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The MHC of the Duck (*Anas platyrhynchos*) Contains Five Differentially Expressed Class I Genes

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MHC class I proteins mediate a variety of functions in antiviral defense. In humans and mice, three MHC class I loci each contribute one or two alleles and each can present a wide variety of peptide Ags. In contrast, many lower vertebrates appear to use a single MHC class I locus. Previously we showed that a single locus was predominantly expressed in the mallard duck (*Anas platyrhynchos*) and that locus was adjacent to the polymorphic transporter for the Ag-processing (*TAP2*) gene. Characterization of a genomic clone from the same duck now allows us to compare genes to account for their differential expression. The clone carried five MHC class I genes and the *TAP* genes in the following gene order: *TAP1, TAP2, UAA, UBA, UCA, UDA, and UEA*. We designated the predominantly expressed gene *UAA*. Transcripts corresponding to the *UDA* locus were expressed at a low level. No transcripts were found for three loci, *UBA*, *UCA*, and *UEA*. *UBA* had a deletion within the promoter sequences. *UCA* carried a stop codon in-frame. *UEA* did not have a polyadenylation signal sequence. All sequences differed primarily in peptide-binding pockets and otherwise had the hallmarks of classical MHC class I alleles. Despite the presence of additional genes in the genome, the duck expresses predominantly one MHC class I gene. The limitation to one expressed MHC class I gene may have functional consequences for the ability of ducks to eliminate viral pathogens, such as influenza. *The Journal of Immunology*, 2005, 175: 6702–6712.

Major histocompatibility complex class I proteins play a fundamental role in the presentation of endogenously derived peptides to CTLs and also serve as self-recognition elements for NK cells. As such, MHC class I genes are critical for immune defenses against viruses. Polygeny and polymorphism of alleles contribute to the breadth of the immune response. Comparison of vertebrate MHC genomic regions shows that it is the most dynamic part of the genome (1). The number of functional and defunct MHC loci can differ greatly in each species, and no orthologous genes can be identified between orders. In humans and mice, three MHC class I loci each contribute one or two alleles, presenting a broad spectrum of diverse peptides. This contrasts with frogs, sharks, fish, and birds, many of which appear to predominantly express one MHC class I gene (2, 3). In many species, additional genes are present in the genome; however, the mechanisms accounting for the loss of expression of additional classical class I genes have not been examined. The limitation to one MHC class I locus is predicted to have wide-reaching functional consequences, influencing the positive and negative selection of the TCR repertoire, the NK cell repertoire, and diversity of peptides that can be displayed. In light of the role of the duck as a reservoir of influenza (4, 5), we are examining MHC class I gene expression to determine their capacity for defense against viruses.

In chickens, there are two clusters of MHC class I genes, the *B* locus and the *Rfp-Y* locus that map to the same microchromosome (6, 7). The *B* locus contributes all of the hallmarks of the MHC, controlling allograft recognition (8, 9), MLRs (10), cellular cooperation (11, 12), and disease resistance (13, 14). In the *Rfp-Y* complex, there are additional MHC class I genes (15), one of which is polymorphic and transcribed (16). However, the *Rfp-Y* genes do not otherwise have the hallmarks of classical MHC class I genes (16).

Within the *B* complex, two MHC class I genes (the minor and major genes) flank either side of the transporter of Ag-processing *TAP1* and *TAP2* genes, with the dominant locus adjacent to *TAP2* (17). Recent nomenclature refers to the minor locus as the *BF1* locus, and the dominant locus is referred to as the *BF2* locus (18). Both loci are considered equivalent to the classical class I MHC genes in other species, and numerous alleles have been identified, particularly for the *BF2* locus (19–23). In chickens, the *TAP1* and *TAP2* genes are also polymorphic (24). Kaufman (2) argues that in this organization, the TAP and MHC class I proteins have an opportunity to coevolve to function coordinately and evolutionary forces select for inactivation of redundant loci.

The MHC class I regions of other birds appear to have undergone extensive duplications, and the “minimal MHC” of the chicken is not the norm for other birds. The MHC of quail has the same overall organization as chickens with MHC class I genes flanking *TAP1* and *TAP2*, but is much more complex, with four MHC class I genes, *Coja-D1, -D2, -B1, and -E* and three pseudogenes -F, -G, and -H (25, 26). Of these, *Coja-B1* adjacent to *TAP2* is the most likely ortholog of *BF2*, and two loci (*D1* and *D2*) appear nonclassical due to weak expression (26). Sequence comparison of the quail MHC class I genes reveals their overall similarity to each other and the authors suggest that they have duplicated since the divergence of these two avian species. In the red jungle fowl, the closest to the evolutionary ancestor of domestic chickens, there are 14 copies of MHC class I-like genes, 3 of which are pseudogenes, in the preliminary analysis of the recently released genome sequence (27). There are reports of extensive duplication within MHC regions of sparrows (28, 29), songbirds (30, 31), and reed warblers (32–34). These data suggest that the MHC of most birds has undergone expansion.

