Dissection of polygenic control of antibody response kinetics in adult chickens

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ABSTRACT

The immune system plays an important role in protecting poultry from diseases. Understanding genetic control of immune response can be utilized to improve chicken health. Adult F₁ hens were generated by mating highly inbred males of two MHC-congenic Fayoumi lines with G-B1 Leghorn hens. Antibody response after *Salmonella enteritidis* (SE) vaccination at day 10, and sheep erythrocyte (SRBC) and killed *Brucella abortus* (BA) immunizations at 19 and 22 weeks were measured. Agglutinating antibody titer at 7 day after primary immunization and mean titer of the final three samples (day 18, 32, and 63 after 2nd immunization) were used as parameters for primary and equilibrium phases, respectively. Secondary phase parameters of minimum (Ymin), maximum titers (Ymax) and time needed to achieve minimum (Tmin) and maximum (Tmax) titers were estimated from seven post-secondary titers using a nonlinear regression model. Both candidate gene and genome scan approaches were used to identify quantitative trait loci (QTL) affecting antibody response to SE vaccination and antibody response kinetics to SRBC and BA. Using the genome scan approach, QTL affecting immune response to SRBC and BA were detected by a stepwise process that culminated in interval mapping. Five significant QTL were detected at the 5% chromosome-wise level on chicken chromosomes 3, 5, 6 and Z, suggesting that regions on chromosomes 3, 5, 6 and Z contain QTL affecting antibody kinetics to SRBC and BA. Single nucleotide polymorphisms (SNP) of 15 immunity related genes were identified. Interferon-γ, immunoglobulin light chain, interleukin-2, MHC class I α1, α2, class II β1, and transforming growth factor-β 2, 3 and 4 SNP were significantly associated with antibody responses to SE vaccination or antibody response kinetics to SRBC and BA or both. For some candidate genes, significant associations were primarily detected in the lineage of the M5.1 grandsire, but in that of the M15.2 grandsire on the others. Strong interactions of the MHC with candidate genes on antibody response were indicated from the study. All the genes characterized in the present study are, therefore, strong potential candidates for application in marker-assisted selection to improve vaccine response and immunocompetence in chickens.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is written in the alternative format in which a literature review (Chapter 1) is followed by eight manuscripts for publication in scientific journals (Chapter 2, 3, 4, 5, 6, 7, 8, 9). The main theme of this dissertation is centered on dissection of polygenic control of antibody response kinetics in adult chickens. The literature review covers three major topics: the linkage disequilibrium approach to detect quantitative trait loci (QTL), the candidate gene approach to investigate associations between traits of interest and genes, and the genetics of antibody response and disease resistance. The final chapter, Chapter 10, contains a general discussion and recommendations for future work. Relevant references are compiled for each chapter, followed by the tables and figures. Chapter 2 focuses on estimating phylogenetic relationships among 23 inbred chicken lines, from which three inbred lines were used as the parental lines to produce an intercrossed F2 population studied in later stages of this overall project (Animal Genetics, 1999, 30, 256-264). Chapter 3 describes DNA polymorphisms among the three chicken inbred lines detected by amplifying cDNA sequences using thirteen immunity related gene-specific primers provided from the U.S. Poultry Genome Coordinators (Poultry Science, 2001, 80, 284-288). Chapters 4, 5, 6, 7, 8, and 9 each cover a specific molecular genetic analysis of DNA samples from an F2 population that was phenotypically characterized for antibody production kinetics. Chapter 4 reports an association study between the promoter polymorphisms of the three candidate genes and antibody response kinetics in chickens (Poultry Science, 2001, 80, 1679-1689). Chapter 5 focuses on associations of interferon-γ genotype and protein level with antibody response kinetics (In press in Avian Disease 2002). Chapter 6 describes a genome scan approach with microsatellites to detect QTL affecting antibody response kinetics in chickens (In press in Poultry Science). Chapter 7 is about associations of major histocompatibility complex class I α1, α2 and II β genes with antibody production in chickens (submitted to Immunogenetics in October 2002). Chapter 8 focuses on associations of transforming growth factor β genes with QTL for antibody response kinetics in chickens (submitted to Animal Genetics in October 2002). Chapter 9 gives general information on the lack of very significant associations of six
candidate genes with antibody kinetics in hens (submitted to Poultry Science in November 2002). The manuscripts were prepared for journal publication. Therefore, the organization and format of chapters 2, 3, 4, 5, 6, 7, 8 and 9 is in accordance with the requirements of those journals. The coauthors of the manuscripts are: Susan J. Lamont and Wei Liu, Department of Animal Science, Iowa State University; A. J. Buitenhuis, Animal Breeding and Genetics Group, Wageningen University, Wageningen, the Netherlands; Steffen Weigend, Institute for Animal Science and Animal Behaviour, Mariensee, Germany; and H. S. Lillehoj, USDA/ARS/PBESL, Beltsville, MD. In all instances, the first author was the primary person responsible for experimental design, laboratory work, statistical analysis, literature review, and drafting the manuscripts.

**Literature Review**

**Quantitative Genetic Variation of Phenotypic Traits**

Immune response, like other economically important traits, such as growth and reproductive traits, shows a continuous distribution of phenotypic values in farm animals, including chickens (Soller and Andersson 1998). The genetic variation in these quantitative traits are usually controlled by multiple genes (Lamont 1998). A quantitative trait locus (QTL) is defined as a region of the genome bearing one or more genes affecting the quantitative trait (Geldermann 1975). It is of importance to detect the genes (QTL) responsible for genetic variation in the traits of interest in livestock, in order to genetically improve animal performance of the economically important traits by marker-assisted selection. The major goal of functional genomics in farm animals is to map and characterize QTL that control the quantitative traits of economical importance (Andersson 2001).

Mapping of QTL offers promise for identifying genes determining complex traits (Korstanje & Paigen 2002). Rapid advances in genomics in the last decade, with a key driving force being the human genome project, provide more and more powerful tools for investigating the molecular basis of quantitative traits. The integrated linkage, physical, and comparative maps in farm animals lay the foundation for exploiting the genes controlling a phenotypic trait (Soller and Andersson 1998). Identification of QTL can be conducted through determining
the associations between phenotypic value and the inheritance of genetic markers in a suitable pedigree (Hillel 1997).

There are two primary strategies for finding QTL: the candidate gene approach through association tests and genome scans based on linkage disequilibrium with anonymous DNA markers.

**The Genome Scan Approach**

The genome scan is one approach to identify QTL affecting phenotypic traits of interest by the use of genetic markers, particularly anonymous markers spaced throughout the genome. The detected QTL are not genetic loci, but genomic regions containing one or more loci affecting the trait. The principle underlying genome scans is to detect linkage disequilibrium (LD) between unidentified QTL alleles and identified marker alleles. The LD can be defined as the nonrandom association of alleles at linked loci (Jorde 2000). Linkage mapping of QTL depends on the LD between markers and trait value detected within population or families, and the size of the genomic region containing QTL is determined by the extent of LD between the gene corresponding to the QTL and marker loci flanking the QTL (Mackay 2001). Three aspects must be considered regarding QTL identification by using the genome scan approach: experimental design, abundance of available polymorphic DNA markers, and statistical analysis.

**Population design** Two general experimental designs are used to map QTL using the genome scan method. One is based on crosses between populations, often inbred lines, primarily including F$_2$ and backcross mapping populations. The other design is implemented within a segregating population, usually on outbred population, primarily including full-sib and half-sib mating (Soller and Andersson 1998). For the first class of design, the two inbred lines are assumed to be fixed for alternate alleles for both DNA markers and QTL alleles affecting the traits of interest. The markers selected for mapping and any QTL segregating in the cross are heterozygous in the F$_1$ population. This design greatly simplifies the analysis method, because an interval flanked by only a pair of markers provides information on transmission of a QTL within that interval (Haley et al. 1994). In general, these designs are
very efficient for detecting marker-trait associations, because crosses between two inbred lines create maximum LD between QTL and marker alleles, and ensure that only two QTL alleles segregate with known linkage phase (Mackay 2001). The degree and direction of dominance of the trait of interest can be determined by comparing phenotypic value of parents with F1 progeny. If the F1 presents intermediate phenotypic value, F2 intercross mating will have greatest statistical power. However, if dominance is present, the F1 animals should be back crossed to the population exhibiting the recessive phenotype (Soller et al. 1976). In the initial genome scan, genetic distance between DNA markers should be no more than 40 cM, but should be more than 10 cM, or little efficiency in experimental work is gained (Darvasi et al. 1993).

In most situations, inbred lines are not available for farm animals. Linkage mapping of QTL is conducted on a within-population basis by means of half-sib or full-sib family designs (Knott and Haley 1992; Mackinnon and Weller 1995; Soller and Genizi 1978). The statistical power of half-sib and full-sib family cross is much less than that of backcross or F1 designs (Soller and Andersson 1998). The two major reasons are: (1) The marker-QTL linkage can be detected only when the parents are heterozygous at the QTL. On the assumption of Hardy-Weinberg equilibrium within the population, at most half of the full-sib or half-sib families can be expected to be heterozygous for a QTL, and only these parents are informative for marker-QTL linkage analysis. (2) Because the parents are selected from a population that is generally in linkage equilibrium, the same marker genotype may be associated with a beneficial allele in some families but with a detrimental allele in other families, depending on the specific linkage phase of marker-allele/QTL-allele in the parents of each given family.

**Molecular Genetic Markers** There are three main categories of genetic DNA markers: bi-allelic dominant, such as RAPD (random amplification of polymorphic DNA), AFLP (amplified fragment length polymorphism); bi-allelic co-dominant, such as RFLP (restriction fragment length polymorphism); SSCP (single stranded conformation polymorphism), and multi-allelic co-dominant markers, such as microsatellites. Multiple alleles per locus and an easy genotyping method [simple polymerase chain reaction (PCR)] has made microsatellites a major genetic marker for genetic linkage mapping in human, mouse and nearly all species
of agriculture interest. In chickens, there are three mapping resource populations: the East Lansing population (Crittenden et al. 1993), the Compton population (Bumstead and Palyga 1992), and the Wageningen population (Groenen et al. 1998). An international collaborative effort was made to integrate the three genetic linkage maps into one consensus linkage map (Groenen et al. 2000). The current chicken genetic linkage map (http://www.thearkdb.org/browser?species=chicken&obttvpe=stats. Date accessed: July 2, 2002), covers most of the genome of 1200-Mb and 4000 cM (Schmid et al. 2000). The map contains over 2408 loci, of which 661 are designed as genes. Over 1000 microsatellite markers have been mapped. All data on markers and maps is being made available through the Arkdb genome databases (http://www.thearkdb.org/. Date accessed: July 2, 2002).

**Statistical analysis of QTL detection** There are two general statistical methods used to implement QTL detection: maximum likelihood estimation (Lander and Botstein 1989) and least-square regression (Haley and Knott 1992). The advantage of the maximum likelihood method is that it can provide more accurate estimates of QTL effect and position and it is more tolerant to the situation of failure of normality assumption of phenotypic traits, compared to the single marker method (Lander and Botstein 1989). Least square analysis can fit many parameters simultaneously, background genetic noise attributable to the other chromosomes can be reduced, and several linked QTLs can be fitted at the same time (Haley and Knott 1992).

A example of a successful linkage mapping study in chickens was conducted by Vallejo et al. (1998). An F2 population, consisting of over 300 birds derived from two inbred lines which differed in resistance to Marek’s disease, was developed. Eight chromosomal regions with statistically significant effects on affecting resistance to Marek’s disease were identified by the genome scan approach. More QTL for resistance to MD were later reported by Yonash et al. (1999). Their results suggest that a limited number of genomic regions play a major role in the genetic control of Marek’s resistance. Yonash et al. (2001) reported that three microsatellite markers were significantly associated with antibody response to Newcastle disease virus and sheep red blood cell (SRBC), or *Escherichia coli* (*E. coli*) and survival, using a half-sib design between two meat-type chicken lines that had been divergently selected for high or low antibody response to *E. coli* vaccine. Van Kaam et al.
(1998; 1999a; 1999b) investigated QTL affecting body weight, growth and feed efficiency in chickens by using a whole genome scan, and found four QTLs affecting growth traits and two QTLs affecting carcass or meat quality traits.

In the present study, a unique genetic cross, the Iowa Antibody Kinetics Resource Population (IAKRP), was produced as an F2 cross from genetically distinct, highly inbred (> 99%) chicken lines, with the Leghorn G-B1 line as the founder dam line and MHC-congenic Fayoumi M5.1 and M15.2 lines as sires. A total of 66 microsatellite markers (40 cM marker spacing) spread over the entire genome were initially used to detect microsatellite markers that were potentially related to antibody response kinetics to sheep red blood cell (SRBC) and *Brucella abortus* antigen (BA) by the DNA pooling technique. Then, selective individual genotyping and whole population genotyping were done. Finally, interval mapping was conducted in specific genomic regions of four chromosomes. The results are presented in Chapter 6.

**The Candidate Gene Approach**

An alternative approach for QTL detection is the candidate gene approach. An association analysis is based on a detected population-wide linkage disequilibrium between a QTL and a DNA polymorphism in the candidate gene. Candidate genes for a given trait may be genes that are directly involved in the physiology or development of the trait (Bryne and McMullen 1996). Therefore, candidate genes can be identified on the basis of their known function. The candidate gene approach requires that genomic or cDNA sequence information for the gene is available. Candidate genes can be divided into three general classes: biological or physiological candidate genes, positional candidate genes, and comparative positional candidate genes. Genes chosen are based on the biological or physiological system involved in the trait of interest. The genes may be based on information from human or mouse, which suggests a role of the gene in the trait of interest (Rothschild and Soller 1997). Once QTL are mapped to specific chromosomal region by linkage-mapping analysis, positional candidate genes can be selected in the specific region where the QTL was detected. Potential candidate genes can be also selected following QTL mapping, and utilizing information on the genes in
chromosomal regions with conserved synteny in other gene-rich species, such as human and mouse. This approach is designated as positional comparative candidate gene analysis (Rothschild and Soller 1997).

Once candidate genes are chosen, the next step for candidate gene analysis is to design primers to amplify the specific region of interest, based on available sequence information from existing database. Primers may be designed directly according to genomic sequence information in this species. On the other hand, if no intron and exon boundary information is available for the gene, then the boundary might be inferred from other species, such as human and mouse. In some instances, if no sequence information is available for the candidate gene in the species of the interest, a deduced consensus sequence for primer design may be generated based on sequence information from several other species. If sequence similarities of the gene between species are very low, degenerate primers can be designed for initial work.

After a specific region of the gene is amplified by designed primers, the amplified fragment should be sequenced to confirm gene identity and to determine if any DNA polymorphism exists in a given resource population. Consequently, a screening procedure must be developed to genotype the whole population. The PCR-restriction fragment length polymorphism (RFLP) is a convenient procedure for large-scale genotyping of the uncovered polymorphisms (Rothschild and Soller 1997).

Many extensive and successful studies have been conducted in farm animals by using the candidate gene approach. Three genes in the chicken MHC genomic region were associated with antibody response to E. coli, SRBC, and Newcastle disease virus in a F₂ cross derived from high and low E. coli antibody lines (Yonash et al. 1999). Resistance to salmonellosis in chicken was linked to NRAMP1 and TNC in a backcross population from a resistant and susceptible chicken line (Hu et al. 1997). In pigs, the estrogen receptor (Rothschild et al. 1996) and the prolactin receptor (Vincent et al. 1998) were discovered to be associated with litter size by the candidate gene approach. In the current study, fifteen candidate genes (twelve physiological candidate genes, and three positional candidate genes) were selected to investigate associations between gene polymorphisms and antibody response kinetics to SRBC and BA in the IAKRP F₂ population. The results are presented in Chapters 4 and 7.
Antibody Response and Disease Resistance

Diseases outbreaks cause substantial costs and losses in commercial chicken meat and egg production (Yunis et al. 2002). Genetic selection for improving chicken immunocompetence and disease resistance can reduce costs due to pathogen invasion. Breeding chickens for higher immunocompetence and disease resistance has been well documented (Hartmann 1989; Gavora 1993; Pinard-Van der Laan et al. 1998). To accomplish successful genetic selection for disease resistance to infectious agents, it would be desirable to select for general resistance rather than specific disease resistance (Gavora 1990), because commercial stocks are exposed to diverse production environments and direct selection for antibody response to each individual pathogen is not feasible. General improvement of immunocompetence by the genetic approach is robust for animals to face numerous and varied environmental challenge (Pinard-van der Laan 1998). Selection for immune response to nonpathogenic antigens such as SRBC and killed BA has long been considered as a supplement to genetic means of disease control in poultry (Parmentier et al. 2001). Low-to-medium realized heritability of antibody level has been estimated in selection experiments for antibody response, and animals selected for high antibody levels tend to be more disease resistant (Gross et al. 1980; Pinard et al. 1993; Boa-Amponsem et al. 1998). The components of the immune system are altered with divergent selection for antibody response to SRBC. Examples include macrophage activity (Biozzi et al. 1979), B cell populations, CD4 and CD8 subpopulations of T lymphocytes (Kreukniet 1996), and T cell populations (Scott et al. 1991; Kreukniet et al. 1994). Gross et al. (1980) also reported that the line selected for high antibody to SRBC exhibited greater antibody response to Newcastle disease, and more resistance to Mycoplasma gallisepticum, Eimeria necatrix, but was more susceptible to Escherichia coli and Staphylococcus aureus. Bidirectional selection for antibody response to Escherichia coli in a broiler line resulted in higher titers to Newcastle disease virus, to vaccine for infectious bursal disease virus and SRBC in the high antibody line than in nonselected control and low antibody chicken lines (Yonash et al. 1996; Heller et al. 1992; Pitcovski et al. 2001; Yunis et al. 2000).
The chicken immune system is a complex system, in which many different cell types and soluble factors such as cytokines act in concert to generate an effective response to pathogenic challenge (Pinard-van der Laan et al. 1998). Immune response traits may be independent of each other, therefore, a global approach should be taken to enhance immune capacity when selecting for immune competence. Pinard-van der Laan (2002) divergently selected for six generations for three different in vivo immune responses in chickens: antibody response to Newcastle disease virus vaccine, cell-mediated immune response, and phagocytic activity. The results showed clearly that the three immune responses are independent of each other. Another study with antibody production to Pasteurella multocida and Mycoplasma gallisepticum vaccine, T-cell mediated immunity and phagocytic activity in two generation of a selection experiment was reported (Cheng et al. 1991). The positive or negative correlations among immune response traits in that study suggested that the total immunocompetence profile is needed for genetic selection. Genetics has a great influence on the modulation of immune responsiveness, through cellular communication and the production of soluble factors, which are involved in antibody production, therefore, the genetic approach provides an opportunity to investigate genetic control of the regulation of complex immune capacity (Lamont 1998). Associations of candidate genes and genetic markers with antibody response to SE vaccination or antibody kinetics to SRBC and BA in the present study lay the foundation to apply marker-assisted selection to improve chicken vaccine efficacy and health.

