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Expression, Linkage, and Polymorphism of MHC-Related Genes in Rainbow Trout, *Oncorhynchus mykiss*¹²

John D. Hansen,³* Pamela Strassburger,* Gary H. Thorgaard,⁷⁺ William P. Young,⁴⁺ and Louis Du Pasquier*⁵

The architecture of the MHC in teleost fish, which display a lack of linkage between class I and II genes, differs from all other vertebrates. Because rainbow trout have been examined for a variety of immunologically relevant genes, they present a good teleost model for examining both the expression and organization of MHC-related genes. Full-length cDNA and partial gDNA clones for proteasome δ, low molecular mass polypeptide (LMP) 2, TAP1, TAP2A, TAP2B, class Ia, and class IIB were isolated for this study. Aside from the expected polymorphisms associated with class I genes, LMP2 and TAP2 are polygenic. More specifically, we found a unique lineage of LMP2 (LMP2/δ) that shares identity to both LMP2 and δ but is expressed like the standard LMP2. Additionally, two very different TAP2 loci were found, one of which encodes polymorphic alleles. In general, the class I pathway genes are expressed in most tissues, with highest levels in lymphoid tissue. We then analyzed the basic genomic organization of the trout MHC in an isogenic backcross. The main class Ia region does not cosegregate with the class IIB locus, but LMP2, LMP2/δ, TAP1A, and TAP2B are linked to the class Ia locus. Interestingly, TAP2A (second TAP2 locus) is a unique lineage in sequence composition that appears not to be linked to this cluster or to class IIB. These results support and extend the recent findings of nonlinkage between class I and II in a different teleost order (cyprinids), suggesting that this unique arrangement is common to all teleosts. The *Journal of Immunology*, 1999, 163: 774–786.

In mammalian immune systems, the essential function of Ag presentation is achieved by a complex process involving different molecules and different pathways. The final result is the presentation of peptides by the MHC-encoded class I and II molecules to cytotoxic (CD8) and helper (CD4) T cell subsets (1, 2). Class I genes can be divided into two categories (classical or nonclassical), based upon structural, functional, and expression criteria (3, 4). Classical class I H chains (Ia) are broadly polymorphic, are expressed on most cell types and tissues, and present endogenously derived peptides to cytotoxic T cells. In contrast, nonclassical class I genes (1b) display limited polymorphism and are restricted in expression with a function that is beginning to be resolved (5, 6). Class I molecules are heterodimers composed of a variable membrane spanning glycosylated H chain that is noncovalently associated with β2-microglobulin (β2m) for the presentation of endogenously derived peptides. The assembly and loading of class I heterodimers with processed peptide is achieved by several other molecules, some encoded within the MHC and some not, in an overall sequence commonly referred to as the class I pathway.

One of the main locations for peptide generation is the cytoplasm, which contains soluble proteases and the multicatalytic proteasome complex (7). Upon IFN-γ induction, the proteasome housekeeping subunits δ, ε, and MC14 are replaced by low molecular mass polypeptides (LMP) δ 2 and LMP7, which are encoded within the mammalian class II region (8), as well as by MECL1, which is located outside of the MHC (9–12). This reciprocal exchange influences the proteasome’s ability for processing proteins into peptides suitable for class I presentation (13, 14). The processed peptides are then transported from the cytosol into the lumen of the endoplasmic reticulum (ER) by the ATP-dependent heterodimeric TAP1 and TAP2 proteins, which are encoded within the mammalian class II region, intermingled with the LMP genes (15–17). Once transported into the lumen, the peptides are held by the TAPs for delivery to the trimolecular β₂m-calcretin-class-I complex (18). Recent studies have also demonstrated that two categories (permissive or restrictive) of TAP2 present in mammals directly influence the types of peptides that are transported and subsequently loaded onto specific class I molecules (19). After a peptide is properly loaded, the class I-β₂m-peptide complex is brought to the surface for peptide presentation to CD8⁺ cytotoxic T cells (1, 20).

In mammals the majority of these molecules are encoded within a single genetic region, the MHC, the organization of which can vary considerably from one order to another, making it difficult to distinguish the essential from the accessory (21). Genes can vary in number and position, and the functional meaning of linkage within the MHC is poorly understood. Chickens offer a variation on the theme by displaying what has been called a minimal MHC.

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⁵Sequences described in this report have been deposited in GenBank under the following accession numbers: AF002171–2180, AF115518–28, AF115533, and AF115536–5541.
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5 Abbreviations used in this paper: LMP, low molecular mass polypeptide; SSCP, single strand conformational polymorphism; LRPCR, long range PCR; PCR-RSA, PCR restriction site analysis; PFGE, pulse field gel electrophoresis; UAA, UBA, and UCA, teleost class I lineages; DAB, class IIB; gDNA, genomic DNA; β₂m, β₂-microglobulin; UTR, untranslated region; ORF, open reading frame; ER, endoplasmic reticulum; OSU, Oregon State University; clonal line-142; HC, Hot Creek; clonal line-E1B.
where a reduced number of elements can be found (22). Some species of Xenopus demonstrate that the physical linkage of LMP2 to the MHC is not necessary for class I expression (23). Now, teleost fish offer yet another chaotic variation on the MHC architectural design since, so far in the species reported, zebrafish class I and II are not linked (24). Whether the teleost situation reflects an evolutionary accident or simply an intermediate step will require studies in more primitive fish, such as the elasmobranchs (cartilaginous), but also within other teleosts, to see whether important insights are gained.

