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Evolution

Contrasting histories of avian and mammalian *Mhc* genes revealed by class II B sequences from songbirds

(ligation-anchored PCR/balancing selection/gene duplication/orthology)

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ABSTRACT **To explore the evolutionary dynamics of genes in the major histocompatibility complex (*Mhc*) in nonmammalian vertebrates, we have amplified complete sequences of the polymorphic second (β1) and third (β2) exons of class II β chain genes of songbirds. The pattern of nucleotide substitution in the antigen-binding site of sequences cloned from three behaviorally and phylogenetically divergent songbirds [scrub jays (*Aphelocoma coerulescens*), red-winged blackbirds (*Agelaius phoeniceus*), and house finches (*Carpodacus mexicanus*)] reveals that class II B genes of songbirds are subject to the same types of diversifying forces as those observed at mammalian class II loci. By contrast, the tree of avian class II B genes reveals that orthologous relationships have not been retained as in placental mammals and that, unlike class II genes in mammals, genes in songbirds and chickens have had very recent common ancestors within their respective groups. Thus, whereas the selective forces diversifying class II B genes of birds are likely similar to those in mammals, their long-term evolutionary dynamics appear to be characterized by much higher rates of concerted evolution.**

Genes of the major histocompatibility complex (*Mhc*) bind peptides from bacteria and other pathogens and present these to T cells for initiation of the immune response. *Mhc* genes are unique among loci in vertebrates in showing extreme polymorphism within populations, high rates of non-synonymous (amino acid changing) substitution in the antigen-binding site (ABS), and long persistence times of alleles (1, 2). Diversity at the population level is thought to arise via interactions of hosts' *Mhc* gene products and parasites [leading to frequency-dependent selection (rare allele advantage) or overdominant selection (heterozygote advantage)], mating preferences, selective abortion, or, more likely, some complex combination of all of these (3).

Class II MHC molecules are heterodimeric receptors consisting of an α and a β chain, each encoded by a separate gene or genes, which have been found in all vertebrates so far investigated. In all cases in which variability in class II β chain (class II B) genes has been examined, the codons in the second (β1) exon encoding the ABS exhibit the major hallmark of balancing selection at *Mhc* loci—namely, an excess of nonsynonymous substitutions over synonymous substitutions in the ABS codons (4, 5). Phylogenetic analyses suggest that diversity for receptors among mammalian class II loci is likely also to be maintained by so-called divergent selection among loci (6–9), resulting in maintenance of duplicated genes over most of mammalian evolution. Although the recent cloning of class II genes from phylogenetically basal groups of vertebrates has yielded insight into conserved features of class II gene evolution (10–13), we do not yet know whether the long-term evolutionary history of class II genes in mammals is different from that in other vertebrate groups because there have been few comparative studies within nonmammalian classes, and the relationships among existing gamebird sequences (14) to one another is not known. To this end we have obtained multiple sequences† of exons 2 and 3 of class II B genes from three songbirds representing the two major divisions within songbirds (Passerida and Corvida; ref. 15). ["Songbirds" are defined as those perching birds (Passeriformes) possessing a complex voice box and song-learning capacity, or Oscine Passerine birds.] The most recent common ancestor of songbirds and chickens is roughly the same age as, or more recent than, that of mice and humans, whereas the divergence of Passerida and Corvida was later, anywhere from 30 to 55 million years ago (MYA) (see *Discussion*). These sequences permit comparison of avian class II genes that diverged around the same time as mammalian orders, allowing us to test the adequacy of models of long-term mammalian *Mhc* evolution when applied to birds.

MATERIALS AND METHODS

Sources of cDNA. Whole spleens (0.5–0.75 g) were collected in the field from one member of the Corvida (western scrub jays, *Aphelocoma coerulescens californica*, $n = 3$) and two Passerida species (house finches; *Carpodacus mexicanus*, $n = 2$; red-winged blackbirds, *Agelaius phoeniceus*, $n = 2$). Spleens were snap-frozen in liquid nitrogen within 5 min of collection. Total RNA and cDNA were extracted and synthesized as described (16, 17).

