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Molecular cloning and mRNA expression of duck invariant chain

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Abstract

In the present study we identified a duck invariant chain (Ii) cDNA, named duck Ii-1, by RT-PCR and RACE. It was 1190 bp in length and contained a 669 bp open reading frame. An alternative transcript encoding a thyroglobulin (Tg)-containing form of Ii, named duck Ii-2, was also found in duck. The putative amino acid sequence of duck Ii-1 showed an 82% similarity to chicken Ii-1 and about 60% similarity to its mammalian homologues. The similarity of the Tg domain between duck and chicken Ii-2 was 96%, and about 70% between duck and mammalian Ii. The result of RT-PCR showed that Ii mRNA was extensively expressed in various tissues. High levels of both Ii-1 and Ii-2 mRNA were observed in the spleen and bursa of Fabricius. The predicted three-dimensional (3D) structures of duck Ii trimerization and Tg domain are similar to the corresponding regions of human Ii analyzed by comparative protein modeling. These findings indicate that the two isoforms of duck Ii, which strongly expressed in the major immune organs, share structural identity with human Ii.
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Keywords: Duck; Invariant chain; Alternative splicing; mRNA expression; Structure prediction

1. Introduction

The invariant chain (Ii) is a non-polymorphic (Jones et al., 1978) type II transmembrane protein and plays a central role in regulating the expression and function of class II major histocompatibility complex (MHC) molecules (Anderson and Miller, 1992; Kropshofer et al., 1995; Serwe et al., 1997). The N-terminal of Ii constitutes a short cytoplasmic tail of 30 amino acids (aa), followed by a single 24 aa transmembrane (TM) region and an approximately 150 aa long luminal domain. The N-terminal cytoplasmic tail of Ii contains two extensively characterized dileucine-based endosomal targeting motifs (Lotteau et al., 1990; Odorizzi et al., 1994; Pond et al., 1995), by which Ii directs associated class II molecules to the endocytic route. The transmembrane domain is the most conserved region in the Ii molecule. It is necessary for efficient delivery of MHC class II to the endocytic pathway (Odorizzi et al.,

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1994; Pieters et al., 1993) and might also contribute to stable homotrimer formation (Newcomb et al., 1996). The luminal domain mainly contains a trimerization region which responsible for its self-association, and a class II-associated Ii-derived peptide (CLIP) region. In acidic endosomal compartments Ii is proteolytically degraded, only leaving CLIP interacted with MHC class II (Freisewinkel et al., 1993; Rudensky et al., 1994). Finally antigenic peptides replaced CLIP and MHC II-peptide complexes are released to the cell surface for recognition by $CD4^+$ T cells.

Several groups have used Ii fusion proteins to target endogenously synthesized antigens to different steps in the MHC class II presentation pathway. Inframe fusion of large protein fragments with the Ii amino terminus directs the antigen to the endocytic pathway, where it can be processed for MHC class II binding (Sanderson et al., 1995). In contrast, Ii fusion construct, in which CLIP region was replaced by short peptides of interest, are used to design recombinant vaccines to stimulate $CD4^+$ T cell responses (Barton and Rudensky, 1998; Fujii et al., 1998; Malcherek et al., 1998).

The immune system and mechanisms of the humoral response have not been well characterized in avian species. Recently, several chicken immune molecules that play key roles in MHC class II antigen presentation have been reported, such as MHC class II beta chain (Jacob et al., 2000), alpha chain (Salomonsen et al., 2003), and the Ii cDNA (Bremnes et al., 2000). The chicken Ii was reported to share some functional properties with its mammalian homologs, such as endosomal sorting, trimerization and binding to MHC class II (Bremnes et al., 2000). Our previous study revealed that Ii exist in two forms in chicken derived from alternative splicing (Zhong et al., 2004). In this study, we present the cloning and mRNA expression of duck Ii cDNAs. We also examine the structural homology between duck and human Ii.

2. Materials and methods

2.1. Separation and culture of spleen lymphocyte fractions

Spleen lymphocytes of a normal adult duck were separated and *cultured* as previously described (Zhong et al., 2004). Briefly, Spleen lymphocytes were separated using lymphocyte separation medium (TBD, China), and stimulated with concanavalin A (Sigma, USA) at a concentration of 15 μg/ml in Dulbecco's modified Eagle's medium supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin at 40 °C for 16 h.

