Classical MHC Class I Genes Composed of Highly Divergent Sequence Lineages Share a Single Locus in Rainbow Trout (Oncorhynchus mykiss)

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The classical MHC class I genes have been known to be highly polymorphic in various vertebrates. To date, putative allelic sequences of the classical MHC class I genes in teleost fish have been reported in several studies. However, the establishment of their allelic status has been hampered in most cases by the lack of appropriate genomic information. In the present study, using heterozygous and homozygous fish, we obtained classical-type MHC class I sequences of rainbow trout (Oncorhynchus mykiss) and investigated their allelic relationship by gene amplification and Southern and Northern hybridization analyses. The results indicated that all MHC class I sequences we obtained were derived from a single locus. Based on this, a unique polymorphic nature of the MHC class I locus of rainbow trout has been revealed. The mosaic combination of highly divergent ancient sequences in the peptide-binding domains is notable, and the variable nature around the boundary between the α3 and transmembrane domains is unprecedented. *The Journal of Immunology, 2002, 168: 260 –273.

The identification of these polymorphic MHC genes and also of TCR and Ig genes (6) indicates that the acquired immune system is already complex at the level of fish, although current data indicate that jawed fish form the most primitive group with an MHC/TCR system (7).

High sequence variability in MHC class I genes has been reported for such fish species as zebrasfish (8), cichlids (9), guppy (10), Atlantic salmon (11), the coelacanth Latimeria chalumnae (12), and shark (13), and apparent sequence lineages could be noticed in some cases. The classical nature of class I molecules includes high allelic polymorphism, the conservation of important amino acid residues revealed in the mammalian classical MHC class I molecules, and expression in most tissues. Due to the lack of appropriate assay systems in fish, classical MHC class I function has not been clearly shown to date. In addition to plausible classical MHC class I genes, apparent nonclassical genes and related pseudogenes were also described for fish (14, 15). Besides the unresolved question about the classical or nonclassical functions of the fish genes, a major impediment in understanding the fish MHC class I system is that most studies do not reveal whether the observed sequence variability is attributed to allelic polymorphism or to variability among sequences derived from different loci. A good exception is the study of Okamura et al. (13), which showed high sequence variability connected to a single locus in shark. Many other studies on the variability of fish classical MHC class I have been limited to the α1 and α2 domains without extensive analysis on allelic relationship.

In rainbow trout, classical MHC class I sequences have been described with several full open reading frame sequences (16–18) and a number of sequences encompassing one or both of the α1 and α2 regions (14, 17). Furthermore, nonclassical MHC class I sequences of rainbow trout have also been reported (18, 19). However, the number of the classical MHC class I loci has not been clearly addressed in the previous studies. Although the existence of at least two loci was expected based on the observation that three classical-type class I genes could be amplified by RT-PCR and also could be isolated from a cDNA library from a single fish, more analyses should be performed to reach the conclusion (17).
The sequence information focusing on the α1 and α2 regions did not address the number of loci clearly, although the existence of two classical loci, A and UA, has been proposed based on the sequence variability (14). Our present study defined a single expressed classical MHC class I locus in rainbow trout that exhibits a unique polymorphic nature.

Materials and Methods

Animals used

Nine outbred rainbow trout (Oncorhynchus mykiss) were used in this analysis: fish W1 and W2 of strain Westralian (20), fish N1 and N2 of strain Nagano (from the Nagano Prefectural Experimental Station of Fisheries, Nagano, Japan), and fish T1, T2, T3, and T4 of strain Tamaru (from the National Research Institute of Aquaculture, Tamaki, Japan). Five different homozygous clones were obtained from strain Nagano: AA1, AB2, AB5, AB7, and C25. The homozygous isogenic trout were produced by gynogenesis in two generations by suppression of mitosis and meiosis in the female. Adult clones were produced by the cross between isogenic trout of the two classical loci, not addressing the number of loci clearly, although the existence of two loci was clearly indicated in previous reports (20). The homozygous clones were obtained from strain Nagano: AA1, AB2, AB5, AB7, and C25. The homozygous isogenic trout were produced by gynogenesis in two generations by suppression of mitosis and meiosis in the female. Adult clones were produced by the cross between isogenic trout of the two classical loci, not addressing the number of loci clearly, although the existence of two loci was clearly indicated in previous reports (20).

PCR primers and probes

The positions of primers and amplified fragments are indicated in Fig. 1. For initial amplification of the α2 domain, degenerate primers were derived from conserved regions for intramolecular disulfide bonds in MHC class I genes in chicken (GenBank accession no. X12780), frog (L.0733), and human (K02883): pMix-α2f, 5'-ACTGTGACCCARRINGHTATG GNTGT (D = A + T + G, H = A + C + T, N = A + C + G + T, R = A + G, Y = C + T) (forward) and pMix-α2r, 5'-ACTGTCAGCYYTNA RCCAYTDATRCA (reverse). The 5'-RACE PCR was performed using primer p4-α2r1 derived from the Onmy-UBA*α401 α2 region, 5'-CTCATCATTCCACCAACCC TACAT (reverse), and anchor primer pVig, 5'-TGGCGCGCCGCTCA GAAACTAGTGGATC derived from vector pZAP-αII (Stratagene, La Jolla, CA).

For full-length cDNA amplification, 3'-RACE PCR was performed with forward primers derived from the 5'- and 3'-untranslated regions (UTR) of Onmy-UBA*α401 or Onmy-UBA*αC32 and anchor primer NorⅠ dT18 (first-strand cDNA synthesis kit; Pharmacia Biotech, Uppsala, Sweden). The forward primers were pIV-5f, 5’-ATAACACCTTACCATGAGGCGGC from Onmy-UBA*α401, p5-6f, 5’-CTTATGGAGGATACATACTTCTG from Onmy-UBA*αC32. PCR products were cloned, and for every amplified fragment 18 clones were sequenced to screen for possible gene or allele variation. The sequences could be divided into lineages and sublineages. Minor differences within a sublineage were not due to sequencing mistakes, as confirmed by an independent PCR analysis performed for all fish.

