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Characterization and expression of a CD40 homolog gene in Japanese flounder *Paralichthys olivaceus*

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Abstract A CD40 homolog cDNA encoding 300 amino acid residues was isolated from a Japanese flounder leukocyte cDNA library. The amino acid sequence identities of Japanese flounder CD40 and previously reported CD40s of cow, human, mouse, and chicken range from 32 to 35%. The positions of cysteine residues, CD40 ligand binding amino acid residues, and four cysteine-rich domains are well conserved in Japanese flounder CD40. The Japanese flounder CD40 gene is composed of nine exons and eight intervening introns spread over 6 kb. The nucleotide sequence of the 5'-flanking region of this gene revealed the presence of several regulatory regions, including a TATA-like box, AP-1-, CEBPB-, IRF-1-, LYF-1-, NF- κ Bp-, SP-1-, and Stat-1-like motifs. In healthy fish, reverse transcriptase–polymerase chain reaction (RT-PCR) detected constitutive expression of CD40 in all tested tissues (leukocytes, kidney, spleen, liver, intestine, brain, gill, and skin). The mRNA of CD40 was predominantly expressed in several tissues that contained lymphocytes (leukocytes, kidney, spleen, intestine, and gill). Expression of the Japanese flounder CD40 molecule was induced in peripheral blood leukocytes from 1 to 6 h following Con A (50 μ g/ml)/phorbol myristate acetate (PMA) (0.35 μ g/ml) stimulation, with a

peak at 1 h after stimulation, and increased at 1, 3, and 6 h after induction by lipopolysaccharide (LPS) (500 μ g/ml) compared with the control.

Keywords CD40 · Con A/PMA · LPS · Japanese flounder · Peripheral blood leukocyte

Introduction

CD40 is a member of the tumor necrosis factor receptor (TNFR) superfamily that is expressed on a wide range of cell types, including B cells (Clark and Ledbetter 1986), dendritic cells (Hart and McKenzie 1988), endothelial cells (Karmann et al. 1995), fibroblasts (Fries et al. 1995), and epithelial cells (Galy and Spits 1992). Since the original isolation and cloning of the first CD40 protein in 1989 (Stamenkovic et al. 1989), much has been learned about the function of CD40 in higher organisms such as human and mouse. CD40 is a receptor involved in cellular signaling and activation and is also involved in inflammation (Schönbeck et al. 1997; Sempowski et al. 1997). Several immune responses are regulated by the interactions of CD40 with its ligand CD40L. CD40L is a 39-kDa glycoprotein of the TNF family that is predominantly expressed on activated CD4⁺ T cells (Kooten and Banchereau 2000). While B and T cell interactions initiate humoral immune responses via the CD40 pathway, the CD40 expressed on dendritic cells influences T cell priming and T-cell-mediated effector functions (Kooten and Banchereau 2000; Ridge et al. 1998). Its amino acid sequence is similar to the nerve growth factor receptors (NGFR), TNF, and to the membrane antigens Fas, CD30, CD27, and OX40 (Braesch-Andersen et al. 1989; Stamenkovic et al. 1989). All TNFR superfamily members are type I membrane glycoproteins characterized by the presence of variable numbers of cysteine-rich domains of about 40 amino acids in length. These domains are distinguished by the presence of a number of cysteine residues with a characteristic spacing pattern. Additional amino acid homologies can be recognized be-

The nucleotide sequence data reported in this paper have been submitted to the DDBJ nucleotide database and have been assigned the accession number BAC87848 (CD40 cDNA)

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Table 1 Oligonucleotide primers used in this study

Name of primer	Primer sequence
CD40 Bac-F	5'-CTGTGCACGGAGGTCACGGTG-3'
CD40 Bac-R	5'-CTGTCCATTGTCACTGAGAAC-3'
CD40 RT-F	5'-GTGACCCACTGACTCAGTATG-3'
CD40 RT-R	5'-GCCAACTGCAACAACACATGC-3'
CD40 Stan-F	5'-CACATGGAGGTCTGATTGCATG-3'
CD40 Stan-R	5'-CTGCTGCCGTTACAGAGG-3'
β-actin Stan-F	5'-TTCCCTCCATTGTTGGTCG-3'
β-actin Stan-R	5'-GCGACTCTCAGCTCGTTGTA-3'
β-actin Real-F	5'-TGATGAAGCCCAGAGCAAGA-3'
β-actin Real-R	5'-CTCCATGTCATCCCAGTTGGT-3'
CD40-Real-F	5'-GTTGTTGCAGTTGGCAGTTTG-3'
CD40-Real-R	5'-GAACCTACAGCGAGAGCCCAG-3'

