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Isolation of carp genes encoding major histocompatibility complex antigens

(polymerase chain reaction/major histocompatibility complex class I gene/major histocompatibility complex class II gene/fish/evolution)

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ABSTRACT **In the evolution of the adaptive immune system unique to vertebrates, teleost fish occupy the critical position. This is the most primitive class of lower vertebrates in which the capacity for acute allograft rejections can be demonstrated, thus suggesting the presence of major histocompatibility complex (MHC) antigens and, therefore, T cells. Here, we report the identification of two putative MHC-antigen-encoding sequences in the carp *Cyprinus carpio*. One, identified as *TLAIα-1*, had reasonable homology to MHC class I heavy chains of mammalian and avian species, while the other, identified as *TLAIIβ-1*, was homologous to MHC class II β chain of the aforementioned higher vertebrates. For these isolations of fish MHC genes, we have identified two highly conserved amino acid sequence blocks surrounding two cysteine residues in the second domain of MHC class II β chains as well as the third domain of class I heavy chains of humans, mice, and chickens. Two kinds of mixed oligonucleotide probes corresponding to these two regions were synthesized. The carp genomic DNA was subjected to amplification by polymerase chain reaction using the above two synthetic DNA fragments as primers. Subsequently, two different DNA sequences sandwiched by these primers were isolated from the amplified products. Their use as secondary probes led to the identification of *TLAIα-1* and *TLAIIβ-1*. We also discuss the applicability of the above approach for isolation from lower vertebrates of other genes belonging to the immunoglobulin superfamily as well as the evolutionary origin of vertebrate MHC antigens.**

The major histocompatibility complex (MHC) of mice and humans has been extensively characterized at the protein and gene levels (reviewed in refs. 1 and 2). In these species, MHC gene loci are clustered in a region spanning more than 2000 kilobases (kb) and they are numbered in the dozens. Each MHC class I molecule is a heterodimer consisting of a heavy chain, which is an integral membrane protein encoded by one of the above-noted MHC gene loci, and a small noncovalently associated β_2-microglobulin, which is encoded by a gene located on another chromosome. MHC class II molecules are also heterodimers but consist of α and β chains, both of which are encoded by genes in the MHC region. Both MHC class I and class II are involved in the immune response at various levels. The MHC class I heavy chain contains three extracellular domains. The membrane-proximal domain consists of multistranded antiparallel β-sheet bilayers in what is called the immunoglobulin fold (3). The two domains distal to the membrane, on the other hand, form a platform of eight antiparallel β-strands topped by α-helices (4, 5). The two domains located at the N termini of MHC class II αβ heterodimers appear to form a platform similar to that of MHC class I molecules (6).

Auffray's group (7, 8) has recently succeeded in isolating MHC class II β-chain genes as well as class I heavy chain genes from the chicken. The tertiary structures of these chicken MHC antigens appeared to be quite similar to those of mammalian MHC, as deduced from their sequences (7, 8). Although their genes are yet to be isolated from amphibians, the presence of MHC molecules has been well documented not only by analyses of biological phenomena but also by immunoprecipitation with alloantisera (9). The presence of MHC molecules in teleost fish, on the other hand, has only been suggested by several indirect results such as acute allograft rejection (10–12), mixed lymphocyte reaction (13–15), and *in vitro* antibody responses (16). Three functionally distinct leukocyte subpopulations were isolated in catfish, which may correspond to B cells, T helper cells, and macrophages (16). The putative presence of T cells also implies the presence of MHC antigens. Elasmobranchs, in sharp contrast, do not show acute allograft rejection (reviewed in ref. 17). Although these observations suggest that the immune system of teleost fish is more mammal-like than that of elasmobranch fish, the presence of MHC genes has not been shown in any of the teleost species.