Sequence analysis

For detection of lineage-specific expression, single-stranded cDNA was synthesized from purified mRNA by using the NorⅠ dT18 primer (Pharmacia Biotech) and a single-strand cDNA synthesis kit (Pharmacia Biotech). For 5'-RACE PCR, a first cDNA library was constructed from isolated mRNA by use of pd(N)6 primer (Pharmacia Biotech), the Time Saver kit (Pharmacia Biotech), and the AZAP II cloning system (Stratagene). In all cases, manufacturers' recommendations were followed.

Genomic DNA of rainbow trout was purified from the pectoral fin tissue by phenol extraction (22).

Conditions of PCR from cDNA or genomic DNA

The general conditions for all PCR amplifications using cDNA were 100-μl volume, 2.5 U of KOD DNA polymerase (TOYOBO, Tokyo, Japan), KO buffer, 0.2 mM dNTPs, and 125 nM of each primer.

Amplification of the α2 domain of MHC class I from an AA1 fish with degenerate primers was performed on single-stranded cDNA from 50 ng of mRNA. PCR conditions were 94°C for 3 min, then 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and finally 72°C for 10 min.

The 5'-RACE PCR was performed on double-stranded cDNA with AZAP II arms obtained from 50 ng of mRNA. PCR conditions were 94°C for 3 min, then 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and finally 72°C for 10 min.

For expression analysis with specific primers, PCR was performed on single-stranded cDNA from 50 ng of mRNA. PCR conditions were 94°C for 3 min, then 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min, and finally 72°C for 10 min.

Sequence analysis

PCR products were cloned into PCR-script Amp SK+ vector (Stratagene). The nucleotide sequences were determined by the dyeoxy chain termination method using a dRhodamine terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and suitable primers; subsequent analysis was performed with an automated sequencer (ABI PRISM 377 DNA sequence; Applied Biosystems). Comparison of nucleotide sequences and deduced amino acid sequences was performed using the programs Search Homology, Multiple Alignment, Create Text Document, and unweighted pair-group method with arithmetic mean (UPGMA) of GENETYX version 9.0 (Software Development, Tokyo, Japan) computer software.

Southern and Northern blot analysis

For both Southern and Northern blot analysis, digoxigenin-labeled probes were made, and hybridization, washing, and detection were performed by using the Dig Lumin Detection Kit (Boehringer). The manufacturer's recommendations were followed.

For Southern blot analysis, genomic DNA was digested by the restriction enzyme PstI, subjected to electrophoresis in 0.8% agarose gels, and blotted onto a nylon membrane. After hybridization with the probes, the stringency of washing was twice for 15 min with 1× SSC/0.1% SDS at 60°C and once with 0.2× SSC/0.1% SDS at 65°C.
68° C. For Northern blot analysis, total RNA from specific tissues was isolated by TRIzol (Life Technologies, Grand Island, NY) and electrophoresed through a 1% agarose/10% formalin gel, and then blotted onto a nylon membrane. After hybridization with the probes, the stringency of washing was twice for 15 min with 2× SSC/0.1% SDS at 68°C.

Results and Discussion
Isolation of MHC class I genes from rainbow trout

When we started this study several years ago, the isolation of MHC class I genes from the rainbow trout was not reported. Thus, we tried to isolate MHC class I genes from this species and initially succeeded to amplify a 240-bp fragment from a kidney cDNA library of the clonal homozygous strain AA1 by use of degenerate primers based on the conserved α2 domain motifs in the classical MHC class I genes of other vertebrates. The amplified fragment, denoted Onmy-UBA*401-α2, showed homology with the α2 domain of the MHC class I genes of other species, and this was regarded as a part of a candidate for a classical MHC class I gene as it encodes, in this domain, the conserved amino acid residues critical for the interaction with a bound peptide. Then we conducted 5′-RACE PCR to obtain the 5′ region, Onmy-UBA*401-5′end and found that the α1 domain sequence of this gene has some characteristic features that are somewhat deviated from those of the typical classical MHC class I sequences (Figs. 1 and 2, discussed later). More investigation was necessary to obtain a clear picture of the classical MHC class I genes of the rainbow trout.

In the present study, nine outbred rainbow trout belonging to three different strains plus five different homozygous clonal fish derived from one of these strains by artificial gynogenesis were analyzed for the MHC class I sequences. Because the comparison of Onmy-UBA*401-5′end (5′ UTR, leader, α1, and α2 domain sequences) with the subsequently published rainbow trout MHC class I sequence Onmy-UCA*352 (16) showed sequence differences in all domains (Fig. 1), we prepared two kinds of 5′ primers, pIV-5′f and pL-5′f, based on our gene and C32, respectively, to conduct 3′-RACE PCR. Using these two primers, we obtained 2- to 2.3-kb fragments from all fish investigated. Unexpectedly, in all cases, only PCR with either pL-5′f or pIV-5′f was successful. For homozygous fish, all 18 clones isolated from each fish appeared to be identical, except for single nucleotide changes in individual clones probably caused by PCR error. In most outbred fish, two truly different sequences were detected. A total of 22 independent PCR products was analyzed. The sequences contain open reading frames of approximately 1.1 kb with homology with known classical MHC class I genes in all regions: the leader peptide; the α1, α2, and α3 domains; and the transmembrane (TM) and the cytoplasmic (CY) regions. The 22 PCR products revealed 10 different sequences. Based on >99% nucleotide identity, they were divided into seven sublineages that were designated Onmy-UBA*1, *2, *3, *4, *5, *6, and *7. The individual sequences of a sublineage were numbered Onmy-UBA*101, *102, and so on. Table I shows the sequences detected in the fish investigated. Fig. 1 compares the nucleotide sequences of single representatives of the sublineages. The homology between sublineages varies between 73 and 98% nucleotide identity. Within one sublineage, the full-length cDNAs show only one to two nucleotide changes in the α2 or 3′ UTR (Fig. 1).

Variability between sublineages seems to occur in all regions and appears to be caused by several mechanisms, including replacement, deletion, and insertion. The most noticeable differences between sublineages in the α3 region, TM/CY region, and 3′ UTR are frequent insertions or deletions. Small direct repeats such as the AC repeats in the 3′ UTR (Fig. 1) seem to be hot spots for mutations.

