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Cloning of Japanese flounder *Paralichthys olivaceus CD3* cDNA and gene, and analysis of its expression

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Abstract Two distinct *CD3* homologue cDNAs, *CD3-1* and *CD3-2*, were isolated from a Japanese flounder leukocyte cDNA library. *CD3-1* consisted of 961 bp encoding 178 amino acid residues, and *CD3-2* consisted of 927 bp encoding 182 amino acid residues. The two deduced amino acid sequences had an identity of 95.1%, and neither had N-linked glycosylation sites. The identities between the Japanese flounder CD3s and previously reported CD3s (CD3ε, CD3γ, or CD3δ) of *Xenopus laevis*, chicken, and various mammals were approximately 25%. The Japanese flounder CD3s had an extracellular domain, a CXXCXE motif, and an immunoreceptor tyrosine-based activation motif (ITAM), each of which are important characteristics of CD3 chains. Furthermore, the positions of four cysteine residues in the extracellular domain were preserved in both of the Japanese flounder CD3s. A phylogenetic tree based on the amino acid sequences confirmed that the Japanese flounder CD3s are closer to CD3ε than to CD3γ and CD3δ. However, the gene structure of Japanese flounder *CD3* is identical to the chicken and *Xenopus CD3*γ/δ genes and the mammalian *CD3*δ gene. Southern blot hybridization and the DNA sequence of the *CD3* gene of homocloned Japanese flounder indicated that the *CD3* gene exists as a single copy. Southern blot hybridization also showed the presence of a polymorphic variant of Japanese flounder *CD3*. An RT-PCR analysis detected Japanese flounder *CD3* mRNA in several organs that contained lymphocytes. The proportion of *CD3*-positive cells in the peripheral blood leukocytes was 34.9%.

Keywords Japanese flounder · CD3 · cDNA · Gene · ITAM · Leukocyte

The nucleotide sequence data reported in this paper have been submitted to the DDBJ nucleotide database and have been assigned the accession numbers AB044572 (*CD3-1*), AB044572 (*CD3-2*), and AB054068 (*CD3* gene).

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Introduction

The antigen recognition signal from the major histocompatibility complex (MHC) is recognized by a T-cell receptor (TCR) in a CD3-TCR complex on T-cell membranes, from where the signal is transmitted to the inside of the cell (Ashwell and Klausner 1990; Klausner et al. 1990). CD3 is indispensable for the expression of the *TCR* genes (Dave et al. 1997; Haks et al. 1998). CD3 has been classified into CD3γ, δ, ε, and ζ chains. The sequence and structural homology of the human and mouse *CD3*γ, *CD3*δ, and *CD3*ε genes, and their chromosomal proximity suggested that they arose by duplication of a common ancestral gene. Glycosylation sites are present in the extracellular domain of the CD3γ and δ chains, but not in the extracellular domain of the ε chain. The CD3γ, δ, and ε chains are members of the immunoglobulin superfamily which have the immunoreceptor tyrosine-based activation motif (ITAM), an extracellular CXXCXE motif, and similarly positioned cysteine residues involved in disulfide bonds (Gold et al. 1987; Williams and Barclay 1988).

Nonmammalian CD3 homologues have been identified in only chicken and *Xenopus* (Bernot and Auffray 1991; Dzialo and Cooper 1997; Göbel and Dangy 2000; Göbel and Fluri 1997). CD3γ/δ chains with a structure similar to the structures of both the γ and δ

chains of mammals have been reported in chicken and *Xenopus* (Bernot and Auffray 1991; Dzialo and Cooper 1997; Göbel and Dangy 2000). The *CD3γ/δ* gene is thought to be an ancestral gene of the mammalian *CD3γ* and *CD3δ* genes (Göbel and Dangy 2000). A chicken CD3ε has also been reported (Göbel and Fluri 1997). However, there have been no reports of CD3 in fish. A comparative analysis of CD3 proteins should include sequences of lower vertebrates such as those of fish for understanding the important amino acid residues and structural features determining the functions of the CD3-TCR complex. Recently, we cloned a partial cDNA fragment of a *CD3* homologue by an expressed sequence tag (EST) analysis of Japanese flounder leukocytes (Nam et al. 2000).