Amplification Strategy and Ligation-Anchored PCR (LA-PCR). LA-PCR (18) was used to obtain sequences of the 5′ ends of reverse-transcribed *Mhc* class II B genes. Songbird sequences determined using primers 1 and 2 (Fig. 1, ref. 16; all primer sequences and references to primers are illustrated in Fig. 1 and its legend) were used to design primer 3, which was in turn used to prime first strand cDNA synthesis. Ten picomoles of a phosphorylated, blocked anchor primer (no. 4) was then ligated onto first strand cDNA with 1 unit of T4 RNA ligase in 10 μl of a 25% PEG solution as described (18). One microliter of this reaction mixture was used as a template in a hemi-nested PCR containing the reverse-complement of primer 4 and another primer (no. 5) located immediately upstream to primer 3. Products of this PCR were cloned into TA vectors (Invitrogen) and sequenced with an Applied Biosystems model 373A automated sequencer as described (16).

Primers 6 (exon 2) and 7 (exon 4) were designed from genomic sequences (16) and published sequences from mammals and chicken (20, 21). These primers were used on

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Abbreviations: *Mhc*, major histocompatibility complex; MYA, millions of years ago; ABS, antigen-binding site; class II B, class II β chain; LA-PCR, ligation-anchored PCR.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U23958–U23976).

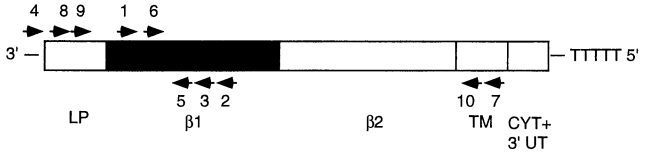

FIG. 1. Amplification strategy for class II B genes of songbirds. All amplifications were performed using first strand cDNA as a template (16, 19). The polymorphic second exon is shaded; names of exons are indicated, as is the poly T primer used to prime cDNA synthesis. Arrows represent primers used for PCR and LA-PCR. The sequences of primers 1 and 2 are given in ref. 16. The sequences of the remaining primers (5′→3′) are as follows: 3, GCCCACATCGCTGTCGAA; 4, CGATGAATTCTCGAGTC; 5, ACAGCTGCCGGTTGTAGA; 6, AGG(C/T)T(G/C)(T/A)TCTACAACCGG; 7, GAAGANGAGC-CCCAGCAC(A/G)AAGCCCCC; 8, GTACTGGTGGCACTGGT-GGTGCT; 9, GGCTGCGGGCGAGAAG; 10, CCGATGCCCGT-CAGCATCTTG. Both strands of the PCR clones were sequenced with a battery of internal primers as well as primers in the vector. LP, leader peptide; TM, transmembrane domain; CYT, cytoplasmic tail; 3′ UT, 3′ untranslated region.

oligo(dT)-primed first strand cDNA (reverse transcription PCR; refs. 19 and 22) to obtain products and clones spanning the entirety of exons 2 and 3. These sequences and those from the LA-PCR experiments were used to design three primers (nos. 9–11) in exons 1 and 4, which were used to amplify 700-bp products from cDNA prepared from each individual. These products were cloned in TA vectors and both strands of a total of 26 clones were sequenced.

Phylogenetic and Statistical Analysis. Exon boundaries and codons in the ABS aligned easily by hand to chicken class II B genes (20) and the β-strand of the human molecule with a known crystal structure (23). The number of synonymous (d_S) and nonsynonymous (d_N) substitutions per site in the ABS codons was calculated using the one-parameter correction and the method of Nei and Gojobori (24, 25). Phylogenetic trees of class II B genes from songbirds and other vertebrates were obtained by the parsimony (26) and neighbor-joining (27) methods. Separate phylogenetic analyses were performed on functional domains (β-sheet, α-helix, exon 3), and a statistical test (28) was used to determine whether the differences in the trees of individual domains were significant.

