of CSF3 genes from three teleost species (Japanese flounder *Paralichthys olivaceus*, fugu *T. rubripes*, and pufferfish

*T. nigroviridis*) and show evidence that the chicken (*G. gallus*) cMGF is in fact the chicken CSF3 orthologue. We also present evolutionary characteristics of CSF3 genes and the constitutive and immunostimulated expression of Japanese flounder CSF3.

## Materials and methods

### cDNA and BAC library screening

The full-length Japanese flounder CSF3 cDNA and gene were determined following Hirono et al. (2000) with minor modifications. A 47-aa-long expressed sequence tag (EST) clone (GenBank accession no. AU260798) showing putative homology to human CSF3 was used as a probe to screen a Japanese flounder kidney cDNA library. Subsequently, the open reading frame (ORF) of the generated putative CSF3 cDNA was used to screen a previously constructed Japanese flounder genomic bacterial artificial chromosome (BAC) library (Katagiri et al. 2000). Specific and overlapping forward and reverse primers, designed using Web primer (http://seq.yeastgenome.org/cgi-bin/web-primer) from the putative CSF3 cDNA, were used to amplify positive BAC clones. The promoter region was sequenced through primer walking of the Japanese flounder genome using a dye-labeled reverse primer designed manually. Sequence and position of the specific primers are shown in Fig. S1.

### In silico search and analysis

CSF3s for fugu, green-spotted pufferfish, and chicken were automatically and manually mined from the existing fugu, green-spotted pufferfish, and chicken genome resources, respectively, accessed through the *Ensembl* genome browser (http://www.ensembl.org/). This was done by using the Japanese flounder putative CSF3 sequence as a query sequence, employing different BLAST algorithms in the Ensembl server and by localized comparisons through the "BLAST 2 SEQUENCES" (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi/). We mapped the CSF3 cluster by identifying the CSF3 gene and the conserved flanking genes in human and mouse and used this cluster as marker to pinpoint the specific position of the CSF3 gene in the fish and chicken genome. For the chicken, the cMGF nucleotide sequence (GenBank accession no. M85034) was used to "BLASTn" query the putative chicken CSF3 locus.

Whenever applicable, the nucleotide sequences, translated amino acids, and average molecular weights of the CSF3 orthologues were analyzed and determined using GENETYX 7.0.3 (GENETYX). SignalP (Center for Biological Sequence Analysis, http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptide cleavage sites. Nucleotide and amino acid sequence identities were calculated using the BLASTn and BLASTp (BLOSUM 62), respectively, implemented in BLAST 2 SEQUENCES while the complete multiple amino acid alignments were carried out in CLUSTAL X 1.81 using default parameters. The MEME/MAST system version 3 (UCSD Computer Science Engineering and San Diego Computer Center, http://meme.imb.uq.edu.au/) for motif discovery and search was used to predict conserved *cis*-regulatory elements. Conserved Domain-Search (CD-Search) at http://www.ncbi.nlm.nih.gov/Structure/ and Conserved Domain Architectural Tool (CDART) at http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps/ were utilized to determined conserved domains.

**Table 1** Nucleotide (*lower grids*) and amino acid sequence (*upper grids*) identities (in percent) of human, mouse, and fish CSF3 orthologues including chicken MGF

| CSF3 Orthologues | Human | Cow | Cat | Pig | Mouse | Rat | Chicken MGF | Flounder poCSF3-2 | Fugu trCSF3-2 | Pufferfish tnCSF3-2 | Fugu trCSF3-1 | Pufferfish tnCSF3-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human | | 82 | 82 | 79 | 72 | 73 | 38 | 26 | 29 | 34 | 26 | – |
| Cow | 88 | | 74 | 72 | 73 | 61 | 40 | 26 | 26 | – | – | – |
| Cat | 89 | 86 | | 67 | 73 | 59 | 37 | 25 | 25 | 30 | 27 | – |
| Pig | 88 | 89 | 87 | | 71 | 60 | 39 | 25 | 25 | – | – | – |
| Mouse | 79 | 79 | 79 | 79 | | 69 | 37 | 31 | – | 26 | – | – |
| Rat | 80 | 80 | 80 | 81 | 89 | | 35 | 36 | – | – | – | – |
| Chicken MGF | 79* | 77* | – | 82* | – | – | | – | – | 23 | 25 | – |
| Flounder poCSF3-2 | – | – | – | – | – | – | – | | 58 | 50 | 34 | 35 |
| Fugu trCSF3-2 | – | – | – | – | – | – | – | 74 | | 76 | 34 | 33 |
| Pufferfish tnCSF3-1 | – | – | – | – | – | – | – | 78 | 83 | | 36 | 30 |
| Fugu trCSF3-1 | – | – | – | – | – | – | – | 74 | – | – | | 50 |
| Pufferfish tnCSF3-1 | – | – | – | – | – | – | – | – | – | – | 75 | |

*Denotes partial local alignment identities detected while (–) means no significant homology

