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Molecular Cloning and Characterization of cDNA Encoding Immunoglobulin Heavy and Light chain Variable Regions from Four Chicken Monoclonal Antibodies Specific to Surface Antigens of Intestinal Parasite, *Eimeria acervulina*

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We have developed four chicken hybridomas secreting monoclonal antibodies to induce a protective immune response against the chicken disease avian coccidiosis, caused by the intestinal parasite *Eimeria acervulina*. However, since the amount of antibodies secreted from these hybridomas is too low or sometimes they lost their ability to produce antibodies, the hybridoma method is not satisfactory in the production of large amounts of chicken monoclonal antibodies. To bypass these problems, we applied the antibody engineering technology using polymerase chain reaction. We cloned and determined the sequences of variable domains of the four chicken monoclonal antibodies, namely, 2-1, 5D11, 13C8 and 8C3. The sequences comparison to germline sequences showed that the gene conversion mechanism might contribute to developing diversification of heavy and λ-light chains in chicken antibodies. Several pseudogene families regarded as donors in gene conversion were identified at each framework region and the complementarity determining region of λ-light chains. In addition, as expected, numerous changes of nucleotide sequences such as nucleotide substitution, insertion and deletion were found predominantly in complementarity determining regions, which are likely to be somatic hypermutations as a result of affinity maturation in antibody-producing cells.

Key words **:** *Eimeria acervulina*, chicken monoclonal antibody, gene conversion, antibody engineering, complementarity determining regions, somatic hypermutation

Avian coccidiosis, caused by intestinal parasites belonging to genus *Eimeria*, is an obligate protozoan disease of chickens, resulting in a significant economic loss in the poultry industry. Despite increasing interest in developing protection strategies, the use of whole parasites or chemotherapy has major drawbacks. For example, due to the complexity of the parasite life-cycle and the existence of multiple species infecting chickens, immunity developed by using whole parasites, in general, is species-specific and cross-species protection has not been observed (20). The application of anti-coccidia drugs is also hindered by high costs and development of drug resistance. Therefore, research has been focused on the development of immunological controls, which is dependent on the identification and characterization of target antigens to induce protective immune responses by the host immune system.

Current efforts to develop an immunological control against coccidiosis involve identification of immunogenic epitope of *Eimeria* parasites to elicit cell mediated immunity (11). In general, two immunological strategies have been envisioned. The first uses recombinant subunit vaccines derived from parasite proteins used to bind to host cell receptors since avian coccidia parasites are known to invade cells of intestinal surface epithelium (1, 9). The second approach involves passive immunization with antibodies that actively block the interaction of parasites with host cells (22). Many coccidial antigens have been identified with mouse antibodies (23) and their cDNAs have been cloned for the development of a subunit vaccine (3, 7). But the efficacy of these antibodies is debatable (25) because of differences in the target antigens recognized by immune sera from chickens and mice (5).

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Therefore, in this regard, chicken antibodies may be more advantageous for the identification of target antigens to cause avian coccidiosis.

Recently, we have developed four chicken monoclonal antibodies (Mabs; 2-1, 5D11, 8C3 and 13C8) which recognize *Eimeria* antigens (10, 22), and characterized their biochemical properties. The immunologic nature of antigens recognized by these antibodies is under the investigation. Recently, we found that the developed chicken Mabs recognize the surface antigens localized in the apical complex of *Eimeria acervulina* (unpublished results). This promising result suggests the possible application of anti-*Eimeria* Mabs for passive immunization. However, chicken hybridomas have some drawbacks such as production of a low amount of antibody and of non-specific IgM, and the loss of ability to produce antibodies (13, 14). Therefore, it is necessary to use recombinant antibody technology to bypass these problems (15). To use this technology, it is a prerequisite to know the sequences of the antigen binding domain, variable regions of heavy and λ-light chains in these antibodies. Unlike mammals such as mice and humans, the immunoglobulin gene diversification in chickens is mainly constructed by gene conversion (16, 17, 18, 19). More specifically, single functional immunoglobulin variable and joining segments at each of the heavy and λ-light chain loci are diversified by conversions with upstream pseudo variable region genes as sequence donors (17, 18, 19, 24). Since the sequences of pseudogenes are highly conserved in the 5'-and 3'-flanking region suggesting that all variable regions in mature B cells or hybridoma have identical ends, gene conversions in chickens make it possible to amplify variable region genes using a single pair of primers per heavy and λ-light chain (4, 26). Therefore, in this study, we have cloned and analyzed the molecular nature of chicken Mabs by polymerase chain reaction (PCR) using a single pair of primers which are specifically designed for the cloning of variable region cDNA of heavy chain and λ-light chain extracted from chicken hybridomas secreting monoclonal antibodies specific to the surface antigen of *Eimeria acervulina*.