Cross-hybridization with available DNA probes has proven effective in isolating closely related genes, yet mammalian as well as avian MHC probes were useless for isolation of teleost MHC genes. Therefore, we adopted a strategy that utilizes the polymerase chain reaction (PCR) (18), and we succeeded in isolating putative MHC genes of the carp.§

MATERIALS AND METHODS

Carp (*Cyprinus carpio*) genomic DNA was prepared from peripheral red blood cells by the published method (ref. 19, chap. 9). Two primers, TGYT(C/A)NGTGACNGRY-TTCTAYCC and AGRCT(T/G)G(T/G)RTGCTCCACNT-GRCA (N = A, T, G, or C; Y = T or C; R = G or A), were produced by a DNA synthesizer (Applied Biosystems). PCR was performed as described in ref. 18. Briefly, a 100-μl reaction mixture contained 1 μg of genomic DNA, 1 μM each primer, 200 μM each dNTP, and 2.5 units of DNA polymerase from *Thermus aquaticus* in 10 mM Tris·HCl (pH 8.4)/50 mM KCl/2.5 mM $MgCl_2$ and gelatin at 200 μg/ml. The mixture was subjected to 50 cycles of amplification in a Perkin–Elmer/Cetus Thermocycler: 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C. After fractionation by electrophoresis through a 4% agarose gel (agarose L and S, 3:1, Wako Pure Chemicals, Osaka, Japan), the amplified DNAs of around 190 base pairs (bp) were cloned in Bluescript vectors (Stratagene). Two kinds of clones were obtained and named

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Abbreviations: MHC, major histocompatibility complex; PCR, polymerase chain reaction.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M37106 and M37107).

KI and KII. A carp genomic library was constructed by partial digestion with *Sau*3AI followed by ligation with λ dash vectors and was screened with KI and KII as probes (ref. 19, chap. 2). The nucleotide sequence was determined by the chain termination method using the Bluescript vectors and Southern hybridization was carried out using the multi-prime-labeling system and nylon membranes (ref. 19, chaps. 9 and 13).

RESULTS

Strategy for Isolation of Carp MHC Genes. We adopted a strategy that utilizes PCR for isolating carp MHC genes. The second domain of MHC class II β chain and the third domain of class I heavy chain molecules contain intramolecular S—S bonds. Fig. 1 compares the amino acid sequences surrounding these two cysteine residues in class I heavy and class II β molecules of chickens, mice, and humans (7, 8, 24). LXCXXXXFYP and YXCXVXHXXL appeared to be the consensus sequences of these two conserved regions. Moreover, random substitutions were not observed at the positions designated here by Xs. In the PCR, even if the sequences of the primers do not perfectly match those of the target gene, a particular DNA can be amplified as long as several nucleotides of the primer 3′ ends are complementary to the template DNA. We considered codons for each amino acid residue to prevent amplification of irrelevant DNA fragments and chemically synthesized two kinds of mixed oligonucleotide primers based mainly on class II β chain sequences as shown in Fig. 1.

PCR was carried out on carp genomic DNA with these two primers. Only one major band, around 190 nucleotides, was observed after 50 cycles of amplification (Fig. 2*a*). The size of this band was similar to that in mice and humans. The band was eluted from the gel and inserted into the Bluescript vector. Nucleotide sequence analysis (Fig. 3) indicated that at least two kinds of DNA fragments had been amplified. Because one, identified as KI, contained a *Hin*fI site, while the other, identified as KII, contained a *Bgl* II site, the amplified fragments were digested with *Bgl* II, *Hin*fI, or both. As shown in Fig. 2*b*, major parts of the amplified 190-bp band consisted of these two kinds of fragments. Southern hybridization analysis of carp DNA with cloned KI and KII fragments as probes indicated that KI and KII represent different sets of DNA fragments and that several DNA fragments whose sequences are similar to those of either KI or KII exist in the carp genome (Fig. 2*c*). Since the putative amino acid sequences deduced from the nucleotide sequences of the KI and KII DNA fragments displayed some homology with those of MHC molecules of higher organisms (see later), the following analyses were carried out, using KI and KII DNAs as probes.

