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A highly conserved apoptotic suppressor gene is located near the chicken T-cell receptor alpha chain constant region

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Abstract A cosmid clone containing the chicken T-cell receptor alpha chain constant region (*TCRAC*) was sequenced. The cosmid contains the *TCRAC* gene, six putative joining gene segments (*TCRAJ*), and surprisingly, a chicken homologue for the human apoptotic suppressor gene, defender against cell death (*DAD1*). The *DAD1* gene is 6.3 kilobases downstream of the *TCRAC* gene and has an inverted transcription orientation with respect to the *TCRAC* gene. The cDNA for the chicken *DAD1* gene is 597 base pairs in length and encodes a highly conserved hydrophobic protein. The proximal location of *DAD1* to the *TCRAC* locus has also been confirmed in both humans and mouse. The location of the *DAD1* gene suggests that *DAD1* may play an important role in T-cell related apoptotic activities.

Introduction

Lymphocytes in the vertebrate immune system can be grouped into two major cell types; B and T cells based on their function and site of maturation. These cells execute the immune responses through their antigen-specific receptors. The antigen receptor for T cells, T-cell receptor (TCR), is a cell surface heterodimeric glycoprotein. The TCR is composed of either TCRA (α) and TCRB (β) or TCRG (γ) and TCRD (δ) polypeptide chains. These chains are encoded by the *TCRA*, *TCRB*, *TCRG*, and *TCRD* gene families (reviewed in Hunkapiller and Hood 1989). Like the B-cell antigen receptor immunoglobulins, the TCR polypeptides are coded by multiple germline separated variable (*V*), diversity (*D*) (for TCRB and TCRD chain genes), joining (*J*) gene segments, and constant region (*C*) genes. During T-cell ontogeny, these gene segments are assembled at the DNA level through a site-specific recombination process.

The expression of the *TCRA* gene is largely regulated by the TCRA chain enhancer which is recognized by a set of complex *trans*-acting regulatory proteins (Diaz et al. 1994; Ho et al. 1990; Winoto and Baltimore 1989). The *TCRA* gene enhancer region is located approximately 4 kilobases (kb) downstream of the *TCRA* constant gene (Ho et al. 1990; Winoto and Baltimore 1989). Between humans and mouse, the 240 base pair (bp) enhancer region shares 86% of their nucleotide sequences (Koop et al. 1994). As well as the well-characterized TCRA chain enhancer, additional highly conserved DNA sequences called conserved sequence blocks (CSB) have been identified in the *TCRAJ* region through a sequence comparison between human and mouse *TCRAJ-TCRAC* region nucleotide sequences (Koop et al. 1994). Although nuclear proteins have been shown to recognize one of the conserved sequence blocks, the functions of these conserved regions remain to be determined (Koop et al. 1992, 1994; Hood et al. 1993).

Unique for T-lymphocyte development, the rearranged and expressed TCR repertoire is subsequently modified in thymus by both positive and negative selection processes (reviewed in Elliott 1993). Specifically, positive selection is the process whereby T cells are selected for maturation when the TCRs can recognize foreign antigens, and the negative selection eliminates self-reactive T cells. In general, more than 98% of the lymphoid cells in thymus are driven to programmed cell death or apoptosis due to the negative selection process (Egerton et al. 1990). Several cellular encoded apoptotic effectors, such as *Bcl-2*, *Bcl-x*, and interleukin-1β-converting enzyme (ICE) have been identified. However, little is known about their roles, if

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any, in the T-cell selection processes occurring in thymus (reviewed in Cory 1995).