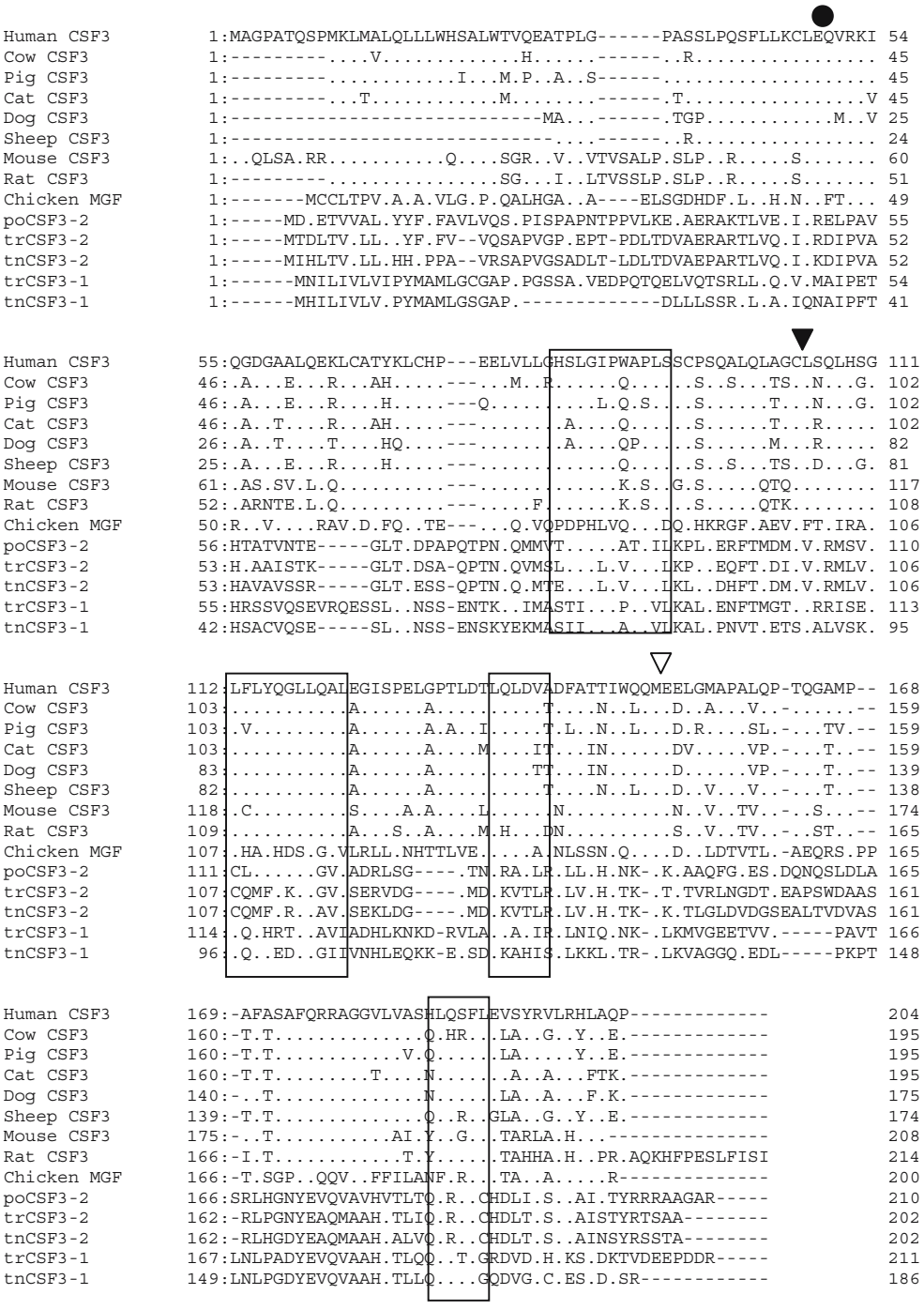


**Fig. 1** Complete multiple amino acid sequence alignment of CSF3 orthologues (including cMGF) using CLUSTAL X. Amino acids similar with human CSF3 are denoted by *dots* (...), apparently conserved motifs are *boxed*, and some gaps were introduced manually to maximize alignment. The important CSF3 receptor binding amino acid residue Glu (E) in mammal (*filled circle*), the conserved Cys (C) residue, except for *tn*CSF3-1 (*filled triangle*), and the conserved Met (M) residue (*unfilled triangle*) are also shown. GenBank accession nos.: human CSF3 (*X03655*), mouse CSF3 (*X05402*), cow CSF3 (*AP092537*), sheep CSF3 (*L07939*), pig CSF3 (*U68482*), cat CSF3 (*AB042552*), dog CSF3 (*P35834*), rat CSF3 (*U37101*), chicken MGF (M85034), Japanese flounder *po*CSF3-2 (AB200968), green-spotted pufferfish *tn*CSF3-1 (Location: *Tn. Chr. 2*, 4493790: 4496325), green-spotted pufferfish *tn*CSF3-2 (Location: *Tn. Chr. Un. Random*, 46234000: 46236000), fugu *tr*CSF3-1 (Location: *Fr. Scaffold_571*, 106000: 110000), and fugu *tr*CSF3-2 (Location: *Fr. scaffold_1637*, 1652: 4555)

Sequences of the members of Pfam IL6/CSF3/cMGF protein family (Pfam00489.11.IL6) were retrieved from the NCBI. Hydropathy plots were drawn using the method of Kyte and Doolittle (1982) in ProtScale (http://tw.expasy.org/tools/protscale.html) using window size 13. The MEGA3 package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA) was used to compute (1) the number of synonymous nucleotide substitutions per synonymous site (Ks), (2) the number of nonsynonymous substitutions per nonsynonymous sites (Ka), (3) their ratio (Ka/Ks) using Jukes-Cantor model, and (4) codon-based Fischer's exact test following Nei and Gojobori's method with complete deletion (<0.05 = positive selection, 1.0 = purifying selection). For phylogenetic analysis, we used the neighbor-joining (NJ) method also implemented in the MEGA3 employing the Poisson method with 1,000 bootstrap tests and with complete deletion of gap sites. Only the bootstrap consensus tree is shown.

## Gene expression analysis using semiquantitative RT-PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) study was carried out following Hirono et al. (2000) with

**Table 2** Comparison of known and unique characteristics of human, mouse, and fish CSF3 orthologues including chicken MGF