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Ducks diverged earlier from the Galloanseriforme lineage (35) and comparison of their MHC class I region may shed light on the primordial avian MHC. Previously we showed that the duck, \textit{Anas platyrhynchos}, like the chicken, also has a predominantly ex-pressed MHC class I gene, and that gene lies adjacent to the poly-morphic \textit{TAP2} gene (36). However, we had evidence of at least four MHC class I genes per haplotype. To resolve the discrepancy between the number of expressed sequences and the number we estimated in the genome, we have undertaken an analysis of the genomic organization of the MHC class I region of the same duck to allow direct identification of expressed and unexpressed genes. In this study, we show that the MHC class I region of the duck contains five genes that show a high level of overall similarity. Surprisingly, the overall organization of MHC class I genes and \textit{TAP} differed from that of chicken or quail and may resemble the locus before a translocation event. Our analysis revealed the de-fects in some of these genes, accounting for their lower expression. Despite an expanded number of genes in the genome, the duck MHC class I region functionally resembles the minimal MHC of the domestic chicken.

Materials and Methods

Screening of a fosmid genomic library for MHC class I genes

A genomic library from erythrocyte DNA from a male duck, \textit{Anas platyrhynchos} (duck 26) was previously constructed in the fosmid vector pCRII as to refract splices immediately 124,488 independent colonies were screened using a probe derived from a conserved region of MHC class I exon 4 common to all cDNA sequences previously identified in the same individual duck (36). Overlapping primers, MHC I, 5'-AGA TCC TGA CCT TGT CCT GCC GTG-3' and MHC I', 5'-GAG TAG AAG CCG CGG GCA CGG CAG-3' (Quagen) were used to generate an "overgo" probe (38). Screening of the library identified only one MHC class I-hybridizing clone, clone Ap26-72A12.

Sequence strategy

Because preliminary sequencing suggested that MHC class I genes in the clone could be very similar, a set of subclones was constructed to completely sequence fosmid Ap26-72A12. EcoRI subcloning produced seven unique clones, which completely covered the original fosmid insert: five MHC class I-hybridizing subclones, 5.8 kb for UBA and 4.2 kb for UCA, 5.9 kb for UDA, and 4.6 kb for UEA to vector arm; a 9-kb subclone containing \textit{TAP1} and \textit{TAP2}; and a 1.5-kb subclone which contained only intergenic sequence. Using the EZ-TN <KAN-2> transposition insertion kit (Epiconcept Technologies) primer islands were inserted into the EcoRI subclones. The 1.5-kb EcoRI subclone was sequenced by primer walking. Sequencing was done using Amersham DYEnamic ET terminator chemistry (Amersham Pharmacia Biotech) on an ABI377 Automatic Se-quence (Applied Biosystems). Contigs were created using AutoAssembler (version 2.1; Applied Biosystems) with coverage of at least 5-fold redundancy and included sequences on both strands. Determining the order of the EcoRI subclones in the final 36.8-kb contig was accomplished by sub-cloning large internal fragments, restriction mapping, and sequencing of overlapping fragments. The sequence of the entire 72A12 fosmid clone is deposited in GenBank under the accession number AY885527.

Sequence analysis

The edited 72A12 final sequence was analyzed for coding regions using GENSCAN (http://genes.mit.edu/GENSCAN.html) and through homology searches using BLAST (www.ncbi.nlm.nih.gov/blast). MHC class I genes within the clone were compared with cDNA and PCR fragments previously published for this individual duck (36) using GeneTool (BioTools) to as-sign haplotype and locus information to expressed genes in this animal as well as to refine splice junctions in predicted cDNA sequences. RepeatMasker (http://repeatmasker.org) and RepeatModeler (http://repeatmodeler.org) identified and classified motifs but not all sequence repeats. Additional WU-BLASTn and WU-BLASTx (http://wublast.org/WU-Blast.html) analyses were needed to refine classification of the embedded retroviral sequences. Chicken repeat se-quences used for comparison were from Repbase (www.girinst.org) and recent analysis of the chicken genome (39). PipMaker (http://bim- ose.psu.edu/pipmaker) was used to produce the dot matrix plot. Gene Rec-ognition and Assembly Internet Link (GRAIL) was used to identify the Cpg islands (http://comphio.ornl.gov/graiexp). Analysis of percent nucle-ootide and amino acid identity was done using GeneTool and PepTool (BioTools).

The program Synonymous/Nonsynonymous Analysis Program (http:// www.hiv.lanl.gov/content/hiv-db/SNP/) was used to calculate rates of nonsynonymous (d\textsubscript{s}) and synonymous (d\textsubscript{S}) nucleotide substitutions between loci and alleles according to the method of Nei and Gojobori (40) as implemented (41). Peptide-binding pocket residues in the Ag recognition site of duck MHC class I genes were predicted by comparison to those predicted for trout (42) and chickens (23). Multiple alignments of MHC class I sequences and a bootstrapped phylogenetic tree was created using ClustalW at DDBJ (http://www.ddbj.nig.ac.jp/search/clustalw-e.html).

Promoter analysis

Regions 3000 bp upstream and ending at the translational start sites of the MHC class I genes were analyzed for potential transcription factor-binding motifs using the following web-based programs: BioInformatics and Mole-cular Analysis Section (BIMAS) WWW Signal Scan (http://bimas.cit.nih.gov/molbio/signal/), Genomatrix MatInspector (http://www.genomatrix.de/products/MatInspector/index.html), and Alibaba2.1 (http://www.alibaba2.com/). Duck sequences were also aligned to known MHC class I promoters (human and chicken) using ClustalW and conserved sites within the promoter regions were identified manually.