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### Materials and Methods

#### Animals

Rainbow trout (Oncorhynchus mykiss, ARO-F1, Idaho origin, 2 females crossed with 1 male) were obtained from Aquatic Research Organisms (Hampton, NH) and maintained in 14°C water at the Basel Institute for Immunology. Killing was accomplished using 100 μg/ml MS-222 (Novartis, Basel, Switzerland). Supplementary media and reagents included 2 mM MgCl₂, 200 μM dNTPs, 100–150 pmols of each degenerate primer (TAP2 sense, 5’-GCITCIACRTANCCRTADAT-3’; and antisense, 5’-CARGARCCIGTIYTNTTY-3’; and LMP2 sense, 5’-ATGCCTCTGATGACGGAG-3’; and antisense, 5’-CATGATAGTGTCGGCTAAG-3’), and 1 U of AmpliTaq (Hoffman-LaRoche). The PCR profile was 94°C for 15 s, 45°C for 30 s, and 72°C for 30 s with an additional extension time of 10 min. PCR products were cloned into the pCRII vector according to the manufacturer’s suggestions (TA cloning kit, Invitrogen, San Diego, CA). Forty random clones were sequenced from each amplification and used in a BLASTX search to identify clones containing TAP2, LMP2, and proteasome δ cDNA probes.

### PCR amplification of trout TAP2, LMP2, and proteasome δ cDNA probes

A portion of TAP2, LMP2, and proteasome δ was amplified using sets of degenerate primers based on alignments of available TAP and LMP2/proteasome δ sequences. Briefly, 100 pg (1 μl) of pronephric or thymic cDNA was added to 24 μl of 1× PCR buffer (GeneAmp, Hoffman-LaRoche, Basel, Switzerland) supplemented with 2 mM MgCl₂, 200 μM dNTPs, 100–150 pmols of each degenerate primer (TAP2 sense, 5’-GCITCIACRTANCCRTADAT-3’; and antisense, 5’-CARGARCCIGTIYTNTTY-3’; and LMP2 sense, 5’-ATGCCTCTGATGACGGAG-3’; and antisense, 5’-CATGATAGTGTCGGCTAAG-3’), and 1 U of AmpliTaq (Hoffman-LaRoche). The PCR profile was 94°C for 15 s, 45°C for 30 s, and 72°C for 30 s with an additional extension time of 10 min. PCR products were cloned into the pCRII vector according to the manufacturer’s suggestions (TA cloning kit, Invitrogen, San Diego, CA). Forty random clones were sequenced from each amplification and used in a BLASTX search to identify clones containing TAP2, LMP2, and proteasome δ. These clones were diluted, amplified, and purified using Qiagen PCR columns (Qiagen, Basel, Switzerland), randomly primed (Life Technologies, Gaithersburg, MD) with [³²P]dCTP (Amersham, Arlington Heights, IL) and then used as homologous probes to screen thymocyte and pronephric cDNA libraries.

#### Table 1. Locus-specific primers utilized within this study

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<td>5’-CTGGCTGTGTTTGGCAGAT-3’</td>
<td>A2</td>
<td>(363–386)</td>
<td>Southern</td>
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<tr>
<td>TAP2B*01 (AF15538)</td>
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<td>(517–537)</td>
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<td>(340–360)</td>
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<td>Aβ2</td>
<td>(595–615)</td>
<td>PCR-RSA/Northern</td>
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</table>

* Southern-L designates the TAP1 reverse primer utilized for generating the segregation analysis probe.
PCR amplification of MHCI and -IIB

The polymorphism for class Ia α1 and -α2 and class IIB β1 and -2 was analyzed by PCR, using cDNA libraries and first strand thymic, spleen, and pronephric cDNAs as templates. For the class I analysis, an anchored 3′ reverse primer (5′-ATGAGGGTATTATCCTGTG-3′) and, L2u, 5′-AT GAGGGTATTATCCTGTG-3′) was used in conjunction with a T3 universal primer for the initial amplification of the 5′UTR, leader, α1, α2, and a portion of α3 from a peripheral blood cDNA library. Two different leader sequences were identified and used for this study (L1c, 5′-ATGAGGGTATTATCCTGTG-3′, and L2u, 5′-AT GAGGGTATTATCCTGTG-3′). To amplify the β1 and β2 domains of OSU and HC class IIB, a reverse primer (32) (5′-CCTCAGCCCTCAAGGAG-3′) residing in the connecting peptide/transmembrane region, coupled with a common β1 forward primer, was used to amplify the β1 domain through the TM region from splenic cDNA. For the PCR polymorphism studies, PFU polymerase (Stratagene, La Jolla, CA) was used to minimize mistakes. OSU and HC parental class I cDNAs (UAA*OSU-01 and *HC-01) were amplified from splenic first strand cDNA using the L1c and a common 3′ UTR primer (5′-GTGTTATGT TCTTGAGAAGGT-3′) in conjunction with PFU. All resulting products (class I and IIB) were cloned into pBlunt (Invitrogen) and sequenced.