RESULTS

Class II B Leader Peptide Sequences via LA-PCR. Because available PCR primers for avian class II genes encompassed only some of the polymorphic segments of the β1 exon (16), further sequences flanking this exon were needed. To this end, cDNAs from a scrub jay and a red-winged blackbird were primed from the middle of exon 2, and the phosphorylated, blocked primer 4 was ligated onto their 3′ ends (Fig. 1). Hemi-nested amplifications of the reverse-transcribed segment, spanning half of exon 2 and the entirety of the leader peptide (exon 1) resulted in a band of appropriate size in both species (Fig. 2*A*). Representative sequences of clones of these products (Fig. 2*B*) indicated that the segment homologous to chicken class II B leader peptides had been amplified and that many of the cDNAs encompassed the initiation codon of these genes, in all cases ATG.

Amplification of β1 and β2 Exons. Using partial sequences of the second exon of various songbirds (16), clones spanning the entire third (exon and part of the transmembrane domain (exon 4) were obtained via reverse transcription PCR (Fig. 1). The base composition of leader peptide sequences was highly GC-rich; only two potential sites for PCR primers directed toward exon 2 were revealed in a computer search (29). Nonetheless, when used with primer 10 (Fig. 1), primer 8 (Fig. 2) readily amplified the appropriate 700-bp class II B segment on first strand cDNA from scrub jays, house finches, and a red-winged blackbird as did primer 9 in the jays and blackbird (Fig. 2*B*).

Balancing Selection at Avian Class II B Genes. To evaluate within-individual diversity of class II B sequences, a total of 18 distinct sequences among 26 cDNA clones spanning the entirety of exons 2 and 3 was obtained from six individual birds sampled from natural populations (Fig. 3). Among scrub jays (three individuals, 19 clones), 12 distinct sequences were found; jay no. 3 yielded 6 different clones from 8 sequenced (Table 1), and there was one case of identical clones found in two different jays (clones 1.3 and 2.1). Importantly, all cloned genes encode cysteine residues in the expected positions in the β1 and α-helix domains for the formation of disulfide bridges in functional class II genes. Because these primers appear to be amplifying cDNAs corresponding to multiple genes, and because the pattern of nucleotide substitution in the 24 ABS codons could be obscured by comparisons of divergent sequences (4, 5), we analyzed groups of sequences within each species that differed from one another by increments of synonymous substitutions in the ABS codons, as recommended (5, 32). The mean d_N for sets of sequences differing by a small number of synonymous substitutions ($d_S < 0.1$) is significantly higher than 1, but, as expected (5, 32), values of d_N begin to plateau in comparisons of more divergent sequences, implying multiple nonsynonymous substitutions at the same codons over time (Fig. 4).

Evolutionary Histories of β Chain Domains. To examine the evolutionary history of the cloned sequences, we performed phylogenetic analyses of functional class II B domains sepa-

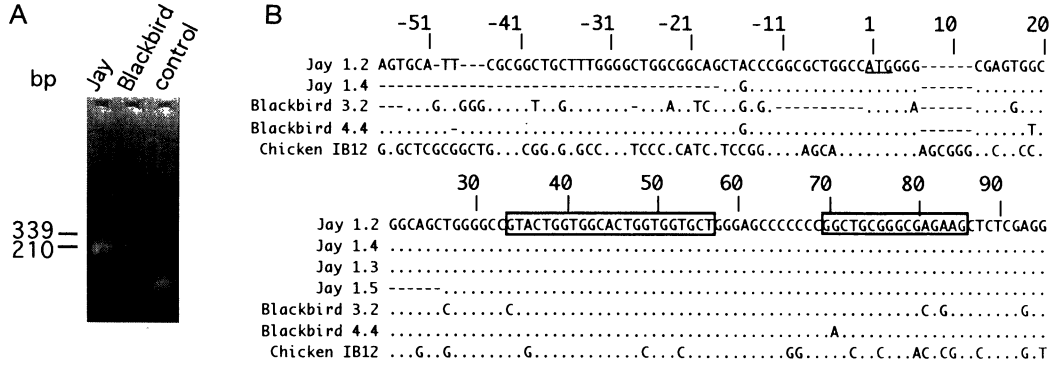

FIG. 2. LA-PCR products and sequences from scrub jay and red-winged blackbird cDNA. (*A*) Agarose gel (2%) showing PCR products generated by amplification of cDNA after ligation of primer 4 (Fig. 1) onto the 3′ end and negative control lane. The primers used in amplification were the reverse complement of primer 4 and primer 5 (Fig. 1). (*B*) Sequences of clones containing exon 1 of *Mhc* class II B genes derived from PCR products in *A*. Sequences are labeled according to species, with individual and clone numbers following. Dashes indicate gaps introduced to improve alignment. The Met start codon (ATG) is underlined in the top sequence; the two priming sites obtained in a computer search of exon 1 are boxed (see Fig. 1). Clones differed in length presumably due to differential efficiency of cDNA synthesis.