Northern blot and allele-specific hybridization

Allele-specific oligonucleotide hybridization was performed to assess the relative transcript abundance. An oligonucleotide probe was designed to be specific for the UBA MHC class I sequence (5'-CGA CAG CGA GAC CAG GAT-3'). The Northern blot was probed to test for expression of transcripts matching the UBA locus using the same blot and conditions previously used to assess transcript abundance (36). The oligonucleotide was end-labeled with [\textgamma\textsuperscript{32}P]ATP (PerkinElmer Canada) to \(>2 \times 10^{8}\) dpm/pmol specific activity, then purified using Sephadex G-25 (Sigma- Aldrich) size-exclusion chromatography. The Northern blot was hy-bridized overnight at 48°C in aqueous hybridization solution (5× Den-hardt's, 6× SSC, 0.1% SDS). Three 15 min washes were conducted at low stringency (6× SSC, 0.1% SDS) at 50°C. The blot was exposed to Kodak X-OMAT film at −80°C for 72 h.

To test the pattern of hybridization of all MHC class I alleles in a tissue, a Northern blot was hybridized with the D26/4p probe amplified from duck 26 using degenerate primers CHE4F1 (5'-GCC CCG GTA CGT TSG TSG-3') and CHE4R1 (5'-CAG GCA GCA CYG GTA CTT GTC-3').

RT-PCR amplification of expressed sequences

To identify expressed MHC class I alleles from other individual ducks, we synthesized first-strand cDNA from 5 μg of duck spleen total RNA using 0.5 μl of an oligo(dT) primer (5'-CTCTGGTTCTTCGTCAGTAC-3') and extending with the Thermoscript RTase H\textsuperscript{-} Reverse Transcriptase kit according to the manufacturer's instructions (InVitrogen Life Technolo-gies). The MHC class I inner primers (SP2, 5'-GGC TGC TGC TGG GGG TCC TG-3' or (E2F1, 5'-GGC CCA CTC CCT GCC GCT-3') and the reverse primer (3' untranslated region, 5'-GCA GAT AGG AGA TGT GAG GTG TGTG-3') were used to amplify MHC class I. Products were cloned into the PCR 2.1 TOPO vector (InVitrogen Life Technologies) and at least three clones for each allele were isolated and completely sequenced. The sequences for these alleles named U*06-U*09 were deposited in GenBank under the accession numbers AY841481-4.

To examine the tissue distribution of expression, we performed allele-specific amplification by reverse transcription PCR using tissues from a duck (D132) that was matched for the MHC alleles of the duck used for the library (D26). First-strand cDNA was synthesized and used for PCR am-plification using Tag proofread (InVitrogen Life Technologies) and TaqStart Ab (Clontech Laboratories). Templates were denatured for 3 min at 96°C followed by amplification for 30 cycles at 96°C for 30 s, 30 s at annealing temperature, followed by extension for 1 min at 72°C. Allele-specific primers had at least three positions of mismatch compared with other sequences, including the 3' end. The UAA locus (U*03 allele) was amplified with forward (5'-ATG GGG AAG CAG CCT TCA CA-3') and reverse (5'-GCA ATT ACT AAT TTT CTG AAA-3') primers, and using the same conditions as UDA primers. The UBA locus (U*04 allele) was amplified using forward (5'-ACT CCC TGC GCT ACT TCT TG-3') and reverse (5'-CTC TGC ATG TGA CCG CGC-3') primers at 67°C, yielding a 391-bp product. All MHC class I sequences were amplified using forward (E2F1, 5'-GCC CCA CTC CCT GCC GCT-3') and reverse (E2R1, 5'-GCC TGC TGT CTA GTG AAT TTT-3') primers using the same conditions as UDA primers. Amplification of β-actin from cDNA using forward (βACT1N1F, 5'-ACC GGC CAA CTC CCC GCC AG-3') and reverse primers (βACTINE2R, 5'-ATA GTC GTC TTT CTG GCC-3') was performed using the same protocol.
were hybridized sequentially with the MHC class I and the TAP2 probes. The MHC class I probe was amplified using forward (E3R2, 5'-CCG GGA GAA CAC CTTG CAT TGG G-3') and reverse (E4R2, 5'-AGC AGC GCA GCA GGA CAA GTT-3') primers on subclones of the genes found on genomic clone 72A12, and PCR amplification products were pooled. All amplifications worked except UCA. The TAP2 probe corresponded to exons 6–8, amplified from cdDNA clone 6.2.2 using forward primer (DT2WAF, 5'-TGA ACG GCA GTG GTA ACG GAG AGA-3') and reverse primer (DT2E8R1, 5'-CAA CAC GCT GCT TCT GCC CA-3') (36). Hybridizations were conducted for 16 h at 60°C in aqueous hybridization solution (5× Denhardt's, 6× SSC, 5% dextran sulfate, 1% SDS, and 100 μg/ml salmon sperm DNA (1× SSC: 0.15 M NaCl, 15 mM Na2HPO4, pH 7.6, and 1 mM EDTA)). Washes were conducted at high stringency in 0.1× SSC/0.1% SDS at 65°C. Probes were radiolabeled with [α-32P]dCTP by random priming (43) (Primelit kit; Stratagene). The blots were exposed to Kodak X-OMAT film at −70°C for 10 days.

