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Immunology

Evolution of the major histocompatibility complex: Isolation of class II *A* cDNA clones from the cartilaginous fish

(polymorphism/primitive vertebrates)

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ABSTRACT **Along with the T-cell receptor and immunoglobulin, the major histocompatibility complex (MHC) plays a key role in mounting immune responses to foreign antigen. To gain insights into the evolution of the MHC, class II *A* cDNA clones were isolated from nurse sharks, a member of the class of cartilaginous fish. Two closely related cDNA clones, which might encode allelic products, were identified; of the three amino acid substitutions found in the α1 domain, two were located at positions postulated to interact with processed peptides. The deduced nurse shark MHC class II α chains showed conspicuous structural similarity to their mammalian counterparts. Isolation of cDNA clones encoding typical MHC class II α chains was unexpected since no direct evidence for T-cell-mediated immune responses has been obtained in the cartilaginous fish. The cartilaginous fish is phylogenetically the most primitive class of vertebrates from which any MHC gene has been isolated.**

Genes of the major histocompatibility complex (MHC) encode two classes of structurally similar, but functionally distinct, glycoproteins that present peptides to T cells (1, 2). In general, MHC class I molecules, consisting of an α chain and β_2-microglobulin, present peptides derived from endogenously synthesized proteins to $CD8^+$ T cells. The peptides are bound by two membrane-distal domains (α1 and α2), which form a deep cleft made up of an eight-stranded β-pleated sheet topped by two long α-helices (3, 4). MHC class II molecules are heterodimers that, as a rule, present peptides derived from exogenously acquired proteins to $CD4^+$ T cells. The peptides are bound by two membrane-distal domains (α1 and β1), assumed to form a cleft structurally similar to that of MHC class I molecules (5).

At which stage of evolution did an ancestral MHC molecule emerge, and when and how did it diversify into two classes of functionally specialized molecules? Was an ancestral MHC molecule class I-like or class II-like? These questions, which are of fundamental importance in understanding the evolution of immunity (6–8), can be addressed only by studying MHC genes of primitive creatures. In addition, the structure of such MHC genes might provide a clue to the origin of membrane-distal, peptide-binding domains (9) and to the primordial function of MHC molecules. With these points in mind, we have embarked upon a project aimed at isolating MHC genes from primitive vertebrates (10).‡

The most primitive class of vertebrates from which MHC genes have been isolated thus far is the bony fish (class Osteichthyes; ref. 11). Consistent with the presence of the MHC, the bony fish display T-cell-dependent immune responses such as acute graft rejection (12), mixed leukocyte reactions (13), and T-/B-cell collaboration for antibody production (14). In contrast, no such T-cell-dependent immune responses have been demonstrated in the cartilaginous fish (class Chondrichthyes), although the presence of a thymus has been documented in some species (reviewed in refs. 7, 8, 15, and 16). Therefore, one might expect that the ancestors of the cartilaginous fish emerged in evolution before the appearance of the T-cell receptor (TCR) or the MHC. In the present article, we show that, contrary to such expectations, nurse sharks, a member of the cartilaginous fish, have a typical MHC class II *A* gene(s).§ This result raises the possibility that not only the emergence of the MHC, but also the subsequent divergence into class I and class II may have predated the appearance of this vertebrate class.

MATERIALS AND METHODS

Animals. A nurse shark, *Ginglymostoma cirratum*, a member of subclass Elasmobranchii, was captured in the Atlantic ocean near Miami, FL. The RNA isolated from this individual was used for the polymerase chain reaction (PCR) and construction of a cDNA library.

Oligonucleotides. The following three oligonucleotides were used in the present study: primer 46, with 32-fold degeneracy (5′-GRI GAI GTI TAY WCI TGY CII GTI SAI CA-3′; where Y is T or C, R is G or A, W is A or T, S is G or C, and I is inosine), an adapter primer (5′-GACTCGAGTCGACATCG-3′), and a $(dT)_{17}$ adapter primer (5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3′). The sequence of primer 46 was designed based on the fact that the region surrounding the second cysteinyl residue of the membrane-proximal domain of MHC proteins is highly conserved across species (for the location of this primer sequence, see Figs. 1 and 2). The sequences of the adapter primer and the $(dT)_{17}$ adapter primer were taken from Frohman *et al.* (17).