tween domains in the same or different receptors so that overall homologies between family members within these domains are in the range of 25%. Recently, CD40Ls have been characterized and cloned in mammals. Studies using monoclonal antibodies to CD40 or recombinant CD40L have shown diverse biologic activities as a result of signaling through CD40. These activities include the proliferation of B cells and induction of immunoglobulin (Ig) secretion in the presence of other cytokines. Furthermore, CD40–CD40L interactions mediate rescue of germinal center centrocytes from apoptosis. CD40 exposure of thymic epithelial cells in the presence of interferon (IFN)-γ and interleukin (IL)-1-induced granulocyte-macrophage colony-stimulating factor (GM-CSF) release. Signals through CD40 up-regulate the expression of LFA-1, B7 ligands, ICAM-1, and CD23, with involvement in both homotypic and heterotypic cell adhesion and costimulation. The CD40–CD40L

interactions are now well established in mammals, but despite the important role that CD40 plays in the immune system, little is known about the CD40 in teleosts. The aim of this present study was to further investigate the roles and mechanisms of the CD40–CD40L complexes of Japanese flounder and to examine the sites of expression for Japanese flounder CD40.

Materials and methods

Cloning of CD40 cDNA

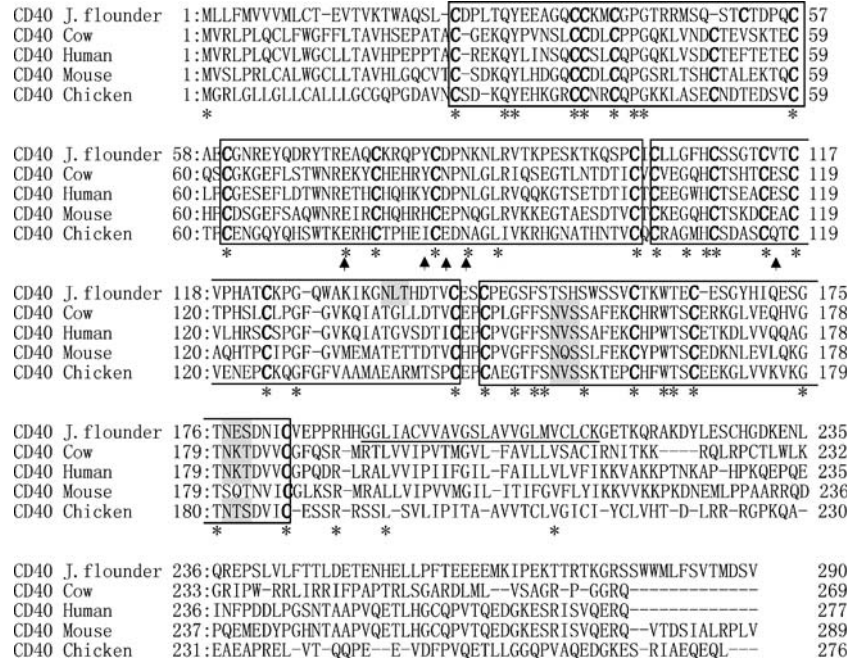
Japanese flounder (*Paralichthys olivaceus*) (600–700 g in weight) that had not been exposed to known antigenic stimulation were used as blood and tissue donors for all in vitro experiments. The cDNA library used in this study have previously been reported (Aoki et al. 1999; Nam et al. 2000). The partial cDNA of Japanese flounder CD40, which was isolated by a previous expressed sequence tag (EST) study, was used as a cDNA probe for screening the full length of Japanese flounder CD40 cDNA. The nucleotide sequence of plasmid DNA was determined using ThermoSequenase (Amersham Biosciences) with M13 forward and M13 reverse primers and an LC200 automated DNA sequencer (LI-COR).

Cloning of CD40 gene

A Japanese founder genomic bacterial artificial chromosome (BAC) library (Katagiri et al. 2000) was screened for the CD40 gene with a specific PCR-derived probe from CD40 cDNA. The oligonucleotide sequences used in this study are given in Table 1 (CD40 Bac-F and CD40 Bac-R).