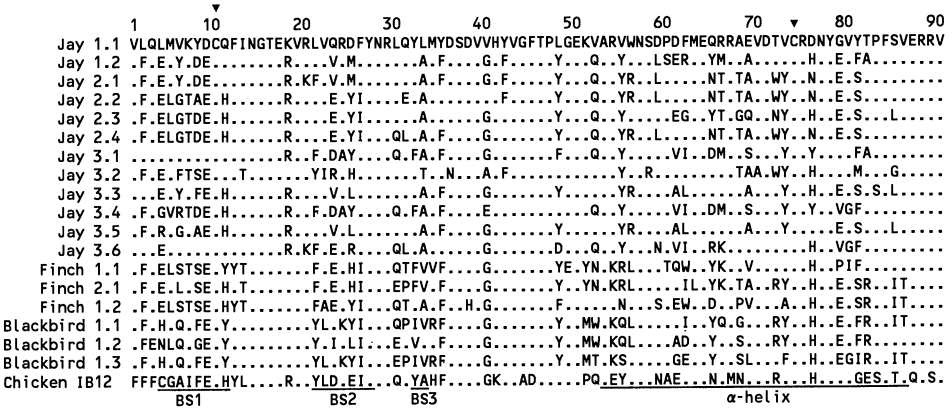

FIG. 3. Amino acid sequences of exon 2 of 18 distinct sequences among 26 class II B clones in songbirds. Sequences are numbered according to species (see text), with individual and clone numbers following. The sequence of the BL-BI gene from the chicken B12 haplotype (30) is shown at the bottom. Cysteine residues conserved in these and other functional class II B genes (11) are indicated with arrows. Amino acids comprising polymorphic subdomains as delimited in the crystal structure of a human DRB allele (23) are underlined.

rately, each time using a chicken sequence as an outgroup. Parsimony and neighbor-joining trees yielded essentially similar results. The trees of songbird class II genes are bush-like; in none of the trees is there strong evidence for clusters of sequences corresponding to orthologous genes in the different species. The tree of alleles of the well-characterized chicken BL-BI/II loci reveals a similarly bush-like tree, one whose internal branches are much shorter than a typical allelic tree incorporating multiple mammalian class II B genes (unpublished data). These results suggest that the loci in question have duplicated just prior to the divergence of alleles due to mutation.

In trees of the β-sheet of the second exon (Fig. 5*A*), the jay sequences frequently do not cluster in a monophyletic group; the trees of the α-helix show even less tendency to cluster according to species (Fig. 5*B*). This is reflected in the fact that there are few fixed amino acid residues clearly distinguishing clones from different species (Fig. 3). By contrast, the non-polymorphic exon 3 sequences cluster according to species in all analyses (Fig. 5*C*), despite little resolution among the jay sequences. Indeed, all blackbird and finch clones share an extra proline codon inserted in frame at amino acid position 105 that is lacking in the jay clones and all other published class II B genes. Thus, this shared-derived codon links the two Passerida species to the exclusion of the Corvida and chicken sequences, in agreement with DNA hybridization evidence (15).

To determine if the phylogenetic information in the three functional domains conflicted to a significant degree, the sequence variation of each domain was overlaid onto the set of parsimony trees implied by that domain and one other. For comparison of two sets of equally parsimonious trees for a given domain, the number of sites undergoing fewer steps on all trees within each set was compared (ref. 28; Table 1). Whereas the variability in the β-sheet could not distinguish between its own best trees and those of exon 3, the variability in the α-helix conflicts significantly with that in the other two domains in jays (Table 1). This pattern is robust to maximum-likelihood tests of discordance (37, 38) and is evident to a lesser degree when analysis is confined to variability in the third positions of codons (Table 1).