References


CHAPTER 2. GENETIC CHARACTERIZATION OF BIODIVERSITY IN HIGHLY INBRED CHICKEN LINES BY MICROSATellite MARKERS

A paper published in Animal Genetics

Huaijun Zhou and Susan J. Lamont

Abstract

Forty-two microsatellite loci were analysed in 23 highly inbred chicken lines derived from Leghorn, Jungle Fowl, Fayoumi and Spanish breeds. Line-specific alleles among breeds and lines were detected. The band-sharing (BS) values were calculated and the proportion of shared alleles distances (Dps) were estimated. The BS values and Dps between sets of MHC-congenic lines ranged from 0.74 to 0.96, and 0.05 to 0.35, respectively. The BS values between each pair of noncongenic Leghorn lines were 0.32 to 0.97, and between Leghorn and exotic (Jungle Fowl, Fayoumi and Spanish) breeds were 0.03 to 0.55. The Dps between Fayoumi lines and other lines were much larger (0.66-1.34) than within Leghorns, and the Jungle Fowl breed had the largest distances with other lines (1.12-5.38). The phylogenetic consensus tree that was constructed grouped these 23 inbred chicken lines into four different clusters. These results are in accordance with the origin and breeding history of these inbred lines, which indicates that the use of microsatellites for the study of genetic biodiversity is accurate and reliable. In addition, the significance and value of inbred chicken lines in molecular genetic research is discussed.

Keywords: microsatellite, inbred chicken lines, phylogenetic consensus tree, genetic biodiversity.

Introduction

Molecular typing methods provide a powerful and reproducible approach of estimating genetic relatedness within and among animal lines based on DNA variation. Many studies have been conducted to determine intraline and interline characteristics in chickens, and

1 Reprinted with permission of Animal Genetics, 1999, 30(4), 256-264.
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comprehensive surveys of relatedness, including Jungle Fowl, layer and broiler strains and White Leghorn, based on DNA fingerprints, have been described (Hillel et al. 1989; Siegel et al. 1992; Kuhnlein et al. 1989 & Ponsuksili et al. 1998). Chen & Lamont (1992) and Plotsky et al. (1995) characterized genetic variation among 13 of the highly inbred chicken lines included in the present study by restriction fragment length polymorphism (RFLP), DNA minisatellite fingerprinting and randomly amplified polymorphic DNA (RAPD). Microsatellite polymorphism in commercial broiler and layer lines has been estimated (Crooijmans et al. 1996). Smith et al. (1996) used RAPD markers to analyze relatedness in chickens and turkeys.

The role of inbred lines in biological research has fundamental importance by providing genetic diversity between lines and constancy over time and place within each individual line (Abplanalp 1992). Inbred lines, after genetic characterization within and among the lines, can be used as resource populations in genome mapping and linkage analysis between DNA markers and qualitative or quantitative traits. Congenic lines differ in single genes of interest but share the genetic background of a common inbred line. This study characterized variation within and among inbred chicken lines, including several sets of congenic lines, by using microsatellite markers both to characterize these inbred lines and to provide tools for future study of linkages between microsatellite markers and quantitative trait loci (QTL).

The primary objectives of this study were to: (1) determine the genetic profile of 23 inbred chicken lines with microsatellites, (2) compare genetic diversity within and among these lines, (3) identify line-specific and breed-specific microsatellite markers, and (4) estimate phylogenetic relationships among these inbred lines, as determined by microsatellite markers.

**Materials and methods**

**Experimental populations**

Thirteen highly inbred lines of chickens with inbreeding coefficients of 99% or greater that originated 33-73 years ago, four inbred lines of chickens with inbreeding coefficients of 60% or greater that originated 34 years ago, and four major histocompatibility complex
MHQ-congenic lines developed from a backcross, which initiated 15 years ago, were used (Lamont et al. 1992; Warner et al. 1989; Knudtson & Lamont 1989) (Table 1). Average generation intervals were 9 to 10 months, resulting in almost 100 generations of continued full-sib mating in the oldest lines. Nineteen lines were derived from the Leghorn breed, two from Fayoumi, one from Spanish and one from Jungle Fowl. The lines 8-42, 19-45 and 19-46, which were initiated in 1925, were among the first set of long-term successful inbred chicken lines in the world (Waters and Lambert 1936; Abplanalp 1992). The M-43 and M-44 belong to the Egyptian Fayoumi breed, and Sp-41 originated from the Spanish Castellana negra breed. Both lines initiated from eggs imported in 1954 (Abplanalp 1992). Several separate lines that are homozygous for different serologically detectable MHC types (B blood groups) were established by selecting within the original genetic base lines. HN-12 and HN-15 were established from the same line which originated from stock obtained from a commercial layer breeding company (H & N Inc. Redmond, WA, USA). The G lines were derived from the GH genetic base which had been created by crossing an HN male with females from the Ghostley (Minnesota) breeding company. The S1 White Leghorn line, originating from U.S. commercial egg stock, is divided into four different MHC-haplotype sublines, based on erythrocyte antigen B (Ea-B) alleles ($B^I B^I$ or $B^{19} B^{19}$), and antibody response to glutamic acid alanine tyrosine (GAT) ($Ir$-GAT$^H$ or $Ir$-GAT$^{lo}$). These four MHC types were also backcrossed for 10 or more generations onto the G line background, before mating *inter se* to produce the four new MHC-congenic lines, G.S1-1L, G.S1-1H, G.S1-19L and G.S1-19H. UCD-001 (Jungle Fowl) and UCD-003 (White Leghorn) are the inbred lines contributing to the East Lansing (EL) reference population (Crittenden et al 1993). Two birds were sampled from each of the highly inbred lines except that four birds (2 males, 2 females) were sampled for the four G.S1 inbred congenic lines and one for UCD-001. Previously, 7 to 10 individuals each from four of the lines were genotyped with 4 microsatellites, and no within-line variation was detected (data not shown). Thus, sampling 2 to 4 individuals from these highly inbred lines was considered to be an adequate sample size to represent the line.

**Microsatellite genotyping**
Forty-two fluorescently labelled microsatellite primer pairs, provided by the Poultry Coordinators of the U.S. National Animal Genome Research Program, were used in the study. Chicken DNA was isolated from venous blood collected in EDTA. The PCR procedure was carried out as described by Cheng et al. (1995). Fluorescent PCR products (1.5 μl) were loaded, in multiplex where applicable, onto a polyacrylamide-sequencing gel and processed on an ABI 377 Sequencing Machine. Gel data were transferred to Genescan Software for analysis. PCR products from individuals were scored by bp size.

Statistical analysis

The data were resampled 100 times with the bootstrapping method, and the band-sharing (BS) values, the proportion of shared alleles distances (Dps), the entropy of allele size distribution and locus and taxa heterozygosity were obtained using the MICROSAT computer program (Minch et al. 1995). A phylogenetic consensus tree was formed using the Fitch-Margoliash method, and this analysis was done with the PHYLIP (Felsenstein 1993) program package.

Results

Genetic variability

Among all microsatellites tested, 41 loci were polymorphic and one locus was monomorphic in all populations. The number of alleles per locus varied from 1 to 6. The allele size range of polymorphic loci was from 2 bp difference (161 bp to 163 bp) in locus ADL0118 to 52 bp difference (262 to 314 bp) in locus LEI0121, and showed large variation across loci (Table 2).

The total heterozygosity per locus ranged over twenty-fold, from 0.040 (locus ADL0120) to 0.781 (locus MCW0134). The entropy of allele size distribution per locus ranged from 0.042 (locus ADL0120) to 0.867 (locus ADL0039) (Table 2). The correlation coefficient between heterozygosity and entropy was 0.559 (P < 0.01). Mean heterozygosities within line were very low and ranged from 0 to 0.1 (Table 1).

Line-specific markers
Most lines had one or more line-specific microsatellites alleles. There were nineteen line-specific alleles in line UCD-001, which was the most among all lines; nine each in UCD-003 and Sp-41; three line-specific alleles in line 8-42 (ADL0184, 130 bp; MCW0137, 266 bp and MCW0301, 274 bp); two in line 19-45 (ADL0020, 100 bp and ADL0145, 149 bp); and one in line 19-46 (MCW0301, 266 bp). There was no unique allele in the MHC-congenic M-43 or M-44, but the Fayoumi breed had seven breed-specific alleles. Birds from line S1-1L, S1-19H and S1-19L had, respectively, two (MCW0088, 298 bp; MCW0137, 248 bp), one (MCW0119, 134 bp) and two unique alleles (MCW0088, 281 bp and MCW0137, 256 bp). The S1 lines with the BІ blood group (S1-1H and S1-1L) shared two line-specific alleles (ADL0114, 163 bp; MCW0301, 278 bp), whereas all four lines (S1-1H, S1-1L, S1-19H and S1-19L) derived from the S1 background shared one unique allele (ADL0188, 131 bp). Some microsatellite alleles differed among congenic lines selected for homozygous MHC serological haplotypes or Ir-GAT from a common genetic background (Table 3).

Band-sharing values and genetic divergence

The BS values and Dps genetic distances for all two-way comparisons among these 23 inbred chicken lines varied greatly (data not shown). The BS values between line pairs with the same genetic background ranged from 0.74 to 0.96. The BS values between each pair of Leghorn lines from different genetic bases were 0.35 to 0.97. Between Leghorn and exotic (Jungle Fowl, Fayoumi and Spanish) breeds, BS values ranged from 0.03 to 0.55, whereas those between Spanish and Fayoumi were 0.36 (M-43) and 0.33 (M-44), between Jungle Fowl and Fayoumi lines were 0.20 to 0.22, and between Jungle Fowl and Spanish was 0.24. The largest Dps genetic distance (5.38) was between UCD-001 and UCD-003. Among all Leghorn lines, distances between UCD-003 and the other lines were largest (0.81-1.29). Distances between Fayoumi lines and other lines were much larger (0.66-1.34) than that of Leghorns with other lines. The Jungle Fowl line had the largest distances with all other lines (1.12-5.38). The Dps distances between lines selected on MHC serological haplotypes from a common genetic background were small to moderate, 0.05 to 0.35.

Phylogenetic tree
The phylogenetic consensus tree, constructed using the Fitch-Margoliash method grouped the lines into four distinct clusters (Fig. 1). The first group was formed of the Fayoumi lines and GH-47; the second of the lines having lineage to GH origin and UCD-001; the third of the SI lines, HN lines, Spanish and UCD-003; and the fourth of the “ancient” inbred lines, 19-45, 19-46 and 8-42.

Discussion

Comparison of microsatellite analysis with RFLP, DNA minisatellite fingerprinting and RAPD-PCR

The genetic variation among 13 of the highly inbred chicken lines used in this study was previously described using RFLP with MHC class I, class II and ev genes (Chen & Lamont. 1992; Lamont et al. 1992; Warner et al. 1989). Pitcovski et al. (1989) estimated genetic polymorphism of the SI lines by RFLP. Fewer polymorphisms were detected in these RFLP studies than in studies using other DNA polymorphism techniques, especially in lines obtained from a single genetic base population. RFLP is usually used to study specific genes with a small number of loci, not the entire genome, so it is likely to be less informative about genome-wide diversity than microsatellite markers. Plotsky et al. (1995) analyzed genetic relationships of the same 13 inbred chicken lines by DNA fingerprinting and RAPD-PCR. The differences estimated among breeds, lines within breed and congenic lines were in general agreement with the current study. But population data on DNA minisatellites do not provide a complete characterization of the genetic variation in terms of allele frequency distributions, because neither the number of loci nor the locus affiliation of alleles is directly observable; and microsatellites are an alternative to these technical or statistical limitations (Ponsuksili et al. 1998). RAPD-PCR is extremely sensitive to assay conditions, so there are some disadvantages in using this technique to analyze genetic divergence and phylogenies which are not inherent in use of microsatellites.

Measurement of genetic diversity
Genetic diversity can be estimated in many different ways. Entropy is a measurement of diversity indices used in molecular biology. The entropy for a specific locus is calculated as $E = -\sum q_i \ln q_i$, where $E$ = the entropy of a locus and $q_i$ = the relative frequency of $i$ allele. Entropy can be interpreted as the information gain expected in the performance of a single measurement from the population under investigation. Entropy is very likely to be highly correlated with an ideal unknown index over the range of its observed variation and will serve as an adequate indicator of genetic diversity (Cowell et al. 1998). The average entropy of allele size distribution of microsatellites in this study was 0.443, which suggests that most microsatellite markers screened were informative. The specific entropy values (Table 2) identify the loci revealing the most diversity.

*Phylogenetic relationships*

Most classical genetic distance measures are based on Kimura and Crow’s (1964) infinite allele model (IAM) without reference to a particular evolutionary model. However, the majority of mutations at microsatellite loci are stepwise in nature, changing allele sizes by one or more repeat units and, thus, these genetic distance analysis models that were designed specifically to apply to microsatellites generally assume Ohta and Kimura’s (1973) stepwise mutation model (SMM) or one of its generalizations. The assumptions of the SMM differ sharply from the assumptions of the IAM, and therefore distances designed to increase linearly under the IAM, such as Nei’s standard distance, are both nonlinear and inaccurate for microsatellite loci (Goldstein & Pollock 1998). But the microsatellites with smaller repeat motifs (1 to 2 bp) do show variations towards the IAM (Shriver et al. 1993). Despite the fact that they follow the SMM or any other evolutionary model, a group of related distances, such as chord distance or allele-sharing distance, perform well for reconstruction of phylogenies, when taxa are closely related (Goldstein & Pollock 1998). Goldstein et al. (1995) showed that Dps reconstructs phylogenies more accurately than other methods, when trees are shorter than 300 generations, so Dps was used to calculate genetic distance in this study in which up to 100 generations are utilized.
One of the primary objectives in this study was to analyze genetic diversity and phylogenetic relationships among different breeds and of lines originating from the same genetic background. From the review of phylogenetic relationships (Dps) and BS values of the 23 inbred chicken lines, the Jungle Fowl line (UCD-001) was most distant from the other lines, which is in accordance with its breed history. The Jungle Fowl is generally accepted to be the ancestor of the domestic chicken (Crittenden et al 1993). In addition, UCD-001 had the largest distance with UCD-003. This distance is in agreement with both lines being chosen as the parents of the East Lansing chicken mapping EL reference population. Choice of parental lines for mapping was based on a wide genetic distance between them that should maximize DNA divergence and thus disclose polymorphism among backcross progeny. The 8-42, 19-45 and 19-46 lines all originated from crosses of ISU inbred lines started in 1925 and these lines were grouped into one cluster, and 19-46 was closer to 19-45 (its MHC-congenic pair) than to 8-42 (Fig. 1). These 3 lines are also known to have been closed lines for the longest time period, and this cluster was far from other clusters. HN-12 and HN-15 were grouped into one subcluster. The distance between them was small (0.07) and the BS value was 0.93, despite closed, separate breeding since 1954.

The BS values and Dps between the lines of GH and HN background were 0.67-0.81 and 0.22-0.41, respectively, which indicates that their genetic relationship is close. This agrees with their origin from North American commercial egg-layer genetic stock in the 1950s. GH-47, GH-48 and GH-49 had little distance among them compared to the other lines, and BS values between them were 0.76 to 0.86. G-6 and G-50 are sublines of GH, and are MHC-congenic sublines that were rederived about 10 years ago by crossing the established congenic lines and selecting homozygotes from an F2, to minimizing background genetic differences between the congenic lines. The distances between G and GH lines were smallest (0.12-0.35), and BS values were 0.71-0.89. The G.S1 lines also were genetically close to the G lines, which had served as the background for establishing the G.S1 recombinant congenic lines. The distances and BS values between G.S1 lines and G lines were 0.05-0.13 and 0.88-0.97, respectively. The four S1 lines and the four G.S1 lines were each grouped into separate subclusters. The distances between S1 lines were greater than the ones between G.S1 lines, whereas BS values were smaller. This is consistent with their respective line-formation
history, which is that the G.S1 lines were formed by introgression of the MHC-bearing micro-chromosome on to the highly inbred G line background, where as the S1 lines are only partly (~65%) inbred. The previous results above suggest that Dps estimated on microsatellite data is a reliable estimate of genetic distance, as confirmed by detailed pedigree information.