| CSF3 Orthologues | Gene size (kb) from start to stop codons | No. of Introns/ Exons | Open Reading Frame (ORF) size (bp) | Predicted protein size (a.a) | Predicted molecular mass (kDa) | Signal peptide length/ cleavage site | Polyade-nylation signal (AATAAA) position | Kozak-type concensus sequence (A/ GCCATG) | Detected concensus CSF box in promoter (GAGRTTCCA/ CC) | Observable, known AU-rich elements (AREs). Nos. in parenthesis | Simple sequence repeats (SSRs) or microsatellites |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Human | 1.49 | 4/5 | 612 | 204 | 22 | 30/Ala$^{30}$ | +2056 | ——— | GAGATTCCCC | AUUUA (6) | —— |
| Mouse | 1.58 | 4/5 | 624 | 208 | 22 | 30/Ala$^{30}$ | +2058 | ——— | GAGATTCCCC | AUUUA (5) | —— |
| Chicken MGF | 1.24 | 4/5 | 603 | 201 | 22 | 23/Gly$^{23}$ | +1560 | ACCATG | GTGAATCCCC | AUUUA (8) | —— |
| Flounder poCSF3-2 | 2.14 | 4/5 | 633 | 211 | 23 | 21/Ser$^{19}$ | +2207 | ——— | GAAAATCCCG | AUUUA (5) | GGGTTCTTC |
| Fugu trCSF3-2 | 1.59 | 4/5 | 609 | 202 | 22 | 19/Ser$^{19}$ | 1977 | ——— | GGAAAACCCG | AUUUA (4) UUAUUUAUU (1) | CA; GT; |
| Pufferfish tnCSF3-2 | 1.86 | 4/5 | 609 | 2002 | 22 | 19/Ser$^{19}$ | +2127 | ——— | GGAAAACCCG | AUUUA (5) | GT; CA; GGTTCCGTACCGGC-CT |
| Fugu trCSF3-1 | 1.50 | 4/5 | 636 | 211 | 23 | 18/Gly$^{18}$ | +1757 | GCCATG | GGGAATCCCA | AUUUA (5) | GT; CT |
| Pufferfish tnCSF3-1 | 1.41 | 4/5 | 561 | 186 | 20 | 18/Gly$^{18}$ | +1304 | GCCATG | GGAAATCCCC | AUUUA (4) | GT; CT |

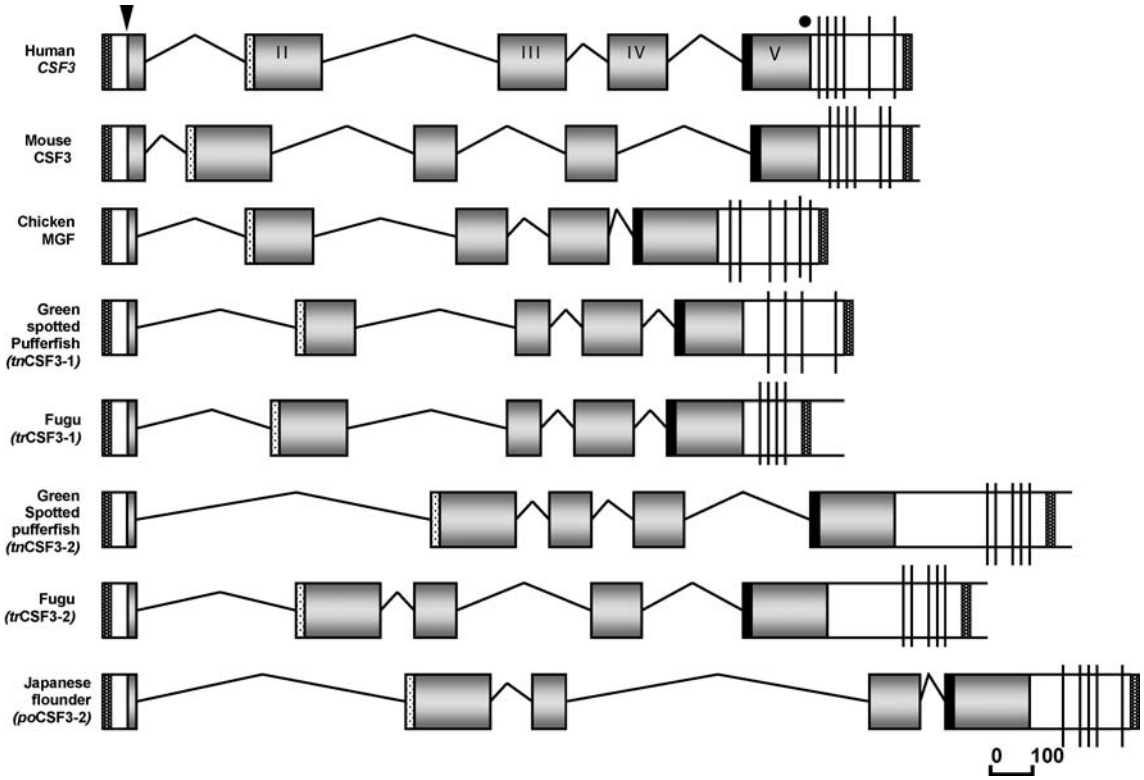


**Fig. 2** Gene organization of human, mouse, teleost CSF3s including cMGF. Known and unique characteristics are shown: exons (*I–V*), introns (*curved lines*), start codon (*filled triangle*), stop codons (*filled circle*), AU-rich elements (*horizontal lines*), "TATA box" and polyadenylation signal "AATAAA" sequences (*dotted filled boxes*), conserved Met (*filled boxes*), and phase 1 intron site (*dotted white boxes*)

minor modifications. Primers used for amplification were designed as above (Fig. S1). For constitutive expression, total RNA was extracted from brain, eyes, gills, kidney, heart, intestine, peripheral blood leukocytes (PBLs), liver, muscle, ovary, skin, spleen, and stomach from three apparently healthy Japanese flounders. PCR conditions were initial denaturation at 95°C for 5 min, 30 cycles (95°C, 30 s; 55°C, 30 s; and 72°C, 1 min), and final elongation at 72°C for 5 min. For the immunostimulation studies, total RNA was extracted from Japanese flounder kidney and PBLs cultured in RPMI and treated with final concentration of 0.5 mg/ml lipopolysaccharide (LPS) and 0.5 mg/ml combination of concanavalin A/phorbol myristate acetate (ConA/PMA) sampled at 1, 3 and 6 h postinduction. β-actin was used as a positive control. PCR conditions were initial denaturation at 95°C for 5 min, 25 cycles (95°C, 30 s; 55°C, 30 s; and 72°C, 1 min), and final elongation at 72°C for 5 min.

All PCR amplicons (5 μl) were visualized on a 1% gel stained with ethidium bromide and photographed with a densitometer (Atto). mRNA bands were semiquantitatively assessed for their relative expression following Lindenstrøm et al. (2004). ImageJ software was used to measure light intensity (Abramoff et al. 2004).