2.2. RNA extraction

Total RNA was extracted from stimulated spleen lymphocytes and various tissues of three normal adult *ducks* using the Trizol reagent (Invitrogen, USA) as previously described (Zhong et al., 2004). Briefly, samples were homogenized in 1 ml Trizol reagent and then 0.2 ml of chloroform was added to each sample, then all samples were centrifuged at 12,000 × *g* for 15 min at 4 °C to separate the mixture. The RNA in the aqueous phase was precipitated with 0.5 ml of isopropyl alcohol, and after centrifugation the pellets were washed with 75% ethanol and used as total RNA.

2.3. Cloning of duck invariant chain cDNA by reverse transcriptase-polymerase chain reaction (RT-PCR)

A first-strand cDNA was synthesized from 1.0 μg of RNA isolated from duck spleen lymphocytes using RevertAid™ First Strand cDNA synthesis kit (Fermentas, CA) according to the manufacture's protocol. A pair of degenerate primers, dpIi-1 (sense) 5′-CA(AG)CG(AGCT)GACCT(CT)ATCT C-3′ and dpIi-2 (antisense) 5′-CAT(CT)TCAAA(G-C)A(AG)(GC)AGCCA-3′, were used in the PCR. Their design was based on regions of high homology among the sequences of human, bovine, mouse, rat and chicken Ii (aa 4/5–9/10 "QRDLIS" and aa 172/176–177/181 "WLLFEM", Figs. 1 and 2A). PCR was performed by using the *Pfu* Taq polymerase (Sangon, China) and PCR conditions (30 cycles) were as follows: denature for 40 s at 94 °C, annealing for 40 s at 50 °C, and extension for 1.5 min at 72 °C. One PCR product of about 500 bp was obtained, and it was subcloned into PMD 18-T vector (Takara, Japan) and confirmed by the ABI Prism automated sequencing method.

```
-41 TTTTCCCCATCGGGTTTGGGTTCGGTGCCGACGGTGCAGCCATGGCCGAGGAGCAGCGGG 19
                                           M  A  E  E  Q  R
 20 ACCTCATCTCCGACCGCGGCAGTGGGGTGGTCCCCATGGGGGACAGCCAAAGGTCTTCCT  79
    dpIi-1
    D  L  I  S  D  R  G  S  G  V  V  P  M  G  D  S  Q  R  S  S
 80 TCGGGCGCAGAGCCGCCCTGTCCACGCTGTCCATCCTGGTGGCCCTGCTGATCGCCGGCC 139
                        A2
    F  G  R  R  A  A  L  S  T  L  S  I  L  V  A  L  L  I  A  G
140 AGGCCGTCACCATCTACTTCGTGTACCAGCAGAGCGGGCAGATCAGCAAGCTGACCAGGA 299
                           d-f-1
    Q  A  V  T  I  Y  F  V  Y  Q  Q  S  G  Q  I  S  K  L  T  R
200 CCTCCCAAAACCTGCAGCTGGAGGCGTTGCAGCGCAAGCTGCCCAAGAGCAGCAAGTCCG 259
    T  S  Q  N  L  Q  L  E  A  L  Q  R  K  L  P  K  S  S  K  S
260 CCGGCAACATGAAGATGTCGATGGTGAACACCCCCCTGGCCATGAGGGTCCTGCCTCTCG 319
    A  G  N  M  K  M  S  M  V  N  T  P  L  A  M  R  V  L  P  L
300 CCCCCTCCCTAGACGACACGCCCGTGAAGGACATGGGGCCCCCCAGCAACAAGACCGAGG 379
                                                        A1
    A  P  S  L  D  D  T  P  V  K  D  M  G  P  P  S  N  K  T  E
380 ACCAAGTCAGGCACCTGCTGCTGCAGGCAGACCCGAAGAAGATGTTCCCGGAGCTGAAGG 439
    D  Q  V  R  H  L  L  L  Q  A  D  P  K  K  M  F  P  E  L  K
440 ACAGCCTGCTGGGCAACCTCAAGAGCCTGAAGAAGACCATGACCGACGCGGACTGGAAGT 499
    D  S  L  L  G  N  L  K  S  L  K  K  T  M  T  D  A  D  W  K
500 CCTTCGAGTCCTGGATGCACAAGTGGCTGCTGTTCGAGATGGCCAAAAGCCCCAAGCCGG 559
                                 dpIi-2
    S  F  E  S  W  M  H  K  W  L  L  F  E  M  A  K  S  P  K  P
560 ACGAGCGTAAAGCCATCCCGGCGGAGAAA GTGCAAACTAAGTGCCAGGCAGAGGCCAATT 619
            S1                  578
    D  E  R  K  A  I  P  A  E  K  V  Q  T  K  C  Q  A  E  A  N

620 TCGGTGGTGTCCATCCGGGTCGCTTCCCCCCCGAGTGCGATGAGAACGGGGACTACCTGC  679
    F  G  G  V  H  P  G  R  F  P  P  E  C  D  E  N  G  D  Y  L
680 CCAAGCAGTGCCACGCCGGCACGGGCTACTGCTGGTGCTGCTACAAAAACGGCACCAAGA  739
    P  K  Q  C  H  A  G  T  G  Y  C  W  C  C  Y  K  N  G  T  K
740 TTGAGGGCACGGCCACCCGGGGAGAGCTGGACTGCTCG GGGGCTGCACTGACGGAGCCCG 799
                                          579                  610
    I  E  G  T  A  T  R  G  E  L  D  C  S  G  A  A  L  T  E  P
                                                       S2
800 ACGAGATGATCTTCTCCGGGGTGGACATGCTCAAGCTGGGCGCTGAGAAAGCCAAGTAGA 859
611                                                   d-r-1      670
    D  E  M  I  F  S  G  V  D  M  L  K  L  G  A  E  K  A  K  *

860 AGAGGACACCTGCAGCTGCCCACACGGCTTTCAGCACTCCAGCTCGGGTTTTTTTTTTGC 919
671                                                              730
```

