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Organisation and diversity of the class II *DM* region of the chicken MHC

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ABSTRACT

In mammals, the DM molecules are encoded by the major histocompatibility complex (MHC) and execute key functions in the class II antigen presentation pathway. Here, we characterised three *DM* genes in the MHC *B* region of the chicken (*Gallus gallus*): *B-DMA*, *B-DMB1* and *B-DMB2*. They encode one class II DM α chain and two β chains, exhibiting motifs of chicken class II molecules as well as specificities of mammal DM proteins. We also studied the expression pattern of those three chicken *B-DM* genes; they are expressed in immune related tissues. Thus we provide the comprehensive description of the genomic sequence of a class II α gene in the chicken and a valuable description of *DM* genes in a non-mammalian vertebrate, reinforcing the hypothesis of the existence of *DM* genes in the primordial MHC, as suggested by previous studies in mammals. We were also able to reconstruct 124 haplotypes corresponding to the 8.8 kb *B-DM* region, in accordance with the 212 SNPs identified in 146 individuals representing a wide range of experimental, commercial, and local breeds from Europe, Asia and Africa, and three wild species of fowl. We also discovered a repeat inside the *B-DMA* second intron, making possible the design and the typing of a new marker for the chicken MHC, linked to the class II region. Therefore this study not only describes three *DM* genes in the chicken, it also provides an overview of MHC diversity in the chicken.

1. Introduction

The *DM* loci, first described by Cho et al. (1991) in the mouse genome, and Kelly et al. (1991) in the human genome, are non-classical MHC class II genes. In the chicken, the MHC is located on microchromosome 16, and is organised in two genetically independent gene complexes, *B* and *Y*. A number of B serotypes, distinguished by classical serological methods (Briles and Briles, 1987) or recently by molecular genotyping techniques (Fulton et al., 2006), are associated with strong differences in resistance or susceptibility to infectious diseases such as Marek's disease. The classical class II molecules of the chicken (BL) are, as for mammals, composed of two chains, α and β, generally expressed by B cells and macrophages. They present peptides, principally derived from exogenous molecules, to CD4+ T lymphocytes. In the chicken, three classical class II genes have been described so far, two encoding β chains and one encoding an α chain in the *B* region, along with at least one non-classical class II gene (*B-LBIII*) in the *Y* region. The cDNA encoding the α chain has been described but not located in the genomic sequence of chromosome 16, which is not completely characterised (Salomonsen et al., 2003).

In mammals, the DM molecules are involved in the formation of classical class II/peptide complexes, before their expression at the surface of the cell. It has been shown that without DM, these complexes are unstable, disrupting antigen presentation (Morris et al., 1994). In fact, classical class II molecules are assembled in the endoplasmic reticulum and stabilised by a third protein, the invariant chain. In endosomes, the invariant chain is cleaved by proteases, and a peptide from the invariant chain, CLIP, remains attached to the binding site of the classical class II molecule. In the presence of DM, CLIP is cleaved and replaced by antigenic peptides, which are derived from proteolysis of exogenous antigens (Sloan et al., 1995). DM also acts as a chaperone protein for classical class II molecules by protecting them from denaturation in acidic compartments. Finally, the DM molecule controls the loading of antigenic peptides by inducing the dissociation of the classical class II/peptide complex of low stability. Thus, only complexes of high stability can be presented on the surface of presenting cells (Kropshofer et al., 1999).

The assembly of the chicken genome, the first map of the *B* region of the chicken MHC (Kaufman et al., 1999) and the extended recent map (Shiina et al., 2007), show, in the *B* class II region, a segment for which *in silico* gene prediction and comparisons with mammals indicates the probable presence of two or three genes orthologous to genes characterised as *DM* in mammals. Ever since,

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it has been assumed that the MHC *B* region of the chicken encoded non-classical class II genes, with only a preliminary promising study by Atkinson et al. (2001) whereas the intracellular localisation and oligomerisation of the invariant chain with classical class II molecules has been recently demonstrated in the chicken (Ye et al., 2009).

This study was initiated as a step towards improving the knowledge of the gene content of the *B* region, in order to determine the individual contribution of genes to disease incidence. In this context, we characterised in detail the molecular organisation, structure, and pattern of expression of the predicted *DM* non-classical class II genes of the chicken. As shown below, we demonstrate that three *DM* genes do exist in the *B* region of the MHC of the chicken. And, in order to assess genetic diversity of these key immune system genes, we also characterised for these genes, a large number of SNPs, and a new marker, from a wide range of experimental, commercial, local breeds and wild related species.

2. Material and methods

2.1. The cDNA library and screening

A set of *B-DM* cDNA were isolated from a serotype B^{13} spleen cDNA library in the pCDM8 vector (Bernot and Auffray, 1991). A clone (C.4.5.6), with high similarities to the mouse and human *DMB* gene, had been previously identified by systematic sequencing of chicken MHC genomic libraries (constructed by subcloning the MHC cosmid clone C.4.5 in the pUC19 plasmid vector). The C.4.5.6 fragment was amplified using the GeneAmp PCR (PerkinElmer) kit, in a total volume of 20 μL with 200 ng of DNA from the cosmid subclone C.4.5.6 and 2 μL of 10× PCR buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 2 μL each of 10 mM dNTP, 0.5 μM of each primer (M13 universal) and 1 U of Taq polymerase). The cycling conditions were an initial denaturation step at 94 °C for 2 min, followed by 30 cycles at 94 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min followed by a final extension step at 72 °C for 7 min. The fragment obtained, encompassing C.4.5.6, was then ^{32}P-labelled using the Multiprime DNA labelling system (Amersham Biosciences), and used as a probe to screen approximately 200,000 clones from the cDNA library. Six clones gave positive hybridisation signals, their nucleotide sequence was determined for both strands by sequencing, and submitted to GenBank under the accession numbers HM545127 to HM545132.