Nucleotide sequences that are conserved through evolution include protein coding exons or important regulatory sequences. Therefore, comparing sequences from evolutionary diverged lines may assist in the identification process of genes and regulatory elements. Birds diverged from mammals approximately 300 to 350 million years ago. To study the evolution of the *TCRAC* gene and shed some light on the possible functions of previously identified conserved sequence blocks in human and mouse *TCRAC* region, a cosmid clone containing the chicken *TCRAC* gene region was isolated, sequenced, and analyzed. In this report, we describe the genomic organization of the chicken *TCRAC* gene and identification of a nearby human apoptotic suppressor gene homologue, defender against cell death (*DAD1*).

Materials and methods

Nucleic acid and libraries

Chicken genomic DNA was prepared from liver using a standard protocol (Maniatis et al. 1989). A cosmid library with ten genome-equivalents was constructed from gnomic DNA in the pWE15A cosmid vector (Wang et al. 1995b). Total chicken thymus RNA was prepared from a freshly killed 3-month-old chicken using the guanidinium isothiocyanate extraction method (Chomczynski and Sacchi 1987). The poly(A) mRNA was selected through an oligo(dT)-cellulose column and cDNA was synthesized and cloned into λ-ZAP directional cloning vector (Stratagene, La Jolla, CA). A total of approximately 1×10^6 independent recombinant phage plaques were obtained and screened.

Probes and hybridization

Polymerase chain reactions [(PCR) (Saiki et al. 1988)] were used to obtain DNA probes. Oligonucleotide primers were synthesized from chicken *TCRAC* mRNA (Gobel et al. 1994) and chicken *DAD1* germline sequences. The primer sequences were as follows:

CHKCA1:	CCTTCAGTCTACAGGCTGACC (for the chicken *TCRAC* gene)
CHKCA1R:	TCAGCATGTTCAGGTTCTCAT (for the chicken *TCRAC* gene)
CHKDAD1:	AGTGGCACGTCGAGCCGTCTCA (for the chicken *DAD1* gene)
CHKDAD1R:	GATGCAGGATGGTGTTGGCAAAG (for the chicken *DAD1* gene)

Approximately 1 ng of chicken thymus cDNA was used in 50 µl of PCR that consisted of 50 mM NaCl, 20 mM Tris.HCl (pH 9.0), 2.5 mM M_gCl_2, 0.5 mM dithiothreitol (DTT), 150 µM of each nucleotide, 1 µM of each gene-specific primer, and 2 units of *Taq* DNA polymerase. The reaction mixtures were subjected to 25 cycles of amplification. Each cycle consisted of 20 s at 94 °C, 45 s at 50 °C, and 90 s at 72 °C. The amplification products were analyzed by gel electrophoresis and the identity of the amplified products was determined by direct sequencing with γ-^{33}P-ATP-labeled amplification primers.

For library screening, the purified fragment was labeled to a specific radioactivity greater than 1×10^8 c.p.m./µg of DNA by random priming (Feinberg and Vogelstein 1983). Membranes were hybridized overnight in 50% formamide (V/V), 5 × standard sodium citrate [(SSC) (1 × SSC = 0.15 M NaCl/0.15 M sodium citrate)], 0.02 M sodium phosphate (pH 6.7), 100 µg/ml denatured salmon sperm DNA, 1% sodium dodecyl sulfate (SDS), 0.5% non-fat dry milk, and 10% dextran sulphate at 37 °C. Following hybridization, these membranes were washed twice in 2 × SSC with 0.1% SDS at room temperature and twice in 0.5 × SSC with 0.1% SDS at 65 °C. The filters were then blotted dry with Whatman 3 MM filter paper and exposed to X-ray film overnight at –70 °C with intensifying screens.

DNA sequencing

The purified cosmid DNA was sonicated and DNA fragments 1.5 to 2 kb in size were purified from agarose gel as described (Wilson et al. 1992; Koop et al. 1994). The DNA fragments were cloned into *Sma* I-digested, dephosphorylated pUC18 vector. Sequencing template preparation and the subsequent sequencing reactions were carried out by PCR and cycle sequencing reactions, respectively (Wang et al. 1995a). Sequences were assembled with a test version of Sequencher DNA assembling program 3.0β4 (Gene Codes Corp., Ann Arbor, MI) kindly provided by Gene Codes Corp prior to its release.