cDNA library screening

The production of rainbow trout thymocyte and pronephros ZAP Express cDNA library screening

The production of rainbow trout thymocyte and pronephros ZAP Express cDNA libraries has been previously described (35). Approximately 1 × 10⁹ PFUs from each amplified library were lifted in duplicate onto BA-855 filters (Schleicher & Schuell, Keene, NH) and hybridized under stringent conditions (4× SSC, 1% SDS, 0.5% sodium pyrophosphate, 0.5% nonfat milk, and 10% dextran sulfate at 65°C), using either the putative TAP2, LMP2, or protosomae β PCR probes. Filters were then washed (0.5× SSC; 0.5% SDS at 65°C) and exposed to x-ray film for 2 days at ~80°C. Positive plaques were cored and subjected to in vivo excision with the Exassist helper phage to release Bluescript phagemids containing putative full-length TAP2, LMP2, and protosomae β cDNAs. For the isolation of trout TAP1A, TAP2B, and the authentic LMP2, new primer sets were synthesized for probe production. A region (180 bp) displaying similarity to both TAP1A, TAP2B, and the authentic LMP2, new primer sets were synthesized in conjunction with PFU. All resulting products (class I and IIB) were cloned into pBlunt (Invitrogen) and sequenced.

Southern and Northern blot analysis

For standard Southern blotting, 15 μg of gDNA was digested overnight with the indicated restriction enzymes, electrophoresed, transferred to nitrocellulose, and hybridized with the appropriate radiolabeled probes (Table I) as previously described (35). RNA preparation and Northern blotting were performed as previously described (36). Before tissue excision, fish were bled out to remove residual blood from tissues. All Southern and Northern blots were washed at a final stringency of 0.5× SSC/0.5% SDS at 65°C. The trout EtTu-I probe has been previously described (36).

SSCP, PCR-RSA, and LR-PCR linkage assays

Before SSCP or PCR-RSA analyses, regions of the various loci were amplified from parental gDNA, cloned, and sequenced for detecting polymorphism(s) between the OSU and HC founders. These clones served as reference for SSCP analyses, while also indicating unique restriction sites for PCR-RSA assays. For SSCP analyses, 200 ng of gDNA was amplified with [γ-³²P]ATP end-labeled loci-specific primers (Table I) in 15 μl of 1× Geneamp buffer (supplemented with 1.5 mM MgCl₂, 200 μM dNTPs, 2 pmols end-labeled sense primer, 8 pmols of “cold” sense primer, 10 pmols of “cold” antisense primer, and 0.35 α Umila) by PCR using the following profile: 94° for 2 min, followed by 35 cycles of PCR using 94°C for 20 s, 58–63°C for 30 s (depending on the primer set), and 72°C for 30 s. Reactions were mixed 1:1 with SSCP loading buffer (95% formamide, xylene cyanol, and bromophenol blue), heated to 90°C for 3 min, and placed on ice. The samples (1 μl) were then loaded onto 8 or 10% acrylamide gels and electrophoresed at 4 W constant power for 3–4 h at 10°C.

![Figure 1. Phylogenetic relationships among the TAP sequences across vertebrate classes.](http://www.jimmunol.org/)

**FIGURE 1.** Phylogenetic relationships among the TAP sequences across vertebrate classes. Amino acid sequences were aligned (Clustal W), and the unrooted neighbor-joining tree was constructed using TreeView-PPC. Overall the tree demonstrates higher conservation for TAP1 than for TAP2, which could be related to coevolution of TAP2 alleles with class Ia in each species (45). Accession numbers were as follows for TAP1: human (Q03518), mouse (U60020), rat (P36370), gorilla (L76468), Atlantic salmon (Z83327), and shark (AF019835). For TAP2: human (M74447), gorilla (L49034), mouse (M90459), rat (X63854), Xenopus (AF062387), Atlantic salmon (AF93328), and shark (AF019836). Scale bar refers to percent divergence.

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<th>424</th>
<th>455</th>
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*The alignment (Clustal W) was made to highlight residues putatively involved in peptide contact and transport (adapted from Elliot (40)). Residue positions refer to the human TAP2 sequence. Residues specific for rat TAP2a or b (+), peptide binding and/or viral inhibition (%), ATP binding ($), and Mg²⁺ cofactor association (++) are indicated below the alignment (40, 46, 47, 50). –, Identity to human sequence.
DNA fragment conformers were visualized by standard autoradiography. For the class Ia, IIB, and LMP2/6 loci, gDNAs were amplified and digested with parental specific restriction enzymes as indicated for PCR-restriction site analysis (RSA). For RFLP analysis using no linkage between loci as the testing criterion: UAA and DAB = 2.08 (accept, 15 recombinants, n = 39); UAA and LMP2 or LMP2/b or TAP2B = 19 (p < 0.001, reject, no recombinants, n = 19); UAA and TAP1 = 10.3 (p < 0.005, reject, 1 recombinant, n = 14); UAA and TAP2A = 0.54 (accept, 17 recombinants, n = 30); and TAP2A and DAB = 0.13 (accept, 16 recombinants, n = 30). LR-PCR was initiated using various combinations of LMP2 and LMP2/6 forward and reverse primers on gDNA in conjunction with the Elongase system (Life Technologies). The LMP2 exon 1 reverse 104–125 (5’-GCTCTAATGAT TCATCTAACAT-3’) and LMP2/6 exon 6 forward 592–611 (5’-CCTG GCTATGGCACAGACA-3’) primer combination resulted in successful amplification of the intervening region between LMP2-LMP2/6. Products were cloned (pBlunt) and sequenced.