Results

The duck MHC class I region has undergone duplications yielding five MHC class I genes

To determine the genomic sequence of the duck MHC class I region and to compare the expressed MHC class I sequences to those in the genome, we screened a genomic library constructed from the same duck used for construction of the cdDNA library. A single hybridizing clone was isolated, 72A12, and subjected to restriction digestion for mapping and subcloning (Fig. 1A). A complete contig of 36.8 kb containing TAP1 and TAP2 and five MHC class I genes (UAA, UBA, UCA, UDA, UEA) was assembled (Fig. 1B). TAP1 and TAP2 are in opposite transcriptional orientation, and the five MHC class I genes are arranged in the same transcriptional orientation. The gene adjacent to TAP2 was previously identified as the predominantly expressed locus and named UAA, and is identical in sequence to the MHC-TAP2 haplotype 2 previously PCR amplified from this duck (Table I) (36). Other genes were given consecutive names based on the proposal for naming vertebrate MHC genes suggested by Klein et al. (44). The relative abundance of transcripts from the MHC class I genes on this haplotype is shown (Fig. 1C), with cdDNA clones U*03 and U*04 previously isolated from this duck matching the UAA and UDA locus sequences exactly (Table I). The overall G + C nucleotide content was 58%. A scan of the sequence using GRAIL identified abundant CpG islands in the 5′ end of most genes extending into intron 1, with UBA being an exception (Fig. 1D). For comparison, we counted 37 CpG motifs in the UAA locus, 41 in UDA, and only 17 near UBA.

We analyzed the complete sequence for the presence of repeats that may provide insight into the duplication events giving rise to the five loci. RepeatMasker detected six simple repeats in intergenic and intronic regions, nine copies of LINES resembling the chicken GGLTR3 family (Fig. 1E). All nine copies of chicken CR1 LINE and the long terminal repeat (LTR) elements were incomplete. The embedded retroviral elements similar to the chicken GGLTR3 longitudinal repeat (GGLTR11) family appeared three times in

Table I. Alleles identified in cdDNA and the corresponding locus

<table>
<thead>
<tr>
<th>MHC-TAP2 Haplotype</th>
<th>UAA</th>
<th>UBA</th>
<th>UCA</th>
<th>UDA</th>
<th>UEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplotype 2</td>
<td>U*03</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>U*04</td>
</tr>
<tr>
<td>Haplotype 1</td>
<td>U*02</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not detected.

3 Abbreviations used in this paper: GGLTR, Gallus gallus long terminal repeat; LTR, long terminal repeat; BSE, IFN-stimulated response element; GRAIL, Gene Recognition and Assembly Internet Link.
the sequence, once between UDA and UEA and once between UAA and UBA, with a small fragment between UBA and UCA. The LTR appears to have inserted within CR1 LINEs before duplication of the locus based on the order CR1-LTR-CR1 within both intergenic regions. The location of this repeat within the UBA to UCA intergenic region suggests that one gene of the duplicated pair giving rise to UCA was lost. The GGLTR5 sequence identified in the intergenic region of UDA to UEA is unique and has inserted subsequent to the duplication events. Further analysis of this element suggests it is closest to the chicken Hitchcock element (39) and is flanked by 7 bp direct repeats, typical of tandem site duplication upon transposon insertion.

A dot plot analysis of the Anpl MHC class I region against itself revealed extended regions of duplication (Fig. 2). Unbroken diagonal lines within the shaded regions indicated that there was conservation of the exons and introns across all five genes. The diagonal lines that extend into the white regions suggested that the region of duplication included large segments of the intergenic regions as well. An extended region spanning the UEA and UDA, forms a line in comparison to the region spanning UBA to UAA, suggesting they are part of an extended region of duplication. The embedded retroelement in this region, which appears between UAA and UBA and also between UDA and UEA, may have preceded the duplication. The only break in the line comparing UEA and UDA to UBA and UAA corresponded to the region encoding the cytoplasmic domains, which are unique and appear disrupted in the UEA locus.

The genes encoding the transporter for Ag processing, TAP1 and TAP2, are directly adjacent to the UAA gene of ducks. To determine whether additional MHC class I genes lie outside the clone, we analyzed restriction fragments hybridizing with the MHC class I and TAP2 genes in genomic DNA from the same duck used for construction of the genomic library (Fig. 3). The clone 72A12 had an EcoRI site between the dominantly expressed MHC class I gene Anpl U*03 and TAP2. A 13-kb TAP2-hybridizing EcoRI fragment does not carry any class I-hybridizing genes, suggesting that there are no class I genes immediately adjacent to TAP1. Most MHC class I genes lie on several EcoRI fragments of 6 and one of 4.5 kb.

The hybridizing EcoRI bands of 32 or 37 kb carry the two allelic forms of the UEA gene. We were unable to determine whether additional MHC class I genes lie on the other side of the UEA locus; however, all of our data taken together is consistent with the clone 72A12 representing an entire haplotype of the MHC class I region. Similarly, from previous analysis of the sequence for haplotype 1, we know there is an XhoI site between U*02 and TAP2. Therefore, the TAP2-hybridizing XhoI fragment of 24 kb belongs to haplotype 1. The fact that this fragment does not hybridize with class I also confirms no class I genes are immediately on the other side of TAP1. Southern blots using other restriction enzymes gave the same conclusion (data not shown).

All five genes within the locus have the hallmarks of classical MHC class I genes

To compare the five MHC class I genes, the deduced amino acid sequences were aligned (Fig. 4). The leaders for UAA, UCA, and UDA were 25 aa, whereas the leader of UBA was 30 aa in length due to a five amino acid insertion. The leader for UEA lies outside the clone. The majority of polymorphic amino acid residues were within these two domains with 42 polymorphic residues in α1 and 30 polymorphic residues within α2. The α3 domain of 91 aa was highly conserved between the five sequences, with only 11 polymorphic residues. The transmembrane and cytoplasmic domains were very similar, except the UBA cytoplasmic domain is one amino acid shorter, and the UEA sequence had an unusual truncated cytoplasmic domain. Within the mature protein, the identity of the amino acid sequences ranges from 81.9% between UAA and UCA to 87.4% between UBA and UDA. Paired amino acid identity matrices for each exon failed to identify loci that are more similar to each other. The loci are most different within the α1 and α2 domains and all are very similar within the α3 domain.