To confirm that in all fish analyzed only one or two of the different sublineages were expressed, five sublineage-specific primer sets were derived from the 5′ and the 3′ UTR, as described in Materials and Methods, and fish mentioned in Table I were tested for fragment amplification. Both positive and negative PCR data obtained (not shown) fully matched the data derived from sequencing full-length cDNA clones, as described above. This means that probably none of the seven sublineages has escaped detection when expressed.

The pink salmon sequence Ongo-UA-(92H) and the Atlantic salmon sequence Sasa-p30 show high homology with the Onmy-UBA sequences in all their domains, including the 3′ UTRs (Fig. 1).

Analysis of deduced amino acid sequences

Fig. 2 shows a comparison of the deduced amino acid sequences of representatives of the seven sublineages described in this study with classical MHC class I molecules from different phylogenetic groups. Atlantic and pink salmon are other salmonid species, carp and zebrafish are cyprinids, and sharks belong to the elasmobranchii; the function of their indicated MHC class I molecules has not been shown, but their classical nature is indicated by sequence analysis. It is noticeable, though, that the depicted zebrafish sequence Brre-UBA*01 lacks the highly conserved Y7 residue (see below) and therefore possibly represents a nonclassical MHC class I molecule.

In all the molecules of Fig. 2, some cysteine residues are conserved, and there is a N-glycosylation site at position 86 (residue numbers mentioned in this section are in agreement with Fig. 2). A similar structure of the depicted proteins is also expected by conservation of some salt bridges described for human MHC class I (23) in the Onmy-UBA molecules: H3-D28, R41-E61 (except in Onmy-UBA*401), H92-D118, K143-D147, K157-E161 (only in Onmy-UBA*601 and *701), and E166-K169 (D166-K169 in Onmy-UBA*201 and *301). Conserved for mammalian classical MHC class I molecules are the amino acids Y7, Y59, Y84, T142, K145, W146, Y159, and Y171, which are involved in binding the termini of the MHC class I-bound peptide (24). The nonmammalian molecules depicted in Fig. 2 show almost complete conservation of these amino acids, except for Y84, which is changed into R84 in fish, amphibian, and bird MHC class I molecules. Other exceptions are the lack of Y59 in Onmy-UBA*401 and the presence of L146 instead of W146 in Onmy-UBA*701. Variability in the residues at position 59 or 146 has also been described for some alleles of human HLA-B and HLA-C (25). Concerning the binding of human CD8 to MHC class I, several investigators described the importance of an acidic stretch in the α3 domain (26, 27). Although not identical to those in humans, the Onmy-UBA sequences all contain an acidic stretch at this position (Fig. 2). Of the three residues (Q114, D121, and E127) in the α2 domain shown to affect CD8 binding in human MHC class I (28), Q114 and D121 have been completely conserved in the trout sublineages. Also, residues that may interact with β2m as F8, T10, V24, Q95, Q114, D118, G119, D121, E229, D235, and Q239 (23) are conserved in mouse (not shown), human, and rainbow trout (Fig. 2).

An additional indication that the Onmy-UBA sequences represent classical MHC class I molecules is the distribution of variability. Whereas the α3 regions of the different sublineages share >73% amino acid identity, within the α1 and α2 regions this can be as low as 49 and 53%, respectively. The variability is especially high at those positions involved in binding antigenic peptides and determining their specificity in humans (indicated as P in Fig. 2) (29). The average number of detected amino acids in the α1 and α2
FIGURE 1. (continues)
FIGURE 1. (continues)
domains of the Onmy-UBA sequences is 1.6 at non-P positions and 2.6 at P positions.

Most interestingly, Onmy-UBA*601 and *701 have 14-aa extensions around the carboxyl terminus of the H9251 domain, which has already been described for the trout sequence Onmy-UCA*C32 (16). For Onmy-UBA*501, a similar small extension is found as described for the Atlantic salmon sequence Sasa-p30. Onmy-UBA*101, *201, *301, and *401 have no extensions as has pink salmon Ongo-UA-(92H). Thus, not only does the length of this region vary between salmonid species, but also probably within one locus in one species. Because the residues of the extensions are largely hydrophilic, they are probably located extracellularly and may influence the distance of the H9251 domain to the membrane.

Whereas the H9251 2 region of the sublineages Onmy-UBA*1, *2, *3, *4, and *5 are more similar to those detected in other salmonids, the H9251 2 regions of Onmy-UBA*6 and *7 are more closely related to those detected in the cyprinid sequences depicted in Fig. 2. They will be discussed below as type I and type II H9251 2 regions, respectively.

Several full-length rainbow trout classical-type MHC class I sequences were reported previously, and they are similar to our Onmy-UBA sequences. Those are Onmy-UCA*C32 (16), FIGURE 1. Nucleotide sequences of the trout MHC class I cDNAs Onmy-UBA*101, *201, *301, *401, *501, *601, *701, and Onmy-UCA*C32 (16); the pink salmon Ongo-UA-(92H) (35); and the Atlantic salmon Sasa-p30 (11). Alignment is performed by computer software and modified by hand. Dots indicate identical nucleotides. Dashes indicate gaps in the sequences. The amino acid sequence of the Onmy-UBA*101-encoded protein is indicated above the second nucleotides of codons. Not indicated are the sequences Onmy-UBA*102 (differs from Onmy-UBA*101 by having an AC insertion at position 1333), Onmy-UBA*502 (differs from Onmy-UBA*501 by substitutions of C and A at positions 509 and 1172 by T and C, respectively), and Onmy-UBA*503 (differs from Onmy-UBA*501 by having the A at position 1172 substituted by C). The locations of various primers are shown, the name and direction of the primers are indicated with arrows, and the sequences of the primers are thinly underlined. Onmy-UBA*401-UTR contains the positions 400–625 of Onmy-UBA*401, and Onmy-UBA*401-5′ end contains the positions 1–433 of Onmy-UBA*401. AC repeats in the 3′ UTRs are underlined and described as such. The trout MHC class I sequence Onmy-UCA*C32 is identical to Onmy-UBA*701, except for extensions at the 3′ and 5′ UTRs. The polyadenylation signals probably used by Onmy-UBA*701 and Onmy-UCA*C32 are boxed. The open reading frames of Onmy-UBA*101 and Onmy-UBA-OSU*01 (17) are identical, but there is a single nucleotide difference in the partly sequenced (65-bp) 3′ UTR of Onmy-UBA-OSU*01 (data not shown). Onmy-UBA*401 appears to be identical to Onmy-UBA*SP3 (17), except that Onmy-UBA*SP3 has a 274-bp insertion in its 3′ UTR and the poly(A) tail is connected at a slightly different (4 nt apart) position (data not shown). The border between the α3 and TM/CY domains as depicted in Fig. 2 is based on the intron-exon organization in this region, as determined for the sequences Onmy-UBA*401, *501, and *701 (data not shown). Near the end of the α3 domain sequence of Onmy-UBA*501, a sequence composed of eight bases (AGACCAAC) was noticed, and the region containing this sequence has been deleted in Onmy-UBA*101, and it appears to be duplicated in Onmy-UBA*701. Thus, the DNA region containing this sequence might have been involved in the formation of some structures to induce a deletion or a duplication of the DNA sequence, resulting in the shortened α3 domain and the elongated one, respectively.