Although microsatellite markers selected in this study evenly cover most of the genome, with an average spacing of 60cM between loci, no microsatellite in chromosome 16 (MHC-containing chromosome) was used because of the current absence of microsatellites in chromosome 16 of the public chicken genetic maps. Among the lines tested, there were several sets of lines congenic for the MHC. Although the microsatellites used were not mapped to the MHC, several showed allelic variation among sets of congenic lines (Table 3). This may be due to random mutation after these lines were separated from each other based upon MHC and then maintained as closed populations. Instability of specific loci is suggested by allelic variation of different allele sizes among congenic sets from multiple, unrelated genetic background. Microsatellites show exceptionally high mutation rates, with minimal rates estimated as high as $10^{-3}$ (Jefferys et al. 1988) to $10^{-4}$ (Henderson & Petes 1992).

Significance of inbred chicken lines in molecular genetic research

Inbred lines provide a powerful research tool because experiments can be conducted with birds having uniform genotypes, thus mitigating background effects (Delany and Pisenti 1998). Congenic lines have proven to be particularly useful for studying the effect of different single genes or groups of genes in backgrounds having as high as 99% genetic identity (Delany and Pisenti 1998). Many of the inbred chicken lines characterized in this study have been used in functional studies of immunogenetics. These highly inbred lines were used to evaluate endogenous viral genes (Lamont et al. 1992), association of Ir-GAT and Ea-B with immune response (Steadham and Lamont 1993), MHC associations with Marek’s disease resistance (Lakshmanan et al. 1996), growth and egg production associations with MHC and with DNA fingerprinting (Cahaner et al. 1996), antibody production to SRBC, BA (Munns and Lamont 1991) and interleukin-2-like activity
(Knudtson et al. 1989, 1990). The S1 lines have served as the population base for study of MHC associations of Marek’s disease (Steadham et al. 1987) and fowl cholera resistance (Lamont et al. 1987), body weight, egg production (Kim et al. 1989), and antibody response to Pasteurella multocida, Mycoplasma gallisepticum, phagocytic activity and T cell-mediated response (Cheng and Lamont 1988). The combination of phenotypic information, molecular genetic marker characterization and estimation of genetic relationships will allow these chicken lines to play an important role in QTL mapping in the future.

In addition, highly inbred lines can be used as parents of mapping reference families, because the parental lines are so inbred that each polymorphism would be represented by only two alleles in the backcross or F2 generations, thereby simplifying the scoring of polymorphic progeny (Crittenden et al. 1993). The defined phylogenetic relationships among the inbred chicken lines will be helpful to select founder populations to maximize the likelihood of finding DNA differences or polymorphisms between the lines (Crittenden et al. 1993). The unique alleles available in the “exotic” (Fayoumi and Spanish) and “ancient” inbred lines may allow study of QTL-trait associations or mapping of regions differing from the parental lines of other reference families.

Acknowledgements

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Table 1. Description of the twenty-three inbred chicken lines studied

<table>
<thead>
<tr>
<th>Line name</th>
<th>Genetic base</th>
<th>Breed</th>
<th>Year of origin</th>
<th>B blood group</th>
<th>Inbreeding coefficient</th>
<th>Heterozygosity$\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-42</td>
<td>8</td>
<td>Leghorn</td>
<td>1925</td>
<td>42</td>
<td>15.1</td>
<td>99</td>
</tr>
<tr>
<td>19-45</td>
<td>19</td>
<td>Leghorn</td>
<td>1925</td>
<td>45</td>
<td>15.1</td>
<td>99</td>
</tr>
<tr>
<td>19-46</td>
<td>19</td>
<td>Leghorn</td>
<td>1925</td>
<td>46</td>
<td>13</td>
<td>99</td>
</tr>
<tr>
<td>HN-12</td>
<td>HN</td>
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<td>12</td>
<td>12</td>
<td>99</td>
</tr>
<tr>
<td>HN-15</td>
<td>HN</td>
<td>Leghorn</td>
<td>1954</td>
<td>15</td>
<td>15</td>
<td>99</td>
</tr>
<tr>
<td>GH-47</td>
<td>GH</td>
<td>Leghorn</td>
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<td>47</td>
<td>15.1</td>
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<td>Leghorn</td>
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<td>99</td>
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<td>G-6 (G-B2)</td>
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<td></td>
<td>Leghorn</td>
<td>1957</td>
<td>0.038</td>
<td></td>
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*New nomenclature proposed by W.E. Briles, 1991 (Northern Illinois University, DeKalb, IL 60115; used in recent publications, e.g. Lamont et al. 1992).
†Nomenclature used in previous publications (e.g. Warner et al. 1989; Knudtson & Lamont 1989)
‡The heterozygosities were calculated using MICRO SAT program (Minch et al. 1995).
Table 2. Location of the microsatellite markers in the East Lansing, Compton, and Wageningen genetic maps; allele size range; total number of alleles, and heterozygosity and entropy of each locus in the 23 lines.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Location</th>
<th>Size range (bp)</th>
<th>Number of alleles</th>
<th>Heterozygosity</th>
<th>Entropy</th>
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</thead>
<tbody>
<tr>
<td>ADL0020</td>
<td>I</td>
<td>96-108</td>
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<td>0.398</td>
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<tr>
<td>ADL0022</td>
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<td>146-164</td>
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<tr>
<td>ADL0038</td>
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<td>0.489</td>
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<td>ADL0039</td>
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<tr>
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<td>166-170</td>
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<tr>
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Table 3. Unique microsatellite allele sizes (bp) among sets of MHC-congenic chicken lines.

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Fig. 1. Phylogenetic relationships among 23 inbred chicken lines, based on 42 microsatellite loci, using Dps genetic distances and the Fitch-Margoliash method of clustering.
CHAPTER 3. GENETIC VARIATION AMONG CHICKEN LINES AND MAMMALIAN SPECIES IN SPECIFIC GENES

A paper published in Poultry Science\(^1\)

Huaijun Zhou, Wei Liu\(^2\), Susan J. Lamont\(^2\)

Abstract

Thirteen gene-specific primer sets provided by the U.S. Poultry Genome Coordinators were used to investigate DNA polymorphisms between two highly inbred chicken lines of Leghorn and Fayoumi origin. Nucleotide and predicted amino acid sequences were then compared among these chicken lines and the Genbank sequences of chicken, mouse, and human. The following genes were selected as candidates for immune response or transcription activation: B2M, DADI, IAP1, IL2, IREB1, LAP18, MAFL, POU1FI, RREB1, TAD, TBPI, TCRG, and ZOV3. Chicken spleen total cDNA obtained from the Leghorn and Fayoumi lines RNA by RT-PCR was used as a template to PCR-amplify gene-specific products. All primers except POU1FI and TCRG generated single PCR products of the predicted 325- to 667-bp size, confirming the efficacy of these gene-specific primers in the chicken. Three and seven of the 11 amplified gene fragments yielded line-specific nucleotide polymorphisms between the Leghorn and Fayoumi, and between the Leghorn and Genbank chicken sequence, respectively. Similarities between inbred Leghorn and mammalian species were 36 to 86% for nucleotide and 25 to 96% for predicted amino acid sequence. The polymorphisms of some gene fragments between the Leghorn and Fayoumi lines will allow for investigation of associations of these genes with immune response and other biological traits.

(Key words: inbred chicken lines, immune response, candidate genes, nucleotide sequence, amino acid sequence)

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\(^2\) Department of Animal Science, Iowa State University, Ames, IA 50011.
Introduction

The development of a comparative map has gained high priority in the poultry genome community due to the relatively limited information on genes available in the poultry genome. Burt et al. (1999) constructed comparative maps of chicken with human and mouse, species that share a common ancestor 300 million years ago. Totally, 223 genes that define 81 autosomal conserved segments were used in the chicken-human comparison and 100 in the chicken-mouse comparison. The number of chromosome rearrangements (72) for the chicken-human comparison are less than for mouse-chicken (128) and mouse-human (171). So they conclude that the organization of the human genome is closer to that of the chicken than the mouse. Studies by Palmer and Jones (1986), Burt et al. (1995) and Groenen et al. (2000) suggest that extensive conserved synteny exists between mammalian genomes and the chicken genome. As the human genome project progresses, more of the genes have been identified through the positional candidate gene approach (Collins 1995). Functional genes have revealed conserved linkage associations among species, and expressed genes are especially informative candidates for conserved synteny mapping. Using anchor loci, syntenic comparisons may provide clues to the location and orientation of orthologous genes (Smith et al. 1997). The vast amount of information collected on the human genome should serve as a resource for agricultural animal species, and the development of a comparative map between the chicken and human genome is a logical course of action (Cheng 1997).

Genes with known function from other species offer an opportunity to explore the genetic variation of these genes in poultry. Primer pairs for genes of known function were developed by the U.S. Poultry Genome Coordinator using primer optimization software from Genbank sequences. Thirteen gene-specific primers (Table 1), selected as potential candidates for immune response or gene transcription, were used to define molecular variation between genetically divergent chicken lines, human, and mouse. These genes were chosen because they have been described in some species to have an important role in antigen identification and processing, molecular interactions and cellular cooperation in the immune response, or to perform a crucial function in regulation of expression and transcription of genes.

Genetic selection, with the aid of proven molecular markers for immune response and disease resistance, may be used to improve health in poultry (Lamont 1998). The highly
inbred chicken lines used in this study have been previously defined for specific characteristics of disease resistance. The G-B1 line originated from U.S. commercial layer stock, and the Fayoumi lines from Egypt (Lamont and Chen 1992). The G-B1 line is resistant to transient paralysis caused by Marek's disease (Parker and Schierman 1983) and against the early lethal effects of highly virulent transplantable Marek's disease lymphomas (Schierman 1984). The Fayoumi lines are the first genetic stocks in which Rous Sarcoma virus resistance on the chorioallantoic membrane was observed (Prince 1958) and are less susceptible to Marek's disease and tumor development than the Leghorn line (Lakshmanan et al. 1996). The Fayoumi lines are more resistant to *Salmonella enteritidis* colonization than the G-B1 line (Lamont, unpublished data). Identifying sequence polymorphisms between the Fayoumi and G-B1 lines will make it possible to determine associations of these genes or linked loci with immunity and resistance to disease in resource populations generated from these lines, an ongoing effort in our laboratory.

The objectives of the current study were to test the efficacy of the gene-specific primers in chickens; define polymorphisms of these gene fragments between Leghorn and Fayoumi lines; and compare the nucleotide sequences and predicted amino acid sequences among Leghorn, Fayoumi, and Genbank sequences of chicken, human, and mouse.

**Materials and methods**

Total RNA was isolated from the spleen of chickens (one male and one female per line) of the Leghorn line (G-B1) and Fayoumi lines (M15.2 and M5.1) using the Totally RNA™ Kit 3, and chicken cDNAs were obtained by RT-PCR (RETRO script™)3, using total RNA as a template. These cDNAs were then used as templates, and PCR-amplifications were carried out in an MJ Research PTC 1004, using 30 cycles with denaturation at 92°C 45 s, annealing at 50°C 40 s, and extension at 72°C 1 min. The first denaturation was performed at 95°C for 5 min, and the last extension was prolonged to 5 min at 72°C. The CENTRI.SPIN™-40 Spin

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3 Ambion Inc., Austin, TX 78744.
4 MJ Research Inc., Watertown, MA 02172.
columns\textsuperscript{5} (Princeton Separations) were employed to purify these PCR products. The nucleotide sequences of the purified products were determined by an ABI 377 sequencer\textsuperscript{6}. The Baylor College of Medicine (BCM) Search Launcher program was used to compare nucleotide sequences and amino acid sequences among chicken, human, and mouse (Smith et al. 1996). In instances of very low identity or large insertions/deletions on the ends of fragments, those sequences were eliminated from the identity comparison.

**Results and discussion**

Eleven of 13 of these gene-specific primers amplified PCR products under the conditions used, of which most were confirmed by direct sequencing of the PCR product as having high similarity to the predicted gene fragment. This demonstrates the efficacy of these gene-specific primers in the chicken. All primers, except POU1F1 and TCRG, which amplified no PCR product under the recommended conditions, generated single PCR products of the predicted sizes of 325 to 667 bp. Sequences of all individuals within each inbred line were identical. No amplified PCR product was obtained from chicken genomic DNA template for all thirteen pairs of primers.

The nucleotide sequences and predicted amino acid sequences among Leghorn, Fayoumi, Genbank chicken data, human, and mouse were compared by using the BCM Search Launcher program (Table 2). The G-B1 Leghorn line was used as a reference line for sequence comparisons, because of its widespread usage in molecular genetics and immune genetics research (Lamont and Chen, 1992, Plotsky et al. 1995). The G-B1 line was previously used as a standard for B blood group alleles by Briles et al. (1982). Sequence polymorphisms were identified for three genes (IAP1, IL2, and ZOV3) between the highly inbred Leghorn and Fayoumi lines, and for seven genes (IAP1, IL2, MAFL, RREB1, TAD, TBPI, and ZOV3) between the Leghorn and Genbank chicken sequences. Similarities of the individual gene fragments between inbred Leghorn and mammalian species in nucleotide sequences were 34 to 86\% and in predicted amino acid sequences were 25 to 96\%. The

\textsuperscript{5} PRINCETON SEPARATIONS, Adelphia, NJ 07710.
\textsuperscript{6} Perkin Elmer, Foster City, CA 94404-1128.
amplified fragments in this study represent only a part of these genes, thus, they may not have included all polymorphic sites and the genetic variation with these chicken lines might be underestimated.

In comparison of the nucleotide sequences of the 10 genes that were available across all 3 species, chicken vs. human identity is 62.7%, while chicken vs. mouse is 62.8%. For amino acid sequence comparisons, chicken vs human is 63.2%, chicken vs mouse is 62.6%. Thus, identities of nucleotide and amino acid sequences between chicken and human were generally close with those between chicken and mouse, in the 10 genes examined. For the four genes with chicken-mammalian amino acid identities above 75%, the nucleotide sequence identities were slightly lower (average 76%) than amino acid (average 86%). The reverse was seen for genes of low mammalian-chicken identity, in which amino acid identity was lower than that of nucleotides.

Among all sequence comparisons, identities of nucleotide and amino acid sequence were lowest (25%-36%) between chicken IL-2 and human/mouse IL-2. This is in agreement with work by several other laboratories. Kaiser and Mariani (1999) reported low identity between chicken IL-2 amino acid sequence and human. Sundick and Gill-Dixon (1997) reported a cloned chicken lymphokine that had 24 and 25% amino acid identity to bovine IL-2 and IL-15, respectively. In the present study, sequence identities between chicken IL-2 and human/mouse IL-2 were slightly higher than those between chicken IL-2 and human/mouse IL-15 (data not shown). The genomic organization of chicken IL-2 is similar to that of mammalian IL-2, not IL-15 genes (Kaiser and Mariani 1999). Choi et al. (1999) identified a cDNA encoding chicken IL-15 that had 46% amino acid identity with bovine IL-15. Also, Tirunagaru et al. (2000) have recently identified a clone showing a significant match to mammalian IL-15 from a chicken liver cDNA library, which suggests that the sequence identified as chicken IL-2 is truly IL-2 or another related cytokine, and not IL-15.

The ZOV3 gene primers were developed from sequence a chicken cDNA library, whose sequence had partial similarity to two different immunoglobulin superfamily proteins: mouse GP-70 and chicken HT7 Saitoh et al. (1993). Later, a similarity with human basigin was noted (Guo et al. 1998). Comparisons presented in Table 2 for ZOV3 are, therefore, with different genes in each species, emphasizing the complexity of comparisons with the
superfamily of immunoglobulin-like genes. No homologous mouse \textit{RREBl} gene was identified in the database search (Table 2).

There are one to four replacement substitutions between the reference G-B1 Leghorn and either Fayoumi or database chicken sequence in seven of the eleven studied genes. These polymorphisms can be utilized in identification of functionally different polymorphisms. The number of replacement substitutions between chicken and human or mouse range from 3-91 and were inversely proportional to the identities, in general, except for three genes (\textit{IL-2}, \textit{TBPI} and \textit{RREBl}) in which there were large internal deletion/insertion occurrences between chicken and human/mouse sequences (Table 2).

Less than half of the studied genes are currently mapped (\textit{B2M, DAD1, IREBl, ZOV3}) (Suzuki et al. 1999; Saitoh et al. 1992; Wang et al. 1997), so the identification of single nucleotide polymorphisms (SNPs) between these chicken lines offer an opportunity to linkage map the remaining polymorphic fragments in chickens. Also, sequence polymorphisms will make it possible to investigate control of biological traits by these genes in resource populations.