Fig. 1 Comparison of the derived amino acid sequence of Japanese flounder, cow, human, mouse, and chicken CD40s.

Asterisks indicate identical amino acid residues, and dashes indicate gaps introduced for maximal alignment. Arrowheads indicate ligand-binding motifs, and cysteine-rich domains are boxed. Extracellular cysteines are shown in bold. Putative N-glycosylation sites are shaded, and the transmembrane region is underlined



High-density replica filters were hybridized as previously reported (Katagiri et al. 2000). Japanese flounder CD40 genomic BAC clones were purified and used for Southern blot and genomic organization analyses of the CD40 gene. Approximately 5 µg of BAC DNA was digested to completion using several restriction enzymes. Southern blot hybridization was conducted as described previously (Hirono et al. 2000). The positive bands were ligated into the pUC119 vector plasmid and transformed to JM109. The nucleotide sequence was determined using Thermo-Sequenase (Amersham Biosciences) with M13 forward and M13 reverse primers and an LC4200 automated DNA sequencer (LI-COR).

Data analysis

The determined nucleotide and deduced amino acid sequences and multiple sequence alignments were analyzed by GENETYX ver. 8.0 (SDC Software Development). Each determined sequence was compared with all sequences available in DNA Database Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank using basic local alignment search tool (BLAST) ver. 2.0 (Altschul et al. 1990, 1997). Phylogenies based on the extracellular regions of TNFR superfamily were inferred using the phylogeny inference package (PHYLIP) program (ver. 3.5) (Felsenstein 1996), and the distance analysis was performed using the neighbor-joining method. The values supporting each node are derived from 1,000 resamplings. Amino acid sequences having the most complete annotation were obtained from the GenBank database: Human TNFR1 (accession no. P19438), Mouse TNFR1 (accession no. P25118), Rat TNFR1 (accession no. P22934), Human TNFR2 (accession no. P20333), Mouse TNFR2 (accession no. P25119), Human Fas (accession no. P25445), Mouse Fas (accession no. P25446), Rat Fas (accession no. Q63199), Human NGFR (accession no. P08138), Rat NGFR (accession no. P07174), Human OX40 (accession no. P43489), Mouse OX40 (accession no. P47741), Rat OX40 (accession no. P15725), Human CD27 (accession no. P26842), Mouse CD27 (accession no. P41272), Human CD40 (accession no. P25942), Mouse CD40 (accession no. P27512), Bovine CD40 (accession no. Q28203), Chicken CD40 (accession no. CAC20218), and Japanese flounder CD40 (accession no. BAC87848).

Reverse transcriptase–polymerase chain reaction

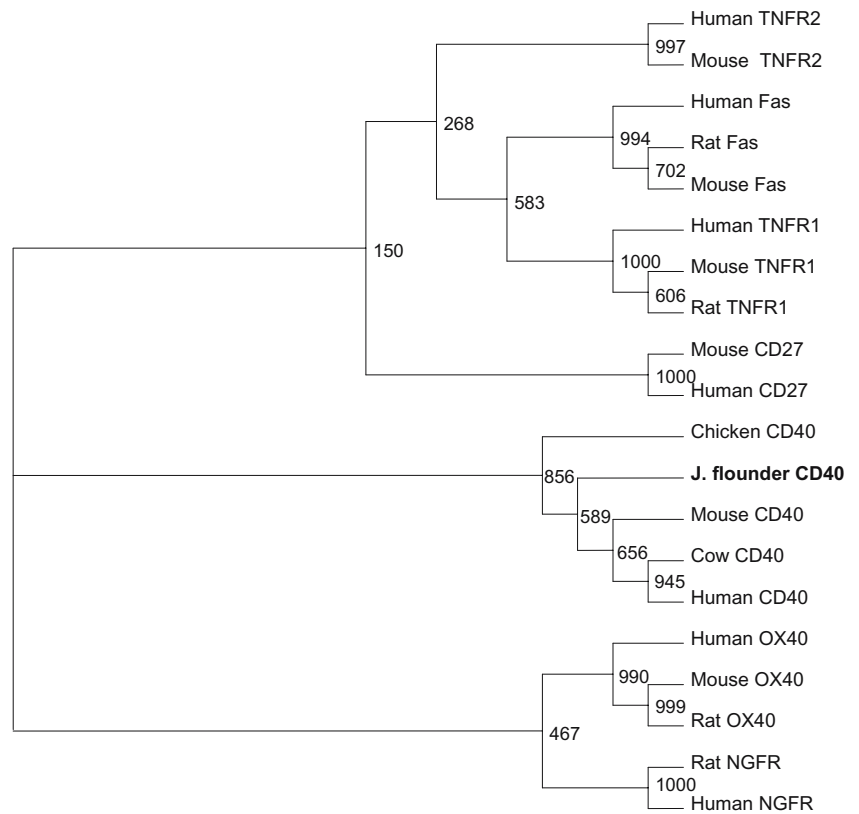
Total RNA (50 ng) from the brain, heart, intestine, kidney, liver, and spleen were reverse-transcribed into cDNA using an AMV Reverse Transcriptase First-Strand cDNA synthesis kit (Life Sciences). PCR was performed on the resulting cDNA using the CD40 RT-F- and CD40 RT-R-specific primers (Table 1). β-actin was amplified as a control using the β-actin-F and β-actin-R primers (Table 1). The mixture was denatured at 94°C for 2 min and subjected to 25 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for