PCR. Nurse shark spleen poly(A)$^+$ RNA (≈80 ng) was reverse-transcribed using the $(dT)_{17}$ adapter primer as described (18). The resultant cDNA was subjected to PCR as described (18). Briefly, the PCR mixture (50 μl) contained 3 μl of the cDNA, 200 μM dNTPs, 1 μM primer 46, 1 μM adapter primer, 10 mM Tris·HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, gelatin (0.2 mg/ml), and 2.5 units of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus). The conditions of amplification were three cycles of 1 min at 94°C, 2 min at

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Abbreviations: MHC, major histocompatibility complex; TCR, T-cell receptor.

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‡The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M89950 (pSα5-1) and M89951 (pSαB-1)].

§According to the convention used in the HLA nomenclature, MHC class II genes encoding α- and β-chains will be referred to as *A* and *B* genes, respectively.

37°C, and 3 min at 72°C; followed by 35 cycles of 1 min at 94°C, 2 min at 48°C, and 3 min at 72°C; and a final extension of 15 min at 72°C.

Construction and Screening of a Nurse Shark Spleen cDNA Library. The nurse shark spleen cDNA library, constructed with the Uni-Zap XR cloning system (Stratagene), was screened according to a standard method (19).

DNA Sequencing. Double-stranded DNA was sequenced by the chain-termination method (20) with Sequenase (United States Biochemical).

DNA Sequence Analysis and Construction of a Phylogenetic Tree. The data bases searched were GenBank nucleotide sequence data base (release 69.0) and Swiss-Prot data base (release 19.0). Pairwise genetic distances between MHC genes of various species were calculated by the method of Nei and Gojobori (21). The distance matrix thus obtained was used to construct neighbor-joining trees (22).

RESULTS

Isolation of Nurse Shark MHC Class II *A* cDNA Clones. To obtain cDNA templates for PCR amplification, nurse shark spleen mRNA was reverse-transcribed using the $(dT)_{17}$ adapter primer. The cDNA thus synthesized was subjected to PCR with primer 46 and the adapter primer. After 38 cycles of amplification, two major bands of ≈650 and ≈600 base pairs (bp) were obtained. These bands were individually reamplified using the same combination of primers and cloned into pBluescript SKII(+). Approximately 10 plasmid clones were sequenced for each DNA band. Two clones derived from the ≈600-bp band were found to encode an identical protein with structural features compatible with those of MHC molecules. One of them, designated clone 62, was used to screen the nurse shark spleen cDNA library at high stringency. Six randomly chosen, strongly positive cDNA clones were plaque purified, and two of them, judged to have the longest inserts, were sequenced in their entirety (Fig. 1). The longer cDNA clone, designated pSα5-1, contained 1206 bp excluding the poly(A) tail. The sequence of the shorter cDNA clone, pSα4-1, was identical to that of pSα5-1. Among the sequences deposited in the GenBank nucleotide sequence data base, the top five genes most similar to pSα5-1 were all mammalian MHC class II *A* genes (≈50% sequence identity; 6.05–8.34 SD above the mean).

A polypeptide made up of 247 amino acids was predicted from the nucleotide sequence of pSα5-1 (Fig. 1). Although there was no in-frame stop codon preceding the first methionine residue (located at nucleotides 88–90), two lines of evidence indicated that this methionine is most likely the translation start site. First, the size of the mRNA as deter-