## Results

### Teleost CSF3 genes

With the use of cDNA and BAC library screening, we isolated a putative Japanese flounder CSF3 cDNA and gene with a decided ORF of 633 bp, encoding for a 211 putative amino acid residues, having a predicted molecular weight of about 21 kDa and a computed signal peptide cleavage site between $Ser_{21}$ and $Val_{22}$ (Table 2 and Fig. S2). It has a 5 exon-4 intron gene configuration as confirmed by the splice donor (cag) and acceptor (gt) sequences (Table 2 and Fig. 2). There were numerous and readily observable regulatory elements in the untranslated regions (UTRs) including the transcription-important "TATA" box in the 5′ region and the polyadenylation signal "AATAAA" and numerous conserved AU-rich elements (AREs) in the 3′ area.

Using putative Japanese flounder CSF3 as a probe to "tBLASTn" fugu and green-spotted pufferfish genomes in Ensembl, we obtained partially predicted CSF3 homologous genes in fugu (*SINFRUG00000157575* on Tr. Chr. Un, scaffold 1,637) and in green-spotted pufferfish CSF3 gene thru Genoscope (*GSTENG00024099001* on Tn. Chr.

2, scaffold 1,478). The fugu CSF3 homologue was initially predicted to have 5 exons, a 723-bp transcript, and 240 amino acid residues. Upon comparison of the sequence with putative Japanese flounder and mammalian CSF3s, we found that the gene is a CSF3 homologue that should have had a shorter first exon fragment in reference to the observed TATA box (Fig. S3). This was similar with the pufferfish CSF3 gene, which was also predicted to have a 4-exon, 858-transcript, and 175-aa feature but should instead have a 5 exon-4 intron gene encoding a predicted 186 amino acids (Fig. S6).

Taking into consideration the whole genome duplication theory in teleosts, we then searched for the duplicate copies of fugu and pufferfish putative CSF3 genes using a combination of automatic and manual BLAST and synteny comparisons (results described below). We were able to isolate the duplicate CSF3 gene copy of the fugu (scaffold 571, ~position 105,000; Fig. S5) and pufferfish (scaffold 13,844, ~position 46,234,000; Fig. S4). We used the above BLAST and synteny procedures to find the CSF3 genes in the zebrafish genome resource but failed.

After this, we looked for the CSF3 duplicate in Japanese flounder using Southern hybridization in the absence of a flounder genome sequence. It is surprising to note that our results only showed one copy number (Fig. S10).

Subsequent comparative analysis of the fish and mammalian CSF3 orthologues revealed that teleost CSF3 genes indeed possess low identities (average of 36%) compared to its mammalian counterpart (Table 1) and that there appears to be little amino acid alignments and not very clear conserved motifs between the nonmammalian CSF3s and their mammalian counterparts (Fig. 1). However, when other features of the CSF3 orthologues were analyzed, significant conservation was revealed and allowed for the classification of the genes to: *tn*CSF3-1 and -2 for fugu, *tr*CSF3-1 and -2 for green-spotted pufferfish, and *po*CSF3-2 for Japanese flounder. The CSF3 gene orthologues have similar nucleotide and amino acid sequence sizes and sequence organization including a 5 exon-4 intron architecture, the short length of the first exon, the phase 1 intron splicing between the first and second exons, the Met (M) residues of the fifth