Isolation of an MHC Class I Heavy Chain Gene from Carp. A carp genomic library was screened with KI DNA as a probe, and one of the positive clones, λTLAI-1, was extensively characterized. It contained two regions which gave strongly positive signals with KI probe. As the restriction maps surrounding these two regions were similar to each other (Fig. 4*a*), we concluded that almost identical sequences were duplicated on this DNA fragment, and we determined the nucleotide sequence of 6.5 kb of DNA, including one of the KI probe-positive regions. This region contained a sequence almost identical with that of KI. To find possible coding regions, we applied the algorithm developed by Fickett (25) to the analysis of the sequence and identified three putative exons. Fig. 4*b* shows the nucleotide sequence of these putative exons and the deduced amino acid sequence. We refer to this gene as *TLAIα-1*. As shall be discussed later, we concluded that this gene encodes an MHC class I heavy chain.

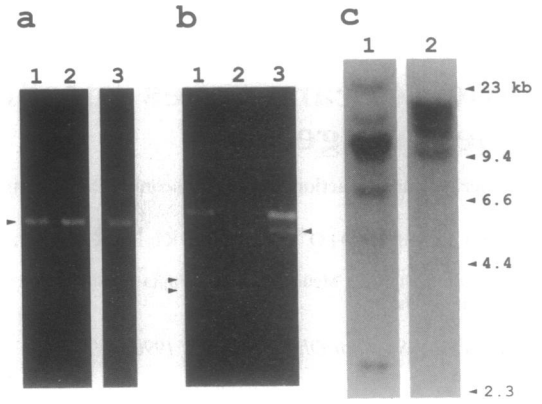

FIG. 2. Characterization of PCR products. (*a*) Human placenta DNA (lane 1), BALB/c mouse liver DNA (lane 2), and carp red blood cell DNA (lane 3) were used as template DNAs for PCR. Major amplified bands, ≈190 bp long, are indicated by an arrowhead. (*b*) Major products, ≈190 bp, amplified from carp DNA were digested with *Hin*fI (lane 1), both *Hin*fI and *Bgl* II (lane 2), or *Bgl* II (lane 3). Arrowheads indicate the positions of the digested DNAs. (*c*) Southern hybridization analysis. Carp genomic DNA was digested with *Eco*RI and electrophoresed through a 0.8% agarose gel. DNA was then blotted onto nylon membranes and Southern hybridization was performed, using KI and KII as probes. The membranes were washed with 4× SSC/0.05% SDS at 42°C (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7). The sizes of fragments from *Hin*dIII-digested λ phage DNA are indicated.

Isolation of an MHC Class II Gene from Carp. Since the putative amino acid sequence encoded by the KII fragment seemed to have greater likeness to amino acid sequences of class II than to those of class I, we expected the KII-containing DNA to encode a class II molecule. The genomic library was screened with KII DNA as a probe and one of the positive clones, λTLAII-1, was characterized. Only one region in this clone gave a strongly positive signal with the KII probe. Determining the nucleotide sequence of 2.5 kb of DNA including the KII probe-positive region, we identified two putative exons in this region (Fig. 5*a*). Fig. 5*b* shows the nucleotide sequence of these exons and the deduced amino acid sequence. We refer to this gene as *TLAIIβ-1*. A comparison of the amino acid sequence with the sequences of