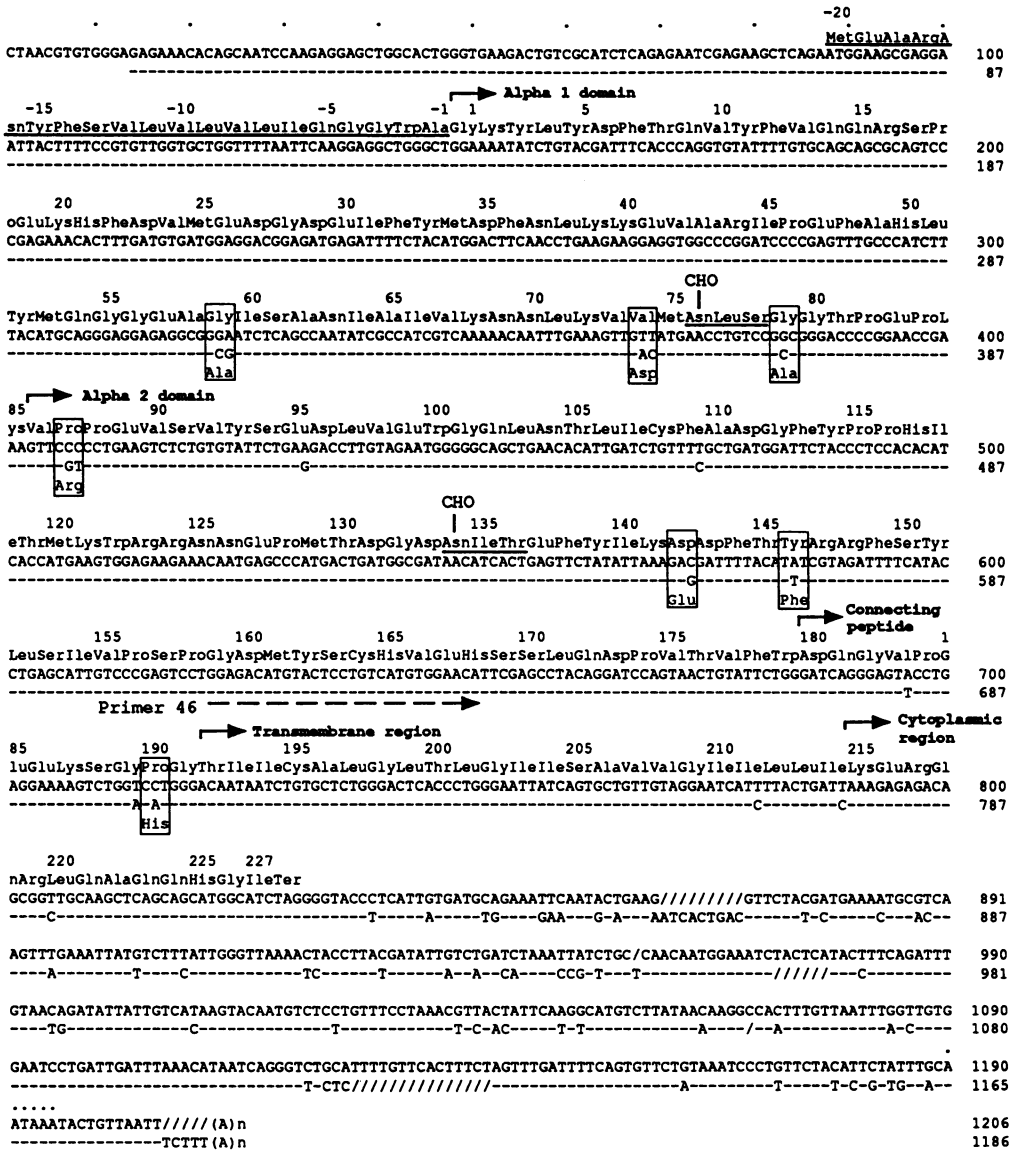

FIG. 1. Nucleotide sequences of nurse shark MHC class II *A* cDNA clones. The nucleotide and deduced amino acid sequence of pSα5-1 is shown in full above the nucleotide sequence of pSαB-1. Clone pSα4-1 starts 21 bp downstream from the start site (nucleotide 1) of pSα5-1. - and / indicate identity to the top sequence and absence of residues, respectively. The putative signal peptide is numbered from −20 to −1 and underlined. Amino acid substitutions found between pSα5-1 and pSαB-1 are boxed. The location of primer 46, the gene-specific primer used for PCR, is indicated by a dashed line. The putative polyadenylylation signal (AATAAA) is dotted. CHO, a potential N-linked glycosylation site; (A)n, a poly(A) tail.

mined from the Northern blot analysis coincided with that of clones pSα4-1 and pSα5-1, suggesting that these cDNA clones are almost full length (data not shown). Second, the nucleotide sequence surrounding this ATG was in good accord with Kozak's consensus sequence (23).