To determine which genes could be involved in Ag presentation, we examined the amino acid sequences for hallmarks of classical MHC class I genes. Among residues predicted to be involved in
peptide anchoring (Y7, Y59, Y84 (R84 in nonmammalian vertebrates), Y123, T143, K146, W147, Y159, and Y171), all were conserved in most loci. The Y123 sequence is replaced by phenylalanine or leucine in all five duck genes, but is also seen in other avian sequences and many other species (45). The UDA did not have the conserved Y171; however, histidine at this residue (H169) was seen in other mammalian classical MHC class I sequences. All sequences have the site for N-linked glycosylation at N85. The negatively charged residues in the two regions of the α3 domain implicated in CD8 binding (46) are conserved, except N250 in UEA replaces aspartic acid. The glutamine directly involved in CD8 accessory function (47) is conserved in all sequences except UCA, in which it is replaced by a premature stop codon.

Evidence for diverse mechanisms of inactivation within additional MHC class I loci

A comparison of the genes in the locus with the sequences isolated from a cDNA library constructed from the same duck allowed us to characterize the expression of these genes (Fig. 1C and Table I). The sequence of the gene adjacent to TAP2 was identical to the sequence of one of the abundantly expressed alleles, AnplU*03. The frequency with which we found transcripts corresponding to UDA was very low, consistent with it being a nonclassical or MHC class Ib gene. The sequence of UDA matched four cDNAs of two different sizes, named AnplU*04. Examination of the 3′ untranslated region of this gene indicated the presence of two polyadenylation sites, one at 152 bp and one at 814 bp from the stop codon. Allele-specific oligonucleotide hybridization suggested that transcripts of either size were expressed at a rate 10-fold less than AnplU*03 (36) (Fig. 1C). To examine the tissue distribution of the two expressed loci (UAU and UDA), we used reverse transcription followed by PCR amplification with allele-specific primers. Although this amplification is not quantitative, it was useful for comparison between tissues. The expression of the UAU (U*03 allele) was 20–25% higher in spleen and liver than in heart, kidney, or testis. The same pattern was seen for amplification of all alleles using universal primers in exon 2. This correlated well with the pattern of hybridization of an exon 4 probe on a Northern blot, expected to hybridize to all sequences (Fig. 5). In contrast, the amplification of UDA was almost equal in all tissues and did not show a significant increase in spleen and liver.

No transcripts were found corresponding to the sequence of UEA. The U*05 cDNA sequence from this duck, differing from UEA by one amino acid in exon 4 and one amino acid in exon 8, is most likely the allele encoded at the UEA locus on the other haplotype. Two transcripts of U*05 were recovered from a cDNA library; however, neither had a polyadenylation signal sequence or poly(A) tail, both arising as unusual clones (36). We noted that UEA does not have a polyadenylation signal sequence anywhere within the intergenic region. The end-labeled-specific oligonucleotide used to examine the expression of the U*05 cDNA sequence would also hybridize perfectly with the UEA sequence. The hybridization of the specific oligonucleotide to mRNA was undetectable in spleen, but two weakly hybridizing bands were detectable...
in intestine, indicating that transcripts from UEA, if present, are also rare (36).

We were unable to detect transcripts for two loci, UCA and UBA. The sequence of UCA encoded a stop codon in-frame in exon 4 and would result in nonsense-mediated mRNA decay. UBA had a unique exon 4 sequence, which had not been amplified from genomic DNA due to mismatches in the priming regions. No cDNA transcripts matching this locus were identified, although the exons of this gene are apparently intact. To examine the expression of this gene, we hybridized the same Northern blot of spleen and intestine mRNA using an allele-specific oligonucleotide hybridization (36). No hybridization to mRNA from spleen or intestine was detectable, indicating this gene is not expressed (data not shown).

To investigate whether differences in promoter cis-elements might be responsible for the differential expression of the class I genes, proximal promoter sequences were aligned for UAA, UBA, UCA, and UDA (Fig. 6). The regulation of MHC class I region genes is mediated by a number of conserved cis-acting regulatory sites including enhancer A (48), an IFN-stimulated response element (ISRE) and site α (homologous to the cAMP response element) (49) and enhancer B (reviewed Ref. 86). Enhancer A is bound by NF-κB/rel family members while the ISRE site is bound by IFN-inducible transactivators (50). We identified putative transcription factor binding sites in the promoter regions using computer analysis and alignment with other promoters. An NF-κB site was identifiable in the UAA, UBA, and UDA promoters, while the sequence of UCA lacked this motif. An ISRE appeared intact in all four sequences, with one nucleotide difference in the ISRE motif for UDA.