The Leghorn line (G-B1) and Fayoumi lines included in this study have been previously genetically characterized by microsatellite markers (Zhou and Lamont, 1999), DNA fingerprinting and randomly amplified polymorphic DNA (Plotsky et al. 1995). Studies using these genomic screening techniques, which were not based on gene-specific sequences, estimated a very large genetic distance between these two lines. MHC Class I genes were also investigated by restriction fragment polymorphism in these chicken lines (Chen and Lamont, 1992). Although gene sequences generally exhibit less polymorphism than anonymous markers, they allow utilization of gene sequence information from other species for comparative genetics study. Thus, the efficacy of use of the readily available gene-specific primers from the U.S. Poultry Genome Coordinator to examine gene sequences in poultry will allow rapid accumulation of structural and functional genomic information in the chicken.
Acknowledgments

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^1NP: no PCR product
TABLE 2. Nucleotide and predicted amino acid sequence identity percentage, and number of amino acid replacements of Fayoumi, Genbank chicken, human, and mouse compared to chicken reference line Leghorn (G-B1)

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Note. Percentage identities were determined using BCM Search Launcher program. GenBank accession numbers for sequences and amino acids, respectively for gene B2M are: AB021288, P01884 (human), Y00441, P07151 (mouse); for gene DAD1 are: D15057, P46966 (human), MMU83628, I49285 (mouse); for gene IAPI are: L49432, AAC41943.1 (human), U88908, Q08863 (mouse); for gene IL2 are: X01586, P01585 (human), K02292, P04351 (mouse); for IREB1 gene are: Z11559, P21399 (human), X61147, P28271 (mouse); for gene LAP18 are: J04991, P16949 (human), J04979, P13668 (mouse); for gene MAFL are: M95925, P54845 (human), L36435, P54841 (mouse); for gene RREB1 are: D49835, BAA23165.1 (human); for TAD gene are: AF001622, AAC80267 (human), AF001104, AAC80266 (mouse); for TBPl gene are: M55654, P20226 (human), D01034, P29037 (mouse); for gene ZOV3 are: X64364, P35613 (human), J03535, P21995 (mouse).
CHAPTER 4. CANDIDATE GENE PROMOTER POLYMORPHISMS AND ANTIBODY RESPONSE KINETICS IN CHICKENS: INTERFERON-\(\gamma\), INTERLEUKIN-2, AND IMMUNOGLOBULIN LIGHT CHAIN

A paper published in Poultry Science

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Abstract

An F\(_2\) population was produced from mating \(G_0\) highly inbred (> 99%) males of two MHC-congenic Fayoumi lines with G-B1 Leghorn hens. The F\(_2\) population was, therefore, essentially a full-sib population with \(F_1\) sire line reflecting MHC effect. Adult F\(_2\) hens (\(n = 158\)) were injected twice with sheep red blood cells (SRBC) and whole fixed \textit{Brucella abortus} (BA). Agglutinating antibody titer at 7 d after primary immunization and mean titer of the final three samples (d 18, 32, and 63 after 2\(^{nd}\) immunization) were used as parameters for primary and equilibrium phases, respectively. Secondary phase parameters of minimum (Y\(_{\text{min}}\)), maximum titers (Y\(_{\text{max}}\)) and time needed to achieve minimum (T\(_{\text{min}}\)) and maximum (T\(_{\text{max}}\)) titers were estimated from seven postsecondary titers using a nonlinear regression model. Three candidate genes, interferon-\(\gamma\) (IFN-\(\gamma\)), interleukin-2 (IL-2), and immunoglobulin G light chain (IgL) were studied. Primers for the promoter regions were designed from EMBL chicken genomic sequences. Polymorphisms between parental lines were detected by direct sequencing. PCR-RFLP methods were then developed to directly detect the polymorphism. There were significant main effects (\(P < 0.05\), general linear model analysis) of IFN-\(\gamma\) polymorphism on Y\(_{\text{max}}\) of BA antibody and interaction of IFN-\(\gamma\) by IgL on primary antibody response to SRBC and BA, and on T\(_{\text{min}}\) and Y\(_{\text{min}}\) of antibody response to SRBC in F\(_2\) offspring of M5.1 grandsires. There were significant main effects of IFN-\(\gamma\)

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polymorphism on Tmax of BA, and interaction of IFN-γ by IL-2 on Ymin to SRBC in F₂ offspring of M15.2 grandsires. The results suggest that IFN-γ genes play an important role in chicken primary and secondary antibody response to both SRBC and BA antigens, and there exists interaction among genes for antibody production.

*(Key words: candidate gene, primary response, secondary response, antibody kinetics, promoter)*

**Introduction**

The immune system plays an important role in protecting poultry from diseases. Disease resistance in poultry has been associated with higher antibody levels against specific pathogens, such as *Escherichia coli* and *Pasteurella multocida* (Hofacre et al., 1986; Leitner et al., 1992). Understanding genetic control of antibody production may therefore provide an opportunity for genetic enhancement of vaccine-mediated immunity and resistance to disease. Candidate genes provide a logical approach to investigate gene associations with antibody production traits.

Cytokines regulate the intensity and duration of the immune response by stimulating or inhibiting the activation, proliferation, and/or differentiation of various cells and by regulating the secretion of antibodies or other cytokines. Interferon-γ (IFN-γ) and interleukin-2 (IL-2) play a critical role in immune system function. IFN-γ increases expression of MHC Class I and Class II molecules, which modulate immune response (Kuby, 1997). IL-2 induces the proliferation and /or differentiation of T, B, and NK cells. IFN-γ has been implicated in immune response to various avian diseases (Kaiser, 1996). Correlation between an IFN-γ promoter polymorphism and resistance to *Escherichia coli* infection in chickens has been reported (Kaiser et al., 1998). In humans, a CA repeat polymorphism of the IFN-γ gene is associated with susceptibility to type I diabetes (Jahromi et al., 2000).

The promoter region located upstream from the initiation site plays an important role in regulating gene expression. Several transcription regulatory elements have been identified in the mammalian IFN-γ gene promoter, such as the TATAATA box, the GATA motif, GM-CSF/MIP motif, and consensus octamer site (ATGCAAAT) (Penix et al., 1993). Some of these elements are conserved in chickens, such as the TATAATA box, GM-CSF/MIP motif,
and potential NF-κB family member-binding site. But the octamer site is absent from chickens (Kaiser et al., 1998). Potential regulatory sequences identified in the chicken IL-2 promoter include a composite NF-AT"AP-1" element, a CD-28 response element, an AP-1 element, an NF-AT element, and the AP-1 part of an AP-1/octamer composite element (Kaiser and Marani, 1999). The immunoglobulin (Ig) promoter region in mammals contains conserved heptamer and octamer elements, which are preferentially involved in immunoglobulin promoter activity (Landolfi et al., 1988; Bemark et al., 1998). The octamer sequence motif has been identified as an important region for promoter activity in the Ig lambda light chain (IgL) in chickens (Heltemes et al., 1997).

Two commonly utilized antigens are often used to elicit antibody production in hens: sheep red blood cells (SRBC), a T-cell-dependent antigen, and Brucella abortus (BA), a T cell-independent antigen (Munns and Lamont, 1991). The utilization of both SRBC and BA allows the evaluation of differences in the induction pathways of antibody formation.

In addition to the level of the antibody response, the reaction kinetics may play a key role in immunological defense. The kinetics of the antibody response to SRBC and BA antigens may be influenced by the different immunological pathways triggered by the two antigens (Weigend et al., 1997). The time course of the humoral immune response is of importance in controlling infection by pathogens. The early high response in antibody production after vaccination may positively affect the survival rate in pathogen challenge in chickens. So, it is of interest to describe such important kinetics features, such as strength and rapidity.

The antibody response in young chickens has been characterized in many experiments (Kreukniet and van der Zijpp 1990; LePage et al., 1996; Karaca et al., 1999; Yang et al., 2000), but is rarely investigated in adults. Antibody production in adults may protect breeder or layer hens and provide protection for chicks from pathogens by passive immunity.

The overall goal of this study was to increase knowledge of the genes controlling antibody response kinetics in adult chickens. The specific objectives were to identify promoter polymorphisms in IFN-γ, IL-2, and IgL genes of the chickens, develop PCR-RFLP methods to detect those DNA polymorphisms, and evaluate associations between promoter polymorphisms and antibody response kinetics in a unique F₂ cross of inbred lines, representing an antibody kinetics resource population for gene mapping.
Materials and methods

Experimental Populations

Genetically distinct, highly inbred (> 99%) chicken lines (Leghorn G-B1 and MHC-congenic Fayoumi M15.2 and M5.1; Warner et al., 1989; Chen and Lamont, 1992; Zhou and Lamont, 1999) were used as parental G<sub>0</sub> lines. The B haplotypes of G-B1, M15.2, and M5.1 are B<sup>50</sup>, B<sup>43</sup>, and B<sup>44</sup>, respectively (Zhou and Lamont, 1999). One male from each Fayoumi line was mated to nine hens of the G-B1 line to produce an F<sub>1</sub> generation. From F<sub>1</sub> offspring of each Fayoumi sire, four sires and eight dams were used to produce an F<sub>2</sub> generation that was, because of the highly inbred nature of the G<sub>0</sub> birds essentially a fullsib-ship (a “clone-ship”), with G<sub>0</sub> sire lineage reflecting MHC effect. Therefore, two separate branches of the F<sub>2</sub> population were generated from the two MHC-congenic Fayoumi sires, M5.1 and M15.2. Because the sires are MHC-congenic, the only difference between the two branches is expected to be the MHC-bearing microchromosome inherited from the Fayoumi sires. Females (n = 158) of the F<sub>2</sub> resource population were used.

Antigen Administration and Sample Collection

At 19 and 23 wk of age, chickens were injected intramuscularly with both 0.1 ml of undiluted BA antigen<sup>5</sup> and 0.1 ml of 20% SRBC in phosphate-buffered saline. Blood samples were obtained from the peripheral vein of the wing of each bird preceding each immunization to determine the baseline antibody level, and at 7 d after primary immunization, and at 4, 7, 10, 18, 32, and 63 d after secondary immunization. Plasma was collected after centrifuging (1000 rpm, 10 min) the blood samples and stored at −20 C until all assays were run simultaneously.

Agglutination Assays

The SRBC and BA antibodies were assayed by agglutination (Nelson et al., 1995). The agglutination titer was expressed as the log<sub>2</sub> of the reciprocal of the greatest dilution showing 50% agglutination.

Development of PCR-RFLP Assays

Chicken genomic DNA was isolated from venous blood collected in EDTA. Polymerase chain reaction (PCR) was carried out with 100 ng genomic DNA from one male and one

<sup>5</sup> Difco Laboratories, Detroit, MI 48232.
female from each of the pure inbred lines (G-B1, M15.2, and M5.1) to detect potential promoter sequence polymorphisms. The PCR products were purified by Centri Spin Column\textsuperscript{6} for DNA sequencing. Purified PCR products were sequenced by the DNA Sequencing and Synthesis Facility\textsuperscript{7}. Sequences were analyzed using Sequence Navigator version 1.0.1 \textsuperscript{8} and compared by BLAST program to detect polymorphisms between the lines. The restriction enzyme sites on these sequences were detected by Wisconsin Package 9.1\textsuperscript{9}.

**IFN-γ gene promoter.** The PCR primers (5' GT AAG GAA CTT CAG CCA TTG 3'; 5' GAC GAA TGA ACT TCA TCT GCC) were designed to amplify a 670-bp fragment by Oligo 5\textsuperscript{10} according to chicken genomic sequence (EMBL accession #: Y079221). The IFN-γ gene promoter DNA fragment was generated from genomic DNA of parental lines by PCR. The reaction conditions were 94 C for 4 min; 30 cycles of 94 C, 1 min, 58 C, for 1 min, 72 C, for 1 min, and an extension at 72 C for 10 min. The reaction included 100 ng of template, 1 x reaction buffer, 10 pmol of each primer, 0.2 mM dNTP, 1.5 mM MgCl\textsubscript{2}, and 1 unit Taq polymerase\textsuperscript{11}.

**IL-2 gene promoter.** Primers (5' TGC TTT TAA CCG TCT TTG 3'; 5' GAT GCT CCA TAA GCT GTA GT 3') were designed to amplify a 659-bp fragment using Oligo 5, based on chicken genomic sequence in EMBL database (accession #: AJ224516). The PCR reaction conditions were the same as for the IFN-γ gene promoter, except that the annealing temperature was 61 C.

**Immunoglobulin lambda light chain (IgL) gene promoter.** Primers (5' GCA GGT GGT TCA AAG AAA CGT 3'; 5' GGC GGA ATC CCA GCA GCT GT 3') were designed based on database sequence (accession #: M24403), and PCR conditions used were developed by Heltemes et al. (1997). Based on the specific sequence of the lines used in this study, new primers (5' TTT ATA CCC GCG TCC TTC 3'; 5' GGG AAA TAC TGG TGA TAG GTG 3') were designed to produce a 354-bp fragment. The PCR conditions were the same as above, except for annealing temperature (57 – 61 C).

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\textsuperscript{6} Princeton Separation, Adelphia, NJ 07710.
\textsuperscript{7} Iowa State University, Ames, IA 50011.
\textsuperscript{8} Applied Biosystem, Perkin-Elmer, Foster City, CA 94404.
\textsuperscript{9} Genetics Computer Group (GCG), Inc., Madison, WI 53711.
\textsuperscript{10} National Bioscience, Inc., Plymouth, MN 55447.
\textsuperscript{11} Promega Co., Madison, WI 53711.
Screening of the F2 Population

A PCR of each individual F2 bird's DNA was performed for each promoter polymorphism according to the conditions described above. For the IFN-γ gene, the PCR product was digested using 2 U Tsp509I at 65°C overnight. The restriction digests were electrophoresed for 2 h at 100 V on a 4% agarose gel with ethidium bromide. For the IL-2 gene, 5 U MnlI was used to digest at 37°C overnight, and the digested products were run for 2 h at 100 V on a 3% agarose gel with ethidium bromide. For the IgL gene, 5 U Sau96I was used to digest at 37°C overnight, and digested amplification products were electrophoresed for 2 h at 80 V on a 3% agarose gel with ethidium bromide. Samples were scored for their individual PCR-RFLP fragment sizes based on standard DNA molecular weight markers for each gene promoter by visualizing the banding pattern under UV light.

Statistical Analysis

The analyses of antibody response were separately conducted by antigen (SRBC, and BA) and by phase (primary, secondary, and equilibrium). For the SRBC and BA primary phase, the single time-point measurement taken at 7 d postprimary immunization (Y) was used, because the maximum primary antibody response to these antigens is generally between 5 and 8 d after primary immunization (Siegel and Gross, 1980; van der Zijpp, 1983; Scott et al., 1994). Secondary phase parameters of maximum titers (Ymax), time (Tmax) needed to achieve maximum titers, minimum titers (Ymin), and time (Tmin) needed to achieve minimum titers were estimated from seven individual time-point postsecondary titer values (taken from d 4 to 63 after secondary immunization) by using a nonlinear regression model (Weigend et al., 1997). For the equilibrium phase, the titers of the last three sample times were used to calculate the mean (Equil.) of the phase.

The GLM test was used to estimate Fayoumi sire effect and interaction between sire and the three candidate genes on the antibody response parameters. Because significant interactions (P < 0.05, data not shown) were found between sire and some antibody parameters, data were thereafter analyzed separately for F2 individuals derived from the two grandsires. This significant interaction is interpreted as being caused by the only genetic difference between the grandsires, their MHC.

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No polymorphism was found in the IL-2 gene promoter region between the G-B1 and M5.1 lines; therefore, different statistical models were used for F2 individuals derived from M15.2 and M5.1 line grandsires.

\[
\text{M15.2 } Y_{ijkl} = \mu + \text{IFN-}y_i + \text{IL-}2_j + \text{IgL}_k + \text{IFN-}y_i * \text{IL-}2_j + \text{IFN-}y_i * \text{IgL}_k + \text{IL-}2_j * \text{IgL}_k + e_{ijkl}
\]

\[
\text{M5.1 } Y_{ijk} = H + \text{IFN-}y_i + \text{IgL}_j + \text{IFN-}y_i * \text{IgL}_j + e_{ijk}
\]

where \(Y_{ijkl}\) and \(Y_{ijk}\) are defined as the dependent traits (\(Y, Y_{\text{max}}, T_{\text{max}}, Y_{\text{min}}, \) and \(T_{\text{min}}\)). The three candidate genes had three levels of genotypes defined as Leghorn homozygote (LL), heterozygote (LF), and Fayoumi homozygote (FF). The JMP® program\(^{13}\) (Sall and Lehman, 1996) was used to conduct the general linear model (GLM) test for association between genotype and antibody response parameters.

**Results**

**Sequence Variation and the PCR-RFLP**

For the IFN-\(\gamma\) gene promoter region, the amplified, 670-bp product was sequenced for each parent line. There were three base pair mutations between the Leghorn and both Fayoumi lines (A for G, C for T, and A for G, at bases -277, -317, and -318, respectively). A line-specific restriction enzyme (Tsp509 I) site at base -318 was identified. The restriction digests produced fragment sizes of 123 bp, 99 bp, 88 bp, 56 bp, 55 bp, 53 bp, and 28 bp for both parental lines, while the Leghorn line had an additional fragment of 168 bp, and the Fayoumi lines both had two extra fragments, 104 bp and 64 bp (Figure 1a).

