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**Jasna Bingulac-Popovic · Felipe Figueroa · Akie Sato
William S. Talbot · Stephen L. Johnson · Michael Gates
John H. Postlethwait · Jan Klein**

Mapping of *Mhc* class I and class II regions to different linkage groups in the zebrafish, *Danio rerio*

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Abstract The mammalian major histocompatibility complex (*Mhc*) consists of three closely linked regions, I, II, and III, occupying a single chromosomal segment. The class I loci in region I and the class II loci in region II are related in their structure, function, and evolution. Region III, which is intercalated between regions I and II, contains loci unrelated to the class I and II loci, and to one another. There are indications that a similar *Mhc* organization exists in birds and amphibians. Here, we demonstrate that in the zebrafish (*Danio rerio*), a representative of the teleost fishes, the class II loci are divided between two linkage groups which are distinct from the linkage group containing the class I loci. The β_2-microglobulin-encoding gene is loosely linked to one of the class II loci. The gene coding for complement factor B, which is one of the region III genes in mammals, is linked neither to the class I nor to the class II loci in the zebrafish. These results, combined with preliminary data suggesting that the class I and class II regions in another order of teleost fish are also in different linkage groups, indicate that close linkage of the two regions is not necessary either for regulation of expression or for coevolution of the class I and class II loci. They also raise the question of whether linkage of the class I and class II loci in tetrapods is a primitive or derived character.

J. Bingulac-Popovic
Croatian Institute of Transfusion Medicine, Department of Molecular Immunogenetics, Petrova 3, 10000 Zagreb, Croatia

F. Figueroa (✉) · A. Sato · J. Klein
Max-Planck-Institut für Biologie, Abteilung Immungenetik, Corrensstrasse 42, D-72076 Tübingen, Germany

J. Klein
Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Florida 33101, USA

J. H. Postlethwait · W. S. Talbot · S. L. Johnson · M. Gates
Institute of Molecular Biology, University of Oregon, Eugene, Oregon, USA

Introduction

The mammalian major histocompatibility complex (*Mhc*) consists of three regions designated I, II, and III (Klein 1986; Trowsdale 1995). Regions I and II contain mostly loci encoding peptide-binding molecules that function in antigen presentation to $CD8^+$ and $CD4^+$ T lymphocytes – the class I and class II loci, respectively (Germain 1994). The class III region is populated by a potpourri of loci, some of which are related to one another, but most of which are not, and none are structurally related to the class I and class II loci (Figueroa 1997). Although the *Mhc* organization varies in detail among the various mammalian taxa (e.g., in the mouse, a few class I loci have assumed an outpost position flanking the class II loci, and in humans, similarly, stray class I loci are located in region II), region III is generally intercalated between regions I and II in a single chromosomal segment (Trowsdale 1995; Kasahara et al. 1995). Genes related to the class I genes have, however, been mapped to chromosomes other than the *Mhc*-bearing chromosome. Thus, for example, the human *CD1* cluster is located on chromosome 1 (Blumberg et al. 1995), the *FCGRT* (Fc receptor, IgG, α-chain transporter) locus is on chromosome 19 (Simister and Mostov 1989), and the *AZGP1* (Zn-α_2-glycoprotein) locus is on chromosome 7 (Ueyama et al. 1991); the human *Mhc* being on chromosome 6.

In birds, as represented by domestic fowl (chicken), the *Mhc* loci reside in two regions – the *B* and the *Rfp-Y* complexes – separated by the nucleolar organizer region (NOR) on microchromosome 16 (Bernot et al. 1994; Miller et al. 1996). Both regions contain class I and class II loci, and since the NOR is a site of frequent recombinations, the regions segregate independently in genetic tests.

In amphibians, represented by the African clawed toad *Xenopus laevis*, the *Mhc* organization is analogous to that found in mammals, the class I and class II loci clusters being separated by the class III region (Flajnik and Du Pasquier 1990). An additional cluster of class I loci exists in *Xenopus*, however, which segregates independently of the

main complex (Flajnik et al. 1993). This additional cluster appears to contain the nonclassical or class Ib loci (Flajnik et al. 1993) that are distinguished from the classical or class Ia loci by low polymorphism and limited functionality (Klein and O'hUigin 1994).