![FIGURE 1. Nucleotide sequences of the trout MHC class I cDNAs Onmy-UBA*101, *201, *301, *401, *501, *601, *701, and Onmy-UCA*C32 (16); the pink salmon Ongo-UA-(92H) (35); and the Atlantic salmon Sasa-p30 (11). Alignment is performed by computer software and modified by hand. Dots indicate identical nucleotides. Dashes indicate gaps in the sequences. The amino acid sequence of the Onmy-UBA*101-encoded protein is indicated above the second nucleotides of codons. Not indicated are the sequences Onmy-UBA*102 (differs from Onmy-UBA*101 by having an AC insertion at position 1333), Onmy-UBA*502 (differs from Onmy-UBA*501 by substitutions of C and A at positions 509 and 1172 by T and C, respectively), and Onmy-UBA*503 (differs from Onmy-UBA*501 by having the A at position 1172 substituted by C). The locations of various primers are shown, the name and direction of the primers are indicated with arrows, and the sequences of the primers are thinly underlined. Onmy-UBA*401-UTR contains the positions 400–625 of Onmy-UBA*401, and Onmy-UBA*401-5′ end contains the positions 1–433 of Onmy-UBA*401. AC repeats in the 3′ UTRs are underlined and described as such. The trout MHC class I sequence Onmy-UCA*C32 is identical to Onmy-UBA*701, except for extensions at the 3′ and 5′ UTRs. The polyadenylation signals probably used by Onmy-UBA*701 and Onmy-UCA*C32 are boxed. The open reading frames of Onmy-UBA*101 and Onmy-UCA-OSU*01 (17) are identical, but there is a single nucleotide difference in the partly sequenced (65-bp) 3′ UTR of Onmy-UAA-OSU*01 (data not shown). Onmy-UBA*401 appears to be identical to Onmy-UBA*SP3 (17), except that Onmy-UBA*SP3 has a 274-bp insertion in its 3′ UTR and the poly(A) tail is connected at a slightly different (4 nt apart) position (data not shown). The border between the α3 and TM/CY domains as depicted in Fig. 2 is based on the intron-exon organization in this region, as determined for the sequences Onmy-UBA*401, *501, and *701 (data not shown). Near the end of the α3 domain sequence of Onmy-UBA*501, a sequence composed of eight bases (AGACCAAC) was noticed, and the region containing this sequence has been deleted in Onmy-UBA*101, and it appears to be duplicated in Onmy-UBA*701. Thus, the DNA region containing this sequence might have been involved in the formation of some structures to induce a deletion or a duplication of the DNA sequence, resulting in the shortened α3 domain and the elongated one, respectively.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)
Onmy-UAA*KD6, *HC-01, *OSU-01, Onmy-UBA*SP3 (17), and Onmy-UBA*0101 (18), and the similarity can be extended to the 3' UTR sequences. The variability in the /H9251 1 and /H9251 2 sequences of rainbow trout class I sequences reported by Miller et al. (14) and by Hansen et al. (17) is also within the range of the variability we detected in our Onmy-UBA sequences. The classical nature of the class I sequences of rainbow trout was examined in the previous studies (16–18) and was suggested based on their expression in various tissues and the presence of predicted key amino acid residues known to be conserved in the classical MHC class I molecules of other vertebrates. The classical nature of our Onmy-UBA sequences is also apparent because of their expression profiles (see below), high variability in the α1 and α2 domains, and the conservation of some critical amino acids.

Ongoing studies of the Onmy-UBA locus, including those of MHC class I-restricted cytotoxicity, up-regulation of mRNA expression by viral infection, and the class I protein expression at the cellular level using a mAb, further support the conclusion that the UBA locus is a classical MHC class I locus.

Phylogenetic comparison

Fig. 3 shows a UPGMA analysis of the extracellular domains of the seven sublineages compared with known probable classical
FIGURE 2. Predicted amino acid sequences of MHC class I molecules of rainbow trout and other vertebrates. Representatives of the Onmy-UBA lineages, Sasa-p30 (11), Ongo-UA*02 (35), Cyca-UA*01 (36), Brre-UA*01 (8), Trsc-UA*101 (13), Xela-UA*01f (37), Gga-B-F12 (38), and HLA-A2 (39). Alignment is performed by computer software and modified by hand. Numbering of amino acids is performed for the Onmy-UBA*101 sequence, starting from the N1 domain. Dashes indicate identical amino acids. Asterisks indicate gaps in the sequence. Downward-pointing arrowheads indicate the conserved positions believed to interact with antigenic peptide termini in mammals (24), and upward-pointing arrowheads indicate conserved positions believed to form salt bridges (23) or to interact with CD8 (28). The positions believed to interact with the antigenic peptide and determine its specificity in mammals (29); C, conserved cysteine residues; the box at position 86, a conserved glycosylation site; the hooked line denoted CD8, the acidic domain believed to interact with CD8 in humans (26). The UBA*401 sequence has a deletion of two amino acid residues, including the conserved Y59 compared with the corresponding region of the mammalian molecules. Some flexibility and compensation may exist in MHC class I molecules in terms of both the structure around this region and the hydrogen bonding to a bound peptide through a conserved residue.