For the IL-2 promoter region, a 659-bp product was obtained from Leghorn and Fayoumi lines, and sequenced. An A/G mutation at base -425 was found between Leghorn and Fayoumi M15.2 lines, and no mutation was found between Leghorn and M5.1. The restriction enzyme Mnl I was used to differentiate between the Leghorn and the M15.2 lines. The digested products had fragments of 165 bp, 115 bp, and 19 bp for both lines, and a line-specific fragment (251 bp) for M15.2 and two line-specific fragments (139 bp, 112 bp) for Leghorn (Figure 1b).

\(^{13}\) SAS Institute, Inc., SAS Campus Drive, Cary, NC 27513.
For the IgL promoter region, a 466-bp fragment containing the TATA-box and the octamer-sequence was obtained for all three lines. Three single base pair mutations (T/C, C/G, and T/C at 30 bp, 46 bp, and 60 bp upstream of the octamer-sequence) were found between the G-B1 and Fayoumi line, respectively. The T/C mutation, 60 bp upstream of the octamer-sequence, could be used to differentiate between the G-B1 and the Fayoumi lines by using the restriction enzyme Sau96 I. For more efficient PCR amplification, new primers were designed to produce a 354-bp DNA fragment. Restriction of the amplified product using Sau96 I resulted in a 161-bp fragment and two predicted 10-bp fragments for both Leghorn and the Fayoumi lines. The Leghorn had an additional line-specific 173-bp fragment. The Fayoumi lines had two additional fragments of 103 and 70 bp (Data not shown).

Association of the Three Candidate Gene Promoter Polymorphisms with Antibody Response

The $P$ values of main effects and interactions of IFN-γ and IgL promoter polymorphisms on chicken antibody parameters of primary, secondary, and equilibrium phase in F2 offspring of M5.1 grandsires, and of IFN-γ, IL-2, and IgL promoter polymorphisms in F2 offspring of M15.2 grandsires are shown in Table 1. The IFN-γ promoter polymorphism had the most frequent associations with antibody response parameters. There were significant main associations ($P < 0.05$) between IFN-γ promoter polymorphism and antibody parameters, and significant two-way interactions ($P < 0.05$) of IFN-γ by IgL on antibody parameters. For F2 offspring of the M5.1 grandsire, there were significant associations ($P < 0.05$) between the IFN-γ polymorphism and primary antibody response to SRBC and Ymax of BA, and between the IgL polymorphism and primary antibody response to both SRBC and BA. There were significant ($P < 0.05$) effects of the two-way interaction of IFN-γ by IgL on primary antibody response to SRBC and BA, and on Tmin and Ymin to SRBC. For F2 the offspring of the M15.2 grandsire, there were significant effects ($P < 0.05$) of the IFN-γ polymorphism on Tmax of BA, and of the two-way interaction of IFN-γ and IL-2 on Ymin of SRBC.

Main Effect of Candidate Gene Alleles on Antibody Response

The allelic effect of IFN-γ and IgL on antibody response is presented in Table 2 for the five instances of significant ($P < 0.05$) main effects of genotype. Effect of the candidate gene
alleles in both MHC-congenic lineages is presented, even though significant differences usually occurred in one only. Most of the candidate gene main effects (four out of five) were detected in the M5.1 grandsire lineage. Of these four instances, the Leghorn homozygous type (LL) of the candidate gene was most frequently significantly different (P< 0.05, Table 2) from the heterozygous (LF) and other homozygous type (FF). For the primary antibody response to both antigens, the mean of the IgL-LL hens was significantly lower than the other two allelic combinations. For the secondary response to BA, however, the hens inheriting both IFN-gamma alleles from the Leghorn line had a significantly higher Ymax. Thus, the Leghorn-derived alleles are not generally detrimental in regards to antibody production, but may vary by specific gene. For primary response to SRBC, the IFN-gamma heterozygous hens had a response significantly higher than the other two genotypes. Only one main effect was detected in the M15.2 lineage, for IFN-gamma effect on Tmax to BA, in which the heterozygous and the Fayoumi-allele homozygous hens had significantly different responses from each other (heterozygote was higher) but neither differed from the Leghorn-allele homozygous group.

**Effect of Candidate Gene Allelic Interactions on Antibody Response**

For primary antibody response of hens of the M5.1 lineage to both antigens (Figures 2a, 2b), the birds inheriting homozygous alleles from the Leghorn line for both the IgL and the IFN-gamma had a significantly lower antibody level than any of the other eight genotypic combinations of these two loci. To display and test the individual two-way interactions, the five significant gene allelic interactions (Table 1) are presented in graphic form in Figure 2. The means and standard errors of these traits, by genotype, and number of individuals in each genotype are given in Table 3. For Ymin to SRBC, the response of hens inheriting two Leghorn alleles for both IgL and IFN-gamma genes was also lower than their counterpart genotypes. This, however, may have been partly influenced by the lower primary response of this group, providing a lower starting level of antibody from which the secondary response was initiated. Birds of the IgL-FF, IFN-γ-LL genotype combination had greater Tmin than any other allelic combination of these two genes in F2 offspring of the M5.1 grandsire (Figure 2e).
For F2 offspring of the M15.2 grandsire, within the IFN-γ-LL genotype, birds from the IL-2-LL genotype had a significantly (P < 0.05) higher minimum secondary antibody titer to SRBC than birds from the IL-2-FF and IL-2-LF genotypes. Birds from the IFN-γ-LL genotype had significantly (P < 0.05) higher minimum antibody titer to SRBC than birds from the IFN-γ-LF genotype, within the IL-2-LL genotype (Figure 2d).

**Secondary antibody response kinetics**

For the candidate genes that showed significant (P < 0.05) main effect or interaction among genes on at least one secondary immune parameter, the secondary antibody response kinetics curves are presented by genotype (Figure 3).

For the IFN-γ gene effect on SRBC antibody response, in F2 offspring of the M5.1 grandsire, although starting from a higher background titer resulting from primary immune response, chickens with the LL genotype did not display higher maximum secondary response levels than the other genotypes. The rates of antibody decline were approximately equal among the genotypes (Figure 3a). For F2 offspring of the M15.2 grandsire, the three genotypes of IFN-γ have similar patterns of secondary antibody response to SRBC (Figure 3b).

For the IFN-γ gene effect on BA antibody response, in F2 offspring of the M5.1 grandsires birds with the LL genotype have a much greater secondary antibody response and sustained higher level than the other two genotypes. The rate of decline of the LL genotype is approximately parallel with the genotype FF. Birds with the LF genotype have an earlier but much lower maximum response than the other two genotypes (Figure 3c). For F2 offspring of the M15.2 grandsires, the three genotypes have similar pattern of secondary responses to BA (Figure 3d).

For IgL gene and IL-2 gene effects on SRBC antibody response, all genotypes display a similar response curve in F2 offspring of the M5.1 grandsire and in F2 offspring of the M15.2 grandsire, respectively (Figure 3e and 3f).

**Discussion**

The IFN-γ, IL-2, and IgL genes play crucial roles in immune response. The promoter region of the gene, especially many of the important transcriptional regulatory elements, can
regulate gene expression. There were three base pair changes in the IFN-γ promoter identified in this study, one of which (base -327) was the same as that identified in White Leghorn inbred lines by Kaiser et al. (1998). There was a mutation found in the IL-2 promoter region in the current study between the Leghorn and M15.2 lines, whereas no polymorphism in this gene promoter was found by Kaiser and Mariani (1999). No mutation was found between the Leghorn and the M5.1 line. Although M15.2 and M5.1 lines are highly inbred MHC-congenic lines, which means that most DNA should be the same except the MHC-bearing microchromosome, it is possible that random mutations occurred over time. Three mutations were detected in the IgL gene promoter region between the Leghorn and Fayoumi lines. None of these nucleotide changes lies in any of the important regulatory elements identified by sequence analysis and comparison to known mammalian IFN-γ, IL-2, and IgL genes promoters. This does not, however, rule out their playing a role in the expression of these genes in chickens, or serving as linked markers to polymorphisms controlling biological traits.

Chicken IL-2 was reported to have 44 to 46% similarities to both bovine IL-2 and IL-15 (Sundick and Gill-Dixon, 1997). Based on the genomic organization, sequence comparisons between chicken and mammalian species, and several other laboratory studies of IL-2 (Kaiser and Mariani, 1999; Tirunagaru et al., 2000; Zhou et al., 2001), the sequence identified as chicken IL-2 is truly IL-2, not IL-15.

The postimmunization time period of this study is longer than most studies, which have generally focused on the primary antibody response to SRBC or BA and sometimes a brief secondary response phase (van der Zijpp et al., 1983, Dunnington et al., 1989, 1990; Larsen et al., 1992; Scott et al., 1994; Boa-Amponsem et al., 1997). The extended time frame of this study (63 d postsecondary immunization) allows detailed analysis of secondary antibody response kinetics and the equilibrium phase, as well as primary response. Investigation of the secondary and equilibrium phases supplies an opportunity to explore how long hens can passively transmit a high level of antibodies to chicks, estimating the capacity for passive immune protection from pathogens via vaccine antibody passage.

Examination of the gene allelic interaction between the IFN-gamma and IgL polymorphisms on primary antibody response (Figure 2) suggests that inheritance of Leghorn
alleles, in homozygous form, for both IgL and IFN-gamma, is detrimental to primary antibody production to both SRBC and BA (Figure 2a, 2b), compared to Fayoumi alleles. Although SRBC and BA have slightly different antibody induction pathways (Nelson et al., 1995), hens of the M5.1 lineage with homozygous Leghorn alleles had lower response to both antigens. The advantage conferred by the Fayoumi alleles appears to be at least partially dominant in that, generally, both homozygous and heterozygous Fayoumi-allele hens outperform the Leghorn homozygotes in primary (7-d post-primary immunization) antibody production. It is evident, however, that the Leghorn-derived alleles do not have a general detrimental effect in regards to antibody production, in that the hens inheriting both IFN-gamma alleles from the Leghorn line had a significantly higher Ymax to BA (Table 2) than the other two IFN genotypes, and hens of M15.2 lineage with homozygous Leghorn alleles for both IFN-gamma and IL-2 had generally greater Ymin to SRBC (Fig 2d). The most striking allelic interaction was not on level of antibody response, but on time required to reach the minimum of the secondary response, in which IgL-FF/IFN-γ-LL hens far exceeded all other genotypic combinations of alleles at these two loci (Figure 2e).

No significant main effects or interactions were consistently shared between the same genes between the primary and secondary antibody response phases. A single time-point (7 d) was used to estimate primary antibody response, whereas multiple time-points were used to estimate secondary antibody response. Additionally, different cell types are involved in antibody production between two antibody response phases. Therefore, differences in apparent gene effects are not unexpected.

SRBC is a T-dependent antigen requiring the help of T cells to produce antibody, whereas BA is a T-independent antigen for which the B cells can produce antibody with little help from the T cells. As illustrated in Figure 3a and 3c, SRBC and BA generated very different secondary antibody response kinetics in F2 offspring of the M5.1 line. The patterns for maximum secondary antibody response and rate of decline among the three genotypes of the IFN-γ gene between two antigens were very different. Birds of the LL genotype have the greatest secondary antibody response with BA, but not with SRBC. The antibody decline rates of three genotypes are different with BA, but are similar with SRBC antigen. This demonstrates that different pathways of antibody formation induced by different types of
antigens affect the secondary antibody response kinetics. Differential effects of the IFN-γ gene on antibody production to the two different antigens were evident.

The chicken MHC plays an important role in antibody production and disease resistance (Bacon, 1987; Lamont 1998a, b; Rothschild et al., 2000). Different kinetic patterns of secondary response effects of the IFN-γ gene in F₂ hens were observed in BA antigen between F₂ offspring of the two congenic grandsires (Figure 3c-d). In contrasting the main effects and interactions between the F₂ offspring of the two MHC congenic grandsires, more significant or near-significant effects were found in the M5.1 than in the M15.2 grandprogeny. The IFN-γ, IL-2, and IgL genes are not linked with MHC. However, it is possible that interactions between expressed products of the MHC and these genes occur during the process of antibody production. Our results suggest that the MHC, as evaluated through the F₂ branches produced from MHC-congenic grandsires, has a gene interaction effect on the effects that other candidate genes exert on antibody production.

The IFN-γ, IgL, and IL-2 genes are reported to be important in immune response in chickens or other species (Kuby, 1997; Kaiser, 1996; Bemark et al., 1998), and were therefore specifically chosen as strong candidate genes for antibody response in chickens. Promoter polymorphisms of three candidate genes were investigated in the current study. We hypothesized that mutations in promoter regions may influence gene expression and thereby regulate immune function.

Benjamini and Hochberg (1995) proposed the false discovery rate (FDR) approach to establish statistical significance in a multiple-test situation. FDR is the proportion of false positive tests among the individual comparisonwise tests that are declared significant. Several studies have applied this approach for QTL mapping with multiple markers (Weller et al., 1998; Zaykin et al., 2000; Mosig et al., 2001). Mosig et al. (2001) used a FDR of 0.10 for the marker-trait association level in a genome-wide approach to scan QTL affecting milk protein percentage in Israeli-Holstein cattle. The significance level α based on FDR value then will be 0.03-0.10. In the present study, the situation should be less strict than Mosig's study because three selected candidate genes were tested, rather than conducting a genome-wide scan. For F₂ offspring of the M5.1 grandsire, 18 statistical tests were conducted for each antigen. At an α = 0.05, one of 18 tests would be expected to falsely appear as significant.
The number of significant associations detected in the current study, however, was much higher: five for SRBC and three for BA response (Table 1). This gives strong confidence in the true significance of most of the detected associations. Additionally, "clusters" of significance were detected as associated with specific genes or specific antibody phases, giving additional support to the non-random nature of the significant gene-trait associations that were found in the M5.1 branch of F₂ hens. This result contrasts with the situation found in the M15.2 branch in which 36 statistical tests were conducted for each antigen. Only one test was significant at $P < 0.05$ for each antigen, which is interpreted as likely being a false discovery. In total, evidence is strong for significant candidate gene-trait associations being detected in the M5.1 branch only. This difference in associations found between the two branches additionally suggests an interaction of the three candidate genes with the only difference between the two branches, the MHC-bearing microchromosome.

The current study demonstrates that IFN-γ promoter polymorphisms had an effect on chicken primary and secondary antibody response to both SRBC and BA antigens. This effect is in agreement with the important role of IFN-γ in immune function. IFN-γ secreted by the T₅₁ subset preferentially inhibits proliferation of the T₅₂ subset (Wang et al. 1997). T₅₂ cells are specialized for B-cell activation to produce antibody. The action of IFN-γ is directly on the B cell and is dependent on IL-2 and B-cell activation through the B-cell immunoglobulin receptor. IFN-γ has the ability to enhance MHC class II expression and antigen-presenting capacity of cells at the time of primary immunization, resulting in higher levels of memory T cells. The class II pathway is strongly inducible by IFN-γ on nearly all cells. All the key genes of the class II antigen-presentation pathway, namely class II α- and β-chains, invariant chain, li, and the DMA- and DMB-chains, are required for normal expression of class II at the cell surface and are regulated by a single IFN-γ-inducible transcription factor CIITA (class II transactivator) (Boehm et al., 1997). The IFN-γ gene is highly conserved, and changes in IFN-γ expression are probably due to the influence of regulatory factors on gene transcription, rather than gene exon polymorphisms (Giedraitis et al., 1999). Lownthal et al. (1998) reported that coadministration of chicken IFN-γ with SRBC antigen at the time of primary immunization in chickens resulted in a significant enhancement of the secondary antibody response, which persisted at high levels for several
weeks. In addition, the coadministration of chicken IFN-γ significantly increased the proportion of birds that effectively responded to SRBC immunization in secondary response from 20 to 90%.

The goal of poultry geneticists is to effect genetic enhancement of chicken health and productivity. The candidate gene approach is a powerful method to identify quantitative trait loci (QTL), such as those controlling immune response and production traits (Rothschild and Soller, 1997). Discovery of the significant effect of the IFN-γ promoter polymorphism on antibody production response in chickens illustrates its potential value for use in marker-assisted selection to improve immune response. This study also demonstrates the general usefulness of the candidate gene approach to discover QTL for immune response traits in the chicken. The population design of a divergent F2 cross as used in the current study is very powerful to detect QTL-linked markers, because of the extensive linkage disequilibrium generated in the F2 population. There is, therefore, a possibility that the investigated candidate gene polymorphisms are linked markers for the actual QTL, rather than the causative mutations of the measured biological effect. Future studies, therefore, will clarify whether the identified IFN-γ promoter polymorphism is causative by investigating its relationship with IFN-γ protein production, and by investigating trait associations of the same DNA polymorphisms in other populations.

Acknowledgments

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TABLE 1. Main effects and interaction ($P$ values) of interferon-$\gamma$ gene (IFN-$\gamma$), interleukin-2 gene (IL-2), and immunoglobulin light chain gene (IgL) promoter polymorphisms on chicken antibody parameters of primary, secondary, and equilibrium phases in F$_2$ females of an inbred cross.