Sequencing, alignments, phylogenies, and modeling
cDNA and gDNA clones were sequenced by dyeoxy chain termination chemistry using universal and gene-specific infrared primers (MWG-Biotech, Ebersberg, Germany) in conjunction with the Thermo Sequenase kit (Amersham). Sequences were processed via an automated sequencer (LI-COR 4000L, MWG). Database searches, alignments, and phylogenies were conducted as previously described (35). Tree drawing was performed with TreeView (http://evolution.genetics.washington.edu) (37), and bootstrapping was accomplished with PHYLIPI V3.572 (http://evolution.genetics.washington.edu) (38). For structural analyses, Swiss PDB (version 3.0 for Power Macintosh) was used to compare putative trout class Ia sequences with the crystal structures of HLA.A28 via the internet server, and models were visualized by RASMOL (R. Sayle; http://expspy.heuge.ch/). Overall, the trout class Ia sequences fold almost identically with the HLA.A28 template, whereas the trout class Ib (31) sequences present a structure with a distorted β sheet floor.

Results and Discussion

Analysis of trout TAP2A, TAP2B, and TAP1A cDNAs

An alignment of available TAP2 sequences was used to identify regions suited for constructing degenerate primers for the amplification of trout TAP2. By using these primers, a portion (357 bp) of trout TAP2 was amplified by PCR from thymic cDNA. This sequence was most similar (e-131) to other TAP2 sequences. The fragment was then labeled and used as a homologous probe to screen a trout thymic cDNA library for a full-length cDNA clone. Four clones were selected for restriction analysis and sequencing. One clone, OnmyTAP2A*01 (AF002180), is full-length (3325 bp) including 90 bp of 5’ untranslated region (UTR) (single TATA and CAAT motif), 713 aa open reading frame (ORF), and 3’ UTR with a polyadenylation signal and tail. Interestingly, exon 1 (135 aa) showed little similarity to the other vertebrate TAP2 sequences (below 20%).

By using a portion of TAP2A*01 that shares identity with the Atlantic salmon and mammalian TAP1 and -2 sequences, two additional TAP2 cDNAs and a single form of TAP1 were isolated by cross-hybridization from a pronephros (anterior kidney) cDNA library (one individual). One of the TAP2 clones appears to be the allele of TAP2A*01, for it is 98.5% and 97% identical in its nucleotide and amino acid composition within the coding region and thus will be designated OnmyTAP2A*02 (AF115537). Overall, TAP2A*02 contains an ORF of 713 aa, which displays 39 nucleotide differences in the coding region, in comparison with 2A*01, which resulted in 12 aa replacements including a 6-bp insertion. The 3’ UTR for both TAP2A genes was 95% identical, and each possessed a single AGo microsatellite repeat at the same location. In addition, a second locus for trout TAP2 was found (OnmyTAP2B*01, AF115538), which is most certainly the homologue of the Atlantic salmon SasaTAP2A locus (39). TAP2B*01 (2, 631 bp) consists of 60 bp 5’ UTR (TATA motif), 724 aa ORF, and 389 bp of 3’ UTR (AG motif not found), including a single polyadenylation site and subsequent tail. TAP2B is 91% identical to the SasaTAP2A sequence whereas OnmyTAP2A was only 60% similar to the Sasa clone. The two putative trout TAP2 loci (A and B) are 61% similar to each other. Overall, TAP2A demonstrates 55% similarity to nonmammalian and 51% to mammalian TAP2 sequences whereas TAP2B shows a higher average similarity (74% and 58%, respectively).

Finally, the full-length OnmyTAP1A*01 (AF115536) cDNA clone (2820 bp) includes 134 bp of 5’ UTR (2 TATA motifs), 739 aa ORF, and 3’ UTR (463 bp) with a single polyadenylation site.

FIGURE 2. Southern blot analysis of trout TAP1A, -2A, and -2B in four individuals. A. The TAP1A probe detects a variety of polymorphic fragments for each enzyme and individual. Arrows indicate bands that overlap with TAP2B. Two to three strong hybridizing bands are evident, suggesting that a second TAP1 locus may be present. B. RFLPs are evident for the TAP2A locus where 1–2 bands per individual are observed, most likely the result of allelic polymorphism. C. The TAP2B-specific probe identifies two basic polymorphic patterns for EcoRI, EcoRV (4,3, and 2-two bands; 1-single band), and HindIII. All probes correspond to exon I of the respective genes, and none of these single domain probes contained internal restriction sites for EcoRI, EcoRV, or HindIII.
just before the poly(A) tail. \textit{OmmyTAP1A}*01 is most similar (e-159) to other TAP1 sequences (55% average similarity to mammalian and 82% to nonmammalian). The strongest homology was to \textit{Sasa}-TAP1A, which is identical in length (ORF) and shares a striking 97% amino acid identity for the coding region. In addition, all trout TAP loci have at least 8–10 predicted ER membrane-spanning domains and a conserved ATP binding region, as well as the highly conserved Walker A and B sites, all of which are consistent with mammalian TAP molecules (40). A phylogenetic analysis using full-length amino acid sequences was used to display the relationships of the TAP sequences among various vertebrate classes. TAP1 and TAP2 sequences group into distinct branches for the mammalian and nonmammalian classes, where each clusters into its own clad (Fig. 1). Overall, the tree is consistent with the expected radiations of these various classes, and the fact that trout TAP2A branches away from the other teleost TAP2 sequences may reflect its possible non-MHC-linked status (see linkage).