Amino Acid Sequence Comparison with Representative MHC Proteins. A computer search of the protein data base showed that the deduced nurse shark protein is most similar to MHC class II α chains of various mammals. The most similar class II α chain was mouse Eα (38% sequence identity). This was followed by rat Bα (37%), HLA-DRα (37%), HLA-DPα (35–37%), mouse Aα (35–37%), HLA-DNα (35%), swine DQα (32–34%), and HLA-DQα (31–34%). Two groups of proteins, which showed the second- and third-highest sequence similarity to the nurse shark protein, were MHC class II β (25–28%), and class I α (23–26%) chains, respectively.

The sequence alignment shown in Fig. 2 provides convincing evidence that pSα5-1 encodes an MHC class II α chain. In addition to the similarity in the overall domain organization, the nurse shark protein had numerous residues characteristic of MHC class II α chains. Such residues were most conspicuous in the membrane-distal domain, where MHC class I α, class II α, and class II β chains shared only a small number of residues. As in its mammalian counterparts, the α1 domain of the nurse shark MHC class II α chain contained no cysteinyl residues that could form an intradomain disulfide bridge. On the basis of the alignment shown in Fig. 2, the first 20 residues of the nurse shark protein (Fig. 1) were predicted to constitute a signal peptide. Two structural features of the nurse shark class II α chain are noteworthy. First, it lacks a salt bridge, made up of His-5 and Asp-27, and a highly conserved Trp-43. Second, it has potential N-linked glycosylation sites at residues 76 and 134. Their mammalian counterparts, located at analogous positions (Asn-79 and Asn-119), are known to be utilized.

Domain by domain sequence comparison between the nurse shark MHC class II α chain and other MHC proteins showed that the region most conserved across species is the transmembrane region. The transmembrane region of the nurse shark MHC class II α chain was 35–48% identical to that of human MHC class II α chains. Neither MHC class II β chain nor class I α chain showed significant sequence similarity in this region. The next most conserved region was the membrane-proximal domain. The sequences that showed significant similarity in this domain were MHC class II α (38–45%), MHC class II β (33–39%), β_2-microglobulin (30–36%), MHC class I α (29–35%), and other members of the