Sequence analysis

Sequence database searches and multi-sequence alignments were performed with BLAST, Inherit (ABI, Foster city, CA) and LaserGene sequence analysis program (DNA star, Madison, WI). The nucleotide and deduced amino acid sequences of the chicken *DAD1* gene were compared with those found in GenBank/EMBL (release 92).

Results

The chicken *TCRAC* gene containing the cosmid, clone 23.1, was sequenced by the shotgun sequencing approach as described (Wilson et al. 1992). The final assembled DNA sequence contains 31793 bp of genomic sequences. The sequence has about 45% of GC and does not contain any di- or tri-nucleotide repeat with significant length (greater then five repeat units). The cosmid contains the entire chicken *TCRAC* gene, six putative *TCRAJ* gene segments, three copies of the chicken *CR1* type repeat, and surprisingly, the chicken homologue of a gene which is thought to be a suppressor of apoptosis, *DAD1* (Nakashima et al. 1993). Two copies of the CR1 repeat are located between the *TCRAC* gene and the *DAD1* gene, and the other one resides between exon 1 and exon 2 of the *DAD1* gene (Fig. 1). The chicken CR1 repeat encodes a retroviral reverse transcriptase pseudogene. The transcriptional orientation of the CR1 repeats are indicated in Fig. 1.

Genomic organization of the chicken TCRAC and TCRAJ genes is similar to human and mouse

The *TCRAC* gene exons were identified by similarity comparisons with the chicken TCRA chain cDNA sequences (Gobel et al. 1994). The *TCRAC* gene in chicken, as in humans and mouse, is encoded by three translated exons, exon 1, 2, and 3, and an untranslated exon 4. The exons are 264, 48, 107, and 474 bp in length and are separated by introns of 442, 1070, and 470 bp, respectively. The chicken *TCRAC* gene exons, like other known *TCRAC* genes, begin with the second codon position which insures

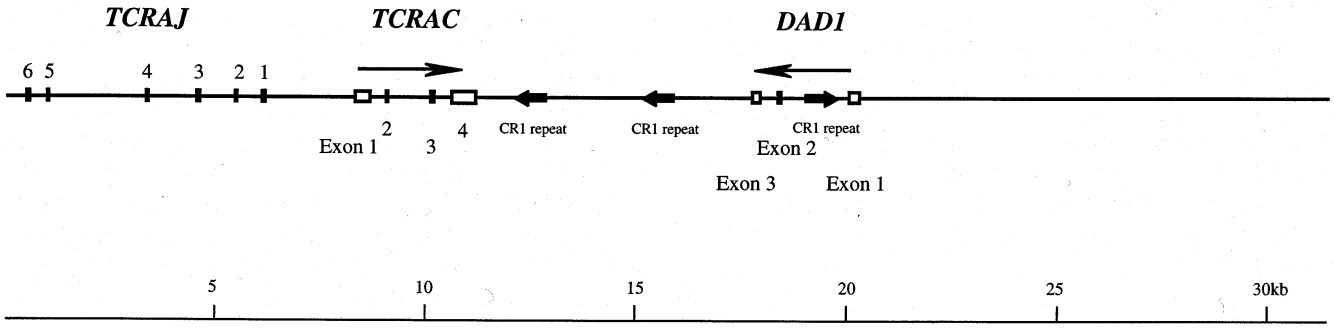

Fig. 1 Genomic organization of the chicken *TCRAJ, TCRAC,* and *DAD1* gene region. The *TCRAJ* gene segments and exons for *TCRAC* and *DAD1* genes are indicated. The CR1 repetitive sequences and its orientations are indicated with solid *arrows*. The *arrows above* the *horizontal line* indicate transcription orientations for the *TCRAJ/TCRAC* and *DAD1* genes

correct RNA splicing between constant gene exons and between the joining gene segment and the constant gene. Even though the chicken *TCRAC* gene share a similar intron/exon organization with human and mouse *TCRAC* genes, it shares little sequence similarity at either the nucleotide or the amino acid levels (Gobel et al. 1994). Amino acid residues that are important for the structural integrity of the TCRAC polypeptide are preserved in birds (Gobel et al. 1994).