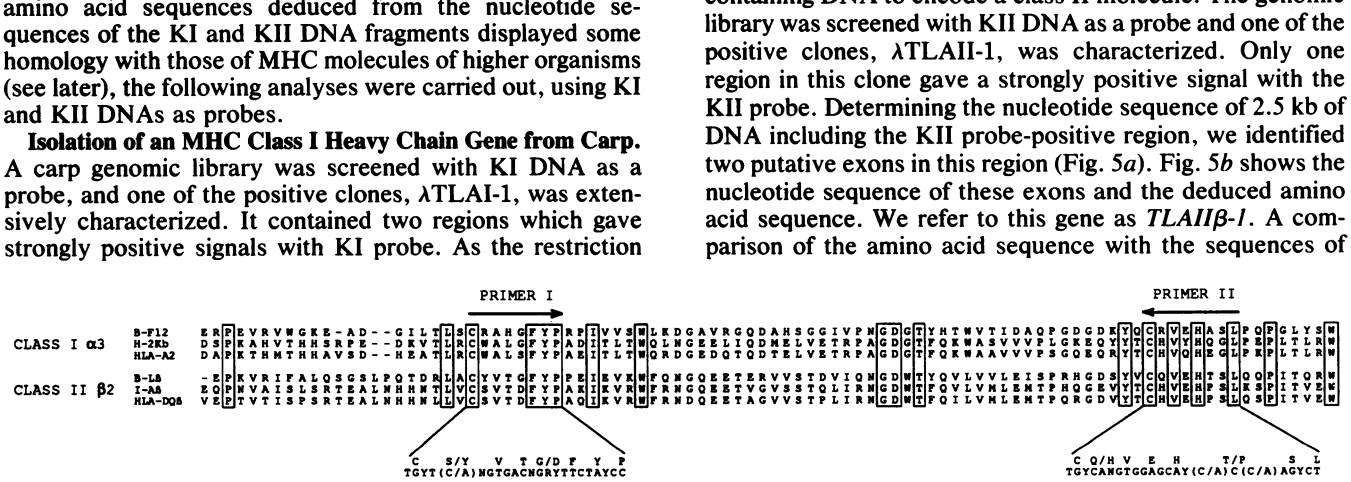

FIG. 1. Primers for PCR. The amino acid sequences of class I α3 and class II β2 domains of chicken, mouse, and human MHC molecules are shown. B-F12 (8) and B-Lβ (7) of chicken, H-2K^b (20) and I-Aβ (21) of mouse, and HLA-A2 (22) and HLA-DQβ (23) of man were chosen as representatives. The amino acid residues that were identical in all these molecules are boxed. The locations of the two primers used in PCR are indicated by arrows and the DNA sequences of the primers and the corresponding amino acids are shown (in the case of primer II, the complementary sequence is shown). The sequences of the two primers were chosen mainly on the basis of class II β sequences.

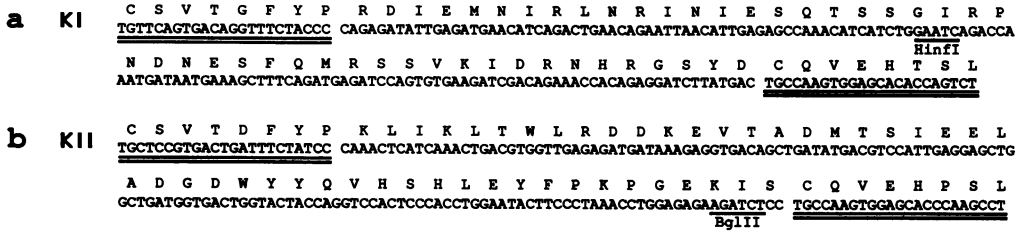

FIG. 3. DNA sequences of relevant PCR products, KI (*a*) and KII (*b*). KI and KII DNAs contain *Hin*fI and *Bgl* II sites, respectively. The primer sequences are doubly underlined. The deduced amino acids are shown above the DNA sequences.

MHC class II molecules of higher vertebrates indicated that this region encodes an MHC class II β chain (see later).