\textbf{Comparison of permissive and restrictive residues in vertebrate TAP2s}

In mammals, a functional TAP (TAP1 and -2) heterodimer is essential for efficient class Ia peptide presentation to cytotoxic CD8\(^+\) T cells (40–42). TAPs generally transport short peptides (8–11mers) from the cytosol to the ER lumen for loading onto recently assembled class I molecules, but recent observations demonstrate that some TAP alleles have the ability to transport large peptides (43, 44). Additionally, functional polymorphism has been described for the two rat TAP2 alleles, a and b, which results in permissive or restricted peptide transport (45). The two alleles differ by 25 aa, and some of these residues (217, 218, 262, 265, and 266), which are thought to interact with peptide in the cytoplasm,
are directly involved in the two TAP functional phenotypes (46, 47). In the permissive situation (rat 2a and human), a broad range of peptides can be efficiently transported and loaded, which can satisfy the fundamental peptide requirements of most class I molecules. On the opposite side, restrictive TAP alleles (rat 2b and mouse) preferentially resist the transport and loading of peptides carrying a polar or charged C-terminal anchor (i.e., R).

In rats, TAP2a and -b are linked specifically to the two different class I H chain groups (RT1-A) such that TAP2b can fulfill the needs of RT1-A molecules possessing a minimally charged F pocket (19), thus suggesting coevolution of specific TAP2s with certain types of class I H chains. In one study, mutation of human TAP2 position 374 (A to D) resulted in a switch from permissive to restrictive transport (48). We then aligned TAP2 sequences from rat (a/b), human, mice, and the two trout loci (2A and 2B) to highlight those residues thought to be involved in the two phenotypic classes (Table II). Each of the trout genes contain residues (e.g., 217, 218, 262, 265, 266, 374, and 376) found at positions implicated in the permissive and restrictive mammalian alleles, suggesting that trout are most likely not restricted in peptide transport and loading, although functional studies are needed to validate this assumption. Interestingly, the putative TAP2A alleles (401/402) show 7 of the 12 coding polymorphisms between the predicted 6th-7th and 8th-9th transmembrane domains and within the ATP-binding region, all of which are predicted to reside in the cytoplasm. The functional significance of these polymorphic residues is currently being addressed.

Identification of trout TAP RFLPs

We next conducted Southern blot analysis on four individuals using the first exon of each gene to estimate the copy number and to identify potential RFLPs. As shown in Fig. 2A, all enzymes gave different polymorphic patterns for all individuals using a TAP1 probe. In most cases, three bands were detected, suggesting the presence of an alternative allelic form of TAP1. Nonidentical banding patterns for TAP2A and TAP2B (Fig. 2, B and C) support the notion that these are indeed distinct loci and that each individual contains both loci as well as possible alleles. A rough estimation of the genomic organization for TAP2A*01 was then obtained by using a set of primers based at the beginning of exon 1 and within exon 3, in conjunction with PCR using gDNA. A single product was amplified, sequenced, and compared with the cDNA clone TAP2A*01 to determine the exon-intron boundaries (data not shown). Both intron positions and splice categories for the first (183 bp, type I) and second (151 bp, type II) intron exist in TAP2A, as is found in mammals and the recently reported Atlantic salmon (Sasa) TAP2 gene (39). Using the same primer combination, we isolated this unique TAP2A region and confirmed the exon-intron structure in two other salmonids, the Chinook and Coho salmon. These two species, which diverged from trout 10–20 million years ago, possessed the unique TAP2A exon (data not shown, 95% identity) and shared the same genomic structure of trout, thus demonstrating its presence in other salmonids.

In summary, as in rats and Atlantic salmon, there are polymorphic alleles and loci (salmon) for TAP2 (39, 49). Secondly, a unique form of TAP2 (TAP2A) was found, in which the first exon is quite different (20% similarity) from that of all other reports describing TAP2 sequences, suggestive of a separate ancient lineage. Considering possible allelic variants at all trout TAP loci (heterozygosity), a total of eight different heterodimers could be formed, which no doubt would influence the peptide repertoire selected for efficient transport and subsequent loading onto recently synthesized class I molecules. Moreover, possession of a variety of TAP alleles and loci, as in trout and Atlantic salmon (39), may be beneficial for disease resistance to certain viral pathogens in which TAP function can be specifically inhibited by virally encoded molecules, as found in mammalian studies (50–52).