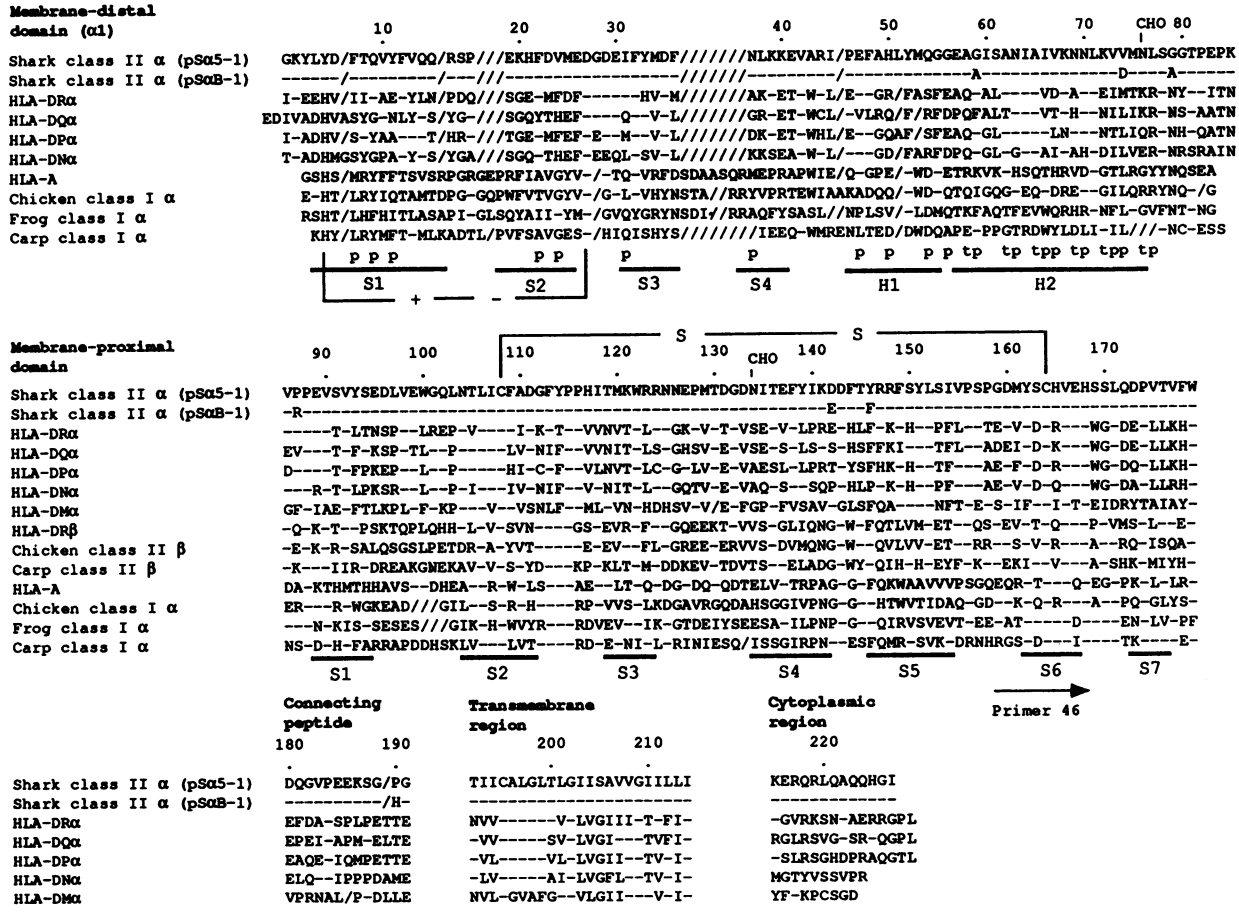

FIG. 2. Amino acid sequence comparison of the nurse shark MHC class II α chains and representative MHC proteins of other species. Sequence alignment was performed manually to maximize sequence similarity and to avoid gaps in the predicted β-strand regions. α1 domain sequences of MHC class I and II proteins were aligned according to Brown *et al.* (5), with minor modifications. - and / are as defined in the legend to Fig. 1. Numbering of residues is based on the shark sequences. The salt bridge (His-5 and Asp-27), conserved in all the sequences except the shark sequences, is indicated. The location of β-strands (S1–S4 in the α1 domain and S1–S7 in the membrane-proximal domain) is based on the crystal structure of the HLA-A2 molecule (3). The predicted location of α-helices (H1 and H2) in the α1 domain of MHC class II molecules was taken from Brown *et al.* (5). Residues assumed to interact with processed peptides and/or the TCR (5) are indicated below the amino acid sequence: p, residues postulated to interact with peptides; t, residues postulated to interact with the TCR. CHO, a potential N-linked glycosylation site. The location of primer 46 is indicated. The sources of the sequences are as follows: shark class II α (this paper; clones pSα5-1 and pSαB-1), HLA-DRα (24), HLA-DQα (25), HLA-DPα (26), HLA-DNα (27), HLA-A (28), chicken class I α (29), frog class I α (10), carp class I α (11), HLA-DMα (30), HLA-DRβ (31), chicken class II β (32), and carp class II β (11).

immunoglobulin superfamily (25–33%). Notably, the membrane-distal domain of the nurse shark molecule showed significant sequence similarity to that of other MHC class II α chains (30–35% sequence identity; >6 SD above the mean), but not to that of MHC class I α chain or class II β chain.