Materials and Methods

Chickens

Embryonated eggs of White Leghorn crosses (SC^R) obtained as fertile eggs from a commercial breeder (Hyline International, Dallas Center, Iowa. U.S.A.) were hatched at the Parasite Immunobiology Laboratory, Beltsville, Maryland, and maintained in brooders until 3 wk of age, at which time they were kept in wire colony cages. Chickens were housed in clean wire-floored cages. Special care was taken not to expose the chickens to specific pathogens. Food and water were available *ad libitum.*

Preparation of Eimeria acervulina sporozoites

Sporulated oocysts of *E. acervulina* (#84 USDA strain, U.S.A.) were collected. Sporozoites were prepared by excysting *E. acervulina* oocysts in a solution containing 0.125% (w/v) trypsin (Sigma, U.S.A.) and 1% taurodeoxycholic acid in Hank's balanced salt solution (HBSS), pH 7.6 for 10 min at 41°C in a 5% CO_2 incubator. Sporozoites were separated from cellular debris on DEAE-cellulose columns (DE52; Whatman Paper Ltd. U.S.A.)

Preparation of sporozoite antigens

Pelleted sporozoites(10^9/ml) in phosphate-buffered saline (PBS) were freeze-thawed 6 times with dry ice and warmed to room temperature, then sonicated at 4°C with a Microson Ultrasonic Cell Disrupter (Heat System, U.S.A.).

Development of Chicken B-cell hybridoma

To produce hybridomas that produce Mabs specific to coccidial antigens, 6-12-wk-old SC chickens were intramuscularly injected with soluble antigen prepared from *E. acervulina* sporozoites (10^7) which was emulsified in Freund's complete adjuvant. A second injection with the same preparation was given in Freund's incomplete adjuvant and additional immunizations were given by intravenous injection with the same preparation without adjuvant at 1-wk intervals. A final boost was given intravenously 3 days before fusion. Spleens from these chickens were used for hybridization. Production of hybridomas was carried out as described by Nishinaka *et al.* (13, 14). Briefly, 3 days after the last immunization, single cell suspensions of spleens were prepared by centrifugation for 20 min at 500 g on a Ficoll-Paque density gradient at 20°C. The cell fusion was carried out as described (10), using the R27H4 nonsecreting chicken myeloma cell line in polyethylene glycol 4000. The fused cells were suspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with with 10% fetal calf serum (FCS) and hypoxanthine-aminopterin-thymidine (HAT; Sigma) and plated in 96-well microculture plates. After 2 weeks, culture supernatants from hybrid clones were screened using an enzyme-linked immunosorbent assay (ELISA) with sporozoite antigens on a solid phase. Hybridomas secreting the Mabs of interest were cloned by limiting dilution using irradiated spleen cells (2×10^6 per well) as feeder cells. Undiluted cultural supernatant from hybridoma was used in all experiments.

Isolation and amplification of heavy and λ-light chain variable domain genes

Total RNA was purified using Trizol™ reagent (Life Technologies Inc, U.S.A.) following the vendor's instruction. Five microgram of total RNA was resuspended in RNase-free water and mixed with 50 ng/μl oligo $(dT)_{12\text{-}15}$ primer. The mixture was heated to 70°C for 10 min and a reaction mixture consisting of 2 μl 10X PCR buffer and 2 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTPs and 2 μl 0.1M

Table 1. Primers used for PCR amplification of heavy and light chain variable regions of chicken monoclonal antibodies[a]

1. Oligonucleotides for PCR	
A. Heavy chain	
CKVHBACK	5'-GCCGTGACGTTGGACGAGTCC-3' A V T L D E S
CKVHFOR	5'-GGAGGAGACGATGACTTCGGT-3' S S V I V E T
B. Light chain	
CKVLBACK	5'-GCGCTGACTCAGCCGTCCTCG-3' A L T Q P S S
CKVLFOR	5'-TAGGACGGTCAGGGTTGTCCC-3' L V T L T T G

[a]CKVHBACK, reverse primer for heavy chain variable region. CKVHFOR, forward primer for heavy chain. CKVLBACK, reverse primer for light chain variable region. CKVLFOR, forward primer for light chain. Amino acid sequences encoded by these primers are shown in a single letter code.