<table>
<thead>
<tr>
<th></th>
<th>SRBC</th>
<th>Brucella abortus</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$Y^1$</td>
<td>$T_{min}^2$</td>
<td>$Y_{min}^3$</td>
</tr>
<tr>
<td>a. M5.1 grandsires</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-$\gamma$</td>
<td>0.04</td>
<td>0.17</td>
<td>NS$^7$</td>
</tr>
<tr>
<td>IgL</td>
<td>0.03</td>
<td>0.08</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-$\gamma$*Ig</td>
<td>0.02</td>
<td>0.02</td>
<td>0.05$^8$</td>
</tr>
<tr>
<td>b. M15.2 grandsires</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-$\gamma$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IL-2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IgL</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-$\gamma$*IL-2</td>
<td>NS</td>
<td>NS</td>
<td>0.04</td>
</tr>
<tr>
<td>IFN-$\gamma$*Ig</td>
<td>NS</td>
<td>NS</td>
<td>0.11</td>
</tr>
<tr>
<td>IL-2*Ig</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1Primary antibody response
2Time required to achieve minimum secondary antibody titers.
3Minimum secondary antibody titers.
4Time required to achieve maximum secondary antibody titers.
5Maximum secondary antibody titers.
6Equilibrium phase antibody titers.
7NS, $P > 0.20$.
8All $P$ values are rounded to two significant digits. Actual value is $P = 0.0486$.
9All $P$ values are rounded to two significant digits. Actual value is $P = 0.0465$. 

TABLE 2. Allelic effect of interferon-γ gene (IFN-γ) and immunoglobulin light chain gene (IgL) genotype on primary antibody response means to SRBC and *Brucella abortus* (BA), Tmax, and Ymax antibody response means to BA in F2 offspring of M5.1 and M15.2 grandsire.

<table>
<thead>
<tr>
<th>Gene Trait</th>
<th>M5.1</th>
<th>M15.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LL</td>
<td>LF</td>
</tr>
<tr>
<td>IgL YS (titer log2)</td>
<td>3.90 (^a) 5.64 (^b) 5.24 (^b)</td>
<td>4.75 (^a) 5.15 (^a) 4.52 (^a)</td>
</tr>
<tr>
<td>IgL YB (titer log2)</td>
<td>9.08 (^a) 10.56 (^b) 10.85 (^b)</td>
<td>9.80 (^a) 10.26 (^a) 10.28 (^a)</td>
</tr>
<tr>
<td>IFN-γ YS (titer log2)</td>
<td>4.21 (^a) 5.84 (^b) 4.72 (^a)</td>
<td>5.37 (^a) 4.65 (^a) 4.40 (^a)</td>
</tr>
<tr>
<td>IFN-γ TmaxB (d)</td>
<td>14.20 (^a) 19.33 (^a) 13.41 (^a)</td>
<td>16.04 (^ab) 20.89 (^b) 10.56 (^a)</td>
</tr>
<tr>
<td>IFN-γ YmaxB (titer log2)</td>
<td>9.71 (^a) 8.02 (^b) 8.67 (^b)</td>
<td>8.53 (^a) 8.88 (^a) 8.57 (^a)</td>
</tr>
</tbody>
</table>

\(^a,b\)Means in a row within a grandsire MHC type with no common superscript differ significantly (P < 0.05).

\(^1\)F2 offspring of M5.1 grandsire.

\(^2\)F2 offspring of M15.2 grandsire.

\(^3\)Leghorn homozygote.

\(^4\)Heterozygote of Leghorn and Fayoumi genotype.

\(^5\)Fayoumi homozygote.

\(^6\)Primary antibody response to SRBC.

\(^7\)Primary antibody response to *Brucella abortus*.

\(^8\)Time required to achieve maximum secondary antibody titers to *Brucella abortus*.

\(^9\)Maximum secondary antibody titers to *Brucella abortus*.
TABLE 3. Mean, standard error, and number of individuals of interaction effects of interferon-\(\gamma\) gene (IFN-\(\gamma\)) and immunoglobulin light chain gene (IgL) on primary antibody response means to *Brucella abortus* (BA), \(T_{\text{min}}\), and \(Y_{\text{min}}\) antibody response means to SRBC in F2 offspring of M5.1 grandsire and interaction effects of IFN-\(\gamma\) and interleukin-2 (IL-2) on \(Y_{\text{min}}\) antibody response means to SRBC in F2 offspring M15.2 grandsire.

<table>
<thead>
<tr>
<th>Gene</th>
<th>genotype</th>
<th>LL (^1)</th>
<th>LF (^2)</th>
<th>FF (^3)</th>
<th>LL</th>
<th>LF</th>
<th>FF</th>
<th>LL</th>
<th>LF</th>
<th>FF</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-(\gamma)</td>
<td>LL</td>
<td>(1.00\pm1.77) (4)</td>
<td>(6.40\pm0.56) (6)</td>
<td>(5.25\pm0.88) (5)</td>
<td>5.00±1.57 (4)</td>
<td>10.60±0.50 (6)</td>
<td>11.75±0.79 (5)</td>
<td>0.24±2.49 (4)</td>
<td>0.71±1.76 (6)</td>
<td>8.38±1.76 (5)</td>
</tr>
<tr>
<td></td>
<td>LF</td>
<td>(6.40\pm0.79) (10)</td>
<td>(5.40\pm0.56) (11)</td>
<td>(5.63\pm0.63) (11)</td>
<td>9.80±0.70 (10)</td>
<td>10.80±0.50 (11)</td>
<td>10.13±0.56 (11)</td>
<td>1.29±1.44 (10)</td>
<td>0.51±1.06 (11)</td>
<td>0.6±1.33 (11)</td>
</tr>
<tr>
<td></td>
<td>FF</td>
<td>(4.20\pm0.79) (4)</td>
<td>(5.13\pm0.46) (8)</td>
<td>(4.83\pm0.72) (6)</td>
<td>10.40±0.70 (4)</td>
<td>10.29±0.42 (8)</td>
<td>10.67±0.64 (6)</td>
<td>0.36±1.58 (4)</td>
<td>1.47±0.99 (8)</td>
<td>0.52±1.44 (6)</td>
</tr>
</tbody>
</table>

**Leghorn homozygote.**  
**Heterozygote of Leghorn and Fayoumi genotype.**  
**Fayoumi homozygote.**  
**Effect ± SEM (n).**
FIGURE 1. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) pattern for candidate genes. AA = Leghorn homozygote, AG = heterozygote, and GG = Fayoumi homozygote. a) Interferon-γ (IFN-γ) gene promoter with Tsp509 I digestion. b) Interleukin-2 (IL-2) gene promoter with Mnl I digestion.
FIGURE 2. Effect of two-way allelic interactions, by grandsire MHC type, on antibody response parameters to SRBC and Brucella abortus (BA). Tmin is time required to achieve the minimum secondary antibody titers, and Ymin is the minimum secondary antibody titers. a) Effect of IFN-γ by IgL genotype on primary antibody response to SRBC in F$_2$ offspring of M5.1 grandsire. b) Effect of IFN-γ by IgL genotype on primary antibody response to BA in F$_2$ offspring of M5.1 grandsire. c) Effect of IFN-γ by IgL genotype on Ymin to SRBC in F$_2$ offspring of M5.1 grandsire. d) Effect of IFN-γ by IL-2 genotype on Ymin to SRBC in F$_2$ offspring of M15.2 grandsire. e) Effect of IFN-γ by IgL genotype on Tmin to SRBC in F$_2$ offspring of M5.1 grandsire. Two statistical contrasts are presented. The first letter above each bar represents the comparison of antibody response means of IgL or IL-2 genotypes within IFN-γ type. The second letter above each bar represents the comparison of antibody response means of IFN-γ type within IgL or IL-2 types. $^{a,b}$ means bearing no common superscript (comparing first letter and second letter separately) are significantly different ($P < 0.05$). IFN = Interferon-γ; LL = Leghorn homozygote; FF = Fayoumi homozygote; LF = heterozygote.
FIGURE 3. Chicken secondary antibody response kinetics of the genotypes of three candidate genes to SRBC or Brucella abortus (BA) in F₂ offspring of M5.1 or M15.2 grandsires. Lines were generated by non-linear model. a) Interferon-γ (IFN-γ) genotypes in F₂ offspring of M5.1 grandsire. b) IFN-γ genotypes in F₂ offspring of M15.2 grandsire. c) IFN-γ genotypes in F₂ offspring of M5.1 grandsire. d) IFN-γ genotypes in F₂ offspring of M15.2 grandsire. e) IgL genotypes in F₂ offspring of M5.1 grandsire. f) IL-2 genotypes in F₂ offspring of M15.2 grandsire.
CHAPTER 5. ASSOCIATIONS OF INTERFERON-γ GENOTYPE AND PROTEIN LEVEL WITH ANTIBODY RESPONSE KINETICS IN CHICKENS

A paper to be in press in Avian Disease

Huaijun Zhou, H. S. Lillehoj, and Susan J. Lamont

Abstract

Although previous studies have demonstrated an association between interferon-gamma (IFN-γ) promoter genotype and antibody response kinetics in chickens, the protein levels that may mediate such a gene-trait association have not been determined. The objective of this study, therefore, was to determine the correlation of circulating IFN-γ levels with both the IFN-γ promoter polymorphisms and with antibody response, in order to evaluate the potential role of IFN-γ protein in mediating genetic control of antibody response in chickens. Antibody response after Salmonella enteritidis (SE) vaccination at day 10, antibody response to sheep erythrocytes (SRBC) and killed Brucella abortus after immunizations at 19 weeks and 22 weeks, and serum IFN-γ protein level, were measured in an F2 population derived from inbred lines. A single nucleotide polymorphism (SNP) in the IFN-γ promoter region was associated with IFN-γ protein expression as measured by an enzyme-linked immunosorbent assay (ELISA) after both primary and secondary immunizations. Higher IFN-γ protein level was correlated with higher antibody level to SE, and with increased maximum level and decreased time to reach the maximum secondary antibody response to SRBC. These results suggest that one of the mechanisms by which promoter polymorphism of IFN-γ affects antibody production in chickens may involve the circulating level of IFN-γ protein.

Key words: interferon gamma, antibody response, protein level, promoter polymorphism, inbred lines

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2 USDA/ARS/Parasite Biology and Epidemiology Laboratory, Building 1040, BARC-EAST, Beltsville, MD 20705.
3 Department of Animal Science, Iowa State University, Ames, IA 50011-3150.
Abbreviation: APC = antigen-presenting cell, BA = *Brucella abortus*, ELISA = enzyme-linked immunosorbent antibody assay, Equil. = equilibrium phase, FF = homozygote from Fayoumi allele, IFN-γ = interferon gamma, LF = heterozygote from Leghorn and Fayoumi alleles, LL = homozygote from Leghorn allele, MHC = major histocompatibility complex, OD = optical density, PBS = phosphate buffered saline, SE = *Salmonella enteritidis*, SNP = single nucleotide polymorphism, SRBC = sheep red blood cell, Tmax = time needed to achieve secondary maximum titers, Tmin = time to achieve secondary minimum titers, Y = primary phase response, Ymax = maximum secondary titers, Ymin = minimum secondary titers.

**Introduction**

Interferon-gamma (IFN-γ), a virus inhibitory molecule, was first reported to be induced from human leukocytes by phytohemagglutinin in 1965 (35). IFN-γ, also known as type II interferon, is an important immunoregulatory protein that exerts multiple effects on the development, maturation, and function of the immune system. IFN-γ acts as a mild inhibitor of proliferation for most cell types, while it stimulates the proliferation of mitogen-activated primary T cells as well as a variety of T cell lines (36). The properties of IFN-γ include regulation of several aspects of the immune response, stimulation of bactericidal activity of phagocytes, stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules, orchestration of leukocyte-endothelium interactions, effects on cell proliferation, and apoptosis (4, 5, 7, 13, 20, 30). IFN-γ also mediates immunoenhancement against tumors, bacteria, viruses, and parasites (8, 10, 19, 25, 26, 27, 31, 40).

An antiviral factor is produced by chicken thymocytes and spleen cells after stimulation with mitogens (34). This factor has been defined as chicken IFN-γ in 1995 (9). Chicken IFN-γ is biochemically and functionally homologous to mammalian IFN-γ, however, the chicken IFN-γ gene has been cloned and characterized, and amino acid sequence identity with human is only 35.2% (9, 18). Chicken IFN-γ plays a primary role in many economically important poultry diseases and antibody response (17).
A single nucleotide polymorphism (SNP) in the promoter of the IFN-γ gene was found between two highly inbred chicken lines. This sequence polymorphism was previously associated with primary and secondary antibody response to both sheep red blood cell (SRBC) and Brucella abortus (BA) antigen in a F₂ population derived from these two inbred lines (38). Resistance to a wide range of diseases may be provided by selection for correlated antibody response to foreign proteins (6). Understanding relationships between general humoral immune response and disease resistance will be very beneficial for commercial breeding populations, which can not be exposed to infectious agents.

*Salmonella* Enteritidis (SE) is one of group of emerging foodborne zoonotic poultry pathogens, which is public health problem to humans in the world. The special biology of the SE infection in the avian host makes standard control measures to SE very difficult. The transmission of SE is not only vertical from parent to chick but also horizontal through environment (28).

An in vitro virus neutralization assay to measure chicken IFN-γ production during cell-mediated immunity requires the use of live virus (33). To avoid use of live virus, a simple, specific, and sensitive *in vitro* chicken IFN-γ assay utilizing mouse monoclonal antibodies in an enzyme-linked immunosorbent assay (ELISA) was developed to measure IFN-γ levels in serum (37). The association of the IFN-γ promoter-region polymorphism with circulating IFN-γ protein level and with SE antibody, as well as associations between circulating IFN-γ protein levels and antibody response to SRBC and BA, are unknown. In the present study, a monoclonal antibody-based ELISA was used to investigate associations among IFN-γ promoter mutation, antibody response to SE, circulating IFN-γ protein levels, and antibody response kinetics to SRBC and BA in chickens.

**Materials and methods**

*Experimental animals.* Genetically distinct, highly inbred (> 99%) chicken lines, the Leghorn G-B1 and MHC-congenic Fayoumi M15.2 and M5.1 lines, were used as parental lines (39). One sire from each Fayoumi line was mated to nine dams each of the G-B1 line to produce an F₁ generation. From F₁ offspring of each Fayoumi sire, four sires and eight dams
were used to produce 158 females of an F\textsubscript{2} generation. The two separate branches, therefore, of the F\textsubscript{2} population reflect the two MHC congenic Fayoumi sires, M5.1 and M15.2 (38).

**Antigen administration and sample collection.** At 10 days of age, birds of the F\textsubscript{2} population were vaccinated with SE, and blood samples were collected at 11 days after vaccination. At 19 and 23 weeks of age, the same chickens were injected intramuscularly with SRBC and BA (38). Blood samples were obtained from the peripheral vein of the wing of each bird preceding each immunization, at 7 days after primary immunization, and at 4, 7, 10, 18, 32, and 63 days after secondary immunization. Sera were collected after centrifuging the blood samples and stored at —20°C until all assays were run simultaneously.

**SE antibody and agglutination assays.** The SE antibodies were measured by using a commercial ELISA with modification as described by Kaiser (16). This ELISA is a competitive assay, thus antibody response to SE was adjusted as follows:

\[
\text{Antibody level} = 1 - \frac{\text{Sample}_{630}}{\text{Neg}_{630}}
\]

where \(\text{Sample}_{630}\) is the sample optical density (OD) measurement at 630 nm, and \(\text{Neg}_{630}\) is the mean of the three negative control’s OD measurements at 630 nm. The transformed data represented the relative SE antibody level in serum. The SRBC and BA antibodies were assayed by agglutination (29).

**IFN-\(\gamma\) ELISA.** Chicken sera collected at 7 days after primary and secondary immunizations were used to measure circulating IFN-\(\gamma\) protein level by the direct binding ELISA, because significantly elevated levels of serum IFN-\(\gamma\) were detected around day 7 postinfection with \(E.\ maxima\) (37). Flat-bottom 96-well microtiter plates were coated with 60 \(\mu\)l of sera in 40 \(\mu\)l of 0.1 M sodium carbonate buffer, pH 9.6 for 18 h at 4 °C, blocked with 200 \(\mu\)l of phosphate buffered saline (PBS) containing 2% (w/v) bovine serum albumin (BSA) for 1 h at room temperature, and washed three times with PBS containing 0.05% Tween-20, pH 7.2 (PBS-T). One hundred microliters of anti-chicken IFN-\(\gamma\) mab (YCI) was added, incubated 18 h at 4 °C, and washed three times with PBS-T, and bound antibody detected at 450 nm with horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (Sigma) and 3,3', 5,5'-tetramethylbenzidine dihydrochloride by an automated microtiter plate reader (37). For each plate, two reference chicken sera and five chicken IFN-\(\gamma\) monoclonal antibody plasmids were measured simultaneously as controls. Plasmid assay measurements were used to validate the
efficacy of the assay system. Circulating IFN-γ protein levels in serum were adjusted by reference samples as follows, due to variations between different measurements:

\[ \text{Protein level} = \frac{\text{Sample}_\text{OD}_{450}}{\text{Ref}_\text{OD}_{450}} \]

where \( \text{Sample}_\text{OD}_{450} \) is the sample OD measurement at 450 nm, and \( \text{Ref}_\text{OD}_{450} \) is the mean of the two reference chicken serum control OD measurements at 450 nm.