Construction of a Phylogenetic Tree. The membrane-distal domain of MHC molecules is subject to overdominant selection (33, 34), which may be driven by pathogens (35). In contrast, the membrane-proximal domain is not under such selection (33, 34). One can therefore expect this domain to evolve at a more constant rate and to better reflect the evolutionary history of the MHC. For this reason, a phylogenetic tree was constructed using the nucleotide sequences of the membrane-proximal domains of the nurse shark and other representative MHC genes (Fig. 3). To obtain this tree, the MHC genes were aligned codon by codon on the basis of the sequence similarity of the deduced proteins. As exemplified in Fig. 2, this alignment required insertion of only a few gaps. All but three genes were found to fall into major clusters designated I, IIA, and IIB (Fig. 3). Cluster I contained MHC class I α-chain genes and human *CD1*, a class I α-like gene that does not map to the MHC (36). Clusters IIB and IIA were made up of MHC class II *B* and *A* genes, respectively. A recently described, highly divergent class II *B* gene *HLA-DMB* (30), and MHC class II *B* genes of frogs and carps did not belong to any major clusters. As expected, the nurse shark MHC class II *A* gene was located in cluster IIA.

Identification of a Second Nurse Shark MHC Class II *A* cDNA Sequence. To isolate cDNA clones encoding a putative allelic sequence of pSα5-1, the nurse shark cDNA library was screened with pSα5-1 under a high-stringency condition. One of the positive cDNA clones, pSαB-1 was found to encode a closely related but distinct sequence (Figs. 1 and 2). The deduced nurse shark MHC class II α chain encoded by pSαB-1 differed from that encoded by pSα5-1 by seven amino acid residues. Three of the substitutions were located at residues 59, 74, and 79 in the α1 domain. The remaining four substitutions were in the α2 domain at residues 87, 142, and 146 and in the connecting peptide at residue 190. At the nucleotide level, the α1 domain contained a total of 5-bp substitutions, and all of them were nonsynonymous. Codons 59 and 74, assumed to encode residues that interact with processed peptides (Fig. 2), contained 2-bp substitutions each. Synonymous nucleotide substitutions were found at residues 95, 109 (α2 domain), 183, 189 (connecting peptide), 211, 214 (transmembrane region), and 220 (cytoplasmic region). The nucleotide sequence similarity between pSα5-1 and pSαB-1 was 98% for the coding region and 83% for the 3′ untranslated region. Repeated low-stringency screening of the cDNA library with an α2 domain-specific probe (the *Pvu* II/*Bam*HI fragment of pSα5-1; nucleotides 454–667) did not yield any clones distinct from pSα5-1 or pSαB-1 (data not shown).

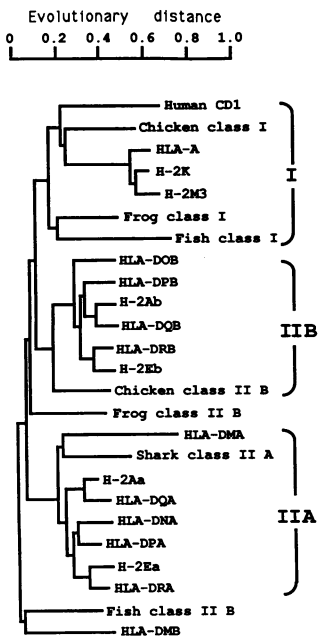

FIG. 3. Phylogenetic tree showing relationship of the nurse shark class II *A* gene (clone pSα5-1) to representative MHC genes of other species. The sources of sequences not described in the legend to Fig. 2 are as follows: human *CD1* (36), *H-2K* (37), *H-2M3* (38), *HLA-DOB* (39), *HLA-DPB* (40), *H-2Ab* (41), *HLA-DQB* (25), *H-2Eb* (42), frog class II *B* (K.S., M.F.F., L. Du Pasquier, M. Katagiri, and M.K., unpublished data), *H-2Aa* (43), *H-2Ea* (44), and *HLA-DMB* (30).

DISCUSSION

The most significant observation made in this work is that the cartilaginous fish have a gene(s) capable of encoding typical, mammalian-like MHC class II α chains. Hughes and Nei (33, 34) showed that nonsynonymous substitutions occur more frequently than synonymous substitutions in the antigen-recognition sites of functional mammalian MHC genes, whereas the opposite is the case for other regions of MHC genes, suggesting that MHC polymorphism is driven by positive Darwinian selection. The pattern and location of nucleotide substitutions observed in the two nurse shark cDNA clones (Figs. 1 and 2) are similar to those found in functional mammalian MHC genes. Thus, these cDNA clones are likely to encode functional MHC molecules. However, available evidence does not allow us to determine whether pSα5-1 and pSαB-1 represent allelic or closely related isotypic sequences.