DTT was added following incubation at 42°C for 5 min. 200 units of Superscript II reverse transcriptase was added and incubated at 42°C for 50 min. The reaction was terminated at 70°C for 15 min. To remove the residual RNA, 1 μl of RNase H was added and incubated for 20 min at 37°C. After RNase H digestion, one-tenth of the cDNA products was used to amplify the heavy and light chain genes. PCR reaction was performed as follows; 1 cycle of 4 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 55°C, 1 min at 72°C, with a final extension step of 7 min at 72°C. Immunoglobulin variable region genes were amplified using the oligonucloetides pairs (Table 1); CKVLBACK (λV_L reverse primer) and CKVLFOR (λV_L forward primer) for the variable region of λ-light chains; CKVHBACK (V_H reverse primer) and CKVHBACK (V_H forward primer) for the variable region of heavy chains. The PCR products were separated on 1% agarose gel in 1X TAE and extracted using QiaEXII DNA extraction kit (Qiagen, U.S.A.). Purified PCR products were cloned into pGEM-T vector (Promega, U.S.A.) and transformed into JM109 as described (21).

Sequencing of the cloned variable domain genes

Plasmid DNA was prepared with a Qiagen plasmid purification kit and sequenced with an ABI 377 automatic sequencer using a big-dye terminator cycles sequencing ready kit (PE Applied Biosystems, U.S.A.). The sequences obtained were analyzed by comparing with germline sequences of heavy and λ-light chains of CB strain (17, 18).

Results and Discussion

PCR amplification of variable regions of heavy and λ-light chain of chicken monoclonal antibodies.

The cloning strategy for the variable regions genes of chicken Mabs is shown in Fig. 1. As shown in Fig. 2, a single band of the correct size for the variable region of heavy chain and λ-light chain was obtained by amplification of the first strand cDNA made from total RNA primed with oligo dT. Because DNase I digestion was performed before cDNA synthesis to remove genomic

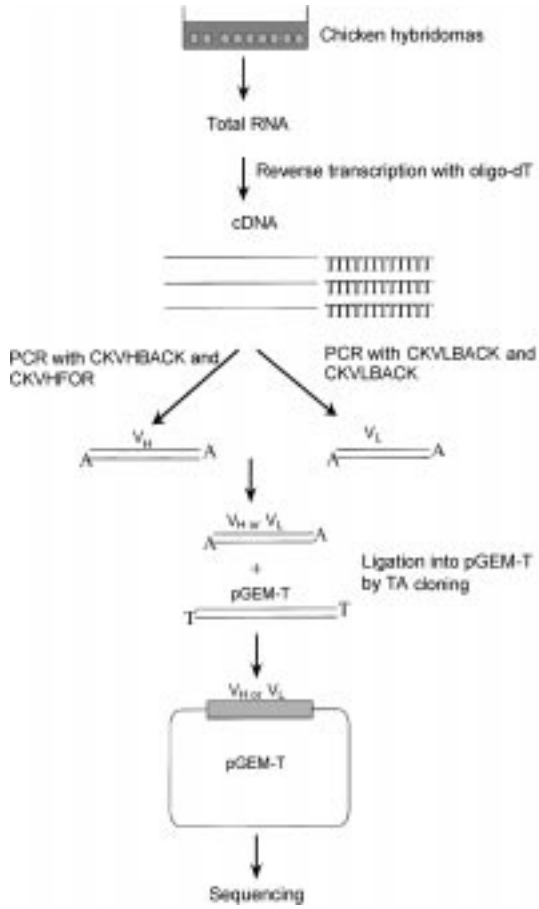

Fig. 1. Cloning strategy of variable region genes from chicken hybridomas secreting *Eimeria* antigen specific monoclonal antibodies.

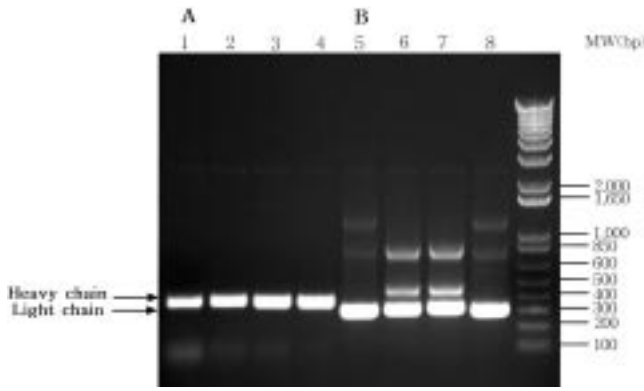

Fig. 2. Amplified PCR products of heavy and λ-light chain variable domains derived from each hybridoma. (A) heavy chain. 5D11 (1), 8C3 (2), 13C8 (3), 2-1 (4). (B) λ-light chain. 5D11 (5), 8C3 (6), 13C8, (7) 2-1 (8).