Characterization of LMP2, LMP2/δ, and δ in trout

Degenerate PCR primers, designed using available LMP2 and δ sequences (human, mouse, rat, and Xenopus), were used to amplify segments from pronephric and thymic first strand cDNAs. A single
FIGURE 5. Class I sequences from trout. A broad range of putative trout class I sequences are polymorphic and display clusters of substitutions. B sheets (β), turns (T), loops (L), and α helices (α) were based upon molecular modeling. Positions putatively involved in N- and C-termini peptide binding for the A and F class Ia pockets are indicated (N or C) above the alignments. Predicted amino acid sequences of four putative class Ia genes from trout aligned with the trout class Ib (Onmy-UAA*-UAA*OK-1, L63542) and human (HLA-A.28, P01891) class Ia sequences. Dots (z) represent identical residues, and dashes (-) define gaps. Residues believed to form salt bridges (p), bind peptides (V), β2 m contact sites (b), or possible CD8 contact sites (s) are indicated.
product of ~460 bp was amplified, cloned, and sequenced. Two distinct sequences emerged from this screening, which displayed 64% identity to each other. Surprisingly, both sequences were most similar to proteasome δ (e-65), followed closely by LMP2 (e-53) cDNA sequences. The two fragments were then used as homologous probes to screen a trout thymocyte cDNA library. Upon restriction analysis of the phagemids, two clones were chosen for further sequence analysis. One, Onmy-δ (819 bp, AF115539), begins with a 671-bp ORF and 145 bp of 3' UTR, including three polyadenylation sites and a poly(A) tail. This clone might not be full-length, though, for, if the first methionine is legitimate, there would be only a short 12-aa leader segment, followed by 333 bp of 3' UTR, with a single polyadenylation site and poly(A) tail. Finally, the coding region of LMP2/δ is polymorphic, for an allelic variant was found that was 94% identical to LMP2/δ (data not shown).

Since the trout LMP2/δ gene appeared to be a distant member of the LMP2 or δ lineages, we constructed new sets of degenerate primers specific for LMP2. A single product of the expected size (~170 bp) was obtained from thymic cDNA, which contained two sequences differing by only 8 noncoding bp. The fragment is most similar (e-220) to the two teleost (medaka and zebrafish, partial) LMP2 sequences, followed by other vertebrate LMP2 genes. This fragment was then used to screen a thymic cDNA library yielding the full-length form of LMP2 from trout. Onmy-LMP2 (1090 bp, AF115541) contains a 654-bp ORF ending in a stop codon followed by 333 bp of 3' UTR, with a single polyadenylation site and tail. Overall, the three trout-coding regions (LMP2, 2/δ, and δ) share ~61% nucleotide and 64% amino acid identity with each other. The 3' UTRs show little similarity among the three genes. A high degree of conservation can be observed among vertebrate LMP2 and δ, including absolute conservation of 62 residues, which increases to 71 if the LMP2/δ sequence is not included (Fig. 3A). Overall, about 16 residues are specific for either LMP2 and/or δ sequences. Interestingly, the LMP2/δ sequence appears to be at a crossroads (Fig. 3B) between LMP2 and δ, for it contains roughly an equivalent number of LMP2, δ, and unique residues. Southern blot analysis of LMP2 and LMP2/δ using probes spanning exons 2–5 (63% identity to each other) revealed a variety of polymorphic bands within the four individuals (Fig. 4). Additionally, several overlapping bands are found for the two genes among the different digests, suggesting a close physical linkage between LMP2 and LMP2/δ. Finally, Southern blot analysis using single domain probes (exon 6 to 3' UTR) suggests the presence of additional loci (data not shown).
Preponderantly we characterized a trout class I H chain gene that by polymorphism, expression, and presence of specific residues appeared to encode an authentic class Ia allele (30). In an effort to identify other polymorphic alleles and to characterize the different class I loci, we used conserved trout class I primers for the isolation of new class I cDNA products. All class I cDNA clones have been classified according to convention, where *Onnmy* defines the genus (*Oncorhynchus*) and species (*mykiss*), UAA (or UBA or UCA) defines class I H chains, and "**XX**" (e.g., "**KD6**" refers to the clone itself (53)). The tentative class I designations (e.g., UAA) refer to sequence lineages and not to whether these sequences are strictly classical or nonclassical in nature. UBA and UCA class I lineages are based upon the presence of specific features, including a unique leader (UBA), a distinct α2 domain (UCA), which is shared with cyprinid fish (30), or further differences found within the connecting peptide (e.g., UCA insertion) and cytoplasmic domains (e.g., UBA truncation).

Overall, 12 different polymorphic class I sequences were obtained from two isogenic lines and four other individuals. To simplify matters, we present an alignment that represents the variety of genes with unique polymorphic α1 and -2 domains (Fig. 5A). Unique deletions, insertions, clusters of replacements, and invariant residues are scattered among the β sheets, turns/loops, and α helices. Thus, in contrast to most vertebrate class I genes where variation is typically limited to peptide-binding domains, the sequences presented here demonstrate variability within both structural and peptide-binding domains. Some of these sequences differ by as much as 50% within an individual for the α1 and -2 domains (e.g. UAA*KD1.5 (AF115523)/UBA*Spu3.1 (AF115526)). In addition, some deletions correspond to regions implicated in peptide binding; thus, these particular alleles may represent lineages similar to the nonclassical genes of mammals. How many class I genes are actually expressed within an individual? We assessed this important question initially by using the two different leader sequences found in UCA*KD2.11 (AF115524) and UBA*Spu3.1 (AF115526) as forward primers in conjunction with a conserved α3 reverse primer. Examination of four individuals for class I expression in the spleen and pronephros by RT-PCR indicates that all individuals express at least two different class I loci per individual, as indicated by the presence of three different sequences. It should be noted that all trout share a common tetraploid ancestor and that multivalents can be observed during meiosis (34, 54); thus, in situ hybridization is needed to clarify whether these different sequences are actually all alleles at a common locus or whether they belong to distinct loci.