Table I. The MHC class I cDNAs detected

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<td>C25</td>
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into lineages Sal-MHCia*A-D by classifying their extracellular regions into different groups. The sequences Onmy-UBA*101, *201, and *301 can be classified into the same lineage as pink salmon Ongo-UA*-92H, and Onmy-UBA*501 and Atlantic salmon Sasa-p30 group together. This definition of lineage is chosen, as recombination events creating different combinations of α1 and α2 groups seem to have been an important cause of polymorphism. For example, the sequences Onmy-UBA*101 and *701 have a completely identical α1 region, but a very different α2 region, with differences beginning at the domain border (Fig. 1). And Onmy-UBA*601 and Onmy-UA*A5.1 (17) have an identical α2 domain, but a very different α1 domain.

**Southern blot analysis**

Even for a homozygous clonal fish, more than nine bands can be detected in Southern blot analysis when using a α3 probe (data not shown), indicating a number of similar genes or pseudogenes. Therefore, the α3 probe was not suited to identify the Onmy-UBA locus, and locus identification was performed with α2 probes.

Genomic DNA of all the nine outbred and the five homozygous fish used in this study was digested with PsI and used for Southern blot analysis. For all sublineages, probes derived from the α2 region were made. The hybridization pattern of all type I α2 probes (from Onmy-UBA*101, *201, *301, *401, or *501) was identical; Fig. 4I was obtained with probe pr3-α2 derived from Onmy-UBA*501. The hybridization pattern of all type II α2 probes (from Onmy-UBA*601 or *701) was also identical; Fig. 4II was obtained with probe pr6-α2 from Onmy-UBA*601. The patterns indicate that under the washing conditions used, all type I α2 probes can recognize all genomic fragments with a type I α2 region, and all type II α2 probes can recognize all type II α2 regions in the genome; there seemed to be no cross-reaction between the two types of α2 regions.

Type I α2 probes only hybridized to one restriction fragment for homozygous fish; in outbred fish, either none, one, or two fragments were detected (Fig. 4I). Hybridization fragments appeared sublineage specific. For fish expressing Onmy-UBA*1 or *2, a 2.4-kb; Onmy-UBA*3, a 2.3-kb; Onmy-UBA*4, a 4-kb; and Onmy-UBA*5, a 1.9-kb fragment was detected, respectively. In cDNA sequences of fish T4, no type I α2 region could be detected, and also Southern blot analysis could not detect such a region. The type II α2 probes detected one fragment of 2.4 kb in every fish (Fig. 4II). For the fish T1, T4, and T5, which were shown by cDNA analysis to express sublineages Onmy-UBA*6 and/or *7, a second fragment of 2.8 kb was found. Because the combined type I and II α2 probes detected only one polymorphic fragment per haploid genome set, the most probable explanation is that all the seven sublineages detected by cDNA analysis belong to the same locus, designated Onmy-UBA.

To clarify the nature of the 2.4-kb band detectable in all fish as shown in Fig. 4II, we tried to amplify novel α2 sequences from genomic DNA by the use of degenerate primers. We succeeded in isolation of a new α2 DNA fragment and named its gene Onmy-UFA. As the new α2 sequence can be classified as a type II α2, it should correspond to the 2.4-kb fragment in Fig. 4II. It turned out to be closely related to reported sequences such as Onis-HN-1 and Sasa-UA*8 (Fig. 5). However, this Onmy-UFA fragment has defects in coding an intact MHC class I protein; a deletion of a DNA segment resulting in a frameshift and the presence of a stop codon could be noticed in the sequence (Fig. 5). We amplified this Onmy-UFA fragment from various fish, and the sequence analyses revealed its nonpolymorphic nature. Thus, every fish possesses this Onmy-UFA, as revealed in the Southern hybridization (Fig. 4II),
and its expression cannot be detected (see below, Fig. 6), in agreement with the mutational defects in the sequence.

Tissue-specific expression
Ubiquitous tissue expression is an indication for the classical nature of MHC class I genes (30). Fig. 6 shows the hybridization pattern of probe pr4-a2 (α2 domain of Onmy-UBA*401) with total RNA isolated from kidney (including head kidney), brain, gill, heart, liver, spleen, intestine, gonad, and muscle of an AA1 fish. For all tissues, expression of a 2.4-kb transcript was detected, but whereas for tissue from gills or intestine a strong signal was found, expression in liver or muscle was very low. The signals around 1.8 kb observed in intestine and gill are likely to be produced by the presence of ribosomal RNA. To date, we could not amplify shorter transcripts. The similarity of the two transcripts recognized by the 3′ probe pr4-a3 and probably pr4-a2 is consistent with the obtained sequence data, except that in an allele-specific way the locus may give rise to two transcripts. A possible explanation for the two transcripts is the use of different polyadenylation signals, as suggested by comparison of Onmy-UBA*401 and Onmy-UBA*701 (Fig. 1).

The observed variability in transcripts is not uncommon for MHC class I; in the frog Xenopus laevis transcripts of divergent lineages differ in size, and alternative use of polyadenylation signals has been found (31).