Fig. 1. Nucleotide sequence of duck Ii-1 and Ii-2 cDNAs. *Arrows* and *solid line* indicate each primer site and its nucleotide sequence. Degenerate primers dpIi-1 and dpIi-2 are used for RT-PCR, d-f-1 are used for 3′ RACE, primers A1, A2, S1, S2 and RT-primer (5′ phosphorylation) for 5′ RACE, S1 and d-r-1 for semi-quantitative RT-PCR. ATG, TAG and ATTAAA are shaded. The 189 bp nucleotide sequence encoding a putative Tg domain is indicated by double underlined. The duck Ii-1 clone was deposited in the GenBank nucleotide sequence database with accession no. AY904336.

```
920  TCTATCCCCTTTCTGCTCTTCCCTCGCTTTCCCAAACACCCCCCGGCACTCCTTCCCCAT 979
731                       RT-primer                                 790
980  GATCACTTACCCTCCCCTGCTCCAGGGCCTGGCCTGGGGTCGATGAGAGTTGGCTGGGGC 1039
791                                                                 850
1040 TGTCCCCAGGATAAATGCTACAGAGGAAACGTTTCCTAAAACTGGAGGTGGAGCAGGCTG 1099
851                                                                 910
1100 GAAGCATTTTCAGCCGCAGCAGATCCCCAGTCCCTTCCCCTGTTGCTGGTGGGGTACGAG 1159
911                                                                 970
1160 CCGCAGTGAGGGCGTTGTGTGAAGGACACCAGCTGGTTCCCAGCCCTGTGCCTGGCCCCC 1119
971                                                                1030
1220 AGGGCACTTCTCCCCCAGCCCAGGCTCTGCGATTGCTCCTGCTGAAGATCGGGCCACTAA 1279
1031                                                               1090
1280 CGCCTGCTTTCTTAGCCTGAACAAACATTAAACGCAGCAAAACCAAAAAAAAAAAAAAA  1338
1091                                                               1149
```

Fig. 1. (*Continued*).

2.4. Rapid amplification of cDNA ends (RACE)

To obtain the 3′-end cDNA of duck Ii, 3′ RACE was performed using a 3′-full RACE core set (Takara, Japan) according to the manufacturer's instructions. Briefly, the cDNA was synthesized using 1.0 μg of total RNA, oligo dT-3 sites adaptor primer and reverse transcriptase for 3′ RACE. PCR for 3′ RACE of duck Ii was performed with d-f-1 5′-GTGTACCAGCA-GAGCGGGCAGA-3′ and three sites adaptor primer (5′-CTGATCTAGAGGTACCGGATCC-3′). PCR conditions (30 cycles) were as follows: denature for 30 s at 94 °C, annealing for 30 s at 55 °C, and extension for 4 min at 72 °C. PCR product was subcloned into the PMD 18-T vector, and then sequenced.