2.2. Total RNA isolation

Total RNA was prepared from tissues of White Leghorn chicks (serotype B^{12}). The tissues were the following: brain, bursa, heart, lung, and spleen. Thirty milligrams of each tissue were disrupted for 4 min at 10–14,000 rpm with an IKA yellowline Dl 18 dispenser. Total RNA was isolated using the RNeasy mini kit from Qiagen. A purification step was added after disruption by pipetting the clear supernatant into a Qiashredder column (Qiagen) and centrifuging at 14,000 rpm for 5 min. Then, the filtrate was used following the RNeasy protocol, including the optional on-column digestion with DNAse (Qiagen RNase-Free DNase). The RNA concentration was measured by UV spectrophotometry.

2.3. Genomic DNA origin and extraction

In the study for genetic polymorphism investigation, 129 animals were used from 47 different populations or lines of domestic chickens (*Gallus gallus*), including 29 populations of various origins and selection history from the European AVIANDIV project (Supplementary Table 1). Detailed information on these populations was reported elsewhere (Hillel et al., 2003). Thirty-four wild *Gallus sp*. were also studied: 25 red Junglefowl (*G. gallus*), 5 grey Junglefowl (*Gallus sonneratii*) and 4 Sri Lanka Junglefowl (*Gallus lafayetii*). Nineteen out of the 25 red Junglefowl were also from the AVIANDIV project.

For the B^{12}, B^{13}, and B^{19} inbred lines from INRA (Nouzilly, France, Miller et al., 2004), the Silkie chickens from the Livestock Research Institute (Sinhua, Taiwan), and the other wild *Gallus sp*. from a zoological park in Clères, France, high molecular weight genomic DNA were obtained from blood samples, as described by Chang et al., 2006.

For four additional lines from INRA, the immune response lines (I.R. lines kept in Nouzilly, France, serotypes B^{12}, B^{15}, B^{19}, B^{21}, B^{34}, Minozzi et al., 2007), and six Taiwanese chicken populations (from NCHU, Taichung, Taiwan), crude DNA extractions were prepared from blood by mixing 2 μL of whole blood with 250 μL of lysis buffer (1 M pH 8 Tris 1%, 1 M $MgCl_2$ 0.1%, 3 M NaCl 5%, 1% Sigma IGEPAL) before precipitation by centrifugation and incubation with 250 μL of NaOH (0.2 M) at 65 °C for at least 2 h followed by neutralisation with 250 μL of Tris (0.1 M)–HCl (0.2 M).

For Benin, Cameroon, Côte d'Ivoire and Ghana samples, DNA were obtained from blood using the DNeasy Blood & Tissue Kit from Qiagen at the Department of Animal Science, University of Ghana (Legon, Ghana).

2.4. Molecular MHC characterisation of the samples

Each animal has been genotyped at the MHC level by PCR amplification with the LEI0258 marker (McConnell et al., 1999; Fulton et al., 2006), and with a new marker also located in the chicken MHC, called GAB0001. Primers for the LEI0258 marker were LEI0258-F: CACGCAGCAGAACTTGGTAAGG forward and LEI0258-R: AGCTGTGCTCAGTCCTCAGTGC reverse. Primers for the GAB0001 marker were designed with the Primer3 software (Rozen and Skaletsky, 2000), using the sequence information obtained during this study: GAB0001-F: CATTCCCCATTAACGCACTC forward and GAB0001-R: AGAGTGTTGGGGTAGCCGAG reverse. Duplexed amplifications were done in 10 μL with 100 ng of DNA, 1× of QIAGEN Multiplex PCR Kit and primer concentrations of 0.1 μM and the following PCR cycle setup: 94 °C for 15 min, then 35 cycles at 94 °C for 45 s, 60 °C for 1 min 30 s, 72 °C for 1 min, with a final extension at 72 °C for 15 min. After a 1:10 dilution, the PCR products were loaded on an Applied 3730XL automated sequencer for electrophoresis and the results were analysed using Genemapper v3.7.

In order to get the real size of the marker LEI0258 amplified fragment, more than half (64%) of the alleles were sequenced, directly for homozygotes or after separation of the two alleles on a 4% agarose gel, band cutting and purification with a QIAquick PCR Purification Kit from Qiagen. Sequencing was carried out by Eurofins MWG Operon, using their standard protocol for purified PCR products.

2.5. 2.5. RT-PCR

Seven oligonucleotides were used in order to study the expression of the genes of the region (Supplementary Table 2, Fig. 1). They were designed using the chicken MHC genomic sequence reported by Shiina et al. (2007) (GenBank accession number AB268588) and verified by the program Primer3 (Rozen and Skaletsky, 2000).

The RT-PCR was carried out in two steps, following the Applera protocol for the GeneAmp RT-PCR kit. Approximately 1 μg of total RNA was used with 4 μL of 25 mM $MgCl_2$, 2 μL 10× PCR buffer, 0.2 mM of each dNTP, 2.5 μM of oligodT (16), 20 U of RNase inhibitor and 50 U of MulV reverse transcriptase in a final volume of 20 μL. The amplification step was carried out by adding 2 μL of 25 mM