Table 1. Statistical tests of conflicts between trees based on different subdomains of songbird class II B genes

Domain	Competing sets of trees		
	β vs. α	β vs. exon 3	α vs. exon 3
All positions			
β-sheet (5)	**25:2**	7:8	—
α-helix (8)	**1:29**	—	**11:2**
exon 3 (6)	—	**0:14**	**18:0**
Third positions			
β-sheet (5)	**11:0**	2:2	—
α-helix (8)	**1:7**	—	2:1
exon 3 (6)	—	**0:6**	**0:7**

For each set of competing trees, the number of sites for the domain indicated undergoing fewer steps on all equally parsimonious trees of each set was counted using the "compare two treefiles" option in MACCLADE (31). The number of equally parsimonious trees in each set is indicated in parentheses (see Fig. 5). Comparisons in boldface type are significant at $P < 0.05$; boldface and underlined type, $P < 0.01$, using a standard G-test. The same set of trees was used to compare the signal in all positions of codons and third positions only. The low rates of *in vitro* recombination of PCR products in our (16) and other (19) studies of amplified *Mhc* genes, the finding of identical sequences within and between individuals, and the fact that several discordances among trees of domains involve sequences amplified in different experiments or individuals all suggest that such discordances are not wholly PCR artifacts.

Contrasting Histories of Class II B Genes in Birds and Mammals. To examine the pattern of orthology of songbird and chicken class II B genes, we aligned exon 3 sequences from songbirds with the six known from chickens BL-B genes and those from various functional genes and pseudogenes from mammals and a bony fish. As expected (8, 9, 39), this tree reflects the retention of orthologous genes produced by gene duplication in the ancestor of mice and humans; thus the

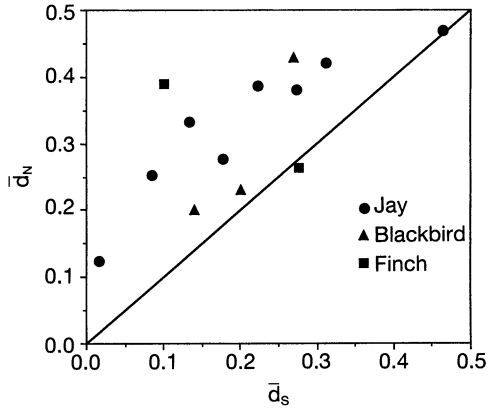

FIG. 4. Patterns of nucleotide substitution in the ABS of songbird class II B genes. Only the 24 residues comprising the ABS of a human allele (23) were analyzed, and only sequences within species were compared. Alleles were placed in groups within which the number of synonymous substitutions in the ABS codons varied from 0.05 to 0.45. Within each of these groups, the mean d_N and d_S were calculated (see text). The line for $d_N = d_S$ is indicated.