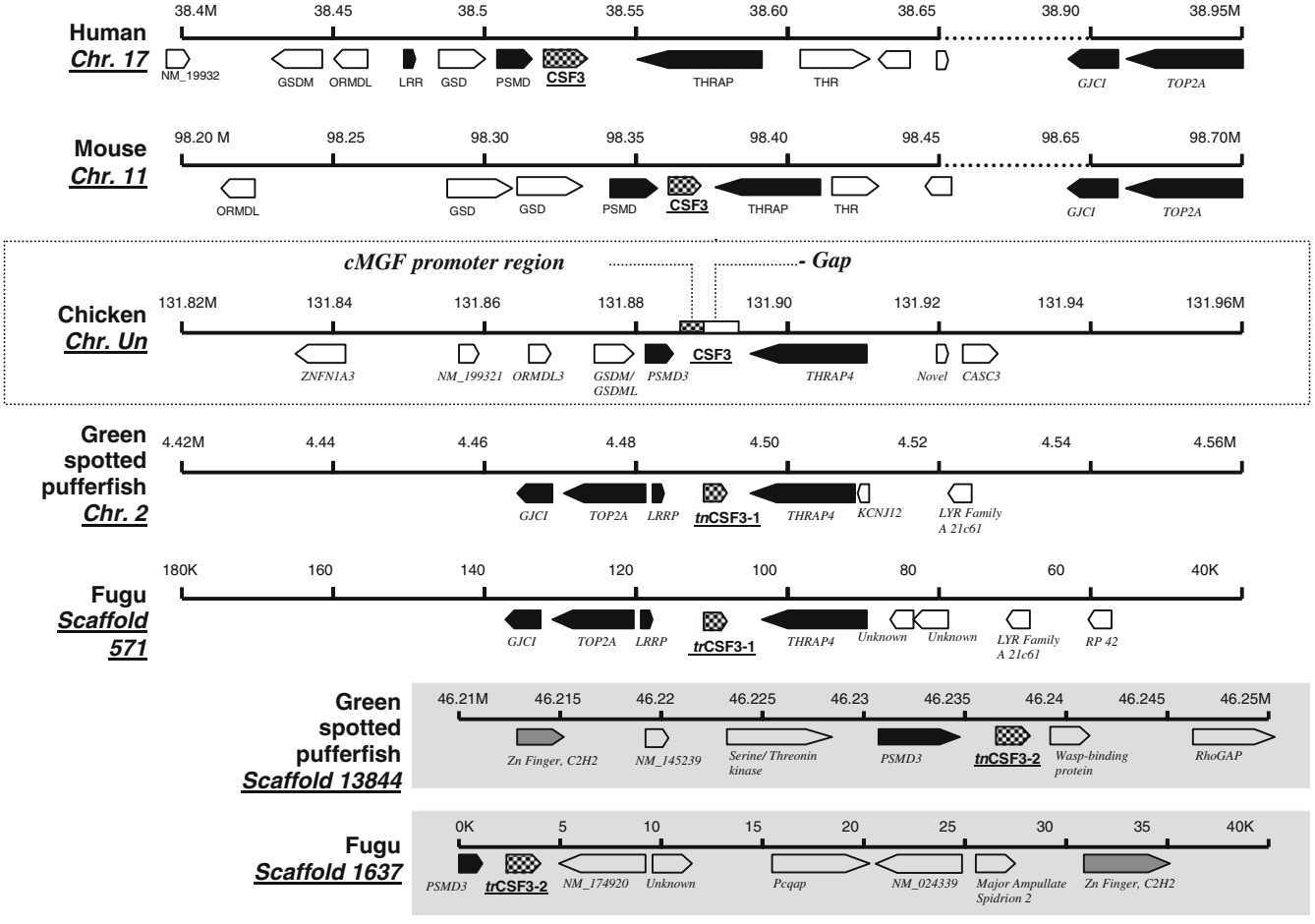


**Fig. 3** Syntenic putative CSF3 locus of human (chromosome 17), mouse (chromosome 11), chicken (Gg Chr Un), green-spotted pufferfish (chromosome 2 and SCAF13844), and fugu (scaffolds 571 and 1,637). The CSF3 genes (*checkered block arrows*) are flanked by conserved genes including THRAP4, PSMD, LRRP, GJCI, and TOP2A (*black block arrows*); by Zn Finger C2H2 (*gray block arrows*); and by nonconserved genes (*unfilled block arrows*). The putative chicken CSF3 locus is also included and shows the cMGF (M85034) promoter site (*checkered rectangular box*) followed by a gap (*unfilled rectangular box*). Figure is not drawn to scale

exon, and the presence of AREs in the 3′ UTR (Table 2 and Fig. 2). CD-search and CDART revealed significant alignment of chicken and teleost CSF3s with the consensus-conserved domain of the Pfam IL6/CSF3/MGF protein family, except for *tn*CSF3-1 (Fig. S7). The Kyte and Doolittle hydropathy plots showed that like mammalian CSF3s, fish and chicken CSF3s were also highly hydrophobic (Table 2 and Fig. S8). In the promoter region, the MEME/MAST motif search was able to detect the presence of the tumor necrosis factor response region across species (Table 2 and Fig. S9).

There were also other observable patterns and features for and among the nonmammalian CSF3s. The gene organization and hydrophobicity plots within the CSF3-1s and CSF3-2s were found to be alike (Figs. 2 and S8). The signal cleavage site of CSF3-1s and cMGF is a Gly (G) residue, while for CSF3-2s it is a Ser (S) and for mammalian CSF3s an Ala (A). In addition, only CSF3-1s and cMGF possess a Kozak consensus sequence, and fish CSF3s, not chicken and mammals, have varied simple sequence repeats (SSRs) or microsatellites (Table 2, Figs. S2, S3, S4, S5, and S6).

A CSF3 cluster synteny map (Fig. 3) showing the CSF3 locus in human (chromosome 17), mouse (chromosome 11), chicken (chromosome unknown), green-spotted pufferfish (/chromosome 2, scaffold 1,478 and 13,844), and fugu (scaffolds 571 and 1,637) was constructed. From this map, we found that the CSF3 locus was conserved in humans, mouse, chicken, and fish CSF3-1s as evidenced by the retention in the locus of THRAP4, LRRP, GJCI, and TOP2A genes. Fish CSF3-2s, on the other hand, are flanked by PSMD3 and Zn Finger C2H2. With this, we were able to confirm the classification of the fugu and pufferfish and the chicken CSF3 orthologues.

## Chicken MGF as CSF3

Because cMGF is remarkably similar in sequence and function to known mammalian CSF3 orthologues (Leutz et al. 1989; Sterneck et al. 1992), we hypothesized that cMGF is a CSF3. To check this, we compared the features of cMGF to mammalian and fish CSF3s. Surprisingly, we observed that cMGF shares the same unique characteristics that the mammalian and fish CSF3 orthologues possess (Table 2 and Fig. 2). Initially, a tBLASTn search of the chicken genome using cMGF amino acid sequence as query did not find any similar sequences. We then specifically analyzed the chicken CSF3 cluster (Fig. 3) using the nucleotide sequences of the full cMGF gene as a query sequence to BLASTn the putative CSF3 locus. This yielded a near exact match between the ~1,500 bp cMGF promoter nucleotide sequence and a region in the putative chicken CSF3 locus from 131,892,000 to 131,894,000 (Fig. S11). This region was found to be followed downstream by a gap (an unfinished sequence), which is where we predict the full length of the cMGF gene will be located. The absence of the cMGF ORF in the contig accounts for why our initial tBLASTn search failed.

## CSF3 evolution

The sequences of the nonmammalian CSF3 genes are indeed in a rapid evolutionary state and by computing its Ka/Ks ratio (Table 3) we further found out that the mammalian CSF3s with Ka/Ks average value of 0.324 and Fisher's test values of 1.0, are clearly under purifying selection while the chicken and fish CSF3 genes, which have a high average value of 0.793, appears to be the