In this study, we cloned and sequenced two distinct *CD3* cDNAs and a gene from Japanese flounder and characterized their expression patterns.

Materials and methods

cDNA library screening and sequencing

The cDNA library used in this study has been previously reported (Aoki et al. 1999; Nam et al. 2000). The cDNA library was screened using a partial cDNA fragment of a *CD3* homologue previously identified by an EST analysis (Nam et al. 2000) as a probe for isolation of full-length *CD3* cDNA. Hybridization was done as previously reported (Aoki et al. 2000). cDNA clones were sequenced using ThermoSequenase (Amersham-Pharmacia) with M13 forward and/or M13 reverse primers and an automated DNA sequencer LC4200 (Li-Cor). Each determined sequence was compared with all sequences available in DDBJ/EMBL/GenBank using BLAST ver. 2.0 (Altschul et al. 1990, 1997).

Phylogenies were inferred using the PHYLIP program (ver. 3.5) (Felsenstein 1996), and by distance analysis using the neighbor-joining method. The values supporting each node are derived from 100 resamplings. The radial tree shown in Fig. 2 was created with TreeView software.

DNA sequencing of a CD3 gene

A *CD3* gene was amplified from genomic DNA of homocloned Japanese flounder by PCR using a set of primers. The PCR primers used in this study were CD3-GF, 5′-ctcagaagacagagaagtgc-3′, and CD3-GR, 5′-tgcatcacacgctgcacatc-3′. An amplified DNA fragment was cloned and sequenced as described above.

Southern blot hybridization

Genomic DNA of a homocloned Japanese flounder and two wild Japanese flounders were isolated as previously reported (Hirono et al., 2000). The isolated genomic DNA was digested with *Eco*RI, *Hin*dIII, or *Pst*I. Southern blot hybridization was conducted as described previously (Hirono et al., 2000).

RT-PCR analysis

Total RNA was extracted from healthy Japanese flounder thymus, peripheral blood leukocytes (PBLs), head kidney, trunk kidney, spleen, intestine, heart, liver, stomach, gill, and brain using Trizol (Life Technologies). The purified total RNA (10 μg) was treated with DNase and then reverse transcribed into cDNA using an AMV Reverse Transcriptase First-Strand cDNA Synthesis kit (Life Sciences). The final volume of the cDNA synthesis reaction was 25 μl. The reverse-transcribed sample (1 μl) was used in 50 μl of PCR reaction mixture. The PCR primers used in this study were CD3-F, 5′-catcactgttgcctgcctg-3′, and CD3-R, 5′-agcggtcgctcggaactt-3′. The *β-actin* primer set and *TCRα* were used for a positive control of RT-PCR (Katagiri et al. 1997; Nam et al. 2000). PCR was performed with an initial denaturation step of 2 min at 95°C, and then 20 cycles were run as follows: 30 s of denaturation at 95°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C. The reacted products were electrophoresed on a 2.0% agarose gel.

In situ hybridization

Digoxigenin-labeled sense and antisense RNA probes for *CD3-1* were generated with a T7 and SP6 Dig RNA labeling kit (Boehringer Mannheim) with digoxigenin-UTP (Boehringer Mannheim).

In situ hybridization was carried out using a commercial kit (Nippon Gene). PBLs were smeared on a glass slide, fixed in PBS buffer containing 10% formalin for 10 min, washed in DEPC-treated water for 1 min, dehydrated in ethanol for 1 min, pretreated with proteinase K (5 μg/ml) for 15 min at 37°C for protein removal, washed in glycine-PBS buffer (2 mg/ml) for 10 min, soaked in 100 mM triethylamine for 15 min for acetylation, immersed in anhydrous acetic acid for 20 min, and prehybridized in 50% formamide with 4×standard sodium citrate at 42°C for 30 min. For the hybridization, the cells were covered with 100 μl of antisense mRNA probe solution (1 μg/ml) and then reacted in a moist chamber at 42°C for 16 h. After the hybridization, the glass slide was kept in RNase-NTE buffer (20 μg/ml) at 37°C for 30 min. The mRNA was detected with a DIG nucleic acid detection kit (Boehringer Mannheim) as described in the technical manual.