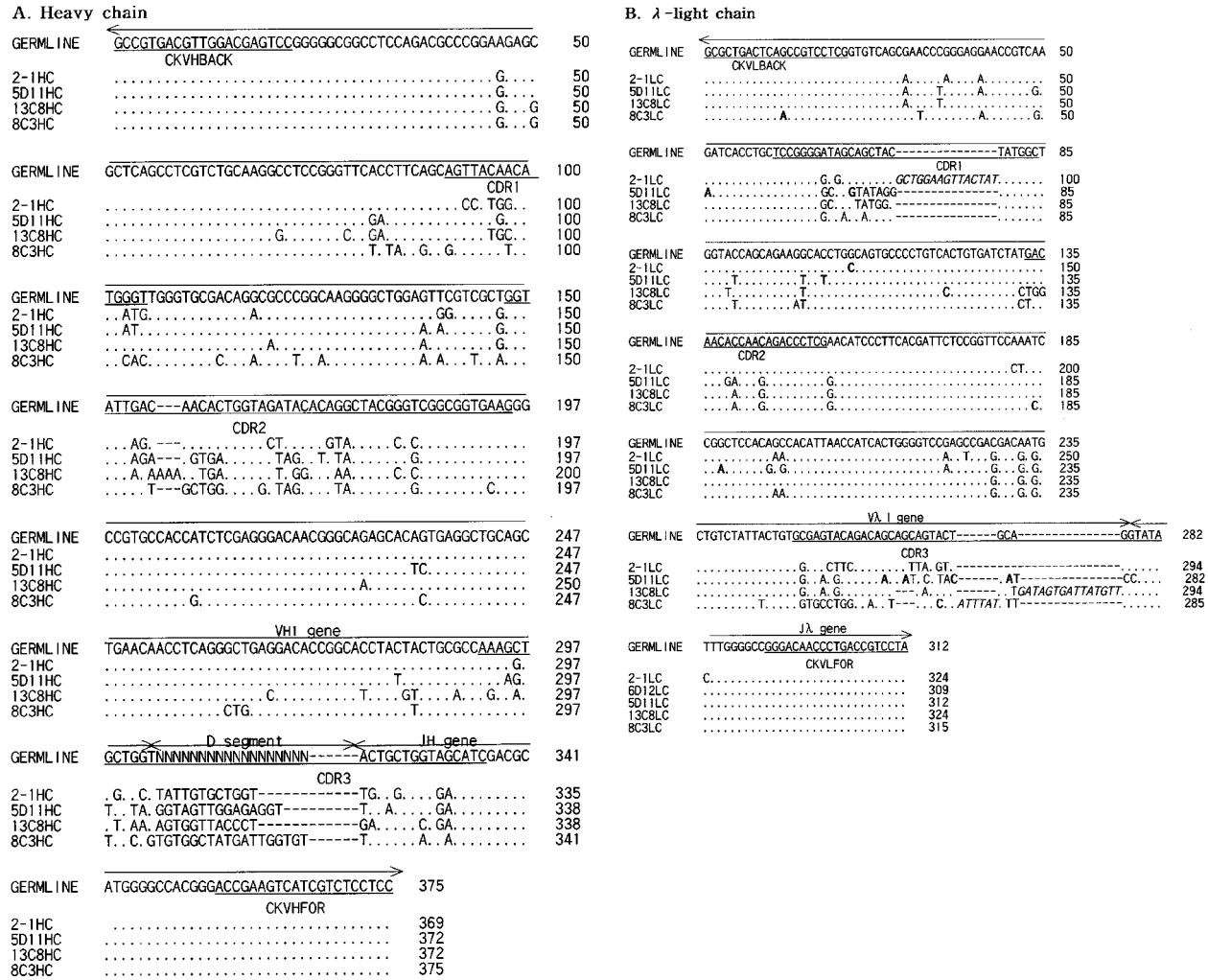

Fig. 3. Nucleotide Sequences of heavy (A) and λ-light chains (B) of anti-*Eimeria* monoclonal antibodies. Germline sequences of CB inbred strain are shown at the top. Nucleotide identity of the germline sequences are shown by dots. The absence of corresponding residues is shown by dashes. The region of CDR and PCR primers are indicated with underlines in the germline sequences. Both VH1 genes, D segments and JH genes in the heavy chain, and $V_{\lambda}1$ genes and J_{λ} genes in the λ-light chain are labelled by arrowbars above the germline sequences. Sequences to be filled with D segment in the germline heavy chain are shown by Ns. Base substitution and nucleotides addition are shown in bold and italic, respectively in λ-light chains.