Table 2 Identities of the deduced amino acid sequences of Japanese flounder CD40 with TNFR superfamily genes of other species

	hCD40	cCD40	mCD40	chCD40	hCD27	mCD27	hOX40	mOX40	mFas	rFas	hNGFR	rNGFR	hTNFR1	mTNFR1	hTNFR2	mTNFR2
Protein structure																
Extracellular region (%)	39	37	38	35	27	29	28	30	27	28	27	27	27	27	29	27
Intracellular region (%)	26	21	27	25	25	23	19	19	21	19	22	22	18	19	19	19
Total protein (%)	35	35	33	32	28	28	27	27	26	26	26	26	25	26	26	25

h Human, *c* bovine, *m* mouse, *ch* chicken, *r* rat

Fig. 2 Phylogenetic tree of the TNFR superfamily. Amino acid sequences were estimated by the neighbor-joining method of clustering in the PHYLIP program



1 min. The products were visualized by separation on a 1.5% agarose gel.

Real-time polymerase chain reaction

Total RNA from normal peripheral blood leukocytes (PBLs) and PBLs treated with lipopolysaccharide (LPS) (500 $\mu\text{g}/\text{ml}$) or Con A (50 $\mu\text{g}/\text{ml}$)/phorbol myristate acetate (PMA) (0.35 $\mu\text{g}/\text{ml}$) were purified as previously reported (Hirono et al. 2000). cDNAs were synthesized for real-time PCR from stimulated and nonstimulated leukocytes. To determine the absolute copy number of the target transcript, a cloned plasmid DNA for each sample was used to generate a standard curve. The cloned plasmid DNAs (0.5 μl) were used in 50 μl of PCR mixture. The primer pairs CD40 Stan-F/CD40 Stan-R and β -actin Stan-F/ β -actin Stan-R (Table 1) were used to amplify the fragments. Amplification was carried out as follows: 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 5 min. Real-time PCR was conducted as previously described (Park et al. 2003). Primers for real-time PCR sequences are also summarized in Table 1. Thermal cycling and fluorescence detection were conducted using the Gene Amp 5700 sequence detection system. All samples were run in triplicates. The ratio between the β -actin in a standard sample (10^6 copies) and the test samples were defined as the normalization factor.

Results and discussion

Cloning of CD40 cDNA

The aligned predicted amino acid sequences of the CD40s of Japanese flounder, human, cow, mouse, and chicken are shown in Fig. 1, and their identities are shown in Table 2. The predicted amino acid sequence is 290 amino acid membrane-bound proteins that consists of a 19-amino acid signal sequence, a 171-amino acid extracellular region rich in cysteines (20 residues), serines (17 residues), and threonines (16 residues), a single 23-amino acid transmembrane domain, and a 77-amino acid cytoplasmic region. The mature protein has a predicted molecular weight (MW) of 32.3 kDa and two potential N-linked glycosylation sites at asparagines 134 and 177. Alignment of the extracellular regions of the Japanese flounder CD40 and four other species demonstrates a higher homology than intracellular regions. In contrast, the cytoplasmic regions of the receptors of different species do not show any significant similarity (Fig. 1 and Table 2). The cytoplasmic region of CD40, however, does not display a close relationship to other members of the family. Like other members of the TNF receptor superfamily in mammals, CD40 is characterized by a repetitive amino acid sequence pattern of four cysteine-rich domains, typically consisting of six cysteines forming three disulfide domains. Binding and mutagenesis analysis, X-ray crystallography, as well as molecular modeling experiments employing single and double amino acid