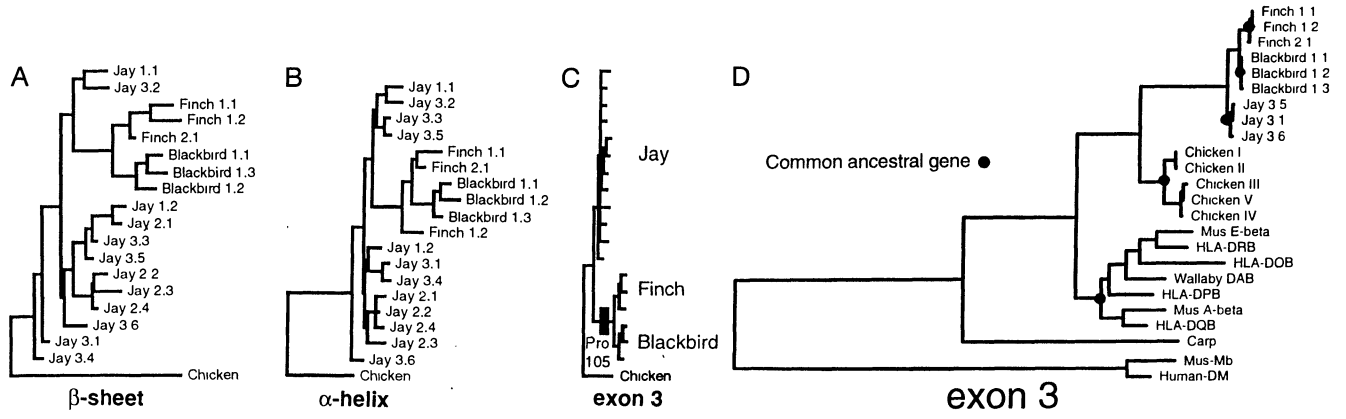

FIG. 5. Phylogenies and relationships of songbird class II B genes with the chicken sequence used as an outgroup. (*A*) Tree obtained using variability in the β-sheet codons (1–56) of exon 2 (see Fig. 3). The tree was one of five equally parsimonious trees found with PAUP (ref. 26; 207 steps) and is identical to the neighbor-joining tree obtained using a Jin–Nei correction (33) with $\alpha = 0.5$. (*B*) Neighbor-joining tree obtained using variability in the α-helix (codons 169–279, Fig. 3) as in *A*. This tree is two steps longer than eight equally parsimonious trees (length = 208). (*C*) Tree of exon 3 sequences, corresponding to one of six equally parsimonious trees (length = 120) and the neighbor-joining tree with distances calculated as in *A*. The extra proline codon at position 105 found in the finch and blackbird sequences is indicated. (*D*) Neighbor-joining tree of representative exon 3 sequences from songbirds, chickens, and other vertebrates. Black dots indicate putative common ancestral genes for duplicated genes within species. A parsimony analysis (26) with 10 replications, sequences added in random order, and treating all substitutions and sites equally yielded six trees of equal length (698 steps) that were similar to the illustrated tree. The branch leading to the songbird sequences is significant by a bootstrap test (34). The mouse and human DM sequences (35, 36) were used as an outgroup; parameters for distance analysis are as in *A*. The five chicken genes for which exon 3 was available are from ref. 30. The other sequences used were from GenBank and accession numbers are available on request. The alignment used was that of ref. 35.

mouse Eβ and human DRβ genes and the Aβ and DQβ genes cluster separately, with related pseudogenes (Fig. 5*D*). In our analysis of exon 3, even a class II B gene from a marsupial, DAB (40), clusters closer to the placental DRB and DOP genes, indicating possible orthology to the ancestor of DRB and DOB. By contrast, none of the songbird exon 3 sequences falls inside the cluster of chicken genes or clusters with any one chicken gene; indeed, the branch leading to the cluster of all songbird sequences is long and statistically significant (Fig. 5*D*). Thus, although the cloned songbird sequences likely stem from multiple genes, none of them is orthologous to any one of the chicken genes.

Recent Ancestry of Chicken Class II B Genes. A recent analysis of variability at chicken BL-B genes (30) revealed a lack of orthology between chicken and mammalian class II B genes. However, the variability in exon 2 was used in these comparisons, and the measure of sequence divergence used and the branch lengths depicted in this tree were unclear. Our analysis (Fig. 5*D*) of exon 3 sequences immediately reveals that the branches leading to the five chicken genes for which exon 3 is known are much shorter than those leading to the mammalian class II genes. For example, whereas the third exon of the mouse Aβ and Eβ genes differ by >40%, chicken class II B genes differ on average by <7%. The similarity and monophyly of sequences cloned from within each of the songbird species recapitulate this pattern. Our analysis also reveals two lineages of chicken genes corresponding to the two linkage groups of the chicken *Mhc*, now known to belong to separate linkage groups (41). Within each of these clusters, exon 3 sequences are identical (e.g., I vs. II) or nearly so.