In all tetrapods studied to date, the class I and class II loci are closely linked in the main cluster that contains highly polymorphic genes involved in the presentation of a wide spectrum of peptides. We were therefore surprised to find hints in our earlier studies that in the zebrafish, *Danio rerio*, this might not be the case. In these studies, we identified the class Ia loci (Takeuchi et al. 1995), the class II *A* and *B* loci coding for the α and β chains of the heterodimeric class II molecules (Ono et al. 1992; Sültmann et al. 1993, 1994), and the *B2m* locus coding for the β_2-microglobulin which forms a heterodimer with the class I α chain (Ono et al. 1993). Chromosomal walking analysis (Sültmann et al. 1994) suggested that the class I and class II loci were either far apart on the same chromosome or not linked at all. The aim of the present study was to determine which of these two possibilities holds true by carrying out genetic segregation analysis.

Materials and methods

Fish

Segregation analysis was carried out in several crosses of zebrafish [*Danio rerio* (formerly *Brachydanio rerio)* Hamilton-Buchanan 1822; Meyer et al. 1993], including the Tübingen wild strain (Tü), and crosses between the wild strains AB and Darjeeling (Postlethwait et al. 1994) or their inbred derivative strains C32 and SJD (Johnson et al. 1996). Haploid embryos were produced essentially as described (Streisinger et al. 1981).

DNA and polymerase chain reaction (PCR)

DNA was collected from three-day-old embryos as described (Fritz 1993) and amplified in the GeneAmp PCR System 9600 (Perkin Elmer, Überlingen, Germany). Two microliters of the embryo lysate were added to a reaction mixture containing 1×PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, and 0.001% gelatin), 0.2 mM each of the four deoxynucleoside triphosphates (Pharmacia, Freiburg, Germany), 1 μM each of the sense and antisense primers (Table 1), and 1 unit of Ampli*Taq* DNA polymerase (Perkin Elmer). The samples were denatured for 1 min at 93 °C, and then subjected to 40 amplification cycles, each consisting of 15 s denaturation at 93 °C, 10 s annealing at the required temperature, a gradual temperature increase (ramp) to 72 °C over 40 s, and 3 min of primer extension at 72 °C. The reactions were completed by a final primer extension at 72 °C for 10 min.

DNA sequencing

Selected DNA fragments were isolated from low-melting-point agarose gels (Gibco/BRL, Eggenstein, Germany) and subcloned in pUC18 plasmid vector with the aid of the SureClone ligation kit (Pharmacia). Double-stranded DNA was prepared with the aid of the Qiagen plasmid kit (Qiagen, Hilden, Germany), diluted to a concentration of 1 μg/μl and sequenced by the dideoxy chain-termination method (Sanger et al. 1977) using the AutoRead sequencing kit (Pharmacia/LKB).

Single-strand conformation polymorphism (SSCP) typing

In some cases, polymorphisms were detected by SSCP (Orita et al. 1989). Four microliters of amplified DNA samples were denatured by incubation for 10 min at 50 °C in the presence of 50 mM NaOH and 1 mM ethylenediaminetetraacetate, and immediately cooled in ice-water. After the addition of 1.8 μl loading buffer containing 100% formamide and xylene cyanol, and bromphenol blue, the samples were loaded in the wells of 10% minipolyacryl CleanGel (ETC, Kirchentellfurt, Germany) and electrophoresed in the Multiphor II system (Pharmacia) for 10 min at 200 V and then for 3–4 h at 373 V and at a constant temperature of 15 °C, using the DELECT kit (ETC). Separated DNA fragments were visualized by silver staining (Orita et al. 1989).