Results and discussion

Nucleotide sequences of Japanese flounder CD3 cDNA and gene

Two distinct *CD3* homologues, designated *CD3-1* and *CD3-2*, were cloned. *CD3-1* consisted of 961 bp encoding 178 amino acid residues, and *CD3-2* consisted of 927 bp encoding 182 amino acid residues (AB044572 and AB044572). The identity of the deduced amino acid sequences of CD3-1 and CD3-2 was 95.1%. In the cases of chicken and mammals, CD3 chains (not including the ζ chains) are thought to have arisen by duplication of a common ancestral gene (Göbel and Dangy 2000). However, the amino acid sequences of different pairs of CD3s are only 25% identical, although some amino acid sequence features are well conserved in CD3γ, δ, and ε of mammals or γ/δ and ε of chicken. The high identity of the two Japanese flounder *CD3*s suggests that they are alleles and not different types of *CD3* chains.

The identities of the deduced amino acid sequences of both Japanese flounder CD3s to other known CD3s were low (~27%) (Fig. 1). The amino acid sequence alignment (Fig. 1) indicates a conservation of the four

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                                              Signal peptide              Extracellular domain

Flounder CD3-1                         1 MKFTSLLPACLLLL--WTLPDTEADDMIKVTSSGDWITLNCNKKSDDASFKVNGVEKKNP    58
Flounder CD3-2                         1 ..............--.........................................-..    57
Chicken CD3 epsilon                    1 .RCEVP..LLG...CVVGAAAQGGQEEFA.EI..TTV.IT.PSSG..IKW.PDPALGD.N    60
Chicken CD3 gamma/delta                1 .WKGRA.GTW...ACVAVAKLGVHGLSMS.KEVSGKVF.Q.QESK.LNTNYLWKKG.EEL    60
Xenopus laevis CD3 gamma/delta         1 ..NHLQIAWM.V.M--V..KA.--CNK.EAFVKNNHLY.T.KVDEKGE..LWTHDT.NID    56
                                                                             #
                                                                                    Transmembrane domain

Flounder CD3-1                        59 LTIRYKDESS-----GLYT-CSLKDENNKFEE---YEIYLKLQTCENCIELNLPTIVGLT   109
Flounder CD3-2                        58 ..........-----....-....V...I..---.........................   108
Chicken CD3 epsilon                   61 KY.IQNHD..-----P.TVS.-----TAGDQ.---HTM..NAKV.A..E..DTF.V..II   107
Chicken CD3 gamma/delta               61 GNM.QL.LGAIYDDPRGTYT.QRDENV.STL----HVH.---RM.Q....VDA...S.IV   113
Xenopus laevis CD3 gamma/delta        57 .FNKTL.LG.-----VWND-PRGNYVCKAT.DGTEAS.EVFVRM.Q....MDTG..S.FI   110
                                                           #                       #   #
                                                                           Cytoplasmic domain

Flounder CD3-1                       110 IGDAVATILLG-LAVYLIASQAGPVISHKK-----SSERPLPNEMRNR-ASND-PYQRLR   161
Flounder CD3-2                       109 ...........-..................IRLSSG............-....-......   165
Chicken CD3 epsilon                  108 AA.--LL.T..V.ILVYYF.KNKKGQ.RAA-----AGS..RAQK.QRPPPVPNPD.EPI.   160
Chicken CD3 gamma/delta              114 VA.V...V--------.L.-I.VYC.TGQD-----KGLMSRASDRQ.LI.-..QL..P.G   158
Xenopus laevis CD3 gamma/delta       111 .A.---I.MI.-----...-I.VYCV.GSE-----TRRPARASDKQ.L-LQ..-L..P.G   154
                                                                                  +         +  +  +