Three different full-length class I genes were then isolated from the pronephros library (one individual), including the previously identified UAA*C32 allele (now termed UCA*C32) and two different genes designated *Onnmy-UAA*KD6 (AF115521) (similar to UAA*HC-01 (AF115519)) and *Onnmy-UBA*SP3 (AF115522). Overall, all trout class I cDNAs (including Fig. 5A, but not UAA*OK-1b (31)) have retained residues (UAA*HC-01 numbering Y-7, Y-59, R-84, F-122, T-142, K-145, W-146, and Y-159) believed to be crucial for N- and C-termini peptide contact in class Ia molecules (27), as well as sites involved in salt bridge formations (H-3, D-28, H-92, and D-118) (Fig. 5B), the exception being UCA*C32, where the highly conserved W-146 is encoded by a L. In mammals, position 84 typically encodes a Y residue, but, in all nonmammalian class Ia H chains, an R is found instead. Positions 84, 142, and 146 are thought to form a hydrogen-bonding meshwork for enhanced binding of the C-terminal end of the peptide in mammals (27). The W to L substitution in C32 would probably not alter this scheme since both possess nonpolar side chains. In addition, some residues (12/17) putatively involved in β2m contact in mammals are conserved in trout. These differences could reflect the fact that trout encode two classes of β2m per individual (31), which may have varying specificity for different trout H chains. An exposed loop containing a small stretch of acidic residues within the α3 domain of mammals facilitates binding with coreceptor
CD8, found on cytotoxic T cells (55). Within the alignment, three acidic residues (220-D/E, D-224, and E-226) within this region are well conserved from trout to man. Interestingly, the conserved D-224 is also thought to play a role in the association of the class Ia H chain with TAP and calreticulin (the conserved α1 N-linked glycosylation (CHO) site needed for the latter) (56). Finally, phosphorylation sites S-323 and S-326 are conserved (57). Overall, looking at the variety of polymorphic sequences there are roughly four to five groups for the α1 domain, four groups for the α2 domain, two different α3 groups (four sequences), and two to three unique connecting peptide/transmembrane/cytoplasmic domain groups, suggesting that a wide range of allelic combinations is possible. Recently, the evolutionary relationships of fish class I H chains have been reviewed, which suggests that the sequences presented within this study were likely derived from two distinct ancestral lineages (58, 59). It should also be noted that particular α1 domains are not restricted to specific α2 domains (Fig. 5A); thus, a broad repertoire of peptide-binding pockets is present in trout. To follow up further on the trout class I-associated polymorphisms, Southern blot analysis was performed. A UAA*KD6 α3 probe detects seven to eight bands on a typical Southern blot (Fig. 5C), supporting the presence of multiple class I loci. Most of these bands also cohybridize with α1 and -2 probes derived from two of these individuals, corresponding probably to other authentic class I loci. In addition, PFGE analysis suggests the presence of independent class I loci not linked to the main class I region (UAA*OSU-01/HC-01, see linkage) (J. D. Hansen, P. Strassburger, and L. Du Pasquier, manuscript in preparation).

**Tissue-specific mRNA expression**

Little is known regarding the expression of MHC-related genes in teleost fish. We previously demonstrated that the class I UAA*C32 gene is expressed in a ubiquitous manner similar to class Ia genes (30) in higher vertebrates. Northern blot and RT-PCR analyses were conducted on a variety of trout tissues using domain-specific probes and primers (Fig. 6). An α1 domain shared by several class I genes, including UAA*C32, -*KD6, -*HC-01, and -*OSU-01, is expressed in most tissues, with highest levels in the intestine followed by the other major lymphoid tissues, with weak expression in the muscle and liver. In this species, the thymus, kidney, (pro)-nephros, and possibly the spleen are the primary lymphoid tissues (36). Using this probe, two messages most likely corresponding to genes such as UAA*C32 and UAA*KD6 were detected, where the full length transcripts differ by ~500 bp. Since the UBA*SP3 α1 domain possesses both a unique leader and a 5-aa deletion corresponding to a peptide-binding site (Y-59), we examined its expression as well. Overall, individuals encoding this allele express it in a manner similar to UAA*KD6 and UCA*C32 (data not shown), suggesting that it is most likely a classical transcript. In the class I alignments (Fig. 5, A and B), three to four distinct α2 lineages are evident; we selected the α2 domain found in UAA*KD6 (UAA*HC-01 type) as an α2 probe since it was associated with a variety of α1 domains. Expression was strongest in the spleen, kidney, and thymus, followed by the intestine. Overall, UAA*KD6, UBA*SP3, and UCA*C32 are expressed in a parallel manner with trout β2-m (31).