#### Table II. Lineage organization of salmonid MHC class I sequences

<table>
<thead>
<tr>
<th>Rainbow trout: Onmy-UBA*101, *201, *301, H9251</th>
<th>a1</th>
<th>a2</th>
<th>a3</th>
<th>Lineage Sal-MHCla*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onmy-UAA*KD6, *HC-01, *OSU-01</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>A</td>
</tr>
<tr>
<td>Rainbow trout: Onmy-UBA<em>401, Onmy-UBA</em>SP3</td>
<td>IV</td>
<td>I</td>
<td>I</td>
<td>B</td>
</tr>
<tr>
<td>Rainbow trout: Onmy-UBA*501</td>
<td>I</td>
<td>I</td>
<td>II</td>
<td>C</td>
</tr>
<tr>
<td>Atlantic salmon: Sasa-p30</td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>D</td>
</tr>
<tr>
<td>Rainbow trout: Onmy-UBA*601, *701, Onmy-</td>
<td>II</td>
<td>I</td>
<td>I</td>
<td>E</td>
</tr>
<tr>
<td>UCA*C32</td>
<td></td>
<td></td>
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<tr>
<td>Rainbow trout: Onmy-UA*b13, *K19</td>
<td>I</td>
<td>I</td>
<td></td>
<td></td>
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<tr>
<td>Rainbow trout: Onmy-UA*b4.10, Onmy-</td>
<td>I</td>
<td>II</td>
<td></td>
<td></td>
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<td>UCA*KD2.11</td>
<td></td>
<td></td>
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<tr>
<td>Rainbow trout: Onmy-UA<em>A4.10, Onmy-UA</em></td>
<td>II</td>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UAA*KD4.5, *SP1.3</td>
<td></td>
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<tr>
<td>Rainbow trout: Onmy-UA*A4.3, Onmy-UA</td>
<td>III</td>
<td>II</td>
<td></td>
<td></td>
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<tr>
<td>UCA*KD2.9</td>
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<tr>
<td>Rainbow trout: Onmy-UA*B3, *K18</td>
<td>IV</td>
<td>I</td>
<td></td>
<td></td>
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<tr>
<td>Onmy-UAA*Spu3.1</td>
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<tr>
<td>Rainbow trout: Onmy-UA*A5.1</td>
<td>IV</td>
<td>II</td>
<td></td>
<td></td>
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<tr>
<td>Rainbow trout: Onmy-UA*A4.11, Onmy-UA</td>
<td>V</td>
<td>I</td>
<td></td>
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<tr>
<td>UAA*KD1.5</td>
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<tr>
<td>*6, *7</td>
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<tr>
<td>Rainbow trout: Onmy-A*1, *2, *3</td>
<td>I</td>
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<tr>
<td>Rainbow trout: Onmy-UA*A1</td>
<td>II</td>
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<tr>
<td>Zebrafish: Brre-UA*01</td>
<td>V</td>
<td>II</td>
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<tr>
<td>Carp: Cyca-UA*01</td>
<td>VI</td>
<td>II</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Zebrafish: Brre-UBA*01</td>
<td>VII</td>
<td>II</td>
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* Roman numbers are derived from Fig. 3. The full-length salmonid sequences are organized into lineages Sal-MHCla*A–D by classifying their extracellular regions into different groups.

Sublineage-specific transcripts
Because sequence analysis indicated length differences between transcripts of the several sublineages, this was investigated further by Northern blot analysis for total RNA of the kidney (including head kidney) of 10 of the fish described. The α3 probe pr4-α3 indeed detected major transcripts of 2.1–2.9 kb (Fig. 7A). Except for the 2.9-kb transcripts for fish T1 and T4, the sizes correlated well with the obtained Onmy-UBA sequence data for each fish. Interestingly, in several fish determined by cDNA analysis to express only one Onmy-UBA sublineage, a minor transcript somewhat larger than the major transcript was detected (in fish N1, N2, AB2, AB7, and C25). Presence and length of the minor transcript also correlated with the Onmy-UBA sublineages detected by cDNA sequence analysis, and therefore might be derived from the same locus. The type I α2 probe pr4-α2 reacted more specifically than in Southern blot analysis, and recognized transcripts of the sublineages Onmy-UBA*2, *3, and *4 (Fig. 7B). The minor and major transcripts of fish AB2 (Fig. 7A) are both recognized by probe pr4-α2 (Fig. 7B), indicating their similarity. The type II α2 probe pr6-α2 recognized two transcripts for sublineages Onmy-UBA*6 and probably Onmy-UBA*7 (Fig. 7C); comparison with fish N1 and N2 indicates there is no hybridization with Onmy-UBA*101 transcripts. The similarity of the two transcripts recognized by the pr6-α2 probe was confirmed by probe pr6-3′ UTR derived from the 3′ UTR of Onmy-UBA*601 (Fig. 7D). The recognized bands in Fig. 7, B–D, were identical to those recognized by the α3 probe (Fig. 7A).

The Northern blot analysis in the present study seems to agree with the sequence data, except that in an allele-specific way the locus may give rise to two transcripts. A possible explanation for the two transcripts is the use of different polyadenylation signals, as suggested by comparison of Onmy-UBA*C32 and Onmy-UBA*701 (Fig. 1).
Allelic relationship of the MHC class I genes isolated from rainbow trout

Despite a great number of MHC class I sequences reported for teleost fish, interpretation of the data with respect to allelic polymorphism has been limited in most cases by lack of locus identification. To date, a few groups reported the MHC class I sequences from rainbow trout (14, 16–18). However, the MHC class I locus (or loci) of this species has not been clearly identified; therefore, allelic relationships of the obtained sequences have not been clarified. Our results defined a single classical MHC class I locus of rainbow trout and its unique allelic polymorphism.

In the present study, we concluded that all our MHC class I sequences are derived from the same locus, namely, Onmy-UBA, based on the following observations: 1) the PCR amplification of cDNAs with appropriate primers revealed a single sequence from a homozygous clonal fish, and essentially two sequences from an outbred fish (Table I); 2) the Southern blot data are basically consistent with the presence of a single expressed classical MHC class I sequence per haploid genome. Namely, the α2 domains of all the MHC class I cDNA sequences isolated by RT-PCR could be assigned to the sublineage-specific polymorphic bands in the Southern blot analysis (Fig. 4); and 3) the Northern blot data revealed that the isolated MHC class I cDNAs could represent the expressed MHC class I genes (that can hybridize with the α3 probe) whose transcripts exhibited sublineage-specific variations in length (Fig. 7). Thus, these results led us to a conclusion that the MHC class I cDNA sequences we obtained belong to a single expressed MHC class I locus, although they include sequences highly divergent to each other.

It should be mentioned that we could not draw our conclusion only through the amplification studies, because we could not strictly exclude a possibility of some fortuitous amplifications for

![FIGURE 4](image-url)  
Southern blot analysis with α2 probes. Genomic DNA of all fish mentioned in Table I was digested by restriction enzyme PstI and electrophoretically separated on 0.8% agarose, 10 µg/lane. After transfer, nylon membranes were either hybridized with a type I α2 probe, pr5-α2 (I), or a type II α2 probe, pr6-α2 (II). Identical fragments were found for fish expressing identical sublineages. Therefore, the type I α2 regions are thought to be located on a 2.4-kb PstI fragment for Onmy-UBA*1 and *2 (1, 2), on a 2.3-kb fragment for Onmy-UBA*3 (3), on a 4-kb fragment for Onmy-UBA*4 (4), and on a 1.9-kb fragment for Onmy-UBA*5 (5). The type II α2 regions of Onmy-UBA*6 and *7 (6, 7) are located on a 2.8-kb PstI fragment. The numbers of the sublineages are indicated at the right of the figure. The probe pr6-α2 also hybridized to a 2.4-kb Onmy-UBA fragment (F).