To identify the chicken *TCRAJ* gene segments, the cosmid sequence was searched for *J* gene segment core sequences, DNA recombination signals, and RNA splice sites as described (Koop et al. 1992, 1994). In addition, the cosmid sequence was compared with a file containing 122 previously identified human and mouse *TCRAJ* gene segment sequences (Koop et al. 1992, 1994). These searches resulted in the identification of six putative *J* gene segments, *TCRAJ1* to *TCRAJ6* (Fig. 1). As in humans and mouse, the chicken *TCRAJ* gene segments are 50 to 63 bp in length and are flanked by a 5′ DNA recombination signal (GGTTTTTGT-11 to 12 bp spacer-CACTGTG) and a 3′ RNA splice site (MAGGTRAG). The chicken *J* gene segments also contain a centrally located conserved amino acid core sequence (Phe-Gly-X-Gly-Thr). All these putative *TCRAJ* gene segments are located upstream of the constant gene and have the same transcription orientation as the TCRA chain constant gene.

Alignment of the nucleotide sequences and deduced amino acid sequences of these *TCRAJ* gene segments are listed in Fig. 2. As expected, nucleotide sequence conservation at recombination signal and RNA splicing sites can be observed in chicken germline *TCRAJ* gene segments (Fig. 2A). Chicken *TCRAJ1* and 2 are probably pseudogenes, since part of the conserved sequence motif does not present in the coding regions (Fig. 2B). However, the functionality of these *J* gene segments can only be determined by expression studies such as cDNA library screening and sequencing.

Extensive nucleotide sequence conservation between the human and mouse *TCRAC-TCRDC* gene region has been

Fig. 2A, B Alignment of the chicken *TCRAJ* gene **A** nucleotide and **B** amino acid sequences. Consensus sequences are adapted from human and mouse *TCRAJ* sequences (Koop et al. 1994). Gaps have been introduced to maximize similarity between the sequences. Recombination signal (GTTTTTGTA-------CACTGTG), *J* gene segment core sequence (TTTGGNNNNGGNAC), and the RNA splice signal (GTAAGT) are indicated

A

	Recombination siganl		Joining region (J)		RNA Splice site
TCRAJ6	GGGGAGATTTCTATTGTGTCCTGAAAGGTTGTG	TAGAATTCAGGGCAAGATC	CAAGCCCATGTTTGTTAGTGGCACCCATCT	TTGGGTGAAA CCCA	GAAGGTGTTAA
TCRAJ5	ATGCAGCATTTTGCAAAGCATGGTGTCACAGTG	TAATACTGGGGGTTACGG	CAAACTCATATTTGGAACTGGAACCAAACT	TACGGTTTCT CTAG	GTATGTCTGAA
TCRAJ4	ACTCTGGATATTGTAGATGCCTGTGTTATTGTG	TGACTATGGATATAGTTA	CAAACTCACGTTTGGATCTGGGAGAAGACT	TCTGGTTCTG CCAG	GTACATGCATC
TCRAJ3	GGCTATGTTTTTGCAGAGATTGGCATCAGTGTG	CCAATCAGGGATATGCA	GAACTGGCATTTGGAGCGGGAACCCAACT	TCTTGTT ACCCCGA	GTAAGTTTAAA
TCRAJ2	CAACAACTGATAGCAAAGTCTTATTGTTGAGTG	AGAAAGAT	ACACT ACATTTGTAAGAGGAACTAGATT	TAAAGTC AGTTCTG	GTGCTTGAGAT
TCRAJ1	GCTGTGTCATTTGTAATGATTTGAATCTGGATG	TCTGGGATACTGGAGCTA	CGGGAGTAATTTTTGGAAATTGCACAAGGCT	GTCTGTA ATCCCAA	GTTATCTAATT
CONSENSUS (Koop et al)	GGTTTTTGTAAAG CACTGTG		AA CT A TTTGG GG AC A CT	GT A CCA	GTAAGT