**Table 3** Pairwise Ka/Ks ratio (*upper grids*) with corresponding codon-based Fisher's exact test values (*lower grids*) of human, mouse, and fish CSF3 orthologues including chicken MGF

| CSF3 Orthologues | Human | Cow | Cat | Pig | Mouse | Rat | Chicken MGF | Flounder poCSF3-2 | Fugu trCSF3-2 | Pufferfish trCSF3-2 | Fugu trCSF3-1 | Pufferfish tnCSF3-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human | | 0.272 | 0.432 | 0.444 | 0.234 | 0.245 | 0.713 | 0.620 | 1.011 | 0.905 | 0.596 | 0.705 |
| Cow | 1.000 | | 0.284 | 0.334 | 0.257 | 0.269 | 0.695 | 0.711 | 0.920 | 0.885 | 0.920 | 0.803 |
| Cat | 1.000 | 1.000 | | 0.476 | .0370 | 0.334 | 1.060 | 0.747 | 1.082 | 1.087 | 0.653 | 0.935 |
| Pig | 1.000 | 1.000 | 1.000 | | 0.345 | 0.345 | 1.105 | 0.714 | 1.127 | 1.208 | 0.756 | 0.992 |
| Mouse | 1.000 | 1.000 | 1.000 | 1.000 | | 0.233 | 0.786 | 0.661 | 0.961 | 0.837 | .0467 | 0.862 |
| Rat | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | | 0.764 | 0.782 | 0.872 | 0.916 | 0.468 | 0.708 |
| Chicken MGF | 1.000 | 1.000 | 0.426 | 0.541 | 1.000 | 1.000 | | 1.077 | 1.344 | 1.254 | 0.832 | 1.084 |
| Flounder poCSF3-2 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 0.364 | | 0.387 | 0.406 | 0.424 | 0.435 |
| Fugu trCSF3-2 | 0.496 | 1.000 | 0.389 | 0.301 | 1.000 | 1.000 | 0.107 | 1.000 | | 0.566 | 0.735 | 0.671 |
| Pufferfish tnCSF3-2 | 1.000 | 1.000 | 0.354 | 0.175 | 1.000 | 1.000 | 0.171 | 1.000 | 1.000 | | 0.537 | 0.558 |
| Fugu trCSF3-1 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | | 0.647 |
| Pufferfish tnCSF3-1 | 1.000 | 1.000 | 0.373 | 0.466 | 1.000 | 1.000 | 0.425 | 1.000 | 1.000 | 1.000 | 1.000 | |

Positive (*filled boxes*) and purifying (*unfilled boxes*) selections are also shown

subject of more nonsilent mutation pressures or positive selection.

The CSF3 cluster synteny map in addition to supporting orthology and paralogy of fish and chicken CSF3s, showed that the CSF3 locus in green-spotted pufferfish (*tn*CSF3-1) at Chr.2 and fugu (*tr*CSF3-1) at scaffold 571 were the loci, which were directly related to the mammalian and avian CSF3 locus. This relationship was clearly seen through the conservation of THRAP4 and LRRP genes and the shifted but conserved position of the GJCI and TOP2A genes of the said loci. Together with our findings that CSF3-1s and chicken posses the same cleavage residue and Kozak sequence, this result indicated that fish CSF3-1s were the "original" or ancestral CSF3 orthologues and the CSF3-2s were the duplicates. The evolutionary significance of PSMD3 in relation to CSF3-2s is, however, unclear.

Phylogenetic analysis likewise revealed some interesting information (Fig. 4). First, all the CSF3s were found to form a single evolutionary clade (that includes cMGF) outside other related cytokine families such as IL6, oncostatin M (OSM), and leukemia inhibiting factor (LIF), suggesting that the CSF3s have a common ancestor and hence are indeed orthologous. Inclusion of cMGF in the CSF3 group provides additional evidence that it is the chicken CSF3 orthologue. Second, the phylogenetic trees are also in accord with our claim that the fish CSF3s genes exist as paralogues. And third, the divergence of the other members of the Pfam IL6/CSF3/MGF protein family is well placed before the mammalian-fish divergence and IL6 is more related to CSF3 than to OSM and LIF.