Table 1 Oligonucleotide primers used in the present study

Designation	Sequence	Location	Expected size of PCR fragment	Identification of alleles[a]
TU865	TCCCTGAGGTACTTCTACACTGGT	I, E2, codon 5–12, sense	} 252 bp	A
TU866	CTGTGTCTGGTTAAACCTCTCCTT	I, E2, codon 81–88, antisense		
TU868	ACCCACAGCTGGAAAGCCTATTAC	I, E2, codon 3–10, sense	} 258 bp	A
TU869	TTGTGTTTGGTTGAAGCGCTCCAT	I, E2, codon 81–88, antisense		
TU1287	CTTGAATTTTGCAGTATGGCGCT	I, E2, codon 67–74, antisense	} 439 bp	A
TU1288	CTTTAGTCAGTTGTGGCGGTTTT	I, intron 1, 148 bp upstream of E2		
TU360	TGCTTTATCACG(G/T)ACAGCTGA	II, *DAB*, E2, codon 89–95, antisense	} 505-574 bp	A, B, C, E
TU385	TGCTGTCG(A/G)CATTTACTGGAAC	II, *DAB*, E1, codon –8 to –1, sense		
TU946	AGTGAACCCATGATTTATGAC	II, *DDB*, E3, codon 87–93, sense	} 158 bp	A
TU947	GAAACCAGATGCCCCCAACAG	II, *DDB*, E4, codon 14–20, antisense		
TU858	CGGTGAGAGTATATGACTAACA	II, *DFB*, intron 1, 80 bp upstream of E2, sense	} 660 bp	A
TU948	AGATCTGGGGAGCATTATTTGTGCA	II, *DFB*, 310 bp dowstream of E2, antisense		
TU461	GCTCAAGCTGAGCACAGG	II, *DAA*, E2, codon 1–6, sense	} 249 bp	E
TU450	TTGCTCTTCTGGAGAGTT	II, *DAA*, E2, codon 78–83, antisense		
TU623	AAGGAGATGAATATACCTGCAGCG	*B2M*, E2 codon 74–71, sense	} 686 bp	D
TU624	ACCTGTAGCAGCTACTTTGGTC	*B2M*, 3′UTR 480–500 bp, antisense		
TU1279	AAGTCCGGGTTTGCCAACCTAATG	*BF*, codon 110–117, sense	} 153 bp	E
TU1280	ACAGTGGTACGTGACTTCATCATC	*BF*, codon 153–160, antisense		

I, II, class I and class II, respectively; *BF*, complement factor B; *B2M*, β_2-microglobulin;
E exon; UTR, untranslated region

[a] A, allele-specific amplification; B, length variation of PCR product; C, sequence variation; D, RFLP, E, SSCP

Table 2 Segregation of *Mhc* genes in haploid zebrafish embryos

Exp.	Locus combination	Gene combination		*N*	*px*	*py*	*qx*	♀*y*	Linkage	χ^2	*P*
1	I-I	*UAA*02*	*UAA*01*	28	12	0	0	16	Yes	29.14	>>0.005
2	I-I	*UAA*02*	*UCA*	19	10	0	0	9	Yes	19.10	>>0.005
3	II-II	*DAB1*	*DDB*	15	9	0	0	6	Yes	16.20	>>0.005
4	II-II	*DAB2*	*DDB*	14	7	0	0	7	Yes	14.00	>>0.005
5	II-II	*DAB*[a]	*DDB*	156	81	0	2	73	Yes	148.90	>>0.005
6	II-II	*DAA*	*DDB*	55	12	0	0	42	Yes	85.80	>>0.005
7	II-II	*DAB1*	*DAB2*	47	28	0	0	19	Yes	50.45	>>0.005
8	II-II	*DAB1*	*DAB4*	38	22	0	0	16	Yes	39.88	>>0.005
9	II-II	*DAB3*	*DAB4*	19	10	0	0	9	Yes	19.10	>>0.005
10	II-II	*DAB*[a]	*DAA*	52	12	0	0	39	Yes	78.07	>>0.005
11	II-II	*DAB1*	*DAA*	38	22	0	0	16	Yes	39.88	>>0.005
12	I-II	*UAA*01*	*DAB1*	86	24	20	22	20	No	0.50	NS
13	I-II	*UAA*02*	*DAB3*	22	6	8	5	3	No	2.35	NS
14	I-II	*UAA*02*	*DAB1*	27	7	4	9	7	No	1.89	NS
15	I-II	*UAA*01*	*DAB*[a]	156	36	45	42	33	No	2.72	NS
16	I-II	*UAA*01*	*DFB*	110	27	29	21	33	No	2.72	NS
17	I-II	*UAA*01*	*DDB*	167	39	43	45	40	No	0.54	NS
18	*B2M*-I	*B2M*	*UAA*02*	22	5	9	3	5	No	3.35	NS
19	*B2M*-I	*B2M*	*UAA*01*	154	38	37	42	37	No	0.44	NS
20	*B2M*-II	*B2M*	*DAB1*	38	10	15	6	7	No	4.73	NS
21	*B2M*-II	*B2M*	*DAB3*	22	7	7	4	4	No	1.63	NS
22	*B2M*-II	*B2M*	*DAB*[a]	163	41	47	40	35	No	1.78	NS
23	*B2M*-II	*B2M*	*DFB*	163	36	44	40	43	No	0.95	NS
24	*B2M-BF*	*B2M*	*BF*	23	7	7	5	4	No	1.17	NS
25	I-*BF*	*UAA*02*	*BF*	22	8	6	3	5	No	2.27	NS
26	II-*BF*	*DAB3*	*BF*	23	6	5	6	6	No	0.13	NS
27	II-II	*DAB*[a]	*DFB*	167	35	49	39	47	No	3.18	NS