substituted proteins implicated the acidic residues E74, Y82, D84, N86, and E117 in human and mouse CD154 binding (Bajorath et al. 1995). Several of these amino acid residues are conserved in Japanese flounder CD40 (Fig. 1). The Japanese flounder CD40 amino acid sequence was identified by its identity to human (35%), cow (35%), mouse (33%), and chicken (32%) (Table 2). In the phylogenetic analysis (Fig. 2), Japanese flounder CD40 is grouped with vertebrate CD40 peptides, including chicken CD40. This grouping was well supported by bootstrapping. The phylogenetic analysis indicated that known human, cow, and mouse CD40s are more closely related than the Japanese flounder and chicken CD40. This result is possibly due to differences between mammalian and nonmammalian species. This clearly shows that the nonmammalian CD40s

differs substantially from mammalian CD40s, although these are closely related.

Based on the conservation of four cysteine-rich domains and the amino acid residues for binding CD40L in the extracellular region of the molecule, we predict that the function of Japanese flounder CD40 is similar to that in mammals.

Analysis of CD40 gene

The Japanese flounder CD40 is encoded on nine exons separated by eight introns (Fig. 3). The gene structure pattern is similar to those in human CD40 (Fig. 4), whereas the human TNFR1, human TNFR2, human NGFR, human

Fig. 3 Full length of the Japanese flounder CD40 gene. The predicted translation of the exon coding regions is given. The poly (A) signal (AAUAAA) is *underlined*. The 5' (GT) and 3' (AG) ends of each intron are in *bold*, and the *boxed* sequence represents a dinucleotide TA repeat sequence

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CTTGAATGCAGCAGACAGTGTGAGTCTGTGAGCAAACCGAATACTGCGAGTGTCCAGCAACATGCTCCTGTTTCATGGTGGTGGTGATG 90
                                     M L L F M V V V M
CTGTGCACGGAGGTCACGgtgagtaaaactgatgatgtgtgttttttaaaaaaagaaaaagaaagattgtttctgacgtgtgatcatcga 180
L C T E V T
tgttggatcagattgtaccactgacactgtgaatgtgtccctctgtgtgagcgttttcttatttacgtgtatttcagcttatttctgt 270
tcttattttttatttcaatgtcttgttctgttactgtctggctcaactttcaactttccacctcagctcagagctgaagctgt 360
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actcagtggtgatgtcagtagtaaaagtttgggctttccactcactgtactctctgaggaagtgaaccaactgtcaacagagagaac 540
ccccccagactacactacatttcaactcagcaggaccttattatcttcaactgttctgttttgagGTGAAGACCTGGGCTCAGTCCCT 630
                                     V K T W A Q S L
GTGTGACCCACTGACTCAGTATGAGGAGGCCGCTCAGTCTGCAAGATGTGTGGTCCACgtaaaccaagtcacgatttctgttttaaatg 720
C D P L T Q Y E E A G C C K M C G P
agccgggaagagaattttccggctctgctttgatgaaacacgtgaaatccctggttttaatttcacatttgagaccgaatttctcaa 810
acgagtcgacattcaacacaattatcttggaggataaacacagaaaagatgcgtattgtctgtttgtctgaattaaagtggatatttct 900
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gtttacttgagatctgactgtgtttagCCACGAGGATGAGTCAAAGCACTGTACGGATCCTCAGTCCGAGAGTGTGGGAAC 153C
G T R R M S Q S T C T D P Q C A E C G N
CGTGAATATCAGGATAAATACACCAGGGAAGCTCAGTGTAAAACGTCAGCCATACTGTGACCCGgtaaagacttgtgacaacaacacaa 162C
R E Y Q D K Y T R E A Q C K R Q P Y C D P
acctgttccaacacagacatccaggagcactgttcagtggttcttcttagATAAAAACCTGCGTGTGACCAAGCCAGAGAGCAA 171C
N K N L R V T K P E S K
GACGAAGCAAAGCCCTGCATCTGCTGCTGGGTTTCCACTGCTCCAGTGGCAGCTGTGTACCTGTGTGCCGACGCAACATGTAAACC 180C
T K Q S P C I C L L G F H C S S G T C V T C V P H A T C K P
AGGCAATGGCCAAAATCAAAGgtaaagcatattctcaaaagctgttttcagacatgcacgctggagacgtccgtgtgatgggtcag 189C
G Q W A K I K
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G N L T H D T V C E
GAATGCCCCGAGGGCTGTTTCCACCAGCCACTCTGGAGCAGCGTGTGCCAAGTGGACGGgtaaagatgggaactctctaaaca 225C
N C P E G S F S T S H S W S S V C T K W T E
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C E S
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G Y H I Q E S G T N E S D N I C
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V E P P
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R H H G G L I A C V V A V G S L A V V G L M V C L C K
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agGAGAAAACAAAAGGGCAAAGgtaaactctgattctcttcttcaattttgtctcgtgtataataacataagtaacagagtagtga 360C
G E T K Q R A K
gccatcaacagattattatttagcacatttaccgtttagagctttagtctcaaatgttttgggtgaaagatgcttattctctcttgcga 369C