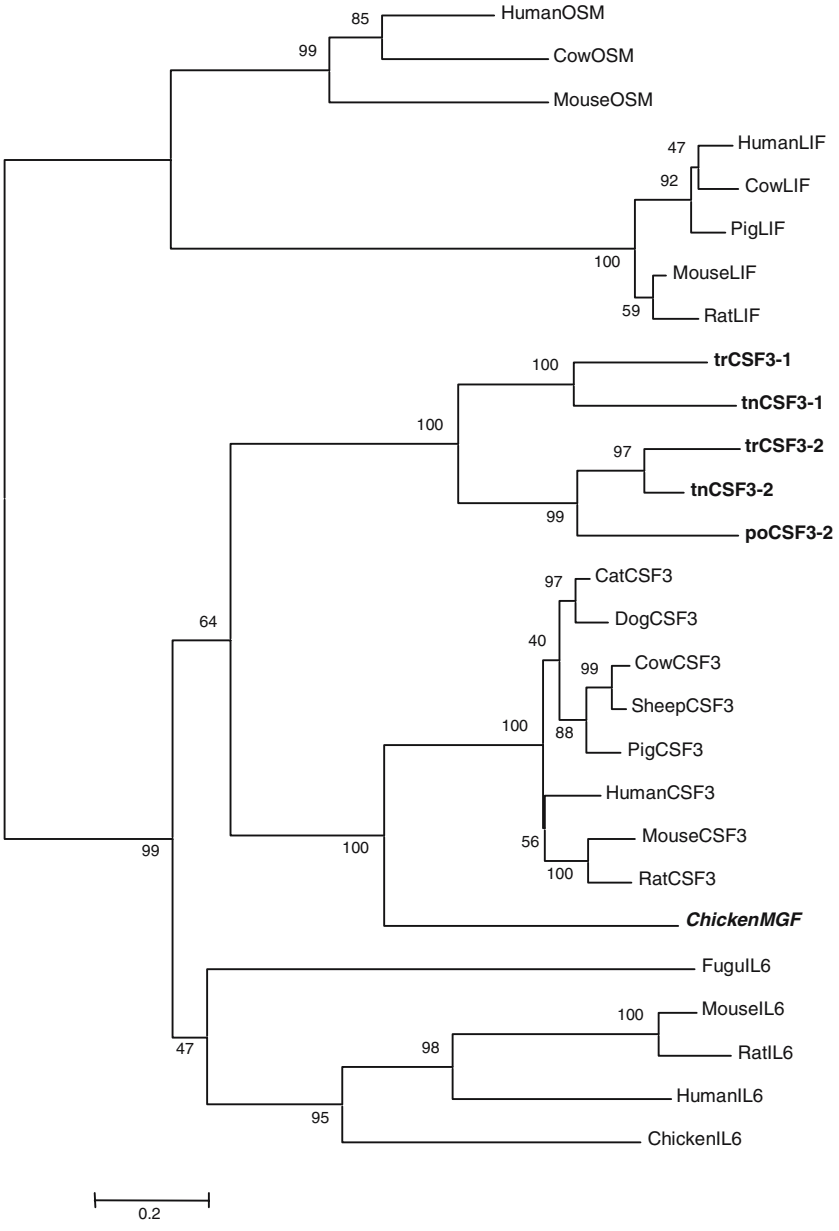


**Fig. 4** NJ tree of the Pfam IL-6/CSF3/MGF protein family. Fish CSF3s and chicken MGF are in *bold* and ***bold italics***, respectively. Additional GenBank accession nos.: human IL-6 (*NP000591*), pig IL-6 (*NM_214399*), mouse IL-6 (*NP112445*), rat IL-6 (*NM_012589*), fugu IL-6 (*NM_001032722*), human OSM (*M27286*), cow OSM (*S78434*), mouse OSM (*D31942*), human LIF (*NM_002309*), cow LIF (*NM_173931*), dog LIF (*AF512028*), mouse LIF (*NM_008501*), and rat LIF (*NM_022196*)

Japanese flounder CSF3 gene expression

The Japanese flounder CSF3 gene (*po*CSF3-2) was highly expressed in tissues known to have immunohematopoietic-related functions, e.g., gills, kidney, and spleen (Fig. 5). It is interesting to note that it was also constitutively expressed in the brain, heart, PBLs, ovary, skin, and stomach although at a lesser degree. Expression of *po*CSF3-2 in kidney and PBLs was significantly upregulated by LPS and ConA/PMA as compared to the control (Fig. 6a,b). However, no significant upregulations were detected between sampling time.

## Discussion

Here, we established the first CSF3 orthologues from three different fish species (Japanese flounder, fugu, and green-spotted pufferfish) based on their conserved features, their synteny, phylogeny, and expression. Such discovery of the fish CSF3 co-orthologues adds to the theory that fish has more genes because of a whole-genome duplication event (Christoffels et al. 2004; Jaillon et al. 2004; Amores et al. 1998) that could have allowed for teleost radiation and biodiversity (Ohno 1970; Postlethwait et al. 1998). It also suggests to some extent that myelopoiesis in fish and mammals, particularly the pathway being induced by CSF3, could be similar in both taxa because they both utilize the CSF3 molecule. Furthermore, our data shows that evolutionarily CSF3-1s would exhibit the ancestral function as compared to CSF3-2s. Studying therefore the function of these duplicate genes could prove to be interesting.

In spite of its rapidly evolving state, numerous CSF3 features were found to be conserved and could be important evolutionary functional elements of the molecule. The consensus Pfam domains, which covers more than 50% of the protein length for the tetraodontids and about 80% for Japanese flounder, suggests that *po*CSF3-2, *tn*CSF3-1 and -2, and *tr*CSF3-1 are likely to share the same secondary protein structure found in mammalian CSF3s and could hence share the same function. The presence of a consensus CK-1 decanucleotide sequence in nonmammalian CSF3s suggests that it may also be responsive to tumor necrosis factor-alpha, IL-β (Shannon et al. 1992), or LPS (Nishizawa and Nagata 1990). However, this needs confirmation because the equally important directly repeated NF-IL6 consensus elements that overlap downstream with CK-1 were not detected in lower vertebrates CSF3s and it is also unknown whether the CK-1 consensus sequence's proximity to the TATA box is relevant to its regulation of chicken and fish CSF3. The easily recognizable 3′ UTR-linked mRNA AREs may play a part in mRNA regulation. It is unclear though which type of AREs is involved in CSF3. UUAUUUAUU motif is said to be the shortest ARE that allows efficient mRNA deadenylation and decay, rather than the pentameric AUUUA sequences (Zubiaga et al. 1995). However, our results show such UUAUUUAUU motif only in the fugu *tr*CSF3-2, the rest have pentamers. It is therefore possible that both these types are functionally relevant because it was reported that there are AREs or mechanisms that could account for or complement mRNA decay (Yang et al. 2003).

This study established that cMGF is actually the chicken CSF3 orthologue and explains the remarkable similarity of cMGF to CSF3. From this, we learned that it is difficult to identify CSF3 genes in lower vertebrates without additional definitive CSF3 sequence or locus data because of their mutations. This may have been the case for cMGF gene, which was identified only on the basis of indirect experiments and not by sequences, synteny or phylogeny. Renaming cMGF to CSF3 have important implications to the study of the CSF3 gene biology. For example, cMGF was shown to induce in vitro proliferation of both granulocytes and macrophages from chicken bone marrow cultures (Leutz et al. 1984; Leutz et al. 1989) and to induce production of the monocyte/macrophage lineage in vivo that resulted in increased nitric oxide (York et al. 1996; Djeraba et al. 2002). These observed functions are different from the neutrophilic-lineage-specific action of the mammalian CSF3 (Basu et al. 2002), which is not surprising as chickens are known to have heterophils, a more primitive granulated cells, rather than neutrophils, eosinophils and basophils of mammals.

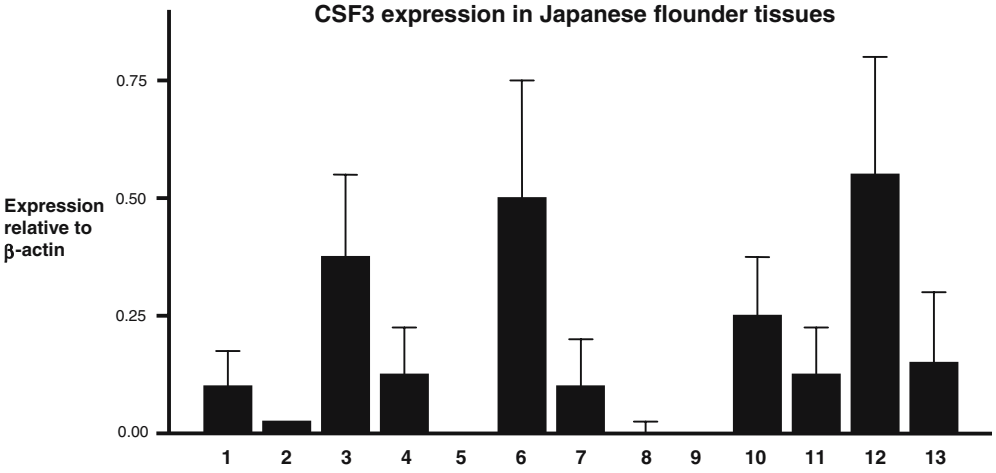


**Fig. 5** Constitutive expression of Japanese flounder CSF3 in various tissues relative to β-actin expression as determined by RT-PCR. *Lanes 1* brain, *2* eyes, *3* gills, *4* heart, *5* intestine, *6* kidney, *7* PBLs, *8* liver, *9* muscle, *10* ovary, *11* skin, *12* spleen, and *13* stomach. *Bars* represent mean values of three samples plus SD