We examined the five duck MHC class I promoters for the presence of the conserved regulatory motif described as the S, X (both X1 and X2), and Y motif within the proximal promoter. The SXY motif is found within MHC class I, MHC class II, invariant chain, DMA and DMB, and β2-microglobulin (51). The SXY module is bound by regulatory factor X, X2 box-binding protein, and NFY bound to X1, X2, and Y respectively, while the S motif does not appear to be occupied in vivo. The

| Ampl1-UAA | tggcagctg caccgcct gtcggct cagggaggg ggggggggg ggggggggg |
| Ampl1-UBA | g.cacttg.g...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgcc...ctgc
requirement for all three motifs and the spacing between them led to the suggestion that this motif functions as an “enhancer-some” in the regulation of MHC genes (52). This motif is intact within the UAA, UCA, and UDA sequences, but there is a deletion within the X1 and X2 box of UBA. A second deletion in the UBA promoter spans a putative CAAT box. The disruptions within the promoter of the UBA sequence may account for the lack of expression of this gene.

**FIGURE 7.** A. Phylogenetic tree showing the relationship of the Anpl MHC class I sequences to the classical and nonclassical MHC class I sequences of other birds. The dendrogram was constructed with the entire mature protein amino acid sequence of each MHC class I sequence. Proteins used were the quail Coja-B1 (BAC82519.1), Coja-C (BAA83671.1), Coja-D1 (82515.1), Coja-D2 (BAC82516.1), and Coja-E (BAC82520.1); the chicken Gaga-BF2 (CA818972.1), Gaga-BF1 (CA818969.1), and Gaga YF (Rib-Y) (AAK115583.1); the Sandhill crane Grus canadensis Grca (AF013106); and the domestic goose Anser anser Anan (AY654899.1) were aligned with ClustalW. Bootstrap values are shown at the branch points. B, Phylogenetic tree showing the relationship of all available Anpl MHC class I sequences to the genomic loci of one haplotype. Sequences were obtained as cDNA clones (alleles U*02–05 with accession numbers AY24416–9 (36) or amplified by RT-PCR in this article (alleles U*06–U*09, accession numbers AY841881–4), or from others AB115241–6 (85) and U*01 AF383511.
The genomic sequences form separate branches on the tree and compared them to the sequences within one haplotype. cDNA libraries and PCR amplification of reverse-transcribed We aligned all expressed sequences, i.e., sequences isolated from orthologous MHC class I loci shared by duck and chicken or quail. Loci are not obvious from comparison of the sequences. No directly lineages. Alternatively, recombination events and/or gene conversion may homogenize loci, so that no locus-specific character has been retained. The duplication events that gave rise to these five loci are not obvious from comparison of the sequences. No directly orthologous MHC class I loci shared by duck and chicken or quail can be definitively identified.

A phylogenetic tree of all available duck MHC class I sequences suggests that alleles are as divergent as locus sequences (Fig. 7B). We aligned all expressed sequences, i.e., sequences isolated from cDNA libraries and PCR amplification of reverse-transcribed cDNA, and compared them to the sequences within one haplotype. The genomic sequences form separate branches on the tree and expressed sequence groups with each locus. Indeed, the two major alleles of duck 26, U*02 and U*03 at the UAA locus, are on different branches of the tree. Locus information cannot be determined from similarity of sequences.

To examine whether only sequences at the major locus would be amplified by RT-PCR, we characterized the expressed and genomic sequences present in a second duck (D64). Two alleles, U*08 and U*09, were isolated by RT-PCR from this duck. Comparison of the genomic sequences, and PCR amplification from MHC class I to TAP2, indicated that U*08 was at the UAA locus, whereas U*09 which groups with UBA, is an allele at a minor locus. This was also reflected in the ratio at which clones were recovered, with U*08 represented by 33 clones and U*09 represented by 3 clones, although this bias could be due to the primers matching one sequence better than another.

To examine the functional diversity present in one duck and that present in the population, we have calculated the rate of synonymous over nonsynonymous substitutions within the proteins (Table II). The ratio of the rate of substitution within the α3 domain is very low at 0.15, suggesting that this part of the protein is conserved. The ratio of change in the α1 and α2 domains, at 0.46, is much higher than that in α3, which suggests that these differences are being selected. When we consider only the pocket residues, the ratio increases to 1.00. Although we cannot argue that a value of 1 is evidence of strong selection, it is strikingly different from the α3 domain and suggests that there is selection to maintain diversity in the peptide-binding pockets. The difference in rates of evolution for the α2 and α3 domains was noted also for chicken and mammalian class I sequences (53). The highest ratio of nonsynonymous to synonymous substitutions between these proteins lies within the pocket residues of both the cDNA and the sequences encoded at individual loci. Although the averages were very similar, the most divergent sequences were the U*02 and U*06 alleles, with a dN/dS ratio calculated at 11.1, whereas within a haplotype the maximum difference between two locus sequences was calculated at 2.7 for the pocket residues. Sequences within a haplotype appear no different than alleles at a locus.

### Functional diversity of alleles and loci

A phylogenetic tree of the mature protein encoded by avian class I sequences shows that the sequences of the duck MHC class I loci group closer to each other than to other avian sequences (Fig. 7A). Phylogenetic trees constructed using the α2 and α3 domain or only the α3 domain gave the same results, showing duck sequences branching separately from chicken and quail (data not shown). This suggests that the individual loci were generated after speciation of the duck from a common ancestor in the galloanseriforme lineage. Alternatively, recombination events and/or gene conversion may account for the lack of expression of the MHC class I sequences encoded at other loci.