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Fig. 3 (continued)

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aagaataagatgataaatttgatatcacttttatactctatgctttttaagaagccagggtcgaaaacttactacctctaaactctacat 378C
ctctatttcgactgtgctcctgtagGATTATCTGGAATCGTGCCATGGAGACAAGGAGAACCTACAGCGAGAGCCAGCCTAGTGTGTT 387C
      D Y L E S C H G D K E N L Q R E P S L V L F
CACCACGCTCGATGAGACCGAAAACCATGAACGTGCGGTTCCACAGAGGAGGAATCACTAGTGGAGATGAAGATACCGGAGAAAAACCA 396C
T T L D E T E N H E L L P F T E E E S L V E M K I P E K T T
GAGGACCAAAGGGAGGAGCAGTTGGTGGATGCTGTTCTCAGTGACAATGGACAGAGTGTGACACAGGAGAGGGGTAAGACGAAATCCAG 405C
R T K G R S S W W M L F S V T M D S V *
CCCCAGCAGGAGACACAAGTGCAGATGTCACAGACTAAAAAAAAAATTCCTCATTGGAATAGTTGATTACATACGATGTTAATGTTA 414C
TGACTCATATTTCACTTGCACCTAATTCTCCTTGTGTGAATGACTTTATGTAATAATATTAGTGTTAACCATACAATTGAAACTGTCTG 423C
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tagaatgagtggaatcaaatgtgctgtagttactctaataatgcctcaggggatcg 5725
    
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Fas, and human OX40 genes consist of ten, ten, six, eight, and seven exons, respectively. Typical intron splice motifs occur at the 5' (GT) and 3' (AG) ends of each intron (Fig. 3). The transmembrane and cytoplasmic regions of the Japanese flounder CD40 are encoded by exons separate from the cyteine-rich domains of the extracellular region. The CD40 gene contains a dinucleotide CA repeat sequence in 5'-flanking region and a dinucleotide TA repeat sequence in the sixth intron (Fig. 3). Dinucleotide repeat sequences are useful markers in genetic studies of disorders affecting immune responses (Ota et al. 1999).

Little is known about the mechanisms underlying CD40 gene activation. Although the CD40 promoter structure is unknown, the fact that IFN- γ can induce CD40 expression in most cell types analyzed suggested the presence of a

signal transducer and activator of transcription (STAT) site (Krszsz et al. 1999). Prompted by this finding (Fig. 5), we analyzed seven putative different transcription factor binding sites (AP-1, CEBPB, IRF-1, LYF-1, NF- κ B, SP-1, and STAT-1) that might be involved in cytokine-inducible CD40 gene expression.

Expression of Japanese flounder CD40

The mRNA expression of CD40 was detected in all tested tissues (leukocytes, kidney, spleen, liver, intestine, brain, gill, and skin) of Japanese flounder. The CD40 gene was predominantly expressed in several tissues that contained lymphocytes (PBLs, head kidney, trunk kidney, spleen,

Fig. 4 a Schematic representation of the genomic DNA structure of human CD40. b Genomic organization of the Japanese flounder CD40 and its derived mRNA, drawn to scale as indicated. The genomic DNA and mRNA are represented at the top and bottom, respectively. Exons are indicated by closed boxes