![FIGURE 5](image-url)  
Comparison of the Onmy-UBA fragment with other α2 type II sequences. Dots indicate identical nucleotides. Dashes indicate gaps in the sequences. An identical fragment could be amplified from each fish investigated (W2, AA1, and C25). Compared with the other α2 type II sequences, Onmy-UBA*101 has a 38-bp deletion, resulting in a frame shift, and a stop codon that interrupts the homologous open reading frame (TAG at position 86). An even higher homology than with the reported rainbow trout α2 type II sequences was found with the α2 domains of the chinook salmon (Oncorhynchus tshawytscha) sequence Ons-HN-1 and the Atlantic salmon (Salmo salar) sequence Sasa-UA*8. These sequences were amplified by Miller et al. (14, 40), and neither expression nor the presence of a full-length open reading frame has been shown. However, those sequences are probably derived from a polymorphic locus, because closely related sequences have been detected in the same species (14, 40).
Mainly based on the sequence characteristics of the exist in the rainbow trout genome by some other investigators. The number of classical MHC class I loci in rainbow trout from different loci (31). Those genes of different lineages were speculated to be derived /H11032 nopus loci may have come together through genetic interactions. In 1). The genes with distinct lineages that used to exist at different /H9251 3, TM/CY, and 3/H11032 pr4-2 probe UTR sequences can be observed at a single locus of rainbow trout. By Hansen et al. (17), three MHC class I sequences were also isolated from a cDNA library made from a single fish (Onmy-UBA*32, -UAA*KD6, and -UBA*SP3) (17). As it has been speculated that the ancestor of salmonid fish has experienced the tetraploidization, and multivalents have been observed at meiosis (33), its possible effect on the number of MHC class I genes was mentioned by these authors, and the allelic relationship of the three sequences remains to be determined. It should be mentioned that the classical-type MHC class I sequences reported by other investigators, including those three just mentioned above, should be detected with the methods used in our study. Thus, it would be interesting to compare more details of our results with those of others to examine the discrepancy. It also should be mentioned that our study does not exclude a possibility of the existence of some other expressed classical MHC class I genes that possess α3 domain sequences distinct from (namely, not cross-hybridized with) those of Onmy-UBA genes.

**Polymorphic nature and ancient lineages at the MHC class I locus of rainbow trout**

With respect to the mode of the variability in the peptide-binding domains, basic mutational mechanisms such as point nucleotide substitutions may be observed by comparing the allelic sequences possessing the same sequence lineage (Fig. 1). We could observe polymorphism at the amino acid positions that could interact with a bound peptide (Figs. 1 and 2). Thus, the basic mutation and selection mechanism found in the MHC class I genes of the other vertebrates also seem to be operating at this locus.

Importantly, however, mosaic combinations of the sequence lineages (discussed later) associated with the α1 or α2 domains were impressively observed in the peptide-binding domains (Table II). We detected in our rainbow trout three types of combinations of sequence groups, namely I/II, I/IV, and IV/I, for the combination of the α1/α2 domain lineages. In Table II, we showed mosaic structures of the peptide-binding domains not only in our sequences, but also in the rainbow trout sequenced reports by others, that may well belong to the Onmy-UBA locus described in the present study. The apparent domain shuffle has plausibly been created by intra- or interlocus recombination between sequences with divergent lineages that had been established by various mutational mechanisms over a long period of time.

In Table II, some ancient lineages can be discerned in the peptide-binding domains. The Atlantic salmon and the Pacific salmon (including rainbow trout and pink salmon) were estimated to diverge from each other approximately 20 million years ago, and the ancestors of rainbow trout and of pink salmon approximately 10 million years ago (14). As discussed by Hansen et al. (16), the group II lineage of the α2 domain could be found both in rainbow trout and in fishes of cypriniformes (carp and zebrafish). Therefore, it was suggested that the formation of the traits of this lineage predated the separation of these two orders of fish groups (16), which may have occurred more than 130 million years ago (34). Subsequently, the existence of this lineage was also confirmed in other salmonid fishes, namely, coho, chinook, and Atlantic salmon (14). Furthermore, it was noticed that this lineage partly shares its characteristics with the coelacanth sequence Lach-UA-01 (14).

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**FIGURE 7.** Sublineage-specific transcripts. Northern blot analysis: 15 μg of total RNA isolated from the kidney (including head kidney) of fish N1, N2, W2, AB2, AA1, AB5, AB7, C25, T1, and T4 was electrophoretically separated in 1% agarose/10% formalin gels. After transfer, the nylon membranes were hybridized with the Onmy-UBA*401-derived α3 probe pr4-α3 (A), the Onmy-UBA*401-derived a2 probe pr4-α2 (B), the Onmy-UBA*601-derived a2 probe pr6-α2 (C), and the Onmy-UBA*601-derived 3’ UTR probe pr6-3’ UTR (D). The same membrane with intermediate washing was used subsequently for C, B, and A, D. Obtained from an independent blot. As size markers, ribosomal RNAs are indicated.