### Discussion

The dominant expression of one MHC class I gene

We screened a duck genomic library, isolated, and sequenced a genomic clone encompassing TAP1 and TAP2 and five MHC class I genes in the following gene order: UAA, UBA, UCA, UDA, and UEA. Previously, we had isolated cDNA clones from this same duck; therefore, we were able to correlate the expressed genes with those present in the genome. The MHC class I gene adjacent to TAP2, that we previously showed was the predominantly expressed gene, we designated UAA. Three loci were not expressed (UBA, UCA, and UEA) and one locus was weakly expressed (UDA) and may be a nonclassical MHC class I gene. All MHC class I sequences resembled alleles of classical MHC class I genes in having the conserved anchor residues for peptide terminal main chain atoms and amino acid polymorphisms located in the α1 and α2 domains responsible for peptide binding.

We have examined the MHC class I genes for evidence of why there is a bias for expression of the UAA locus. UCA carried a stop codon in-frame, which would result in nonsense-mediated decay of mRNA transcripts from this locus. In an analysis of human MHC class I null alleles, any stop codon found (54) or introduced (55) within the first five exons of the MHC class I gene, resulted in no detectable transcripts. The exon 4 sequence carrying the stop codon in-frame was identified in five ducks examined. There were no obvious defects in the MHC class I sequences encoded at other loci, although the polyadenylation signal sequence lacking in UEA would mean that this sequence, even if transcribed, is not translated into protein.

An examination of the proximal promoters identified a defect that may account for the lack of expression of UBA. UBA lacked the X2 box of the MHC class I region SYX regulatory element, important for constitutive expression of MHC class I and class II genes. The UBA promoter also appeared to lack the enrichment of CpG typical of functional promoters (56). The promoters for UAA and UDA were virtually identical, both in sequence and CpG distribution; therefore, there was no obvious reason for the nearly 10-fold lower expression of UDA. One difference of UDA relative to UAA, a 9-bp insertion in the sequence between the NF-κB site and the ISRE, may place the NF-κB element out of register with the other transcriptional machinery on the DNA helix. Alternatively, DNA methylation or other epigenetic mechanisms are responsible for this differential expression. DNA methylation is important in establishing and maintaining the diverse repertoires of killer cell Ig-like receptors on NK cells (57, 58) despite their similar promoters.

### Table II. Rate of dN over dS nucleotide substitutions

<table>
<thead>
<tr>
<th>dN/dS Values MHC Class I genes</th>
<th>α1/α2 (180 codons)</th>
<th>α1/α2 Pockets (37 codons)</th>
<th>α1/α2 Nonpockets (143 codons)</th>
<th>α3 (91 codons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All sequences (18)a</td>
<td>0.46</td>
<td>1.00</td>
<td>0.33</td>
<td>0.15</td>
</tr>
<tr>
<td>All cDNA (12)</td>
<td>0.43</td>
<td>0.87</td>
<td>0.31</td>
<td>0.12</td>
</tr>
<tr>
<td>MHC locus sequences (5)</td>
<td>0.42</td>
<td>0.97</td>
<td>0.30</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*a In parentheses are the number of sequences analyzed for each group.
The additional MHC class I loci in ducks are disabled through defects in the promoters, a stop codon in-frame, a missing polyadenylation site, and potentially epigenetic effects such as methylation. The diversity of these mechanisms suggests that these processes evolved independently. This would suggest that despite duplication, there is strong selection for inactivation of additional loci over time. This is counter to an intuitive reasoning that additional loci could contribute greatly to an immune response by having the potential to present a greater diversity of peptides. Additional MHC would also result in the positive selection of a greater TCR repertoire, as well as NK cell repertoire, since every NK cell must have an inhibitory receptor for self-MHC class I (59). Others have argued that additional MHC would have a detrimental effect on immunity, because it would result in the deletion of more of the TCR repertoire during negative selection (60, 61). This could be significant, since the TCR repertoire may already be limited in birds (62). There are natural examples where MHC genes have been reduced over time. In polyploid frogs, the MHC tends toward diplodity and the duplicated copies are silenced (63). Similarly, in rainbow trout, with a tetraploid ancestry, there is evidence that the MHC class I region is diploid (42). The reasons for the deletion of MHC class I genes is unknown.

The expression of a single classical MHC class I gene has been seen in other lower vertebrates. Frogs have only one single classical class I gene and a large family of nonclassical MHC class Ib genes not involved in Ag presentation (64, 65). The picture is more complicated in other vertebrates in which there are additional genes present in the genome that resemble classical MHC class I loci. In sharks, most individuals expressed a single gene although other genes were present (66, 67). Screening of cDNA libraries from cichlid (68) and pufferfish (69) produced a maximum of four sequences in one individual. In catfish, isolation of four classical MHC class I sequences suggested three loci, but most transcripts corresponded to one sequence (70). Similarly, rainbow trout have additional genes in the genome (71), although some appear to be nonclassical (45, 72). Analysis of expressed sequences showed trout predominantly use one MHC class I locus (42, 73, 74).

Two lower vertebrates appear to be exceptions to the expression of one MHC class I locus, the axolotl and the cod. In the axolotl, between 10 and 17 unique sequences were amplified by RT-PCR of one MHC class I locus, the axolotl and the cod. In the axolotl, trout predominantly use one MHC class I locus (42, 73, 74). In rainbow trout, with a tetraploid ancestry, there is evidence that the MHC class I region is diploid (42). The reasons for the deletion of MHC class I genes is unknown.