B

	K L F G G T L V P	CONSENSUS
	10 20	
E F R A R S K P M F V S G T H L W V K P		TCRAJ-6P
N T G G Y G K L I F G T G T K L T V S L		TCRAJ-5P
D Y G Y S Y K L T F G S G R R L L V L P		TCRAJ-4P
N Q G Y A E L A F G A G T Q L L V T P		TCRAJ-3P
E R Y T T F V R G T R F K V S S		TCRAJ-2P
W D T G A T G V I F G N C T R L S V I P		TCRAJ-1P

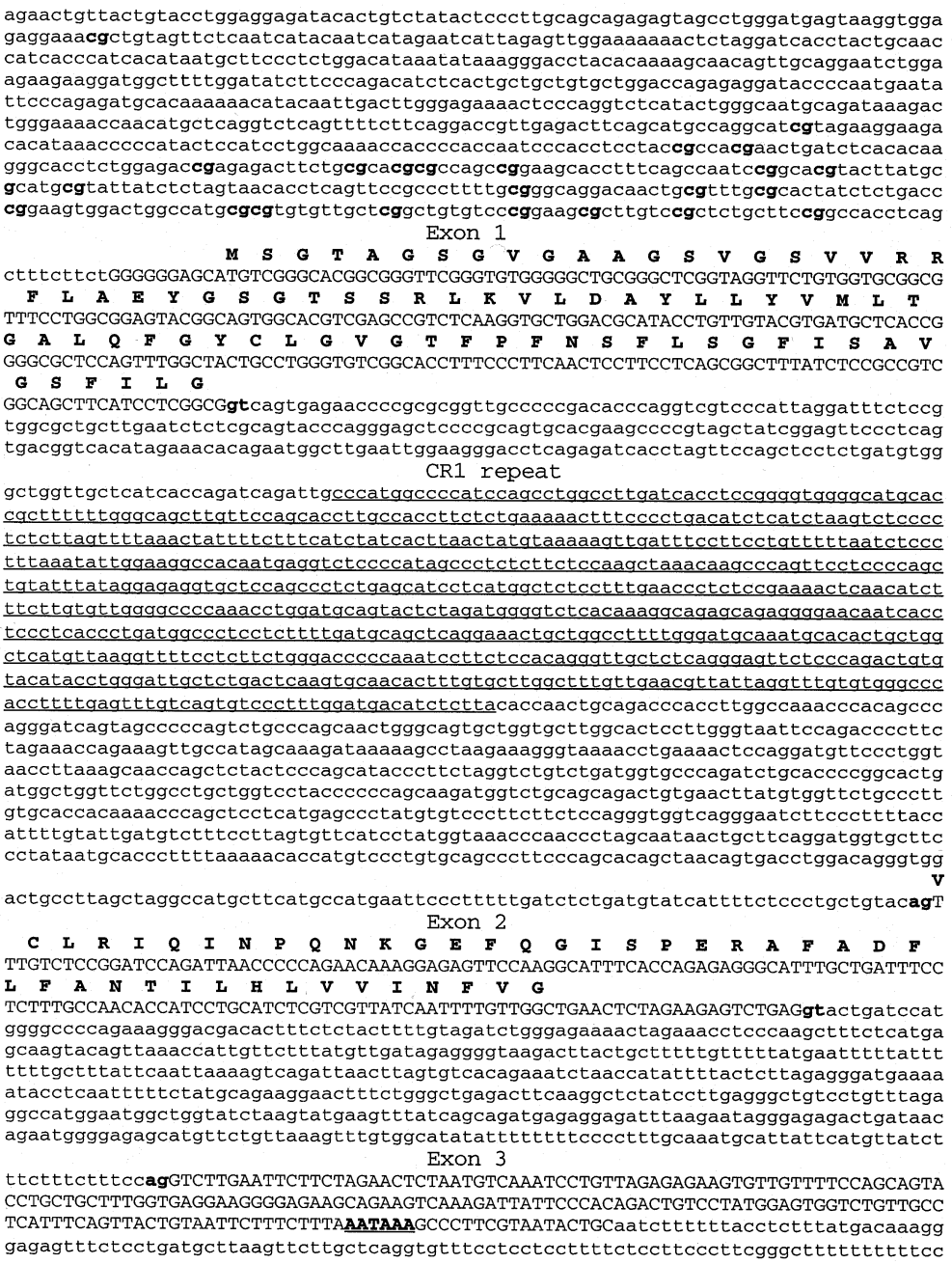

Fig. 3 Germline nucleotide and deduced amino acid sequences of the chicken *DAD1* gene. The *DAD1* gene exons are identified by similarity comparison with the chicken *DAD1* cDNA sequences. The exons are indicated and listed in *capital letters*. The deduced amino acids are listed *above* of the exons. Polyadenylation signals are in *underlined boldface* letters. The CpG islands and the RNA splice donor and acceptor sites are indicated in *boldface* characters. The chicken CR1 repeat sequence is *underlined*

observed (Koop et al. 1994). However, no significant sequence conservation on the orthologous regions has been identified through careful sequence comparison among the chicken, humans, and mouse.

A highly conserved apoptotic suppressor gene, DAD1 is located downstream of the TCRAC gene

Using BLAST and GRAIL search, a chicken homologue for the human apoptotic suppressor gene, *DAD1* (Nakashima et al. 1993) was identified on the *TCRAC* gene containing cosmid sequence (Fig. 1). The chicken *DAD1* gene is 6.3 kb downstream of the *TCRAC* gene and has an inverted transcription orientation with *TCRAC* and *TCRA J* gene segments.

Chicken *DAD1* cDNA is 697 bp in length and encodes a highly hydrophobic protein with 123 amino acid residues. Like its human orthologue, chicken *DAD1* is encoded by two translated exons, 1 and 2, and an untranslated exon 3. The chicken *DAD1* gene exons are 250, 149, and 198 bp in length and are separated by introns of 1659 and 506 bp, respectively. The germline nucleotide sequence and deduced amino acid sequence of the chicken *DAD1* gene is listed in Fig. 3. The chicken *DAD1* gene cDNA sequence contains a short 9 bp 5′ untranslated (UT) region and a 216 bp 3′ UT region. Similar to the *DAD1* gene in humans, expression of the *DAD1* gene in chicken can also be observed in all the tissues that have been studied thus far