The number of classical MHC class I loci in rainbow trout

To date, two classical MHC class I loci have been considered to exist in the rainbow trout genome by some other investigators. Mainly based on the sequence characteristics of the α2 domain, one group expected two classical type class I loci, A and UA in salmonid fish genome (14), which have been originally found by Grimholt et al. (11) in Atlantic salmon and by Hansen et al. (16) in rainbow trout, respectively. However, our present results revealed that MHC class I sequences with highly distinct lineages can be observed at a single locus of rainbow trout. By Hansen et al. (17), three MHC class I sequences were also amplified from a single fish in RT-PCR experiments, suggesting the existence of at least two loci. Furthermore, it was reported that three MHC class I sequences were also isolated from a cDNA library made from a single fish (Onmy-UCA*32, -UAA*KD6, and -UBA*SP3) (17). As it has been speculated that the ancestor of salmonid fish has experienced the tetraploidization, and multivalents have been observed at meiosis (33), its possible effect on the number of MHC class I genes was mentioned by these authors, and the allelic relationship of the three sequences remains to be determined. It should be mentioned that the classical-type MHC class I sequences reported by other investigators, including those three just mentioned above, should be detected with the methods used in our study. Thus, it would be interesting to compare more details of our results with those of others to examine the discrepancy. It also should be mentioned that our study does not exclude a possibility of the existence of some other expressed classical MHC class I genes that possess α3 domain sequences distinct from (namely, not cross-hybridized with) those of Onmy-UBA genes.

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**TABLE II.** Some ancient lineages can be observed at a single locus of rainbow trout. The genes with distinct lineages that used to exist at different loci have come together through genetic interactions. In X. 1o r...
Hansen’s group (17) also reported two sequences with the group V lineage of the α1 domain (Onmy-UA*A4.11 and Onmy-UA*A*KD1.5) from rainbow trout. We noticed that this lineage corresponds to that of the α1 domain of zebrafish Brre-UAA*01 (Fig. 3 and Table II), although not mentioned previously (17). We expected that the sequences with the group V lineage in the α1 domain would also belong to the Onmy-UBA locus reported in the present study, as the reported rainbow trout sequences with this lineage possess the group I α2 sequences that are exclusively associated with the Onmy-UBA locus in our study. Whereas these rainbow trout sequences possess a V/I combination for the α1/α2 domain lineages though, the sequence Brre-UAA*01 exhibits a V/III combination. Considering the mosaic pattern of the α1 and α2 lineages in UBA, we anticipated that a V/II combination could be found in rainbow trout as well, which was confirmed by our recent data (our unpublished observation).

Close relationship between the group I of the α1 domain in the salmonid fish and those in the neoteleost fish, such as guppy (the order cyprinodontiformes), cichlid (perciformes), rockfish (scorpaeiformes), and stickleback (Gasterosteiformes), has been noticed (reviewed in Ref. 14). The group I of the α2 domain also shares the sequence characteristics with the α2 domain sequences from the neoteleost fish (also reviewed in Ref. 14). Therefore, it is supposed that these lineage groups were present in the common ancestor of the salmonid and the neoteleost fish.

Although the group IV of the rainbow trout α1 domain has not been observed in cyprinid fishes or in fishes of other orders, this group may be ancient because of its divergent characteristics compared with the other lineage groups in this domain. Actually, the group IV sequence shares some characteristic amino acid residues, namely, G14, I15, E16, I32, D33, and Y34 with Cyca-UA*A*01 sequence (Fig. 2), and it also shares these residues, except D33, with the sequence from a wild guppy (Pore-UA-W4.4) (10). Some of these residues could also be found in the group II, III, and V, but not in the group I sequences of the α1 domain.

Because the study of the variability of MHC class I genes in cyprinid fishes has still been limited, it is possible that groups apparently related to the group I or the group IV in the α1 domain and those related to the group I in the α2 domain are present in cyprinid fishes.

The observation that highly divergent lineages exist at a MHC class I locus has precedents in other vertebrates. Namely, in banded houndshark (Triakis scyllia), two divergent lineages of α2 sequences are present at an MHC class I locus (13). One of them can be observed at another locus, and the interaction between the two loci has been suggested. At the Xenopus MHC class I locus, the existence of two ancient lineages has been reported (31), and the divergence of those lineages, supposedly originally belonging to different loci, was estimated to occur more than 100 million years ago comparing with the divergence between the human and mouse MHC class I sequences. It is also conceivable that, in rainbow trout or in its ancestors, highly distinct sequence lineages had been produced at different loci, then those lineages came to exist at a single locus, namely Onmy-UBA, through the genetic interaction between those loci. Thus, at a single MHC class I locus of various vertebrates, the appearance of ancient distinct lineages that possibly had been originally formed at distinct loci has been observed. And in some cases, mosaic structures of the peptide-binding regions have been produced with distinct domain lineages or sequence motifs derived from them.

**Comparison with a recently reported study**

After the present study was submitted, a study by Shum et al. (41) reporting the diversity of the rainbow and brown trout MHC class I genes appeared. The most important claim of their study relevant to the present one is that a single trout MHC class I locus exhibits high polymorphism with some ancient divergent lineages and the apparent shuffling of the α1 and α2 domains, as observed in our study. However, the most critical point, that the MHC class I sequences from the respective fish species obtained in their study are derived from a single locus, appears not to be substantiated. The result that they did not obtain by PCR more than two sequences from an individual fish (actually only a single sequence in 12 of 25 rainbow or brown trout) is compatible with, but does not demonstrate, the allelic relationship of the sequences. In the present study, we stressed the importance of the allelic relationship of the isolated sequences on which the sequence comparison was based.

**Concluding remarks**

We established the allelic relationship of the isolated rainbow trout MHC class I sequences by PCR and Southern and Northern blot analyses, and also by using homozygous fish. Because highly divergent sequence lineages turned out to exist at this locus, such various extensive analyses should be necessary to draw a conclusion concerning allelic relationship of the isolated genes. The genes derived from this single locus, Onmy-UBA, essentially exhibit characteristics of classical MHC class I genes, although some members show divergent features. These rainbow trout MHC class I genes exhibit a broad range of variability. In addition to the polymorphism caused by nucleotide substitutions and insertions/deletions of nucleotides, the polymorphism based on mosaic combinations of the α1 and the α2 domain sequences with ancient lineages has been revealed. Thus, the present study clarified interesting combinatorial variations at a single rainbow trout MHC class I locus that should be an important characteristic of polymorphism in the peptide-binding domains and presumably broaden peptide-binding repertoire in this species. The example of rainbow trout presents a notable unique case of extensive domain shuffle at the MHC class I peptide-binding region in the vertebrates. The antiquity of some of the sequence lineages has been directly indicated by the presence of those sequence lineages in distantly related orders of fish groups. Finally, unprecedented variability at the α3/TM boundary region of rainbow trout MHC class I genes is noteworthy.

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