DISCUSSION

In this study we utilized the PCR method to amplify the MHC gene-encoding DNA segments of the carp genome. Two kinds of DNAs responded to amplification. Using these amplified DNAs as probes, we isolated genomic DNA clones. Fig. 6 compares the amino acid sequences of *TLAIα-1* and *TLAIIβ-1* genes with those of chicken, mouse, and human MHC class I heavy chains and MHC class II α and β chains (7, 8, 24). Since the *TLAIα-1* gene consisted of at least three exons, each of which corresponded to a domain, it was very likely that this gene encoded an MHC class I heavy chain. The *TLAIα-1* product of the carp shared identical residues with class I heavy chains of humans, mice, and chickens at 11, 10, and 17 positions of first, second, and third exons, respectively. When the *TLAIα-1* sequence was compared with all the published sequence data of human and mouse MHC class I (24, 28–30), 3–4 times more amino acid residues encoded by *TLAIα-1* were identical to at least one of the mammalian molecules. Moreover, key amino acid residues, such as cysteine and tryptophan, were conserved. When we superimposed the predicted tertiary structure of this molecule onto the proposed structure of mammalian MHC class I (4, 5), the conserved amino acid residues in the first and second domains were mainly located in the antigen-presenting groove. Disparities in the pattern of amino acid residues were located at regions which would not cause a drastic change in these fundamental structures. For example, seven amino acid residues were missing in the middle region of the first domain, but this region was located at the loop between β strands, S3 and S4, according to refs. 4–6. The amino acid sequence of the *TLAIIβ-1* gene was rather similar to the sequences of chicken, mouse, and human MHC class II β chains as shown in Fig. 6. The sequence conservation of exon 2 was especially remarkable. Thirty amino acid residues remained identical in this exon of the four species. We concluded that *TLAIα-1* and *TLAIIβ-1* encode carp MHC class I and class II β molecules, respectively. The results of Southern hybridization with KI and KII probes (Fig. 2*c*) suggested that several MHC class I and class II β genes exist in the carp. In Fig. 2*c*, λTLAI-1 corresponds to the 10-kb intense band in lane 1 and λTLAII-1 to the 9.4-kb band in lane 2. Since the intense bands may consist of multiple copies, MHC class I and class II β genes in the carp should, thus, number quite a few. Presence of an MHC and T-cell receptor recognition system in teleost fish has been suggested, as described in the introduction. Our study clearly shows that the carp has at least two classes of MHC genes: class I and class II. This strongly supports our view that teleost fish possess a highly organized immune system of the kind characterized for mammals.

In immune systems of higher vertebrates, immunoglobulin, T-cell receptor, and MHC molecules play major roles in recognizing antigens. Although each molecule has a different specialized function, they feature a similar basic structure. In