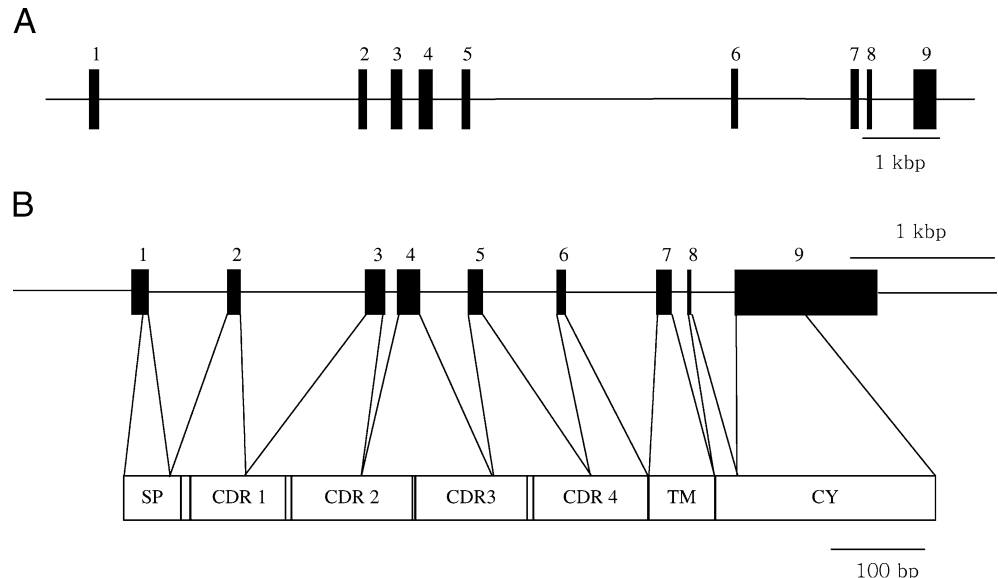


Fig. 5 5'-flanking region of Japanese flounder CD40. The underlined sequences represent the transcription elements predicted by TRANSFAC software, and the **boxed** sequence represents a dinucleotide CA repeat sequence