**TAP1A** is expressed in all tissues, with highest levels in the spleen, kidney, thymus, intestine, heart, and testis. Surprisingly, a truncated message is apparent in the liver, which could be due to an alternative polyadenylation site, although none are present in TAP1A*01. Northern blot and RT-PCR analyses of TAP2A and -B demonstrate that TAP2B is expressed in all tissues at much higher levels than TAP2A (data not shown). We next examined the expression of the three proteasome-related loci: LMP2, LMP2/δ, and δ. Both LMP2 and LMP2/δ are expressed in a nearly identical fashion, with highest expression in the trout lymphoid tissues, similar to the class I alleles, which is consistent with LMP expression in other model systems. As expected, δ is expressed in a ubiquitous manner (although high in the testis, like TAP), agreeing with its general role as a housekeeping proteasome gene. Finally, we show that the trout class IIB genes are mainly expressed in lymphoid tissue, with highest expression in the spleen and intestine, followed by the thymus and kidney. The different transcripts most likely correspond to DAB alleles possessing long and short 3′ UTRs (32). Looking at the expression patterns of UAA*KD6, UBA*SP3, and UCA*C32 (30), coupled with the presence of specific residues and polymorphic variants of each, these three genes appear to meet the general criteria of classical class I molecules. As a whole, members of the class I pathway (UAA, TAP, and LMP) and DAB are expressed in a similar manner, basically being limited to the major teleost lymphoid tissues, consistent with their biological roles.

**Linkage analysis of the trout MHC**

We employed several methods of linkage analysis to obtain an initial representation of the MHC architecture in trout. In the previous section, we outlined that trout possess a vast array of class I loci and alleles. We therefore used the major class Ia splenic transcripts (UAA*OSU-01 and -*HC-01, 91% nt coding region identity) of the OSU and HC parental strains (diploid, isogenic (33, 34)) as the class I reference markers for our segregation study. As a starting point, we analyzed the linkage of the class I (UAA) and IIB (DAB) loci using both SSCP and PCR-RSA cosegregation analysis from F1 gDNAs that were generated by a backcross (OSU female × OSU/HC male hybrid). As shown in Fig. 7A, cosegregation of Onmy-UAA*OSU/HC-01 and Onmy-DAB was not
found by using either SSCP or PCR-RSA analyses (>37% recombinants, n = 39). As a control, Onmy-DAB β1 and β2 domains cosegregated for every individual as expected. We have not determined whether UAA*OSU-01 and -HC-01 compose a single locus (i.e., allelic partners in heterozygotes) or whether they represent distinct loci, which is a possibility.

We were then able to demonstrate that LMP2 and LMP2/δ do cosegregate with the class I region (Fig. 7A). Coupled with the observation of overlapping RFLPs (Fig. 4), it was quite possible that LMP2 and LMP2/δ were physically linked to one another in relation to the class Ia region and that LMP2/δ or LMP2 arose via a gene duplication event. Using LR-PCR, we determined that LMP2/δ and LMP2 are ~1.5 kbp apart in the same transcriptional orientation, suggestive of an ancient, tandem duplication event. We then determined that TAP2B (Fig. 7A) is linked to the class Ia region and that TAP1A (Fig. 7B) is also part of this region. Interestingly, TAP2A is not closely linked to the class I region nor is B2 (J. O. Sunyer, J. D. Hansen, and J. D. Lambris, unpublished observations), a putative representative of the trout class III region (66). From linkage data alone, class I (UAA*OSU/HC-01), TAP2A (>50% recombinants to both UAA and DAB), and DAB could all be possibly located on different linkage groups. Finally, δ is not linked to the class I or II regions, as determined by segregation (data not shown). A summary of the linkage analysis is shown in Fig. 8.

From amphibians through mammals, the class I, II, and III regions are physically linked, forming the MHC proper. In mammals, the LMP and TAP loci are within the class II region (17) whereas, in Xenopus, analysis of F1 recombinants suggest that these genes are situated in closer proximity to the single class Ia locus than to class II (67). Recently, MHC mapping studies in the teleost Danio rerio (zebrafish) demonstrated that the zebrafish class I and II regions mapped to different linkage groups (24) and possibly to different chromosomes, although formal proof (in situ hybridization) of the latter has yet to be demonstrated. In addition, the zebrafish LMP2/7 and TAP2 genes were found to be linked to the class I region, in contrast to that which is found in mammals (68, 69). In medaka, LMP2 and -7 are closely linked, but not to B2, a class III member (70, 71). Combining the zebrafish, medaka, and, now, trout data, it appears that the LMP2/7 and TAP1/2 complex has been maintained as a linkage entity for more than 350 million years. Thus, there must be a selective advantage for expression and coevolution as a unit where the whole locus may be more transcriptionally active following induction (i.e., IFNs) during an infectious response (44).

The close proximity of genes involved in the class I processing is conserved in teleost fish, anuran amphibians, and perhaps birds (23, 67, 68, 72–74). It makes better sense that components of the class I pathway segregate and coevolve as a physical unit, as is found in teleosts, Xenopus, and birds. This observation makes the mammalian gene organization look like an accident. Recently, we found in teleosts, medaka, and, now, trout data, that it appears that the LMP2/7 and TAP1/2 complex has been maintained as a linkage entity for more than 350 million years. Thus, there must be a selective advantage for expression and coevolution as a unit where the whole locus may be more transcriptionally active following induction (i.e., IFNs) during an infectious response (44).

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Note added in proof. It came to our attention that two other teleosts display a lack of linkage between class I and Iib loci (79, 80).

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References