To obtain the 5′-end cDNA of duck Ii, a 5′-full RACE core set (Takara, Japan) was employed to perform the 5′ RACE according to the manufacturer's instructions. cDNA was generated with 1.0 μg of total RNA and RT primer (5′-GGAAAGCGAGGGA-3′), intact 5′-end cDNA was obtained with nested PCR using S1 (5′-ACGAGCGTAAAGCCATCCCG-3′), A1 (5′-TGACTTGGTCCTCGGTCTTGTT-3′), S2 (5′-GCTGAGAAAGCCAAGTAGAAGAGG-3′) and A2 (5′-GGATGGACAGCGTGGACAGG-3′), which were designed based on the sequence obtained by 3′ RACE (Fig. 1). One PCR product of about 300 bp was obtained and it was subcloned into PMD 18-T vector, and then sequenced.

2.5. Cloning of duck Ii-2

To determine whether an alternative transcript of duck Ii gene exists, we designed primers S1, 5′-ACGAGCGTAAAGCCATCCCG-3′ (sense) and d-r-1, 5′-CCTCTTCTACTTGGCTTTCTCAG-3′ (antisense), to amplify a fragment not containing the Tg domain of 116 bp or an alternatively spliced product containing the Tg domain of about 310 bp (Fig. 1). Synthesis of first-strand cDNA was preformed as described above. PCR conditions were as follows: 30 cycles of (denature for 1 min at 94 °C, annealing for 1 min at 62 °C, and extension for 1 min at 72 °C), and then 72 °C for 8 min.