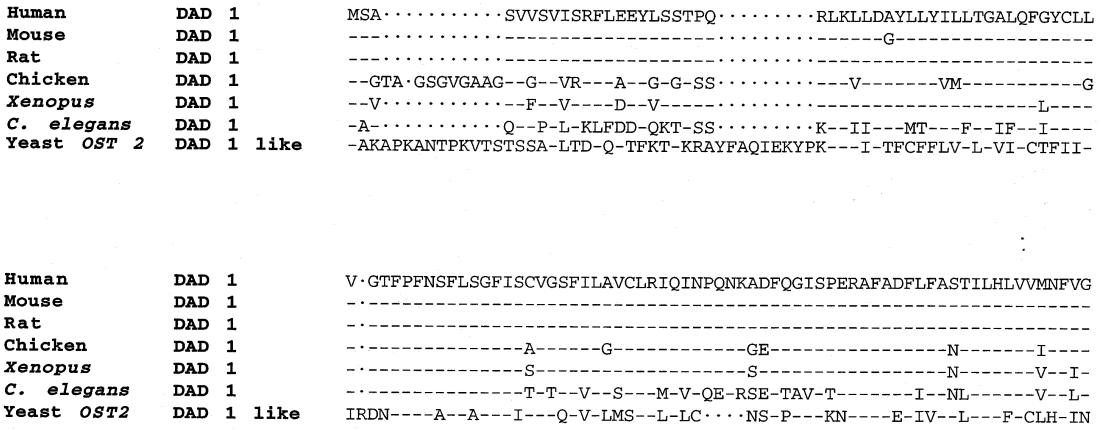

Fig. 4 Amino acid sequence alignment for *DAD1* genes and its yeast homologue. *Dashes* (-) are used to indicate sequence identity with the human *DAD1* sequence. *Dots* (·) are used to indicate gaps that were introduced by alignment program to maximize sequence similarity. The GenBank accession numbers for the sequences used in the alignment are: human D15057 (Nakashima et al. 1993), mouse U83628 (K. Wang and co-workers, in preparation), rat D15058 (Nakashima et al. 1993), chicken U83627, *Xenopus* B54437 (Nakashima et al. 1993), *C. elegans* X89080 (Sugimoto et al. 1996), and yeast *OST2* U32307 (Silberstein et al. 1995)

including spleen, thymus, brain, liver, and pancreas (data not shown).

Like other widely expressed genes, CpG islands can be identified in the *DAD1* promoter region (Fig. 3). Apart from the CpG islands, no other obvious regulatory sequences can be identified based on direct sequence analyses.

The DAD1 gene is highly conserved through evolution

Sequence alignment of chicken *DAD1* against other known *DAD1* genes and an yeast *DAD1* homologue, epsilon-subunit of the oligosaccharyltransferase (OST2) gene (Silberstein et al. 1995), is shown in Fig. 4. Over 60% of the *DAD1* amino acid sequences are conserved from *Caenorhabditis elegans* to human. The chicken *DAD1* gene shares 80% of its amino acid sequence identity (Fig. 4) and 77% of coding region nucleotide sequence identity with the human *DAD1* gene (data not shown). In contrast, the nearby chicken *TCRAC* gene shares only 25% amino acid identity with its human counterpart (data not shown). The chicken *DAD1* gene has an insert of 10 codons in its 5′ region, unlike other *DAD1* genes (Fig. 4). The yeast *OST2* gene shares approximately 40% and 30% of its peptide sequence with human and chicken *DAD1* sequences, respectively (Fig. 4).

Discussion

Comparing the coding region sequences of the *TCRAJ* and *TCRAC* gene between chicken and humans revealed only limited overall nucleotide sequence conservation. Regulatory sequences required for *TCR* gene assembly are conserved between chicken and human. These include recombination signals as well as RNA splice donor and acceptor sites. Other sequences such as the core sequence motif for *J* gene segments and key amino acid residues which are required for maintaining structural integrity of the TCRA chain polypeptide are also preserved through evolution. In contrast to the TCRA chain gene, the apoptotic suppressor gene, *DAD1* revealed extreme sequence conservation at both the nucleotide and the amino acid levels (Fig. 4).

Analyzing the genomic organization of the TCRAC gene in bird revealed a striking conservation in intron and exon structure

Even with 300 to 350 million years' separation and low overall nucleotide sequence conservation, the chicken *TCRAC* gene, like its human counterpart, is still encoded by four separated exons, three coding (exon 1 to 3), and one noncoding (exon 4). The protein translation frame in the chicken *TCRAC* gene, as in humans and mouse, begins with the second codon position which insures correct RNA splicing between constant gene exons and between *TCRAJ* and constant genes. The conservation of intron/exon structure and protein translation reading frame clearly indicates that the *TCRAC* genes in birds and mammals are evolved from a common origin.