Flounder CD3-1                       162 FNSGARKDTYDVINHNR                                            178
Flounder CD3-2                       166 .................                                            182
Chicken CD3 epsilon                  161 KGQRDV---.AGLE.RGF
175Chicken CD3 gamma/delta           159 ERNDGQ---.SQLATAKARK                                         175
Xenopus laevis CD3 gamma/delta       155 QR.---E...SHL.SR                                             167
                                                  +  +
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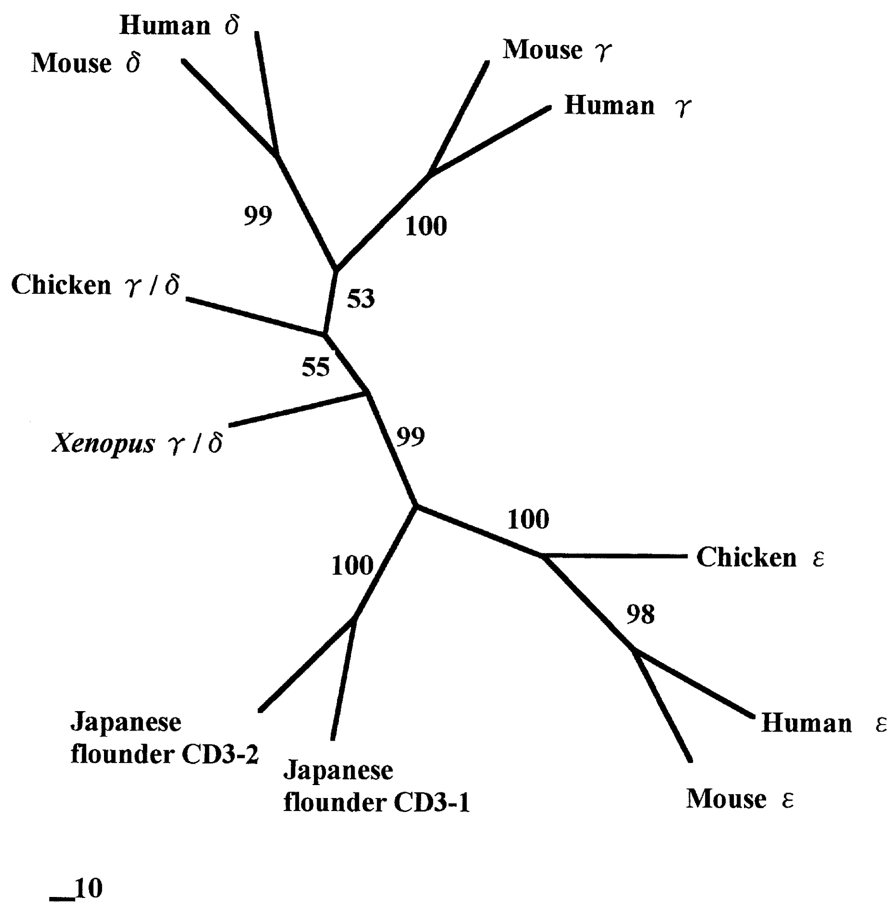

Fig. 2 Phylogenetic tree of amino acid sequences of CD3γ, δ, and ε. Sequences were obtained from DDBJ/EMBL/GenBank. The phylogeny of CD3 was estimated by the neighbor-joining method of clustering in the PHYLIP program

Fig. 1 Amino acid sequence alignment of CD3s. Sequences were obtained from DDBJ/EMBL/GenBank. Amino acids identical to the Japanese flounder CD3-1 are shown by *dots*. The positions of cysteine residues identical in all sequences are shown by #. The conserved amino acid residues in ITAMs are indicated by +. Gaps (*dashes*) have been placed to maximize the identity

cysteine residues involved in disulfide bonds, the glutamic acid residue following the two cysteines (CXXCXE motif), and the ITAM, all of which are thought to be important characteristics of CD3 chains. There was no N-linked glycosylation site, which is one of the important characteristics of the glycoprotein CD3γ and δ chains, in either of the Japanese flounder CD3 sequences. A phylogenetic tree based on the amino acid sequence alignment indicates that the Japanese flounder CD3s are closer to the CD3ε chain than to the mammalian CD3γ and δ chains (Fig. 2). The distance of Japanese flounder CD3 and *Xenopus* CD3γ/δ and the distance between Japanese flounder CD3 and chicken CD3ε are almost the same.