Fig. 2. (A) Alignment of the predicted duck Ii-1 translation product with chicken Ii-1 (GenBank accession no. AY597053.1), and Ii p31 from bovine (GenBank accession no. D83962), mouse (GenBank accession no. BC030458), rat (GenBank accession no. NM_013069), and humans (GenBank accession no. K01144). Identical (*Asterisks*) and similar (*colons*) amino acid residues are indicated. The TM (transmembrane) and the trimerization domains are shaded, and the CLIP (class II-associated invariant chain peptide) domain is indicated by a double underline. See text for detailed explanation. Sequence alignment was performed with the Align software at the Clustal W network server (http://www.ebi.ac.uk/clustalw/). (B) Alignment of the predicted Tg (thyroglobulin type-1 repeat) domain of duck Ii-2 with Tg domain from chicken (GenBank accession no. AY597054), mouse (GenBank accession no. HLHMSG), rat (GenBank accession no. S04362), and human (Strubin et al., 1986). The six cysteines that are characteristic of the Tg domain are highlighted.

```
             <   cytoplasmic domain      ><          TM            >
DuckIi       MAEEQRDLISDRGS-GVVPMGDSQRSSFGRRAALST-LSILVALLIAGQAVTIYFVYQQS 58
ChickenIi    MAEEQRDLISSDGSSGVLPIGNSERSSLGRRTALSA-LSILVALLIAGQAVTIYYVYQQS 59
BovineIi     -MEDQRDLISNHEQLPMLGQRPGAQESKCSRGALYTGFSVLVALLLAGQATTAYFLYQQQ 59
MouseIi      -MDDQRDLISNHEQLPILGNRPREPE-RCSRGALYTGVSVLVALLLAGQATTAYFLYQQQ 58
RatIi        -MDDQRDLISNHEQLPILGQRARAPESNCNRGVLYTSVSVLVALLLAGQATTAYFLYQQQ 59
HumanIi      -MDDQRDLISNNEQLPMLGRRPGAPESKCSRGALYTGFSILVTLLLAGQATTAYFLYQQQ 59
               ::******.  .  ::        .    * .* : .*:**:**:****.* *::***.
                               <      CLIP      >
DuckIi       GQISKLTRTSQNLQLEALQRKLPKSSKSAGNMKMSMVNTPLAMRVLPLAPSLDDTPVKDM 118
ChickenIi    GQISKLTKTSQTLKLESLQRKMPIGTQPANKMSMSTMNMPMAMKVLPLAPSVGDMPMEAM 119
BovineIi     GRLDKLTVTSQNLQLENLRMKLPKPAKPMSQMRMAT---PMLMRALPMAG---PEPMKNA 113
MouseIi      GRLDKLTITSQNLQLESLRMKLPKSAKPVSQMRMAT---PLLMRPMSMDNML-LGPVKNV 114
RatIi        GRLDKLTVTSQNLQLENLRMKLPKSAKPVSPMRMAT---PLLMRPLSMDNML-QAPVKNV 115
HumanIi      GRLDKLTVTSQNLQLENLRMKLPKPPKPVSKMRMAT---PLLMQALPMGALP-QGPMQNA 115
             *::.*** ***.*:** *: *:*  .:. . * *:      *: *: :.:       *::
               <                        trimerization domain
DuckIi       GPPSNKTEDQVRHLLLQADPKKMFPELKDSLLGNLKSLKKTMTDADWKSFESWMHKWLLF 178
ChickenIi    EPRSNKTEDQIRHLLLKSDPRKTFPDLKDDMLGNLKRLKKTMSAMDWQDFETWMHKWLLF 179
BovineIi     TKYGNMTQDHVMHLLLKADPLKVYPQLKGSLPENLKHLKDSMDGLDWKLFESWLHQWLLF 173
MouseIi      TKYGNMTQDHVMHLLTRSGPLE-YPQLKGTFPENLKHLKNSMDGVNWKIFESWMKQWLLF 173
RatIi        TKYGNMTQDHVMHLLTKSGPVN-YPQLKGSFPENLKHLKNSMNGLDWKVFESWMKQWLLF 174
HumanIi      TKYGNMTEDHVMHLLQNADPLKVYPPLKGSFPENLRHLKNTMETIDWKVFESWMHHWLLF 175
              .* *:*:: *** .:.* : :* **.  :   **: **.:*   :*: **:*:::****
                                  >  exon 6b
DuckIi       EMAKSPKPDERKAIPAEKGAALT-----EPDEMIFSGVDMLKLGAEKAK- 222
ChickenIi    EMAKGPKMEEQNTIPAEKAPAPTQPPSAEPEEVIFSGVDMVKAK------ 223
BovineIi     EMSKNSLEEK----PFEGPPKDP-----MEMEYPSSGLGV---------- 204
MouseIi      EMSKNSLEEKK---PTEAPPKEP-----LDMEDLSSGLGVTRQELGQVTL 215
RatIi        EMSKNSLEEKQ---PTQTPPKEP-----LDMEDPSSGLGVTKQDMGQMFL 216
HumanIi      EMSRHSLEQK----PTDAPPKES-----LELEDPSSGLGVTKQDLGPVPM 216
(A)          **:: .  ::    * :  .  .         *   **:.:
```

```
DuckTg       VQTKCQAEAN-FGGVHPGRFPPECDENGDYLPKQCHAGTGYCWCCYKNGTKIEGTATRGE 59
ChickenTg    VQTKCQAEAS-FGGVHPGRFRPECDENGDYLPKQCYASTGYCWCCYKNGTRIEGTATRGQ 59
MouseTg      VLTKCQEEVSHIPAVYPGAFRPKCDENGNYLPLQCHGSTGYCWCVFPNGTEVPHTKSRGR 60
ratTg        VLTKCQEEVSHIPDVHPGAFRPKCDENGNYMPLQCHGSTGYCWCVFPNGTEVPHTKSRGR 60
HumanTg      VLTKCQEEVSHIPAVHPGSFRPKCDENGNYLPLQCYGSIGYCWCVFPNGTEVPNTRSRGH 60
             * **** *.. :   *:** * *:*****:*:* **:..  ***** : ***.:  * :**.

DuckTg       LDCS 63
ChickenTg    LDCS 63
MouseTg      HNCS 64
ratTg        HNCS 64
HumanTg      HNCS 64
(B)           :**
```

Two PCR products of about 110 and 310 bp were amplified. The 310 bp PCR product was subcloned into the PMD 18-T vector, and then sequenced.

2.6. Sequence analysis

Sequences were aligned by CLUSTAL W (1.82) multiple sequence alignment online analysis system.

2.7. Semi-quantitative RT-PCR

First-strand cDNAs were synthesized from 1.0 μg of RNA isolated from different duck tissues using RevertAid™ first strand cDNA synthesis kit. For PCR amplification, different PCR cycle numbers and different amounts of synthesized cDNA were first examined to evaluate the linearity of the reaction. PCR amplifications in 25 μl reaction mixtures were carried out using 4 μl of the synthesized first-strand cDNAs of different tissues as templates with primers S1 and d-r-1. PCR conditions were as follows: 25 cycles of (denature for 1 min at 94 °C, annealing for 1 min at 62 °C, and extension for 1 min at 72 °C), and then 72 °C for 8 min. The endogenously expressed duck β-actin mRNA was used as an internal control. A pair of chicken β-actin primers, chβ-1 (sense), 5′-ATGGCTCCGGTATGTGCAAGG-3′, and chβ-2 (antisense), 5′-AGCTTCTCC TTGATGTCACGC-3′ were used to amplify a 608 bp fragment of duck β-actin. The same schedule was used for β-actin amplification except that only 23 cycle repeats were performed. The PCR products were electrophoretically separated on 2.2% agarose gels, and stained with ethidium bromide, and then the cDNA bands were visualized and analyzed by Eaglesight software (Stratagene, USA).