Sequence analyses of the *TCRAC* gene region in chicken failed to recognize the previously identified TCRA chain enhancer and conserved sequence blocks (CSB). This may imply that 1) the enhancer region and CSBs may not have any regulatory roles in the expression of the TCRA chain gene, 2) the avian TCRA chain gene may use different set of regulatory elements, 3) we may not be comparing the orthologous region, and more likely, 4) the core sequences for TCRA chain enhancer and other putative regulatory regions such as CSBs are much smaller then previously suggested and are not revealed by direct sequence comparison. Thus, biological studies such as transfection and deletional analysis may be required to locate the avian *TCRA* locus regulatory regions.

Extensive sequence conservation through evolution suggests that the function of DAD1 is fundamental for the cell

Unlike *TCRAC*, the *DAD1* gene is small and highly conserved. Between humans and rat, the *DAD1* gene is 99% identical at the protein level. Other apoptotic proteins such as Bcl-2 do not show such extreme sequence conservation. Recently, the *DAD1* orthologue has been identified in *C. elegans* and even in plants (Sugimoto et al. 1996). The *DAD1* polypeptide is highly hydrophobic, which suggests that *DAD1*, like the Bcl-2 gene product, may be associated with sub-cellular compartmental membranes. As in humans, chicken *DAD1* gene expression was detected in all the tissues tested. Ubiquitous expression and extreme sequence conservation suggests that the *DAD1* gene may play an essential and fundamental role in the survival of eukaryotic cells.

Yeast epsilon-subunit of the oligosaccharyltransferase has been cloned recently (Silberstein et al. 1995). Surprisingly, the yeast epsilon-subunit of the oligosaccharyltransferase protein shared 40% of its peptide sequence identity with the human *DAD1* protein. This finding suggests that *DAD1*-mediated apoptotic suppression may be involved in the glycosylation and mannose transport pathways in the cell.

The human DAD1 gene is also located downstream of the TCRA chain constant gene

The human *DAD1* gene has been mapped to the chromosome 14q11-12 region by YAC and fluorescent in situ hybridization (Ylug et al. 1995). Our sequencing data in chicken raises the possibility that the *DAD1* gene may also be located near the TCRA chain constant gene in human. Genomic cloning and long range PCR confirm this suggestion (K. Wang and co-workers, manuscript in preparation). Since the coding region of the *DAD1* gene in humans and chicken shares more than 80% of their sequence identity, it will be interesting to see whether this sequence conservation extends to introns and the 5′ and 3′ flanking regions of this gene.

The location of the DAD1 gene implies that it may play some role in the T-cell selection process

Negative selection processes play a key role in the maturation of cellular immunity. A cluster of cytotoxic T-cell-specific serine proteases, granzymes, have been mapped to human chromosome 14q11 near the *TCRA* locus (Harper et al. 1988; Hanson et al. 1990; Caughey et al. 1993). It is not unusual in cells that functionally related genes are closely linked in the genome. From studies of the serine protease cluster, it has been proposed that this chromosomal region, from *TCRA* to the granzyme cluster, is coordinately regulated during early stages of T-cell development (reviewed in Cory 1995). The identification of an apoptotic suppressor *DAD1* gene downstream of the *TCRAC* gene suggests that *DAD1* may also play role in the early T-cell development processes.

The isolation of TCRA chain genes from evolutionary diverged species usually depends on cross-species hybridization or utilizes degenerate primers in PCR. These methods in general are difficult, unspecific, and time-consuming due to the low sequence conservation of the TCRA chain gene. The nearby *DAD1* gene, in contrast, is highly conserved through evolution. Thus, it may be possible to obtain the *TCRAC* gene from other species by taking advantage of the *DAD1* gene′s extreme sequence conservation and proximal location to the *TCRA* locus.

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