I, II, class I and II, respectively; *B2M*, β_2-microglobulin; BF, complement factor B; *N*, numbers of embryos tested; NS, non-significant

[a] alleles not discriminated; all χ^2 values were calculated using the formula $\Sigma\ (o\text{-}e)^2/e$, where *o* is observed and *e* is expected number of embryos under the assumption of no linkage between tested genes

Arrangement of segregant classes:

	x	*y*
p	*px*	*py*
q	*qx*	*qy*

Results

In our earlier zebrafish studies, we identified three *Mhc* class I loci (*Dare-UAA*, *-UBA*, and *-UCA*; Takeuchi et al. 1995), four class II *A* loci (*Dare-DAA*, *-DBA*, *-DCA*, and *-DEA*; Sültmann et al. 1993, 1994; Ono et al. 1992), six class II *B* loci (*Dare-DAB*, *-DBB*, *-DCB*, *-DDB*, *-DEB*, and *-DFB*; Sültmann et al. 1994; Ono et al. 1992), and one β_2m-encoding locus (*Dare-BM2;* Ono et al. 1993), as well as the locus coding for the complement factor B (*Dare-BF;* Seeger et al. 1996), which is one of the class III loci in mammals (Klein 1986). Of these, however, the *Dare-DCA*, *-DEA*, *-DBB*, *-DCB*, and *-DEB* loci only occur in some stocks of zebrafish and were absent in the stocks used in the present study (Sültmann et al. 1994). The β_2m constitutes the β chain of the class I heterodimers; the class II *A* and class II *B* loci code for the α and β chains of the class II heterodimers (Klein 1986).

To determine the linkage relationships among the *Mhc* loci present in the standard laboratory stocks of zebrafish, we produced haploid embryos from eggs of heterozygous mothers, isolated DNA from them, and then amplified the DNA by PCR using primers specific for the individual loci (Table 1). DNA polymorphisms segregating in these crosses were identified by one of four methods: allele-specific oligonucleotides which amplify a specific band from one allele but not from another; variation in PCR product size revealed by agarose gel electrophoresis; sequence variants detected by SSCP; and restriction site polymorphisms in amplified fragments. Sequencing confirmed SSCP patterns or PCR product variants. Key loci were located on the genetic linkage map with respect to markers defined previously (Postlethwait et al. 1994; Johnson et al. 1996).

The segregation results establish several points concerning the linkage relationships among the *Mhc* loci. First, the three class I sequences are closely linked (Table 2, lines 1, 2). The *UAA* and *UCA* sequences are not alleles because they segregate in coupling, i.e., both loci are amplified in individual haploid animals. Since no recombinants have been found between them, they are presumably rather closely linked. It is possible that the *UAA* and *UBA* sequences may be alleles at one locus (*UAA*) because the two sequences appear to segregate in repulsion with the primers used, i.e., both genes were never amplified in individual haploids. *UAA* and *UBA* were originally assigned different family designations because of the large sequence difference between them (Takeuchi et al. 1995) but an alternative possibility is that they actually represent ancient, widely divergent allelic sequences. Correspondingly, we have renamed the genes *UAA* and *UBA* as *UAA*01* and *UAA*02*, respectively.