2.8. Three-dimensional (3D) modeling

The predicted 3D structures of trimerization domain and Tg domain of duck Ii were determined by "comparative protein modeling" on the Swiss-Model server (http://www.expasy.ch/swissmod/SWISS-MODEL.html). Molecular models for duck Ii were created using as template the crystallographic structure of two strands of human Ii liieB.pdb and 1l3hA.pdb. Models were visualized and manipulated using RasWin Molecular Graphics Program (RasMol, version 2.7.2), software package (http://www.umass.edu/microbio/rasmol/).

3. Results

3.1. Identification of duck Ii

The duck Ii PCR product, which at 528 bp has a high sequence similarity to mammalian and chicken Ii, was obtained from total RNA by RT-PCR using a pair of degenerate primers, dpIi-1 and dpIi-2. To identify full-length duck Ii cDNA, sense primers were designed from the sequence of 528 bp for RACE. Both 3′ RACE and 5′ RACE were performed and 990 and 278 bp products, respectively, were obtained and sequenced. After confirmation of the presence of a poly (A) site and ATG codon, a duck Ii cDNA sequence with 1190 nucleotides was identified (GenBank accession no. AY904336). It was named duck Ii-1, which contained a 669 bp open reading frame encoding a putative 222 amino acid protein (Figs. 1 and 2A). As an aim to look for an alternative spliced product of duck Ii, we identified a 189 bp nucleotide sequence encoding a putative Tg domain (Figs. 1 and 2B). We named the alternative transcript as duck Ii-2.

3.2. Analysis of the predicted duck Ii

The putative amino acid sequence of duck Ii-1 showed an 82% similarity to chicken Ii-1, 67% similarity to bovine Ii p31, 62% similarity to mouse p31, 60% similarity to rat p31 and 58% similarity to human p33, as determined by BLAST searches. An alignment of the putative peptide sequence of duck Ii-1 with chicken Ii-1, bovine p31, mouse p31, rat p31 and human p33 showed that some certain regions, like the TM region, N-terminal part of the cytoplasmic tail and the region responsible for self-association (aa163–183) were well conserved (Fig. 2A). A Gln-Arg-Asp-Leu-Ile-Ser (Q-R-D-L-I-S) motif found in the cytoplasmic tail and a Try-Leu-Leu-Phe-Glu-Met (W-L-L-F-E-M) motif found in the trimerisation domain were well conserved between avian and mammal Ii. The cytoplasmic tail of duck Ii was found to contain only one Leucine sorting signal (Leu 8/Ile 9). Another sorting signal (Met 16/Leu 17) which was conserved in chicken and mammal was replaced by

"Pro 16/Val 17/Val 18". An alanine in position 2 in duck and chicken Ii is absent in mammalian Ii, and the two aspartic acid residues (or a glutamic acid and an aspartic acid) in position 2 and 3 in mammalian Ii are exchanged with glutamic acid in avian Ii (Fig. 2A).

The similarity of the Ii-2 Tg domain to those of the chicken Ii-2, mouse p41 and human p41 domains was 96%, 71% and 70%, respectively (Fig. 2B). The six conserved cysteine residues in mammalian Tg domain were well conserved in duck Ii-2. However, A Cys-Trp-Cys-Val motif which was found in most of the type I repetitive units of human thyroglobulin (Malthiery and Lissitzky, 1987; Veneziani et al., 1999), was replaced by Cys-Trp-Cys-Cys in Ii-2 of duck and chicken (Fig. 2B).

3.3. Expression analysis of duck Ii-1 and Ii-2

In order to examine the tissue distribution of duck Ii, RT-PCR was performed on total RNA from various tissues of three individuals using primers S1 and d-r-1, known to amplify both forms of duck Ii. The expected sizes of the product were 116 and 308 bp, respectively. Both transcripts of duck Ii can be detected in all of the tested seven tissues. Similar results for the specific tissues of each duck were observed (Fig. 3).