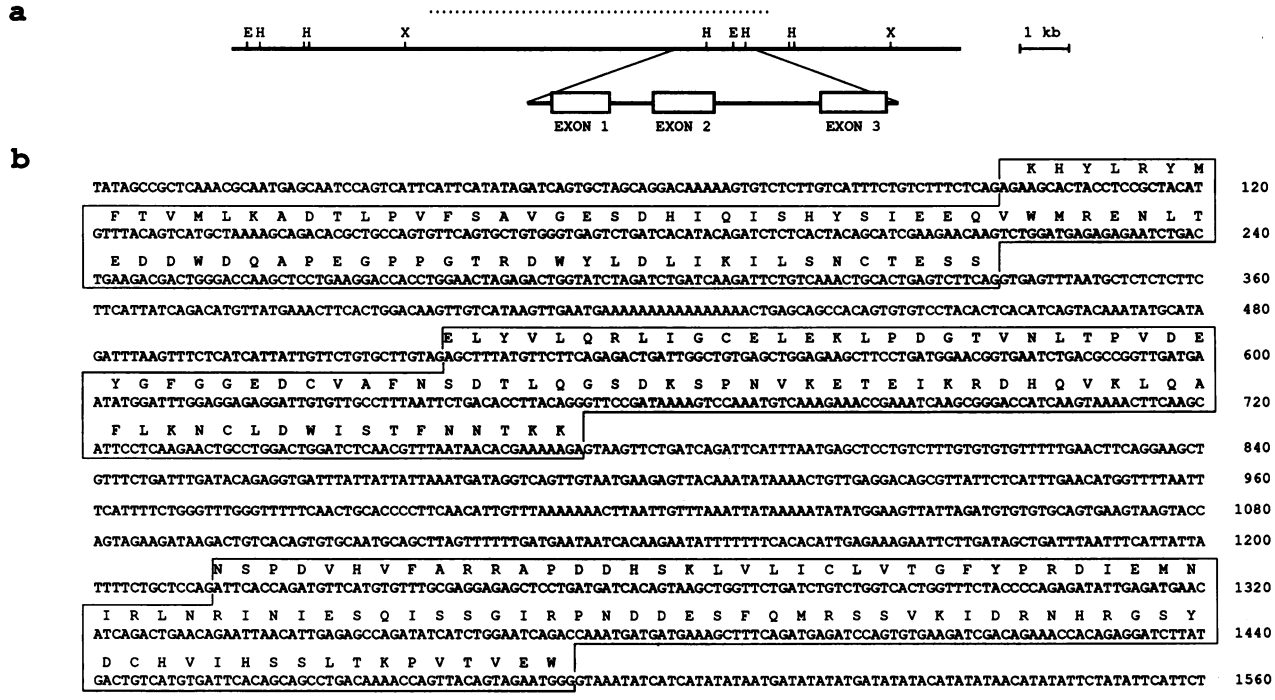

FIG. 4. Analysis of λTLAI-1. (*a*) Restriction map of λTLAI-1. The dotted line indicates the region whose sequence was determined. Three putative exons were identified and are shown by boxes. Restriction enzymes are E, *Eco*RI; H, *Hin*dIII; and X, *Xba* I. (*b*) DNA sequence of the *TLAIα-1* gene. The three putative exons are boxed. The deduced amino acids are shown above the DNA sequence.

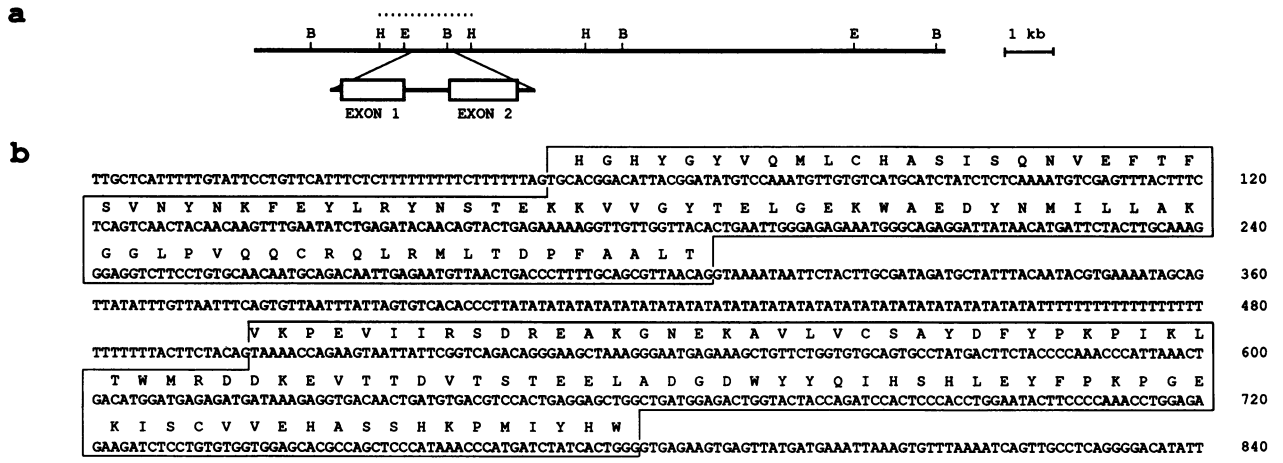

FIG. 5. Analysis of λTLAII-1. (*a*) Restriction map of λTLAII-1. The dotted line indicates the region whose sequence was determined. Two putative exons were identified and are shown by boxes. Restriction enzymes are B, *Bgl* II; H, *Hin*dIII; and E, *Eco*RI. (*b*) DNA sequence of the *TLAIIβ-1* gene. The two putative exons are boxed. The deduced amino acids are shown above the DNA sequence.