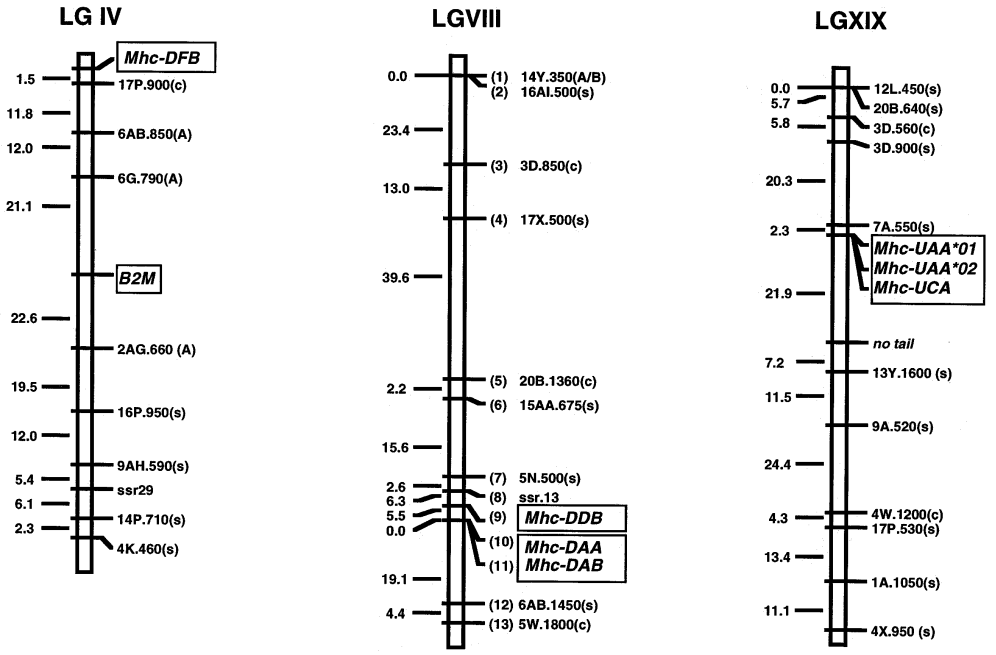

Fig. 1 Distribution of *Mhc* loci in the zebrafish genome. LG, linkage group. Numbers on the *left* are genetic distances in centimorgans, symbols on the *right* are DNA markers (genes)

Second, some of the class II *B* sequences cosegregate, while others do not (Table 2, lines 3–9). The former include the *DDB* gene and the *DAB* family sequences. The demonstration of *DDB* and *DAB* sequences in individual haploid embryos shows that these represent distinct loci at a distance of 1.3 cM from each other. The class II *B* gene *DFB*, on the other hand, segregates independently of the class II *B DAB* gene (Table 2, line 27).

Third, the class II α chain-encoding gene *DAA* cosegregates with the class II β chain-encoding gene *DAB* (Table 2, line 10–11). This result is consistent with the mammalian gene organization in which *Mhc* class II *A* and *B* loci are closely linked. Chromosomal walking reported earlier (Sültmann et al. 1994) established the *DCA* and *DCB* loci to be one pair of neighbors and the *DEA* and *DEB* loci to be another. However, because these loci are absent in the stocks used in the present study, the relationships of the two pairs to each other and to the *DAA*, *DAB*, and *DDB* cluster could not be tested.

Fourth, the class I genes *UAA*01* and **02* segregate independently of the class II *B* genes *DAB* and *DDB*, as well as of the class II *A* gene *DAA* (Table 2, lines 12–15, 17). This is a key result because it demonstrates that class I and class II genes are not linked in zebrafish, in contrast to their arrangement in mammals. The class II *B* gene *DFB* segregates independently of the class I gene *UAA*01* (line 16).

Fifth, the *B2M* gene segregates independently of the class I genes *UAA*01* and **02* (Table 2, lines 18–19), as well as of the class II *DAB* and *DFB* genes (Table 2, lines 20–23). Sixth, the *BF* gene, the orthologue of which is in region III in the mammalian *Mhc*, segregates independently of the class I gene *UAA*02*, of the class II gene *DAB3*, and of the *BM2* gene (Table 2, lines 24–26).

Segregation analysis alone has insufficient resolution power to demonstrate that loci are unlinked; genetically unlinked loci can reside on the same chromosome, given the meiotic length of many zebrafish chromosomes as being over 100 cM (Johnson et al. 1996). Because a key result of the segregation analysis was that the class I and class II loci are unlinked, it was important to determine whether they still reside on the same chromosome. Mapping studies showed that *DAB*, *DDB*, and *DAA* genes cluster in linkage group 8, between the markers *6M.850* and *SSR13*; the class II gene *DFB* maps to linkage group 4; and the class I genes *UAA*01*, *UAA*02*, and *UCA* map to linkage group 19 between the *no tail* mutation and *13Y.1700* (Fig. 1). As each linkage group represents a different chromosome (Johnson et al. 1996), we can conclude that *Mhc* class I *A* genes reside on a different chromosome than the clusters of the class II *A* and *B* genes.