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Two lower vertebrates appear to be exceptions to the expression of one MHC class I locus, the axolotl and the cod. In the axolotl, between 10 and 17 unique sequences were amplified by RT-PCR from cDNA (75). Many appeared to be classical, but the analysis was limited to the α3 domain; therefore, this remains inconclusive. In cod, 17 different cDNA sequences were identified in one individual (76). Unfortunately, most were partial clones, truncated within the α2 domain, making it difficult to determine whether they were all capable of peptide binding. Nonetheless, the expression of a large number of genes is unique in these animals.

Kaufman (2) argues that the dominant expression of a single MHC class I locus in most lower vertebrates is attributable to the proximity of MHC class I to the TAP genes, which provides an opportunity for coevolution of these proteins involved in interrelated functions in Ag presentation. In sharks, fish, and frogs the MHC class I genes are in close proximity to the Ag-processing genes (LMP2 and LMP7), proteasome genes, and the Ag transporter genes (TAP1 and TAP2) in a “true class I region” (2). In chickens, class I genes flank either side of the TAP genes and the LMP genes have been lost from the genome (17, 27). The major locus of chickens is believed to be evolving in concert with the transporters, while the minor locus is expressed at a lower level reported to be due to deletions within their promoters (77). In rainbow trout, the MHC class I genes appear constrained to evolve within deep lineages, whereas the genetically unlinked MHC class II evolves rapidly (42). This is potentially due to linkage to the Ag-processing and presentation genes. Allelic lineages have evolved in *Xenopus*, the MHC class I genes presumably constrained by linkage to TAP and the proteasome gene LMP7, since these genes are known to exist within extended haplotypes (78–80). Similarly, evidence of extended haplotypes of class I, TAP, and LMP genes exists in the nurse shark (67). The evidence from these lower vertebrates implies that the haplotypes are stable and the proteins encoded in the MHC class I region may have an opportunity to coevolve. Indeed, in ducks we had evidence that the TAP2 gene is significantly polymorphic (36), with alleles differing primarily in the regions thought to confer peptide specificity. Additional MHC class I loci might then become useless because they cannot accept peptides from the mismatched TAP transporters. Although this provides a compelling argument for inactivation of redundant loci, the same selective forces should favor homozygous expression from the major locus, which is never seen.

Evidence from other birds suggests that the minimal MHC of the domestic chicken is not a paradigm for all birds. Indeed, the wild ancestor of the chicken, the red jungle fowl has 14 MHC class I genes (27). The quail has duplicated genes within the MHC class I region and has seven genes, including three partial pseudogenes and two weakly expressed loci (26). Similarly, in rainbow trout, 14 cDNA clones representing eight different sequences encoding MHC class I were isolated from one individual, and all appeared classical (32). Relative expression has not been examined; however, 5 of 14 cDNA clones were identical, suggesting a bias for this locus. It has been argued that migratory birds have the need for more MHC class I and II (both allelic diversity and number of loci) for greater protection against the diverse pathogens that would be encountered than birds in a sedentary lifestyle (33). The mallard duck is also a migratory bird that would encounter many pathogens in its environment. Our data on ducks would suggest that despite the evident duplication of the locus, one gene contributes predominantly to Ag presentation.

**Organization and evolution of the locus**

The MHC class I genes were organized in the same transcriptional orientation and more closely resembled each other than the MHC class I genes of the chicken or quail. This suggested that the genes arose recently in successive rounds of duplication since the divergence of these species. Evidence from a dot plot suggested that one unit of duplication included a pair of duck MHC class I genes, since the entire intergenic region spanning the UAA and UBA genes was similar to the region spanning the UDA and UEA genes. The duplicated region includes fragments of CR1 flanking either side of a GGLTR11 element. The conservation of the location of this retroelement in the intergenic sequence in three separate locations suggests that a pair of genes were involved in duplication events. UAA and UBA represent one pair and UDA and UEA represent another pair. It is likely that UCA was originally part of a third pair; however, one gene and part of the intergenic region have been lost. Similarly, units of duplication were evident in the quail MHC class I region (26). Previously, no repetitive DNA elements were identified in chicken or quail MHC class I regions. Our reanalysis of the quail MHC class I region with RepeatMasker detected a retroelement GGLTR-12C, which is most similar to the Soprano LTR element, immediately upstream of the pseudogene *Coja-F*. This was different from the two retroelements identified in the duck MHC class I region, including GGLTR-11 and GGLTR-5 (Hitchcock). Therefore, both the MHC class I genes and the repetitive DNA elements are unique within each species. This suggests that the primordial MHC class I region of birds was a simple...
MHC, which has undergone duplication independently within each species.

The duck MHC class I genes appear to be confined to the TAP2 side of the transporter genes. Using Southern blot analysis, we saw no evidence of MHC class I genes on the TAP1 side of the locus, unlike the chicken and quail (26). The duck MHC gene organization may resemble the ancestral gene organization before a translocation event that placed MHC class I genes on either side of the TAP genes. The few hybridizing bands observed on Southern blots of duck MHC class I genes suggested that there are no other MHC genes located in the genome. This differs from chickens in which TAP1 was weakly hybridizing bands that were identified as re-arrangement of the transporter genes. Using Southern blot analysis, we saw no evidence of MHC class I genes on the TAP1 side of the locus. The neighboring genes, encoding proteins with all the hallmarks of capable Ag-presenting proteins, may preserve genetic diversity, which can be exchanged when the species comes under duress from a new pathogen.

**Disclosures**

The authors have no financial conflict of interest.

**References**


**Why duplicate, then inactivate extra copies of the MHC class I loci?** The answer must lie in the ability to access the information provided by the chicken major histocompatibility complex (B and Y) complex.

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