The Japanese flounder *CD3* gene is approximately 2.8 kb long and consists of five exons and four introns (Fig. 3). The positions of the exon-intron junctions are identical to those of the chicken and *Xenopus CD3*γ/δ genes and the mammalian *CD3*δ gene. The characteristics of the cloned gene in this study, i.e., its gene structure and amino acid sequence, suggest that the Japanese flounder *CD3* is an ancestral gene of the *CD3*γ, *CD3*δ, and *CD3*ε genes. Of course, the possibility remains that another type of CD3 chain exists in Japanese flounder. To address this question, the entire Japanese flounder *CD3* locus needs to be sequenced.

Polymorphism and copy number of CD3

Southern blot hybridization showed two positive reaction bands in the DNA digested with *Eco*RI or *Pst*I

Fig. 3 Nucleotide and deduced amino acid sequences of the Japanese flounder *CD3* gene

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   1  ctcagaagacagagaagtgcacacacaATGAAATTTACATCACTGTTGCCTGCCTGCTTGCTGCTGCTTTGGACGCTGCCAGgtaaggcc  90
                                 M  K  F  T  S  L  L  P  A  C  L  L  L  L  W  T  L  P  D
  91  tctgtcaaaatgttttcacttagatgaggattataacgaggtcttcaaacatcttatattaaatgggtttagcgaaaaagaaaacctttc 180
 181  aatattttaatggcagctaattgtgtgtcagcttcattaaagtaaacacttttaacagcttgtaaagataaagggagttgtgcaatagt 270
 271  gactgcatgagtgaaattgtgtttacatttgttttaatgaaggtttgcctgcagagtttttatcaagaagccgttcagtaaggagcacgg 360
 361  gtttaatgtgcatgattctgttttctacacagACACCGAAGCAGATAATATGATTAAAGTCACATCATCACGAGATTGGATTACTCTGAA 450
                                        T  E  A  D  N  M  I  K  V  T  S  S  R  D  W  I  T  L  N
 451  TTGCAACAAGAAGTCCGACGATGCATCTTTCAAAGTAAATGGTGTAGAGAAAAATCCTCTGACAATACGTTACAAAGATGAATCGTCAGG 540
       C  N  K  K  S  D  D  A  S  F  K  V  N  G  V  E  K  N  P  L  T  I  R  Y  K  D  E  S  S  G
 541  ACTCTACACATGCTCCTTGAAAGTCGAAAACAACAAAATCGAGGAATACGAAATCTATTTGAAATTACAAAgtaagttccaaagcagcgt 630
       L  Y  T  C  S  L  K  V  E  N  N  K  I  E  E  Y  E  I  Y  L  K  L  Q  T
 631  cttgttatgaaagcactagtgttgttgtgcgtcatgggttttatgtgcaatgcatgttacatctattacttgtcttcacactgtgtgata 720
 721  atgtttacagcatgttacagcatttctttatattaatcaaatacttttcaaaatctcagattctcaagactctctctctctctctctc 810
 811  tctctctgtgtgtgtgtgtgtcaagCCTGTGAAAACTGCATCGAGCTCAACCTACCTACCATAGTCGGCCTCACTATTGGAGATGCGGTG 900
                                 C  E  N  C  I  E  L  N  L  P  T  I  V  G  L  T  I  G  D  A  V
 901  GCAACAATCTTGTTAGGATTGGCTGTCTACCTTATCGCGTCTCAGGCTGGTCCAGTCATCTCTCACAAGAAAAgtatctatctatctatg 990