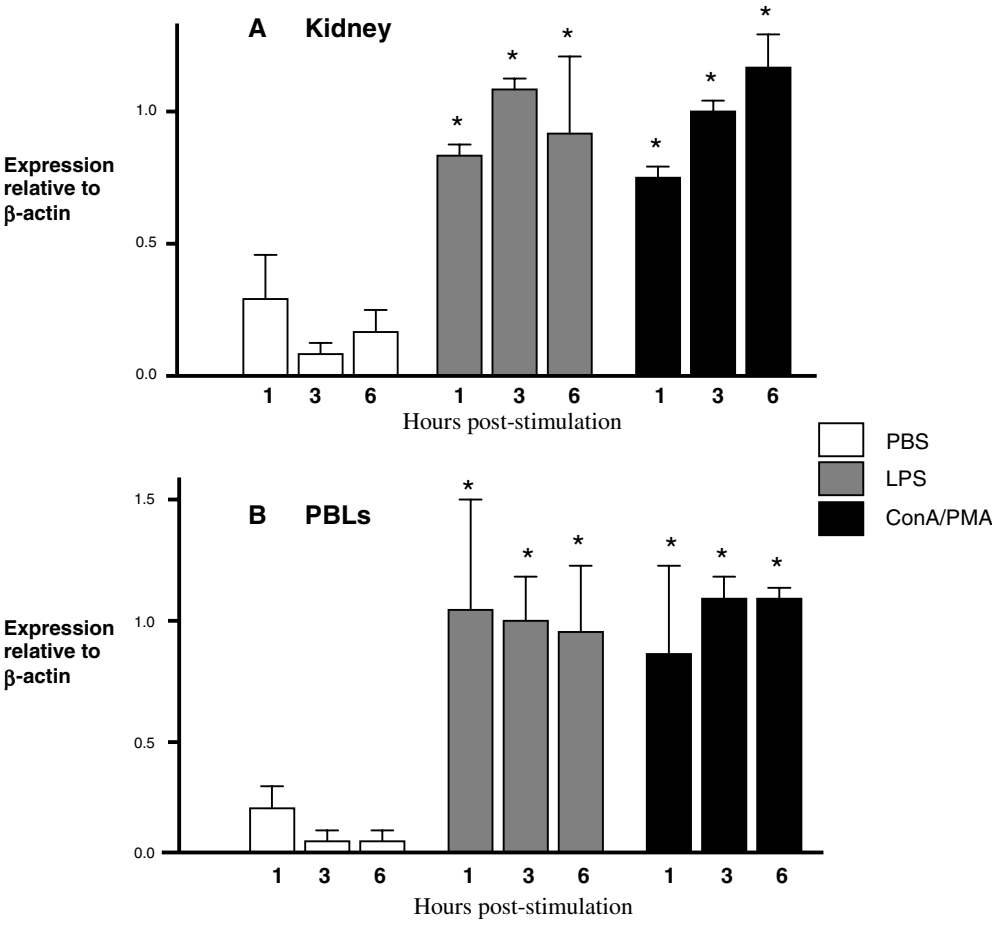


**Fig. 6** Expression of Japanese flounder CSF3 gene in response to immunostimulants. CSF3 gene expression relative to β-actin expression in Japanese flounder kidney (**a**) and PBLs (**b**) in vitro at 1, 3, and 6 h poststimulation with phosphate-buffered saline (PBS), LPS, and a combination of conA/PMA. PBS was used as negative control. *Bars* represent mean values of three samples plus SD. *Asterisks* denote significant difference from corresponding control ($P<0.05$)

The observation that immune-related proteins are evolving more rapidly in lower vertebrates than in mammals because of higher Ka/Ks ratio (International Chicken Genome Sequencing Consortium 2004) is equally true for the CSF3 gene. The presence of numerous SSRs or microsatellites interspersed in the UTRs and introns of the fish CSF3 genes may have contributed to this dynamic evolutionary state. As reviewed by Li et al. (2004), various reports suggest that these SSRs are nonrandomly distributed in the genes and that they could interfere with correct gene transcription and translation through frameshift mutations, slippage, SSR expansion, and/or contractions, which can inactivate or alter gene function and eventually allow for phenotypic changes. This limited identity could explain why the duplicate copy of *po*CSF3-2 was not detected by DNA-based Southern hybridization and could very well be true with similar experiments in many genes in fish.

The poCSF3-2 constitutive expression in known immune-related organs of fish (kidney, gills, and spleen) (Iwama and Nakanishi 1996) provides additional evidence that this cytokine could be actively involved in at least the fish immune system. This is further confirmed by its inducibility by known mammalian CSF3 immunostimulants, endotoxin LPS (Sallerfors and Olofsson 1992; Hartung 1999; Mathiak et al. 2003), conA (Whitin et al. 1987), and PMA (Oster et al. 1989; Kothari et al. 1995). In human, LPS started to induce monocytes to synthesize CSF3 3–6 h poststimulation (Sallerfors and Olofsson 1992). We therefore see a general similarity of fish and mammalian CSF3s activity in response to bacterial endotoxins, indicating that at the least the gene's immune-related function is conserved.

**Acknowledgement** This study was supported in part by the Grants-in-Aid for Scientific Research (S) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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