addition to these molecules, many other proteins have been shown to share common features and are grouped into the immunoglobulin superfamily (reviewed in refs. 31–33). It is widely accepted that most proteins belonging to the immunoglobulin superfamily are expressed on cell surfaces, that their functions are related to cell adhesion or binding to other molecules, and that all of the immunoglobulin-related domains are derived from one primordial domain. The sequences of the domains of contemporary proteins are grouped into three categories, called V-set, C1-set, and C2-set (31). The origin of the first domains of MHC class II α and β chains and the first and second domains of MHC class I heavy chain, however, remains problematical. The three-dimensional structure of these domains is quite different from the immunoglobulin fold (4–6), and there is no significant sequence homology between these MHC-specific domains of mammalian and avian species and the immunoglobulin-related domains except for the presence of two cysteine residues in the first domain of MHC class II β chain and the second domain of MHC class I heavy chain (32, 33). However, we found sequence similarity between the amino acid sequence encoded by the first exon of *TLAIIβ-1* and that of the CD8 β chain, in spite of the fact that this domain of the CD8 β chain has been classified as a V-set according to Williams' definition (32, 34). Fig. 7 summarizes the comparison of amino acid sequences between the first domain of carp MHC class II β chain and the V-set domains. In the case of rat CD8 β chain, around 50% of the amino acid residues are identical with those of TLAIIβ-1 in the middle portion. Interestingly, this region corresponds to the S3′ and S4′ peptides of the proposed model for MHC molecules (4–6) and to the C′ and C″ peptides in the immunoglobulin fold (3) as shown in Fig. 7. The sequence similarity between these two sequences seems to be too high to be ascribed to evolutionary convergence. Furthermore, Fig. 7 shows that this region of TLAIIβ-1 also shares several identical residues with various members of the V-set. Thus, it is possible that antigen-presenting domains with characteristic α-helices of class I as well as class II MHC antigens also arose from members of the V-set. It so happened that this domain of carp class II β chain still retains recognizable homology with members of the V-set, while this homology was lost in higher vertebrates. This unifies MHC with immunoglobulin and T-cell receptor molecules, all originating from similar heterodimers composed of V and C domains. Preliminary analysis of the secondary structure of TLAIα-1 and TLAIIβ-1 amino acid sequences by the method of Chou and Fasman (37) and Garnier *et al.* (38) suggested the presence of α-helical structures in the second half of the first and second domains of TLAIα-1 as well as in the second half of the first domain of TLAIIβ-1. However, the presence of α-helices is still a