**PCR-RFLP for the chicken IFN-γ promoter region.** There were three base-pair mutations in the chicken IFN-γ promoter region between the Leghorn and both Fayoumi lines. A SNP at site -318 was evaluated, by PCR restriction fragment length polymorphism (RFLP) assay with restriction enzyme digestion in the F₂ population (38). The three levels of IFN-γ genotype were defined as homozygote from Leghorn allele (LL), heterozygote of both Leghorn and Fayoumi alleles (LF), and homozygote from Fayoumi allele (FF).

**Statistical analysis.** The analyses of antibody response were separately conducted by antigen (SE, SRBC, and BA) and by phase (primary, secondary, and equilibrium) for SRBC and BA. The single time-point measurement taken at 7 days postprimary immunization was used for the primary phase parameters (\( Y \)) for SRBC and BA. The secondary phase parameters of maximum titers (\( Y_{\text{max}} \)), time (\( T_{\text{max}} \)) needed to achieve maximum titers, minimum titers (\( Y_{\text{min}} \)), and time (\( T_{\text{min}} \)) needed to achieve minimum titers were estimated from seven individual time-point, postsecondary titer values by using a nonlinear regression model (38). The equilibrium phase parameter (Equil.) was calculated as the mean of the titers of the last three sample times.

The JMP® program (SAS Institute, Cary, NC) was used to conduct the general linear model test for associations of IFN-γ genotype, with IFN-γ serum protein level and SE antibody level; the multivariate pairwise correlation test between IFN-γ protein level and SE antibody level or antibody parameters to SRBC and BA antigens. Because grandsire by IFN-γ protein level and grandsire by SE antibody interactions (\( P < 0.05 \), data not shown) were found, data were thereafter analyzed separately for F₂ individuals derived from the two MHC-congenic grandsires.
Results

*Main effect of IFN-γ promoter genotype on antibody response to SE.* The IFN-γ promoter polymorphism was associated with SE antibody level \((P = 0.04)\) of \(F_2\) offspring of the M15.2 grandsire. The allelic effect of IFN-γ genotype on SE antibody level is presented in Table 1. For \(F_2\) offspring of the M15.2 grandsire, the SE antibody level of Fayoumi homozygous IFN-γ promoter SNP hens \((0.35)\) was higher than the other two allelic combinations \((0.30\) and \(0.25)\) \((P = 0.05)\). There were 17 % and 40 % increases of the SE antibody level for Fayoumi homozygous individuals compared to Leghorn homozygous and heterozygous birds, respectively.

*Main effect of IFN-γ promoter genotype on circulating IFN-γ protein level.* The ranges, means, and standard errors of the mean of IFN-γ protein level with sample sera at 7 days after primary and secondary immunization and reference chicken serum, are presented in Table 2.

The IFN-γ promoter polymorphism was associated with the IFN-γ protein level at 7 days after primary immunization \((P = 0.002)\) of \(F_2\) offspring of the M15.2 grandsire, and at 7 days after secondary immunization \((P = 0.02)\) of \(F_2\) offspring of the M5.1 grandsire. The allelic effect of IFN-γ genotype on IFN-γ protein level is presented in Table 1. For \(F_2\) offspring of the M15.2 grandsire, the IFN-γ protein level of Leghorn homozygous IFN-γ promoter SNP (LL) hens \((0.81)\) at 7 days after primary immunization was higher (33 %) than the other two allelic combinations \((0.60\) and \(0.61)\) \((P = 0.002)\). For \(F_2\) offspring of the M5.1 grandsire, in contrast, the Leghorn homozygous IFN-γ promoter type (LL) \((1.09)\) had a higher (18 %) IFN-γ protein level at 7 days after secondary immunization than hens from the other two allelic combinations \((0.93\) and \(0.92)\) \((P = 0.05)\).

*Phenotypic correlation of chicken IFN-γ protein level with antibody production.* IFN-γ protein level after primary immunization was positively correlated with IFN-γ protein level after postsecondary immunization \((0.31, P = 0.0002)\). Phenotypic correlations between chicken IFN-γ protein level and SE antibody level or antibody parameters of primary,
secondary, and equilibrium phases to SRBC and BA of all F₂ females are shown in Table 3. The IFN-γ protein level at 7 days postsecondary immunization was positively correlated with SE antibody level (P = 0.01), maximum titer to SRBC (P = 0.02), and equilibrium antibody response to SRBC (P = 0.08), and negatively correlated with time required to achieve maximum titer to SRBC (P = 0.07) and primary antibody response to BA (P = 0.04). The level of IFN-γ protein level at 7 days postprimary immunization was positively correlated with time to reach the minimum titer to SRBC (P = 0.08) and BA (P = 0.09), and negatively correlated with time to achieve the maximum titer to SRBC (P = 0.03).

Discussion

The important role of IFN-γ has been documented in many diseases in poultry (8, 17, 19, 25, 31). Sequence polymorphisms in the promoter of mammalian IFN-γ genes have also been shown to play a role in resistance to disease (1, 32). Disease resistance is often associated with higher antibody levels against specific pathogens, such as *Escherichia coli* and *Pasteurella multocida* (15, 24). Also, after divergent selection for response to SRBC, the high antibody line displayed higher antibody to Newcastle disease virus, and greater resistance to Marek's disease virus, *Eimeria tenella*, *Mycoplasma gallisepticum*, *Eimeria necatrix* than the low antibody selected line (11, 12, 14). In chickens, IFN-γ promoter polymorphisms have previously been demonstrated to be associated with chicken primary and secondary antibody response to both SRBC and BA antigens (38). Thus, the potential role of promoter polymorphisms of the chicken IFN-γ gene in chicken disease resistance, led us to investigate the role of the IFN-γ polymorphism in modulating antibody level.

Salmonella bacteria are a major food-borne infectious pathogen in humans. IFN-γ plays a critical role in host defense and intestinal immunity against *Salmonella typhimurium* in the mouse (2, 3). There was positive correlation between antibody response to SE and circulating IFN-γ protein level at both post-primary and post-secondary times in the current study. Serum antibody level to SE vaccine has a high negative genetic correlation with SE burden in cecal content (16). Therefore, high IFN-γ protein level in serum may be associated with reduced gastrointestinal SE burden in chickens.
Both IFN-γ mRNA and protein are expressed predominantly by T cells and large granular lymphocytes (36). IFN-γ affects antigen-presenting cells, T cells, and B cells, each of which participates in the complex chain of events following antigen exposure to the immune system. In mice, blockage of endogenous IFN-γ reduces the level of primary IgM antibody responses to sheep erythrocytes in vitro. IFN-γ is a necessary component of T cell-derived helper factors for antibody induction in in vitro immunization systems, and IFN-γ stimulates polyclonal Ig production by resting or activated human B cells (36). Therefore, expression of IFN-γ may affect antibody response via modulation of antigen presentation. Lymphocytes need to be activated to produce IFN-γ, which involves cooperation of accessory cells, mostly mononuclear phagocytes (4). Many diverse, convergent signal transduction pathways have been implicated in the transcriptional control of IFN-γ (36). Therefore, there are many different genetic and cellular factors that control the production of IFN-γ. Because the promoter region of a gene can regulate gene expression, we hypothesized that mutation in the promoter region would affect IFN-γ gene expression. The allelic effects of promoter polymorphism on IFN-γ protein level were very consistent, in that birds with IFN-γ LL genotype had higher IFN-γ protein levels than both other genotypes after both primary and secondary immunization and in F₂ offspring of both M5.1 and M15.2 grandsires.

IFN-γ production, as a quantitative trait, is affected by many genetic and non-genetic factors. Based on the general linear model analysis of IFN-γ genotype with protein level in serum, the IFN-γ promoter polymorphism determined 15.8 % of the phenotypic variation in IFN-γ protein level at 7 day after primary immunization in F₂ offspring of the M15.2 grandsire, and 8.5 % for IFN-γ production at 7 day after secondary immunization in F₂ offspring of the M5.1 grandsire. Accounting for this large percent of variation strongly suggests that DNA sequence polymorphism in or near the IFN-γ promoter region might influence IFN-γ protein level after both primary and secondary immunizations.

There were consistently negative correlations of IFN-γ protein level after secondary immunization with primary antibody response to SRBC and BA. However, there was a positive correlation of IFN-γ protein level after both primary and secondary immunizations with maximum secondary antibody response to SRBC, and negative correlations (P = 0.03,
0.07) with the time to required to achieve maximum secondary antibody response to SRBC. These results suggest that augmented IFN-γ protein levels can increase maximum secondary antibody response to SRBC and decrease the time needed to reach maximum secondary antibody response to SRBC. Thus, exogenous administration of IFN-γ might be an effective method to increase antibody response to booster (second or later) vaccinations.

The different correlation directions of IFN-γ protein level between the two antibody response phases with SRBC antigen might arise from the different cell types involved in primary and secondary antibody production. When a naïve T cell recognizes an antigen-MHC complex on an appropriate antigen-presenting cell or target, an activated T cell will initiate a primary immune response. The memory T-cell and B-cell, however, are involved in the secondary immune response (21). The factors related to IFN-γ expression and antibody production might thus have different effects between primary and secondary phases of immune response.

The chicken MHC plays an important role in antibody production (22, 23). Interferon-stimulated response elements mediate the induction of MHC class I expression by type I and type II IFN. In particular, IFN-γ regulates the expression of multiple components in the MHC class I-restricted antigen presentation circuit in human cell line (13). The direction of allelic effects of the IFN-γ promoter genotype on chicken antibody response to SE was different between the F2 branches produced from the two MHC-congenic grandsires. This result suggests a genetic interaction between the chicken IFN-γ promoter polymorphism and MHC haplotype on antibody response to SE vaccine. Therefore, effects of IFN-γ polymorphism must be defined in the context of the population’s MHC alleles.

Adequate functioning of the IFN-γ and macrophage system is essential for innate, as well as acquired, resistance to infection (4). The results of the current study, which clearly demonstrated the relationship of a promoter polymorphism with circulating IFN-γ levels, and also of IFN-γ protein levels with antibody response parameters, sets the stage for enhancement of humoral immunity via genetic selection of optimal IFN-γ genotypes.
References


Acknowledgments
The authors thank Ervin Johnson, A. J. Buitenhuis, and Michael Kaiser for their excellent technical assistance.
TABLE 1. IFN-γ promoter allelic effect (mean ± SEM) on IFN-γ protein level and antibody response to *Salmonella enteritidis* in F_2_ offspring of M5.1 and M15.2 grandsires.

<table>
<thead>
<tr>
<th>Trait</th>
<th>M5.1 Grandsire</th>
<th>M15.2 Grandsire</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LL^A</td>
<td>LF^B</td>
</tr>
<tr>
<td>PL1^D</td>
<td>0.84 ± 0.07^a</td>
<td>0.74 ± 0.05^a</td>
</tr>
<tr>
<td>PL2^E</td>
<td>1.09 ± 0.05^a</td>
<td>0.93 ± 0.04^b</td>
</tr>
<tr>
<td>SE^F</td>
<td>0.42 ± 0.03^a</td>
<td>0.37 ± 0.02^a</td>
</tr>
</tbody>
</table>

^a^Means in a row within a grandsire MHC type with no common superscript differ significantly (*P* < 0.05).

^A^Leghorn homozygote.

^B^Heterozygote of Leghorn and Fayoumi genotype.

^C^Fayoumi homozygote.

^D^IFN-γ protein level at 7 days after 1st injection.

^E^IFN-γ protein level at 7 days after 2nd injection.

^F^Antibody level to *Salmonella enteritidis* vaccination.
TABLE 2. Serum IFN-γ protein level (optical density at 450 nm) in F2 females of an inbred cross.

<table>
<thead>
<tr>
<th></th>
<th>Sample chicken serum</th>
<th>Reference chicken serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary</td>
<td>Secondary</td>
</tr>
<tr>
<td>Range</td>
<td>0.12 – 0.66</td>
<td>0.06 – 0.27</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.29 ± 0.01</td>
<td>0.14 ± 0.003</td>
</tr>
</tbody>
</table>
TABLE 3. Phenotypic correlation between chicken IFN-γ protein level and antibody response to *Salmonella enteritidis*, sheep red blood cells, and *Brucella abortus* in F₂ females of an inbred cross.

<table>
<thead>
<tr>
<th></th>
<th>Sheep red blood cells</th>
<th>Brucella abortus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SE²</td>
<td>Y¹</td>
</tr>
<tr>
<td>PL₁[^h]</td>
<td>Correlation</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>NS[^i]</td>
</tr>
<tr>
<td>PL₂[^i]</td>
<td>Correlation</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.01</td>
</tr>
</tbody>
</table>

|             | Y                   | Tmin⁴          | Ymin¹    | Tmax⁴      | Ymax¹    | Equil.² |
| PL₁[^h]    |                     | 0.06           | 0.16     | -0.05      | 0.06     | 0.03    | -0.11   |
|             | P value              | NS[^i]        | 0.09     | NS         | NS       | NS      | 0.17    |
| PL₂[^i]    |                     | -0.17          | -0.02    | -0.07      | -0.08    | -0.04   | -0.02   |
|             | P value              | 0.04           | NS       | NS         | NS       | NS      | NS      |

[^A]: Antibody response to *Salmonella enteritidis*.
[^B]: Primary antibody response.
[^C]: Time required to achieve minimum secondary antibody titers.
[^D]: Minimum secondary antibody titers.
[^E]: Time required to achieve maximum secondary antibody titers.
[^F]: Maximum secondary antibody titers.
[^G]: Equilibrium phase antibody titers.
[^h]: IFN-γ protein level at 7 days after primary injection.
[^i]: IFN-γ protein level at 7 days after secondary injection.
CHAPTER 6. GENETIC MARKERS ASSOCIATED WITH ANTIBODY RESPONSE KINETICS IN ADULT CHICKENS

A paper accepted for publication in Poultry Science

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Abstract

A linkage disequilibrium approach with microsatellites was employed to investigate QTL affecting immune response. Highly inbred males of two MHC-congenic Fayoumi chicken lines were mated with highly inbred G-B1 Leghorn hens. Adult F\textsubscript{2} hens (n=158) were injected twice with SRBC and fixed \textit{Brucella abortus} (BA). Agglutinating antibody titers were measured. Secondary phase parameters of maximum titers (Y\textsubscript{max}) and time (T\textsubscript{max}) needed to achieve Y\textsubscript{max} were estimated from post-secondary titers by using a non-linear regression model. A three-step genotype strategy (DNA pooling, selective genotyping, and whole population genotyping) was used to identify microsatellite markers that are associated with immune response to SRBC and BA. The linkage distances between adjacent markers in the F\textsubscript{2} population were estimated by Crimap. The QTL affecting immune response to SRBC and BA were detected based on F statistic by interval mapping. A total of five significant QTL, as determined by a permutation test, were detected at the 5\% chromosome-wise level on Chromosomes 3, 5, 6, and Z. Two (Chromosome 3 and 6) of five QTL were significant at the 1\% chromosome-wise level. The variance explained by the QTL ranged from 6.46\% to 7.50\%. The results suggest that regions on Chromosomes 3, 5, 6, and Z contain QTL that affect antibody kinetics in the hen.

(Key words: genome scan, immune response kinetics, quantitative trait loci, interval mapping, permutation test)

Abbreviation Key: BA = \textit{Brucella abortus}; SNP = single nucleotide polymorphism; TGF\textbeta{}\textsubscript{2} = transforming growth factor \textbeta{} 2 gene; TGF\textbeta{}\textsubscript{3} = transforming growth factor \textbeta{} 3 gene; T\textsubscript{max} = time to achieve maximum secondary antibody titers; Y = primary antibody response; Y\textsubscript{max} = maximum secondary antibody titers; ZOV3 = immunoglobulin superfamily gene.

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Introduction

A major current goal in the animal breeding industry is to improve animal health, which can be accomplished through selection for genetic resistance to disease. Several previous studies revealed that artificial genetic selection for antibody response parameters efficiently improves disease resistance in chickens (Siegel and Gross 1980; Gross et al., 1980; Dunnington et al., 1986; Pitcovski et al., 1993; Yonash et al., 1996). Antibody response in chickens, which has low to medium heritability, is controlled by QTL. The development of molecular genetic techniques and their application in farm animals provide an opportunity to investigate QTL that control quantitative traits. A comprehensive genetic linkage map in chickens has been developed over the last decade (Bumstead and Palyga 1992; Burt et al., 1995; Cheng et al., 1995; Groenen et al., 1998; 2000; and Schmid et al., 2000). Currently, there are approximately 2400 genes and genetic markers mapped in the chicken, with a majority of these being anonymous genetic markers (http://www.genome.iastate.edu/chickmap/ Date accessed: June 16, 2002).

The linkage disequilibrium, genome scan approach using anonymous molecular markers is one of the major strategies used to identify QTL affecting economic traits. Many studies have mapped QTL affecting several economic important traits in farm animals (Andersson et al., 1994; Paszek et al., 1999; De Koning et al., 1999; Lien et al., 2000; Van Tassell et al., 2000; and Malek et al., 2001). Van Kaam et al. (1998; 1999a, b) investigated QTL affecting body weight, growth and feed efficiency in chickens by using a whole genome scan. The QTL affecting susceptibility to Marek's disease virus-induced tumors in F2 intercross chickens were mapped by Vallejo et al. (1998) and Yonash et al. (2001). Yonash et al. (2001) detected QTL affecting antibody response and survival rate in meat-type chickens.