The result showed both Ii-1 and Ii-2 mRNA were extensively expressed in various tissues, whereas high levels of expression of the two Ii isoforms was only observed in the spleen and bursa of Fabricius (Fig. 3). Expression of Ii-1 was stronger than Ii-2 in every specific tissue, and Ii-2 was weakly detected in the heart, brain, liver, thymus and kidney. This suggested that duck Ii-1 was the predominant form expressed in

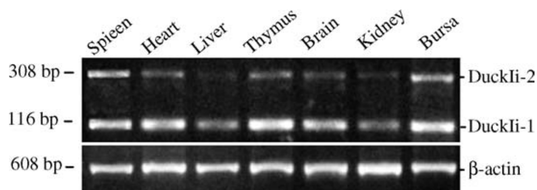

Fig. 3. mRNA expression of duck Ii-1 and Ii-2 in different tissues. One microgram of total RNA from each sample was subjected to RT-PCR and 5 μl of the 25-(Ii) and 23-cycle amplicons were separated by electrophoresis on 2.2% agarose gels and stained with ethidium bromide. Expected products were 116 (duck Ii-1), 308 (duck Ii-2) and 608 (β-actin) bp in size. β-actin mRNA was used as an internal control.

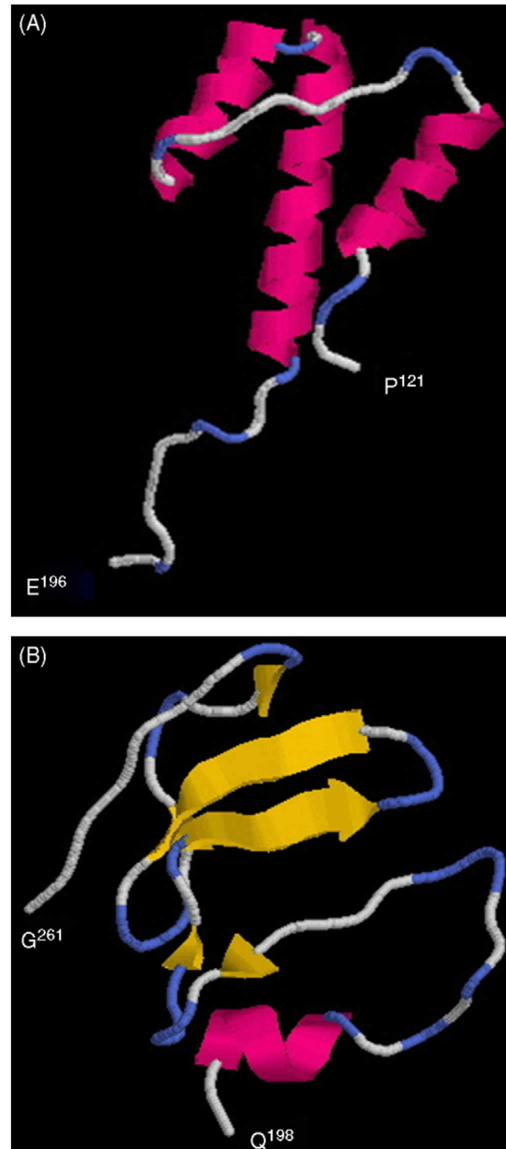

Fig. 4. 3D (three-dimensional) structures of trimerization and Tg domain of duck and chicken Ii. Cartoon displays of structure models of duck and chicken Ii are shown. *Yellow arrows* represent β strands and *magenta spiral* represent α helices. (A) Trimerization domain of duck Ii-1. (B) Tg domain of duck Ii-2. These hypothetical structure models were generated by the Swiss-Model server (http://www.expasy.ch/swissmod/SWISS-MODEL.html), and the three-dimensional structure were viewed and colored with the RASMOL, version 2.7.2, software package (http://www.umass.edu/microbio/rasmol/).

duck tissues, which was coordinate with the pattern of Ii-1 expression in chicken (Zhong et al., 2004) and p31 expression in mammals (Koch and Harris, 1984). RT-PCR for β-actin generated specific bands of similar intensity in all tissues. All controls for non-specific amplification were negative (not shown).

3.4. Structural homology between duck and human Ii

Trimerization domain of duck Ii-1 was modeled using liieB.pdb template which shares 61.67% identity with duck Ii-1. It included 75 residues, corresponding to Pro^{121}-Glu^{196} (P^{121}-E^{196}, Fig. 4A) and contained three α-helix and loops of various lengths that connect helices. Tg domain of duck Ii-2 was modeled using 1l3hA.pdb template which shares 54.84% identity with duck Ii-2. It included 64 residues, corresponding to Gln^{198}-Gly^{261} (Q^{198}-G^{261}, Fig. 4B) and contained one α-helix, six strands, ten turns and loops of various lengths. The predicted 3D structures of trimerization and Tg domains were similar between duck and human despite their differences at amino acid level.