      A  T  I  L  L  G  L  A  V  Y  L  I  A  S  Q  A  G  P  V  I  S  H  K  K  I
 991  aacattcattcttatctaaaatctgacttggccaggtagtgttatgtaagtggctaacttctcaaagataagaaaaatccctgtattgtg 1080
1081  ttttaaagtaataggaattactttctacggtggctctgagggtcaaagcaacatttcaagatgacacctcggaaaattcagcagcataat 1170
1171  actgatcatcaacattacattcagttgatgtgttcactctggttgatgattggttatttccagtggcagatttttctgaataaggggcc 1260
1261  ataaaggtgccacaatttacacagacatccctgattgacatcattgacaaagaattaaccttcttggcagagatagttatatgaatgccc 1350
1351  ttggtttaaatatgttctaatggtttccatctcctcttccatccattcacactcctgcaaggatgctgctaggataagtgacaatagaaa 1440
1441  cagggaatgacatagagttgttgtcaggaacttggattctgtctgggaacaaaaagccatgactcacctttccgtaatctgaaatgttg 1530
1531  acgtgttttgtgaaatgctgttgtgtttggtcaaactttgtgcgtcttttaagttgttgttattattattataatgttaatttttattgt 1620
1621  tgtgttatataattcagtgtcagtttctacaaatgcaccagattaaaaaatctgtttatattatgtaatatagccgtattcagagtgacg 1710
1711  aaaatatctgtttcacttagatgtagcacttggctttttgaagctcatgtacttttgtacactcatggttgaattcacttattggaagt 1800
1801  tgctttgtataaaagcatcagctaaattatgtgtaatgtttacaacctttgtgttgggtacgctaacctgcctgtcctgagactacgagc 1890
1891  atatttattgtatcatagatgaacaggaacaggaagttctttattaactagttcactaattattattattttcacttgccacatacacta 1980
1981  tatatacatatatcaatcatccaaattaataacttaaataaaatgtactcattatgaaagatccaatctttaaataaaacaattactaat 2070
2071  actagtatttgtgcaatcatcagTTCGTCTGTCCTCAGGTTCCGAGCGACCGCTTCCAAATGAGATGCGCAACAGAGCCTCAAACGACCC 2160
                                R  L  S  S  G  S  E  R  P  L  P  N  E  M  R  N  R  A  S  N  D  P
2161  ATACCAGgtgagatgacatgtgacggcctttccactgcaatatgtgataatcacatttaaaggtccagtgtgtaagatttaggtgaaagg 2250
       Y  Q
2251  gaacgattggcagaaagttaatgtagaataatcctcatgatgttttcactagttcgtttcatctaagttttatgaattgtagttttcttt 2340
2341  accccagaaaaggccctttatatttaaatactttatatttacatccaggggaccctctctacggaggccgccatgttttttacattagtc 2430
2431  cagactggacaaactaaacgccttttgagttttaagacaactgaagtttaccacaggttctttgtcgtgtttgtaaggagagtgtgagg 2520
2521  tgaggggtgttccgctgcaacatgtaatttcaacactagaaatcacacaattctacacccttacctttaaatacaatgaaggaatgatga 2610
2611  ttttgattcatttaactattaattatttcttcttttaattcatatattcaatccctcctccctcagCGTCTGAGATTCAACAGTGGTGCC 2700
                                                                        R  L  R  F  N  S  G  A
2701  CGGAAGGACACGTATGATGTCATTAACCACAATAGATAGaggggtggaccgccccatctccgcccccagatgtgcagcgtgtgatgca 2788
      R  K  D  T  Y  D  V  I  N  H  N  R  *
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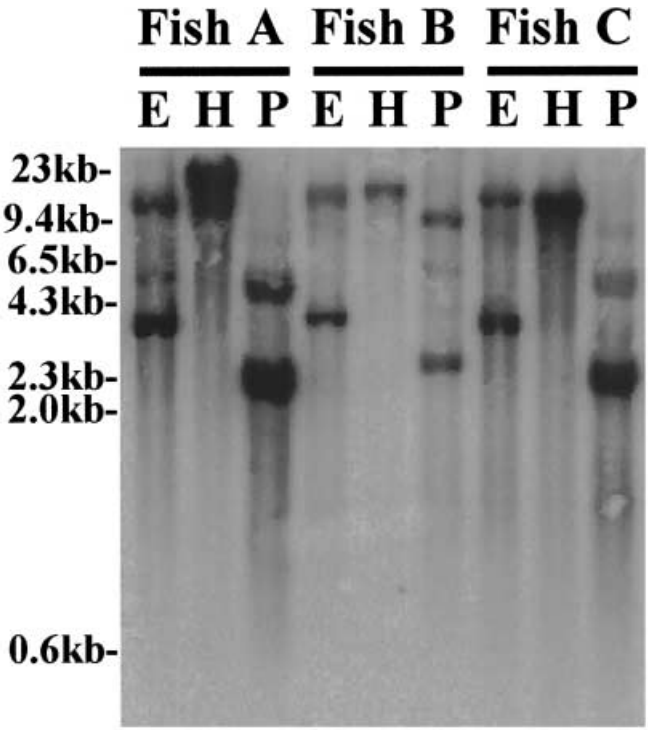