DISCUSSION

Using variability in the complete second and third exons obtained via the LA-PCR technique, we have been able to quantify the effects of balancing selection at avian *Mhc* class II B genes and to determine the pattern of orthology of these genes within birds. These comparisons show that lack of orthology in the *Mhc* class II region may occur *within* non-mammalian groups, in contrast to the pattern observed in mammals, in which duplicated class II genes are retained for long time periods. Nonetheless, variability in the ABS codons of songbird class II B genes is the result of diversity-enhancing selection similar to that observed in mammals. Thus, although the short-term evolutionary histories of class II genes in birds are likely similar to that in mammals, their long-term evolutionary dynamics appear to be different.

The *Mhc* class II region of mammals is well known for its stability over long time scales, as compared to the class I region, which experiences higher rates of gene duplication, deletion, and pseudogene formation (9, 42, 43). The *Mhc* of marsupials contains novel β chain genes of dubious orthology to known eutherian genes. But the precise placement of these sequences in the mammalian class II B tree varies depending on the domain and analysis used (refs. 40 and 44; Fig. 5*D*), and orthologous relationships at some class II α chain loci have persisted since the divergence of therian [marsupial and monotreme] and eutherian mammals (40). Thus, at least some duplicated mammalian class II genes have been retained for >100 MYA of mammalian evolution (45). The clustering of duplicated genes within each songbird species (indicating lack of orthology), rather than as lineages spanning multiple species, suggests that orthologous relationships such as those between mammalian DRB genes are not present among *Mhc* class II B genes even within the songbirds. The most likely explanation for this pattern is that chicken and songbird class II genes stem from much more recent ancestral genes within their respective groups than their mammalian counterparts. Our results suggest that rounds of gene duplication and homogenization in the class II B genes of birds occur on a faster time scale than in mammals, implying in turn that evolution in the avian class II region may in fact resemble that in the class I region of mammals more so than that in the mammalian class II region. Although we have amplified multiple class II B genes, the primers used here may not have amplified some genes that are orthologous to chicken genes. However, the existence of such genes in songbirds is unlikely given how recently the chicken genes appear to have diverged.

Our results conform to general models of long-term *Mhc* evolution (7, 43, 46) in which ancestral genes undergo duplication and deletion independently in divergent vertebrate lineages. The timing of these processes suggested by studies in

mammals would produce a pattern in which orthologous class II genes from different species will cluster together over relatively short time scales within major taxonomic groups (e.g., within mammals) but will not exhibit clear orthologous relationships over longer time scales (e.g., between mammals and other vertebrates). While this model likely holds for *Mhc* class II genes in all classes of vertebrates, we suggest that the values of parameters of this model—rates of gene duplication and deletion and the strength, mode, or consistency of diversifying selection among loci—may differ drastically between mammals and birds. The finding that rates of such processes differ between birds and mammals at other immune system loci (57) suggests a general difference in the immunogenetics of these lineages.

The absence of orthologous class II B genes between chickens and songbirds and within songbirds might be less surprising if these lineages diverged about the same time as marsupials and placental mammals (100 MYA). Older estimates of divergence between gamebirds/waterfowl (Galloanseriformes) and other neognathous birds (>100 MYA), between Passerida and Corvida (55–60 MYA; refs. 15 and 47), and between Passerida lineages soon after this rest primarily on DNA hybridization evidence. However, a variety of other data, molecular (48–50) and fossil (51), suggest that these dates could be much more recent. Refinement of these dates will be important for a more informed interpretation of the long-term evolution of the *Mhc* in birds.

The pattern of discordances among polymorphic subdomains found in the bird class II B sequences (Table 1; Fig. 5 *A–C*) is similar to that found in a similar analysis of human DRB sequences (52). Different histories of the domains in exons 2 and 3 could be the result of recombination within species, different degrees of trans-species evolution between domains facilitated by recombination, or convergence within and between species. Only the last hypothesis posits incorrectly reconstructed trees. Because we are dealing with an unknown number of songbird genes, and because the divergence times of songbird lineages are uncertain, it is difficult to evaluate these alternatives at this time, although we note that mammalian ABS regions are likely subject to convergence (53–55), and the extremely biased base composition of third positions of codons in the ABS of songbird class II genes makes even these sites susceptible to convergence (ref. 56; this study). More detailed characterization of these genes in songbirds and other avian groups will help discriminate among these hypotheses.

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