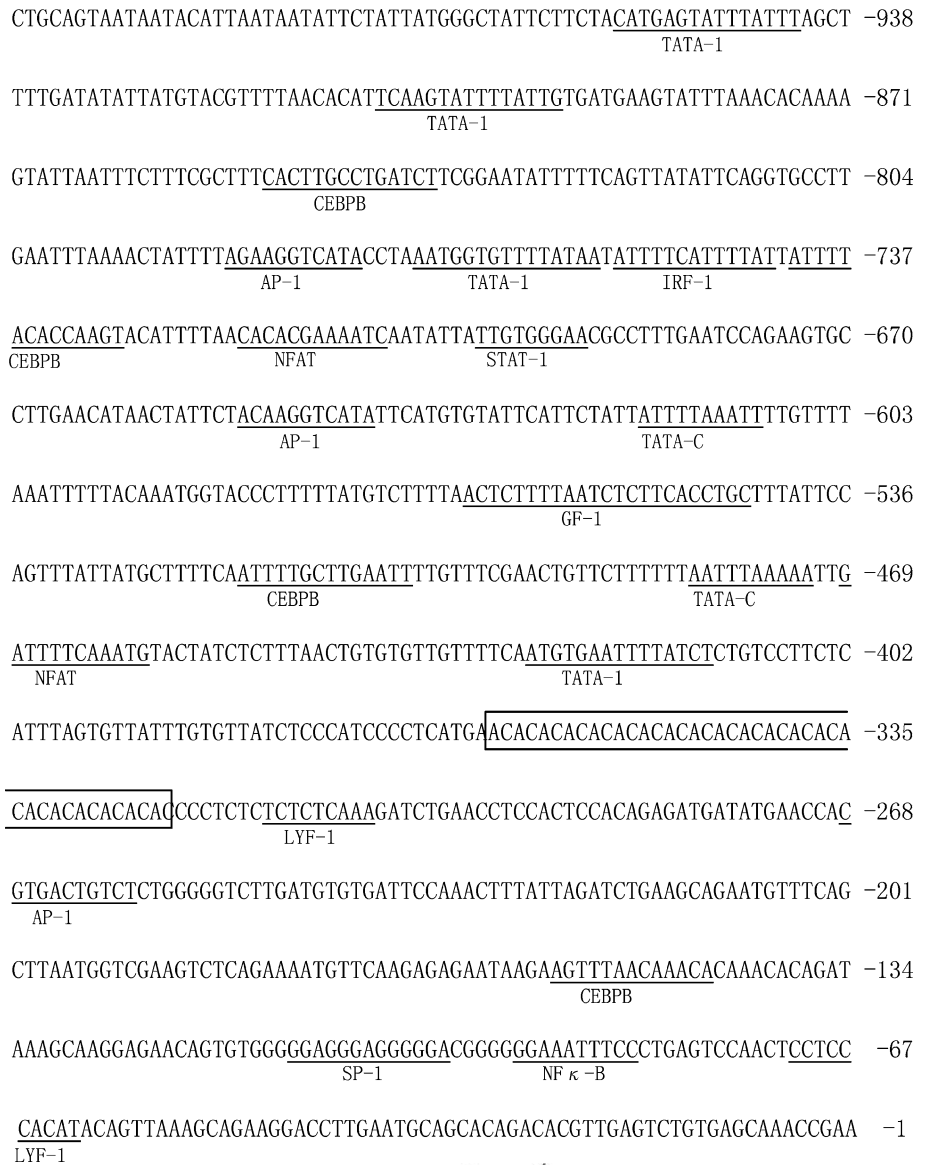


Fig. 6 Detection of CD40 mRNA levels from various tissues of healthy Japanese flounder by RT-PCR. *M* indicates a 100-bp ladder marker

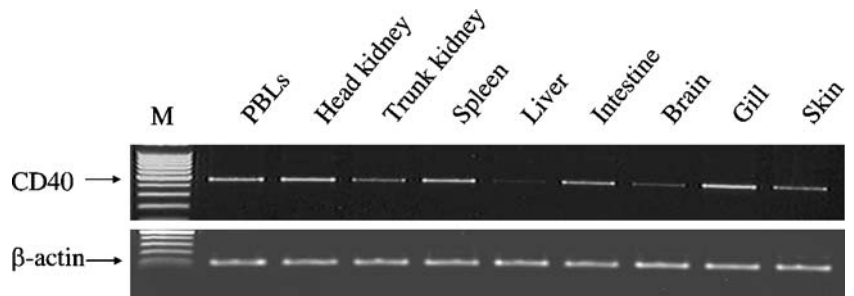
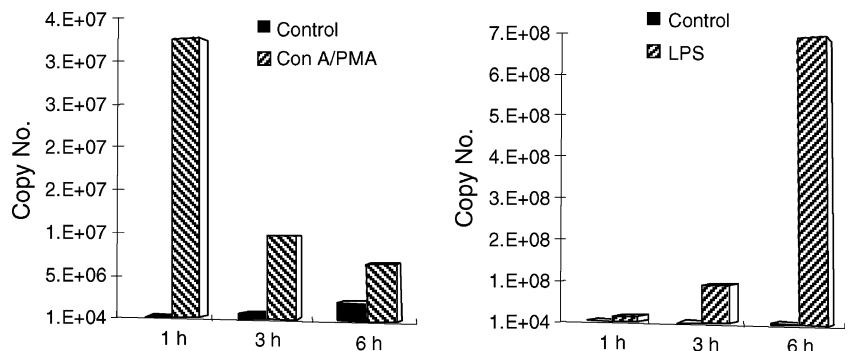


Fig. 7 Quantitative real-time PCR analysis of the CD40 expression in Japanese flounder normal PBLs, and LPS- (500 µg/ml) or Con A- (50 µg/ml)/PMA (0.35 µg/ml)-treated PBLs at 1, 3, and 6 h



intestine, and gill) (Fig. 6), thus suggesting that this gene plays important roles in the immune system.

The Con A, PMA, and LPS activation system has previously been used for the analysis of molecular determinants of fish leukocyte proliferation (Graham and Secombes 1988; Johnson et al. 1987; Murphy and Norton 1993; Takeshita et al. 1988). Japanese flounder CD40 expression was induced from 1 to 6 h following Con A/PMA stimulation, peaking at 1 h, and increased at 1, 3, and 6 h after induction by LPS compared with the control (Fig. 7). Although the CD40 gene is constitutively expressed in most cell types, expression of CD40 protein can be up-regulated. Stimuli for CD40 expression include cytokines such as IFN- γ , IL-1, IL-3, IL-4, TNF- α , *Mycobacterium tuberculosis*, PMA, 12-*O*-tetradecanoylphorbol-13-acetate and antibodies against IgM or CD20 (Schönbeck and Libby 2001).

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