4. Discussion

The human and murine Ii have been extensively studied and functionally important regions have been revealed. However, Ii is not well characterized in avian and other species. In this study, we have identified two isoforms of duck Ii. Ii-1 showed 82% sequence similarity to chicken Ii, and about 58–67% similarity to mammalian Ii; An alternative transcript encoding a thyroglobulin (Tg)-containing form of Ii, was also found in duck. In mammals, an important function of Ii is to ensure the targeting of newly synthesized MHC class II to the endocytic pathway. The endosomal localization signals were identified as two independent motifs, Leu 7/Ile 8 and Pro 15/Met 16/Leu 17 in the Ii cytoplasmic tail (Bremnes et al., 1994; Odorizzi et al., 1994; Pieters et al., 1993). The Met 16/Leu 17 signal was less efficient for endocytosis (Kang et al., 1998), mutational analysis suggested that the motifs were recognized as part of a larger secondary structure (Motta et al., 1995; Pond et al., 1995). Only one Leucine sorting signal (Leu 8/Ile 9) was found in duck Ii, whether this will influence its sorting and internalization efficiency is unknown.

In the transmembrane region there was a cleavage site (at aa 42 in human Ii) that might cleave the cytoplasmic tail of Ii (Lipp and Dobberstein, 1986). This cleavage site is conserved between avian and mammalian Ii. The CLIP region is responsible for the binding of Ii to class II molecules in mammals. This region showed a low homology of sequence between avian and mammal Ii. However, the Met 91 and Met 93 residues required for binding MHC class II in CLIP region (Ghosh et al., 1995) were well conserved. In mammals, Ii chains have two N-linked glycosylation sites (aa 118/120 and 124/126, for review see (Stumptner-Cuvelette and Benaroch, 2002)), only the second one is present in duck and chicken.

In the mouse, Ii exists in two forms, namely, p31 and p41, resulting from alternative splicing of exon 6b, which encodes a cysteine-rich domain of 64 amino acids and forms a thyroglobulin type-I domain (Koch et al., 1987). Both p31 and p41 can enhance antigen presentation (Serwe et al., 1997); however, p41 is confirmed to be involved in proteolysis, and functions as a protease inhibitor (Bevec et al., 1996; Fineschi et al., 1995). Our previous study revealed that Ii exist in two forms in chicken derived from alternative splicing (Zhong et al., 2004) and is strongly expressed in the main immune organs. In this study we examine the tissue distribution of duck Ii-1 and Ii-2 by RT-PCR. The result showed both Ii-1 and Ii-2 mRNA were extensively expressed in various tissues, and strongly expressed in the spleen and bursa of Fabricius. In mammals, Ii and MHC class II are always coordinately expressed in cells and tissues (Hua et al., 1998; Momburg et al., 1986). However, in normal mucosa, epithelial cells and non-immune cells of some organs (lung, kidney, testis and pancreas), the expression of Ii was observed in the absence of MHC class II (Badve et al., 2002; Momburg and Moller, 1988). Therefore, Ii may have functional roles in addition to antigen presentation (Badve et al., 2002). In fact, previous studies show that Ii is also involved in the selection of $CD4^+$ T-cells (Bikoff et al., 1993; Wright et al., 1998), enhancing the MHC class I cell surface expression (Reber et al., 2002; Vigna et al., 1996), and inducing B-cell maturation as a signaling molecule (Matza et al., 2002a,b, 2001; Shachar and Flavell, 1996). The extensively distribution of Ii in different tissues in

the present study could be related with its multiple roles in immune system.

In conclusion, our results indicate that, similar to chicken and mammalian Ii, duck Ii exist in two forms resulting from alternative splicing. The two isoforms are extensively expressed in various tissues, while duck Ii-1 was the predominant form expressed in duck tissues. The predicted 3D structures of trimerization and Tg domain of duck Ii are similar to human Ii. Therefore, based on the structural characterization, mRNA expression pattern and the predicted 3D structures of the two duck Ii, we presume that Ii-1 and Ii-2 may have similar functions to their mammalian homologues in MHC class II antigen presentation.

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