DNA, the possibility of PCR products templated from genomic DNA was excluded.

Sequence analysis of cloned variable regions of chicken heavy and λ-light chain

Nucleotide sequences of the cloned variable regions of heavy and λ-light chains of the four Mabs were determined and compared with germline VH1-JH sequences (Fig. 3A) and $V_{\lambda}1$-J_{λ} (Fig. 3B) of the CB strain (17, 18), respectively. Framework (FR) and complementarity determining regions (CDRs) were also determined according to Kabat *et al* (6). The sequence comparisons between the four clones and germline show that differences between the cloned genes and the germline were predominantly in the CDRs in the heavy and λ-light chains as expected (Fig. 3). For example, the insertion of 15 nucleotides (GCTGGAAGTTACTAT) was observed in the CDR1 in the λ-light chain of 2-1 clone. The CDR3 of the 13C8 clone and 8C3 clone also contain the insertion of 15 nucleotides (GATAGTGATTATGTT) and 6 nucleotides (ATTTAT), respectively. The deletions were found in 3 different clones. For example, 3 nucleotides (GCA) in the CDR3 were deleted in 2-1 clone. In the case of 13C8 and 8C3 clones, 3 different nucleotides (AGC) in CDR3 were deleted.

Gene conversion was traced by comparing the variable region of λ-light chain nucleotide sequences with 25 pseudogenes of the CB strain (17) and other known pseudogenes in different chicken strains (8). For example, both the CDR1 and CDR2 of the 2-1 clone was derived from ΨVλ8. These CDRs were derived from ΨVλ14 and ΨVλ7 in the 5D11 clone, ΨV23 and ΨV12 in the 8C3 clone, and ΨV14 and, ΨV14 and ΨV12 or ΨV13 in the 13C8 clone (Fig. 5). The gene conversion found here showed

A. Heavy chain

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Germline    AVTLDESGGGLQTPGRALSLVCKASGFTFSSYNMGWVRQAPGKGLEFVAG   50
                                          CDR1
2-1HC       ..............G..............HG.M....T......W...    50
5D11 HC     ..............G..........D....D.I..........Y...    50
8C3 HC      ..............GG.........SIGG.I.H....T......Y...    50
13C8 HC     ..............GG......G..LD....A.................    50

Germline    IDNT-GRYTGYGSAVKGRATISRDNGQSTVRLQLNNLRAEDTGTYYCAKA   99
              CDR2
2-1 HC      .S..-.T..Y.AP.....................................-    98
5D11 HC     .RSD-.SSIY..A.................L..................S    99
8C3 HC      ..AG-.GS.Y..A..Q....V.........L.........L......F....  99
13C8 HC     .KKND.SW.N.AP.........................D...I.V.TRD   100

Germline    AGXXX--XX-----XTAGSIDAWGHGTEVIVSS      124
              CDR3
2-1 HC      -GGAY--CA-----GCG.D.............       122
5D11 HC     ---SY-----GSWRGST.D.............       123
8C3 HC      SRCGYDWCS-------.DN.............       124
13C8 HC     VNSGY---------PD.AD.............       123
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B. λ-light chain

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Germline    ALTQPSSVSANPGGTVKITCSGDSSY-----YGWYQQKAPGSAPVTVIY    44
                                    CDR1
2-1 LC      ............E........GG..AGSYY.........A........    49
5D11 LC     ...........L.E..E.....-----GRYR.......SS.........    44
8C3 LC      ...........I..E..E.....GNN.-----.......S.........    44
13C8 LC     ...........L..........G-----SYG...F...S......P...    44

Germline    DNTNRPSNIPSRFSGSKSGSTATLTITGVRADDEAVYYCASTDSSST---  91
              CDR2                                        CDR3
2-1 LC      ...............L....N.......QVE.......G.F...YV---  94
5D11 LC     ..DK...D..........D..G.......Q.E.......GNA.NNTY---  91
8C3 LC      Y.NK...D.........P...N.........E.....F.GAWE..PI---  91
13C8 LC     W-NK...D.......................E.......GNA..NTADSD  94