Fig. 4 Southern blot hybridization of genomic DNA of three individual Japanese flounders. *Fish A* cloned fish; *Fish B, Fish C* wild fish. Genomic DNA was digested with *Eco*RI, *Hin*dIII, or *Pst*I

(Fig. 4). This result was expected because there is one *Eco*RI site and one *Pst*I site in the *CD3* gene (Fig. 3). These results indicated that the *CD3* gene exists as a single copy, and suggest that the two distinct *CD3*s of Japanese flounder are alleles and not a duplicated gene. Southern blot hybridization of *Pst*I-digested DNA of three fish showed the presence of a polymorphic variant of the Japanese flounder *CD3* gene (Fig. 4).

Expression of CD3 in PBLs

We tested several organs of healthy Japanese flounder for the presence of both *CD3* mRNAs by RT-PCR. The Japanese flounder *CD3* mRNAs were detected in thymus, PBLs, head kidney, trunk kidney, spleen, intestine, heart, gill, and brain after 20 cycles of PCR (Fig. 5). The organs and cells in which *CD3* mRNA was detected were the same as those in which TCR-α was detected.

A representative in situ hybridization is shown in Fig. 6. Of 358 PBLs examined, 125 cells (34.9%) expressed the *CD3* gene. In humans, lymphocytes account for 20–45% of all PBLs, and T lymphocytes account for 55–75% of the lymphocytes (Klein and Horejsí 1997). The proportion of Japanese flounder *CD3*-positive cells that are thought to be T lymphocytes is similar to that of humans. These results suggest that the cloned *CD3* cDNA could be useful for identification and characterization of T lymphocytes of Japanese flounder. We recently cloned the *CD8*α, *TCR*α and *TCR*δ chains, and membrane *IgM* and *IgD* cDNAs by EST analysis of a hirame rhabdovirus-infected Japanese flounder leukocyte cDNA library (Aoki et al. 1999; Nam et al. 2000). In the near future, we hope to identify the types of lymphocytes in Japanese flounder by an in situ hybridization analysis using these cloned cDNAs as probes.

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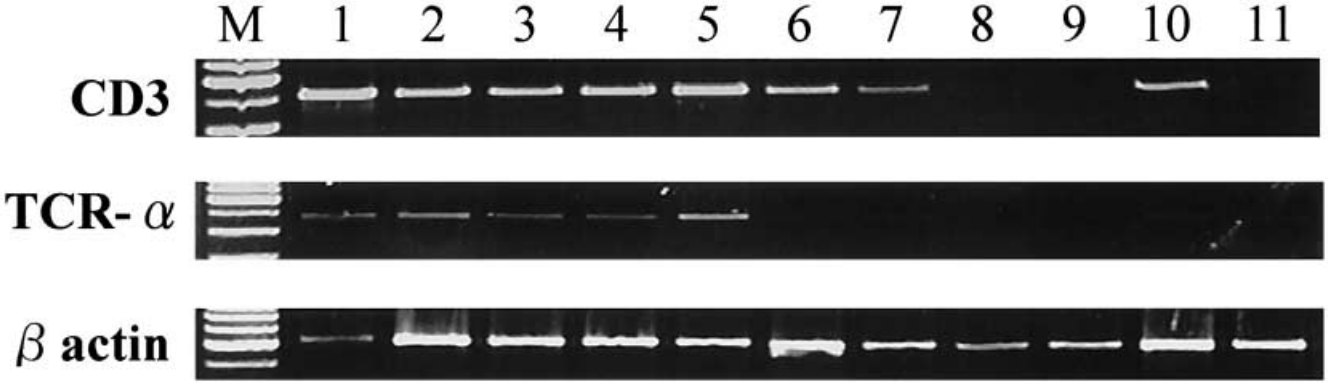
Fig. 5 Detection of mRNAs of the Japanese flounder *CD3* by RT-PCR. *Lane M* 100-bp ladder, *lane 1* thymus, *lane 2* PBLs, *lane 3* head kidney, *lane 4* trunk kidney, *lane 5* spleen, *lane 6* intestine, *lane 7* heart, *lane 8* liver; *lane 9* stomach, *lane 10* gill, *lane 11* brain