The phylogenetic tree shown in Fig. 3 needs to be viewed with certain reservations because the number of sequences derived from different classes of lower vertebrates is small. Nevertheless, the fact that the nurse shark sequence was located in one of the three major clusters, each made up exclusively of class I α-chain, class II *A*, or class II *B* genes, indicates strongly that prototypic class I α, class II α, and class II β chains were established long before the emergence of the ancestors of nurse sharks. Consistent with this deduction, the nurse shark MHC class II α chain showed neither transitional features that might be found in evolutionary intermediates between MHC class I and II, nor atypical features that might be expected for very primitive MHC molecules (Fig. 2). Indeed, a recent study has shown that sharks may have an MHC class I α-like gene (45). Therefore, not only the emergence of the MHC, but also the subsequent divergence into class I and II may have predated the appearance of the cartilaginous fish.

Several features of this tree are noteworthy. First, the tree is unrooted and therefore does not allow one to determine which cluster (or gene) is the oldest. Although the *HLA-DMB* gene, whose β2 domain appears to be equidistant to that of class I and II (30), occurs on a separate branch, the branch lengths connecting this gene to the three major clusters as well as those interconnecting the three major clusters are short. This might suggest that the split into class I and II took place within a short period of time after the emergence of a primordial MHC molecule. Second, the fact that the nurse shark class II *A* gene and the *HLA-DMA* gene are on the same branch does not necessarily imply that the nurse shark gene is homologous to *HLA-DMA*. This clustering most likely results from the fact that these genes are more distantly related to the other mammalian MHC class II *A* genes than the latter are to one another. Third, the frog MHC class II *B* gene was located outside cluster IIB. The significance of this observation is not clear, since the branch length connecting this gene to the node leading to clusters I and IIB is very short. Inclusion of reptile MHC class II *B* sequences (when they become available) is likely to provide a more reliable branching pattern in this part of the tree. Fourth, the carp MHC class II *B* gene was located outside the three major clusters together with *HLA-DMB*. Since there is no evidence that this carp gene is functional (11), its apparently abnormal location might be accounted for by assuming that it is a pseudogene that diverged faster than the functional MHC

class II *B* genes located in cluster IIB. Finally, the tree shown in Fig. 3 differs significantly from the one presented in our previous paper (10). Although numerous factors are likely to account for this difference, the single most important factor appears to be the way the sequences were aligned. In our previous tree, sequence alignment was performed solely at the nucleotide level, without any regard for matching the codons; in contrast, the tree shown in Fig. 3 was constructed after aligning the sequences at the predicted amino acid level. The tree presented in this paper is more streamlined in the sense that the intermingling of MHC class I and II genes observed in our previous tree is almost absent and, hence, appears to better reflect the evolutionary history of the MHC.

It is interesting that cDNA clones encoding an apparently bona fide MHC class II gene(s) have been isolated from an organism in which no T-cell effector functions as defined in mammals have been demonstrated (reviewed in refs. 15, 16, and 47). This apparent paradox can be accounted for in two ways. First, the nurse shark MHC class II molecule might perform an as yet undefined, primordial function other than presentation of peptides to T cells. For example, spontaneous cytotoxic responses by shark peripheral blood lymphocytes have been well documented (46); shark class II molecules might serve as recognition elements for such cytotoxic cells. Alternatively, nurse shark T cells might not yet have developed the necessary accessory molecules and signals to mount full-fledged T-cell-mediated immune responses (16). The fact that the nurse shark and mammalian MHC class II α chains share a number of residues postulated to interact with the TCR and that amino acid substitutions between the two nurse shark class II α chains are found at positions postulated to interact with peptides argues strongly that the nurse shark has T cells with the TCR fundamentally similar to that of mammals. Therefore, the second explanation appears to be more likely. The cDNA clones isolated in this study offer a powerful tool with which to define the hitherto poorly characterized immune system of the cartilaginous fish. An attempt to raise antibodies against nurse shark MHC class II α chains remains to be done.

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