Germline    -AGIFGAGTTLTVL    104
2-1 LC      --..L..........   106
5D11 LC     -DP............   104
8C3 LC      YV.............   105
13C8 LC     YV.............   108
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Fig. 4. Deduced amino acid sequences of heavy chain (A) and λ-light chain (B) variable regions with germline sequences. Germline sequences of the CB strain are shown at the top. The CDRs are indicated with underlines in the germline sequences. Amino acid identity to the germline sequences is shown by dots. The absence of corresponding residues is shown by dashes. Amino acid residues derived from D gene in the heavy chain are shown by an X.

characteristics similar to those reported previously for the number of gene conversion events in rearranged variable genes (12). The boundary of the donor pseudogene and germline gene was not clear, and sometimes more than one candidate pseudogene was found (Fig. 5), indicating the multiple gene conversion events in one variable region.

These data clearly suggest that most of the distinct differences between the cloned genes and the most closely matching known germline sequences of the λ-light chain can be accounted for by conversions with the pseudo-VL gene sequences (17). In addition, sixteen single nucleotide substitutions were found after identification of donor pseudogenes (Fig. 3). Although we cannot completely exclude PCR errors or DNA polymorphism in the pseudogene in our line of chickens (White Leghorn), the data suggest possible somatic hypermutation. Among 16 mutations found in the Vλ1 genes, 8 mutations were located in the CDRs and 8 mutations were located in the FR in all four clones. Since clusters (7 out of 8) of point mutations in CDR are found in CDR3, the base substitutions in CDR3 are likely to be somatic hypermutations. Since the results shown here are those of mature immunoglobulin molecules from chicken hybridoma, we assume that more mutations could be accumulated in CDRs as a result of affinity selection of B cells. The sequence analysis cannot be made with the heavy chains as the complete set of pseudo-VH sequences and germline D region sequences was not determined (18, 19). However, as shown in Fig. 3 and Fig. 4, large number of sequence differences were found between the four clones and germline, specially in the CDRs of heavy chains.

A vestige of gene conversion was also detected by comparing nucleotide sequences with germlines and 18 known pseudogene sequences of the CB inbred strain (18; data not shown).

Although germline and pseudogene sequences of the White Leghorn strain have not yet been analyzed, it suggests that the primers used in this study can be effective for obtaining chicken variable region genes by PCR. In fact, for most White Leghorn lines, DNA polymorphism is negligible in the 5' and 3' ends of the variable region in both heavy and λ-light chains (2).

Amino acid sequence differences between the cloned

λ-light chain

Antibody	FR1	CDR1	FR2	CDR2	FR3	CDR3
5D11	ΨV4 and ΨV2/7/18/23/24	ΨV14	ΨV7	ΨV7	ΨV7/12	V12 and V11
8C3	ΨV24	ΨV23	ΨV7	ΨV12	ΨV7/10/12, ΨV2/8/17, and ΨV13	ΨV13
13C8	ΨV4	ΨV14	ΨV6 and ΨV11	ΨV14 and ΨV12/13	ΨV11	ΨV12 and ΨV20
2-1	ΨV4 and ΨV5/8/14	ΨV8	ΨV8	ΨV8	ΨV8 and ΨV14	ΨV14 and ΨV3

Fig. 5. Donor pseudogenes used in variable regions of λ-light chain. Boundaries between inserted pseudogenes and the germline sequences cannot be specified. If the number of candidate pseudogenes exceeded one, all are listed, divided by slashes.

genes and germline of the CB strain shown in Fig. 4 are consistent with Fig. 3 indicating that differences between the cloned genes and germline were predominantly in the CDRs in both heavy and λ-light chains.

Although all four clones have similar antigen binding specificity in Fig. 4, their deduced variable amino acid sequences of the CDRs are very different in each clone. It suggests that they may recognize the different epitopes of *Eimeria* surface antigens since antigen binding specificity is based on the encoded combining site specificity mostly dominated by the CDR regions in the heavy and light chains.

In conclusion, all the sequences analyzed in this report show enough evidence of extensive and varied gene conversion of the single rearranged variable gene in both heavy and λ-light chains. Moreover, the gene conversion contributing to immunoglobulin gene diversification in chickens can simplify the production of the chicken recombinant antibody fragments using a single pair of primers as used in this report. Therefore, we are now in a position to evaluate whether the sequence information could be used to produce the recombinant chicken antibody fragments expressed in *Esherichia coli*.

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