Discussion

The present study documents the first instance in which the class I and class II loci are not found in a single chromosomal region. Instead, in the zebrafish, the class I and class II genes are located in different linkage groups and the class II genes are divided between at least two linkage groups. A gene that maps to the *Mhc* region III in mammals (complement factor B, *BF*) is not linked to any of the three clusters. Before considering the implications of these results, two issues must be addressed – the possibility that we may have missed a set of either class I or class II loci in one of the three clusters, and the generality of the observation.

At least three arguments contradict the notion of a missing gene set. First, both the class I and class II loci we have identified in the zebrafish fulfill the two criteria for classical *Mhc* genes (Klein and O'hUigin 1994) – high-level expression of apparently nondefective transcripts in

lymphoid tissues and high polymorphism (Ono et al. 1992; Sültmann et al. 1993, 1994; Takeuchi et al. 1995). Furthermore, some of the zebrafish *Mhc* polymorphism is of ancient origin (Graser et al. 1996), indicating that the loci are under the influence of balancing selection and hence functional. Second, an extensive search for additional class I and class II loci in genomic DNA, genomic libraries, and cDNA libraries failed to provide evidence for the existence of such loci. Although the possibility that distantly related *Mhc* genes have been overlooked cannot be excluded entirely, it is highly improbable that these, rather than those identified, are the primary *Mhc* genes in the zebrafish. Third, recent segregation analysis has revealed the *LMP2* and *LMP7* genes to be part of the class I cluster in the zebrafish (K. Takami, R. Grasser, V. Vincek, F. Figueroa, and J. Klein, unpublished data). The *LMP* genes code for subunits of the proteasome that, in the cell, processes proteins into peptides which then bind to the class I molecules (Brown et al. 1993). Since in mammals, the *LMP* genes are part of the *Mhc* (Monaco and McDevitt 1982), their class I linkage in the zebrafish certifies the authenticity of the identified cluster and its homology to the mammalian *Mhc*. Fourth, parallel analysis of zebrafish *Mhc*-bearing BAC clones has confirmed that each of the three clusters identified by segregation analysis contains only one class of *Mhc* loci (R. Graser, J. Klein, and V. Vincek, unpublished data). We are therefore confident that in the zebrafish, the main functional class I and class II loci are not linked.

As for the generality of the finding, segregation analysis of representatives of another fish order, Perciformes, indicates that in these species the functional class I and class II loci are not linked either (E. Malaga, H. Sültmann, and J. Klein, unpublished data). Perciformes and Cypriniformes (the order represented by the zebrafish) are estimated to have diverged more than 50 million years ago (Lauder and Liem 1983). Hence, the separation of the class I and class II regions is not the recent quirk of a single species but a primitive character for two very large orders of teleostean fishes at least.

The findings reported in this communication have implications for the significance of the clustering of *Mhc* loci and for the origin of the complex. The observation that in tetrapods the two *Mhc* classes of loci have remained together despite being separated by unrelated loci has been interpreted as having a functional or evolutionary significance (Trowsdale 1995). It has been suggested that the loci are located together in a single chromosomal region because of a need to coordinate their expression or their evolution. The observation that in the fish, the class I and class II loci have existed separately from each other for at least 50 million years can be used as an argument against this proposition. The *Mhc*, like the immunoglobulin and T-cell receptor complexes of the immune system, apparently functions normally in situations in which the distinct classes of loci are positioned on different chromosomes.

The class I and class II genes are structurally and functionally related and presumably arose from a common ancestor by gene duplication and exon shuffling (Klein and O'hUigin 1993). Until now, it has been assumed that the ancestral locus subsequently duplicated and that the two classes of *Mhc* loci arose from the two (or more) copies. The data described in the present communication raise the possibility of a different origin of the *Mhc* classes. If separate class I and class II regions were to exist in other teleosts as well as in cartilaginous fishes, it would indicate that the separateness is a primitive and the linkage a derived character. One could imagine, for example, that the chromosome carrying the ancestral, undifferentiated *Mhc* locus or loci duplicated, perhaps in one of the tetraploidization events postulated to have occurred during vertebrate evolution (Ohno 1970; Lundin 1993), and that the duplicated *Mhc* genes subsequently differentiated into class I and class II loci. In the ancestors of the tetrapods, the two classes were then brought together by chromosomal translocation in some of the vertebrate taxa. This hypothesis would account for the existence of *Mhc* region III and for the presence of different loci in this region in some of the vertebrate classes. To decide between the two hypotheses, it is essential to map the *Mhc* loci in other teleosts, sarcopterygians, and, in particular, cartilaginous fishes.

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