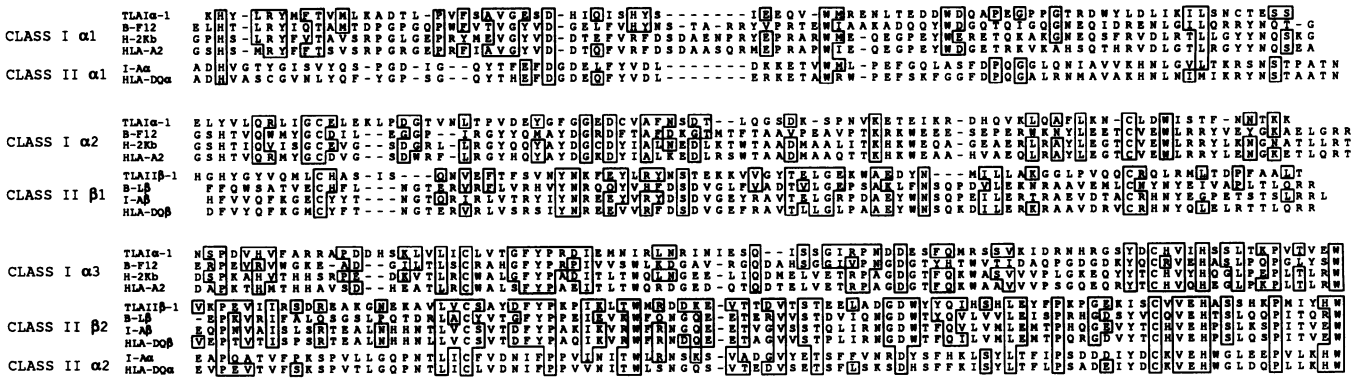

FIG. 6. Comparison of amino acid sequences encoded by *TLAIα-1* and *TLAIIβ-1* with those of mammalian and avian representative MHC class I and class II chains. (*Top*) Deduced amino acids of *TLAIα-1* exon 1 are compared with those of class I α1 and class II α1 domains of other species. (*Middle*) *TLAIα-1* exon 2 and *TLAIIβ-1* exon 1 are compared with class I α2 domains and class II β1 domains, respectively. Alignments of class II α1 with class I α1 and of class II β1 with class I α2 are according to Brown *et al.* (6). (*Bottom*) *TLAIα-1* exon 3 is compared with class I α3 domains and *TLAIIβ-1* exon 2 is compared with class II β2 and α2 domains. For each comparison, the amino acid residues identical with those encoded by *TLAIα-1* or *TLAIIβ-1* are boxed. Amino acid residues used in this figure are quoted from the following refs.: B-F12 (8), H-2K^b (20), HLA-A2 (22), I-Aα (26), HLA-DQα (27), B-Lβ (7), I-Aβ (21), and HLA-DQβ (23).

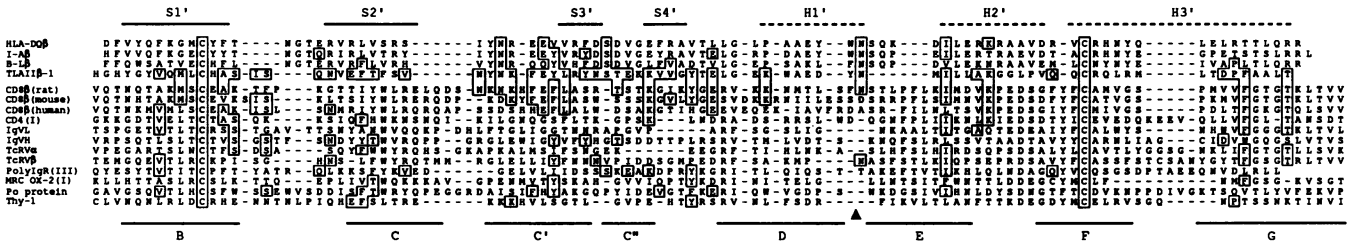

FIG. 7. Comparison of the amino acid sequence of *TLAIIβ-1* exon 1 with immunoglobulin superfamily V-set sequences. V-set sequences are quoted from refs. 31, 32, and 35 and the references cited therein and aligned according to refs. 31 and 32. Alignment of the last part of the sequences (corresponding to the β-strand G in the immunoglobulin V domain) were in accordance with refs. 34 and 36. Alignments were not attempted in this region for poly immunoglobulin receptor and P_0 protein. The amino acid sequences of MHC class II β1 domains of other species are shown above the *TLAIIβ-1* sequence. The amino acid residues are boxed when they are shared by *TLAIIβ-1* and V-set member(s). β-Strands and α-helices observed in the MHC class I HLA-A2 α2 domain are indicated by solid lines and broken lines, respectively, above the MHC class II sequences according to refs. 4–6, and β-strands in the immunoglobulin V domain (3) are indicated in solid lines below the V-set sequences. Comparison of the amino acid sequences between V-set and C-set suggested that several amino acids located between D and E are missing in the V-set (32). An arrowhead indicates this position.

conjecture awaiting the verification by future x-ray crystallographic study.

In this study, we isolated carp MHC genes by using the PCR method (18). We propose that the basic strategy described in this paper should be applicable to the cloning of other genes of the immunoglobulin superfamily to be found in lower vertebrates. These proteins consist of domains which are 90–110 amino acids long each, and with a few exceptions, each domain is encoded by a single exon (31–33). Phylogenetically conserved amino acid residues are not evenly distributed within their domains but tend to cluster in specific regions. Two conserved regions used in this study are found in many immunoglobulin-related domains classified as C1-set. As long as proper amino acids are chosen, the target genes would respond to amplification as described in this paper. To elucidate the origin and evolution of immunoglobulin, T-cell receptor, and MHC genes, it is essential to clone these genes from progressively more primitive vertebrates, until one finally approaches the boundary between invertebrates and vertebrates.

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