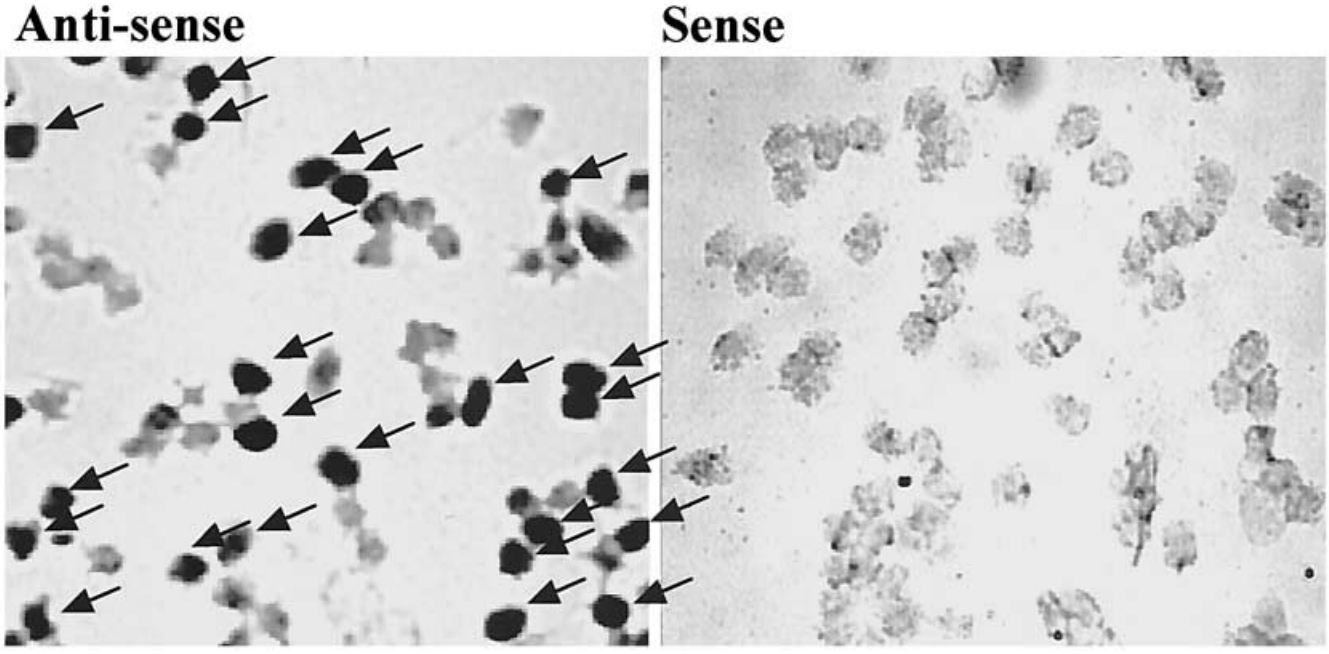


Fig. 6 Detection of CD3-expressing cells in PBLs by in situ hybridization. *Arrows* indicate the cells that were reacted with digoxigenin-labeled *CD3* cRNA

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