To achieve sufficient power to identify linkage between marker loci and QTL with low to moderate effects requires a large sample of animals to be genotyped (Darvasi et al. 1993). The relatively high cost of marker genotyping limits these applications for genetic analysis and genetic improvement. The DNA pooling, also called bulk segregant analysis, is an efficient method to reduce costs in marker-QTL linkage determination by pooling DNA from selected individuals at each of the two phenotypic extremes, which are the most informative individuals (Darvasi and Soller 1994). A whole genome scan for QTL affecting milk protein
percentage in Israeli-Holstein cattle by selective milk DNA pooling was conducted successfully by Mosig et al. (2001).

The overall objective of the current study was to investigate the efficiency of a three-step procedure to detect linkages between DNA markers and QTL affecting antibody response kinetics to SRBC and Brucella abortus (BA) in hens of a unique F2 resource population (Zhou et al., 2001a), derived from an intercross between two genetically distant and highly inbred lines. First, the DNA pooling technique was employed to identify suggestive microsatellite markers potentially associated with QTL affecting antibody response, based on relative frequency differences of microsatellite alleles between the two phenotypic extremes. Second, the individuals of each of the two phenotypic extreme pools were genotyped with the suggestive markers that had been associated with multiple traits in the first step. Third, the whole population was genotyped with a small number of markers, based on positive results of the individual selective genotyping.

Materials and methods

Experimental Population

Genetically distinct, highly inbred (> 99%) chicken lines, the Leghorn G-B1 and the MHC-congenic Fayoumi M15.2 and M5.1 lines (Zhou and Lamont 1999), were used as parental lines. One sire from each Fayoumi line was mated to nine dams of the G-B1 line to produce the F1 generations. From the F1 offspring of each Fayoumi sire, four males and eight females were used to produce a total of 158 F2 females (Zhou et al., 2001a).

Antigen Administration, Sample Collection, and Agglutination Assays

At 19 and 23 wk of age, F2 chickens were intramuscularly injected with SRBC and BA (Zhou et al., 2001a). Blood samples were obtained from the peripheral vein of the wing of each bird preceding each immunization to determine the baseline antibody level, and at 7 d after primary immunization, and at 4, 7, 10, 18, 32, and 63 d after secondary immunization. Blood was allowed to clot for approximately four hours. Sera were collected after centrifuging the blood samples, and stored at -20 C until all assays were run simultaneously. The SRBC and BA antibodies were assayed by agglutination (Zhou et al., 2001a).

Genotyping Procedures for Microsatellite Markers
Chicken DNA was isolated from venous blood. The PCR procedure was carried out as described by Cheng et al. (1995) by using fluorescently labeled primers (courtesy of the U.S. National Animal Genome Research Program Poultry Coordinators). The PCR products were pooled in multiplex where applicable, then loaded onto a polyacrylamide-sequencing gel and processed on an ABI 377 DNA Sequencer. GenScan 2.1 software\(^2\) was used to determine the alleles.

**Pooling of DNA Samples**

After DNA extraction, an aliquot from each individual was diluted in water to give a calculated concentration of 50 ng/\(\mu\)L based on optical density at 260 nm determined by spectrophotometer. The concentration of diluted DNA samples was also verified to be consistent by visualization after agarose gel electrophoresis. Each DNA pool was independently formed in duplicate by combining equal amounts of DNA from the 20% of the individuals constituting each phenotypic extreme of the F\(_2\) population for each of the eight traits. This resulted in a total of 32 DNA pools (2 duplicates \(\times\) 2 phenotypic extremes \(\times\) 8 traits).

**Determination of Microsatellite Allele Frequency and Criteria for Determining Suggestive Microsatellite Markers**

Each inbred parental line had only one allele for each microsatellite. The height of each GenScan peak was used to estimate the approximate relative allele frequencies in pools, based on the assumption that peak height is directly proportional to the amount of DNA for that allele (Kirov et al., 2000). The mean of the two values of the duplicate pools was used to estimate the allele frequency for each pool. The criterion to designate a suggestive difference microsatellite allele frequency was set to a greater than 0.15 allele frequency difference between high and low pool for a trait, based on the report by Khatib et al., (1994).

**Genotyping for the Chicken Transforming Growth Factor \(\beta\)-2 (TGFB2) and \(\beta\)-3 (TGFB3) Genes and Ig Superfamily Gene ZOV3**

For interval mapping, the studied regions were on GGA3, 5, 6, and Z. The genes TGFB2, TGFB3, and ZOV3 are located on chicken Chromosome 3 (GGA3), GGA5, and GGAZ, respectively, near the microsatellite markers used in these chromosomes for interval

\(^{2}\)GenScan 2.1, 1989-1996, Perkin Elmer Corp., Foster City, CA 94404
mapping. The genes, therefore, were used as additional genetic markers for linkage mapping
and interval mapping analysis. There were single nucleotide polymorphisms (SNP) in the
chicken TGFβ2 promoter region, TGFβ3 intron 4 region, and ZOV3 exon region between the
G-B1 Leghorn line and both Fayoumi lines (Zhou et al., 2001b). A PCR-RFLP assay was
conducted with these SNP to genotype the F2 population (Li et al., 2002; Zhou et al., 2001c).

Statistical Analysis

The analyses of antibody response were separately conducted by antigen (SRBC and BA)
and by phase (primary, secondary, and equilibrium). For the SRBC and BA primary phase,
the single time-point measurement taken at 7 d postprimary immunization (Y) was used.
Secondary phase parameters of maximum titers (Ymax), and time (Tmax) needed to achieve
maximum titers were estimated from seven individual time-point postsecondary titer values
by using a nonlinear model (Weigend et al., 1997). For the equilibrium phase, the titers of the
last three sample times were used to calculate the mean of the phase (Zhou et al., 2001a).
This yielded a total of 8 antibody kinetics traits analyzed in the population.

The general linear model tests for associations between individual marker and antibody
response parameters were conducted using the JMP® program3 (Sall and Lehman, 1996).
For the chromosome or linkage group with more than three microsatellites or genes, marker
linkage maps for this F2 resource population were computed with Crimap version 2.4
software (Green et al., 1990), by using the flips and all options to get the best order of the
markers and the fixed option to obtain the map distances. The maps were then used
for QTL analysis of the three autosomes and the Z chromosome by using the line cross
least square regression interval mapping program (Haley et al. 1994).

Marker information was used to calculate the probabilities that an F2 offspring inherited
none, one, or two alleles from each line for a putative QTL at each 1-cM position in the
genome. Based on these probabilities, additive and dominance coefficients were obtained for
the putative QTL, contrasting average QTL alleles from the two line origins.

Detection of QTL was based on an F statistic that was computed from sums of squares
explained by the additive and dominance coefficients for the QTL. Significance thresholds
of the F statistic were derived at the chromosome-wise levels on a single-trait basis by the

permutation test of Churchill and Doerge (1994). A total of 5,000 random permutations of the data were performed. Percentage of F₂ variance explained by model was calculated as following:

\[ \text{variance \%} = 100 \times \frac{(\text{RMS}-\text{FMS})}{\text{RMS}} \]

where RMS is defined as residual mean square from the reduced model omitting QTL but including all fixed effects, and FMS is defined as residual mean square from the full model including QTL and all fixed effects. The \( \chi^2 \) test was performed with genotype distribution between the low pool and high pool for the suggestive microsatellite markers at the selective genotyping stage.

**Results**

**Trait Parameter Means and SD**

The means and SD of each antibody response parameter of the F₂ hens are presented in Table 1. For the secondary antibody response phase, parameters occasionally could not be estimated if the seven time points of an individual did not fit the non-linear model curve.

**Genome Coverage and Three-stage Genotyping Procedure**

The East Lansing chicken genome map was used to select microsatellites at the initiation of the present study. Approximately 200 microsatellites across the genome were selected to identify polymorphisms between the founder lines. Of these, sixty-six evenly distributed informative microsatellite markers were selected. The 66 markers covered 22 of the 41 linkage groups of the East Lansing chicken genome map: eight macrochromosomes, the Z chromosome, and 13 small linkage groups. With an average 40 cM interval between adjacent microsatellites, the total genome coverage of the current study was about 70%.

There were three stages in the present study. Stage 1 was DNA pooling. There were a total of eight phenotypic traits (four for each antigen). The DNA from the 20% of individuals with high phenotypic value and 20% of low phenotypic value in the F₂ resource population were separately pooled. Replicate DNA pools were made independently. The 66 microsatellite markers were used to genotype the total of 32 DNA pools (2 antigens * 4 antibody traits * 2 phenotypic extremes * 2 replicate pools = 32 pools). Of a total of 528 marker-trait combinations (66 microsatellites * 2 antigens * 4 antibody traits), 38 suggestive
microsatellite markers were found for the eight traits (Table 2). Stage 2 was selective genotyping. Individuals from the phenotypic high pool and low pool were genotyped. Focus was on only eight suggestive markers that were associated with multiple antibody parameters or located on a chromosome with multiple suggestive markers. The LEI0043 and MCW0083 markers on Chromosome 3, the ADL0023 and MCW0078 markers on Chromosome 5, the LEI0097 marker on Chromosome 6, the ADL0201 and MCW0294 markers on Chromosome Z, and the ADL0184 marker on E31 were chosen for selective genotyping. Stage 3 was whole population genotyping. For the microsatellites that were positive at the first two stages, polymorphic flanking microsatellites around 20 to 30 cM were screened by individually genotyping the entire F2 population. A total of 16 microsatellites were used to genotype the whole population: MCW0083 and LEI0043 on GGA3, MCW0078, ADL0023, ADL0292, and LEI0145 on GGA5, LEI0097, LEI0196, ADL0138, and ADL0230 on GGA6, MCW0294, ADL0201, LEI0111, and ADL0250 on GGAZ, and ADL0184, and ADL0290 on E31.

**Comparison of Allele Frequency of Microsatellites Estimated from DNA Pooling with that Determined by Selective Individual Genotyping and χ2 Test for Genotype Distribution from Selective Genotyping.**

For the suggestive markers that had more than one marker-trait association at the DNA pooling stage, allele frequencies of microsatellites from the Leghorn line of the low and high pools for both DNA pooling and selective genotyping stages are presented in Table 3. The allele frequency differences between the low pool and high pool were very consistent at both stages, except for the MCW0078 marker with T_max to SRBC and the ADL0184 marker with T_max to BA. There were significant ($P < 0.05$) differences of genotype distribution between the low and high pools with the LEI0043 marker and the MCW0083 marker on T_max to BA, with the LEI0097 marker on Y_max to SRBC, and the MCW0294 and ADL0201 markers on equilibrium antibody response to BA based on the $χ^2$ Test of the selective genotyping allele frequency.

**Main Effects ($P$ values) of Microsatellite Markers on Chicken Antibody Response to SRBC and BA**
The P-values of main effects are shown in Table 4. There were significant (P < 0.05) associations of MCW0083 and LEI0043 on GGA3 with primary antibody response to BA and equilibrium antibody response to SRBC, respectively. There were significant main associations between MCW0078 and Tmax to SRBC and primary antibody response to BA, and between ADL0023 and Tmax and Ymax to SRBC on GGA5. There were significant main effects (P < 0.05) of LEI0097 on GGA6 on Ymax to SRBC and BA and equilibrium phase antibody response to BA. Significant associations (P < 0.05) between MCW0294 and ADL0201 on GGAZ and Tmax to SRBC and equilibrium phase antibody response to BA were found. There were significant associations (P < 0.05) between ADL0184 and primary antibody response to SRBC and Tmax to BA and between ADL0290 and Tmax to SRBC and BA on E31.

**Significance Thresholds**

The significance levels at the 5% and 1% level for individual chromosomes, as determined by the permutation test, differed slightly by trait, but more substantially by chromosome. Therefore, the average thresholds across the eight investigated traits for each chromosome were used. Average 5% and 1% chromosome-wise thresholds ranged from 3.97 to 5.92, and 6.37 to 8.67, respectively.

**Antibody Response Kinetics QTL Mapping**

Estimated QTL that are significant at the 5% chromosome-wise level, location, gene effect, and percentage of F² variance explained by each QTL are presented in Table 5. The QTL plots of the F statistic across chromosomes are shown in Figure 1 with QTL significance at the 5% chromosome-wise level. Some plots suggested evidence for multiple QTL in adjacent intervals for the same trait (Figure 1d), however, only results for the most significant position were included in Table 5 because only a single QTL model was conducted.

A total of five QTL were detected at the chromosome-wise level for the eight traits evaluated in the present study, not counting potential multiple QTL in adjacent intervals. There were QTL identified affecting equilibrium phase antibody response to SRBC (between LEI0043 and MCW0083) and primary antibody response to BA (between MCW0083 and TGFB2) on GGA3, with QTL accounting for 6.76% and 7.50% of the F² variance, respectively. A QTL affecting Tmax to SRBC (between TGFB3 and LEI0145) at
the 5% chromosome level on GGA5 was detected, with the QTL accounting for 6.46% of the F₂ variance. There were QTL identified for equilibrium antibody response to BA on GGA6 (very close to LEI0097) and GGAZ (close to ADL0201), with the QTL explaining 6.98% and 6.55% of the variance, respectively. For the five QTL, two QTL (primary antibody response to BA on GGA3 and equilibrium antibody response to BA on GGA6) were significant at the $P < 0.01$ level, and the significance level of another two QTL (equilibrium antibody response to SRBC on GGA3 and Tmax to SRBC on GGA5) were close to the 0.01 level. Based on the additive effects and dominance effects of five QTL, all QTL showed overdominance, except the equilibrium phase antibody response to BA on GGA6.

**Discussion**

The genetic linkage distances between markers in the F₂ population (data not shown) were nearly consistent with the chicken consensus map. The map order of the markers was the same in the two maps, except for a reversal in order between ADL0250 and MCW0294 on GGAZ (Groenen et al., 2000).

Mapping of QTL is one of the major research interests in poultry genomics (Burt 2002). Microsatellite markers that are distributed evenly throughout the genome provide an opportunity to detect QTL, given an appropriate resource population. The principle behind QTL mapping is based on linkage disequilibrium between alleles at a marker locus and alleles at the linked QTL. The F₂ population design is more powerful than the backcross design to detect additive and dominant effects (Falconer and Mackay 1996). Therefore, a resource population derived from two genetically distant, highly inbred parental lines with differences in the trait of interest will be an optimal one. Yonash et al. (1999) and Vallejo et al. (1998) detected significant QTL affecting susceptibility to Marek's disease in egg-type chickens with such a population design. Marker spacing between 10 and 40 cM is an appropriate choice at the initial scan for QTL (Soller and Andersson 1998). Therefore, the current study, with unique population design, was able to support the detection of the QTL affecting antibody response kinetics in adult chickens.
A very large number of genotyped individuals with a large number of genetic markers are required to achieve the theoretical high power needed for detecting QTL (Soller 1990). The use of selective DNA pooling can greatly reduce the costs and the amount of laboratory work for screening large number of individuals. Mosig et al. (2001) demonstrated that 4400 pool data points had the equivalent statistical power of 600,000 individual data points. Therefore, selective DNA pooling was applied for the initial genotyping of the current study to identify suggestive microsatellite markers for the second stage of selective genotyping. The rather liberal criterion of 0.15 allele frequency difference between the high pool and the low pool for the first stage was based on the premise that subsequent stages would reject false positive markers accepted at the first stage. The criterion for the second stage was more conservative. Only the suggestive markers that had multiple marker-trait associations were selected for this stage. To evaluate the accuracy of the DNA pooling technique, allele frequency differences between the low and high pools at the DNA pooling stage and the selective genotyping stage were compared. More than 90% (19 of 21) marker-trait allele frequencies were very consistent between the two stages, or allele frequency differences were close to 0.15. This result indicated that the DNA pooling method was very accurate in the present study. For these selected markers, there were significant \((P < 0.05)\) associations between all markers and at least one trait, based on the whole population genotyping results. This result indicates that the three-step procedure of the present study to identify microsatellite affecting antibody response was efficient and successful. The efficiency of the pooling and selective genotyping of the first two stages reduced total cost and time needed.

The DNA pooling technique is based on the assumption that differences in marker allele frequencies between high phenotypic value pool and low phenotypic value pool are highly correlated with differences in frequencies of homozygotes (Dekkers 2000). No such limitation was required, however, to estimate additive effects. Estimates of dominance effect might be biased, based on contrasting the low pool or high pool to the mean of all individuals rather than contrasting the low pool to the high pool.

There existed some inconsistency with associations among the three step procedures, such as MCW0083 and LEI0043 on GGA3. The allele frequency differences between high pool and low pool at DNA pooling stage (0.16 and 0.16) for MCW0083 and LEI0043 with Ymax
to BA were consistent with the value (0.18) at selective genotyping stage. But the $\chi^2$ tests
with genotype distribution between the high pool and low pool were not significant ($P > 0.2$).
There were, however, also no significant associations between both markers and Ymax to
BA at $\alpha = 0.05$ level. Several factors might affect the association results: first, the allele
frequency difference between the high pool and low pool might not be big enough to result in
a significant association with the whole population. Second, the two markers might be false
positives (Type I error), or the statistical power not be enough to detect significance using the
whole population, because more individuals with intermediate phenotypic value were added.

Allele frequency difference could not be used as the only criteria to effectively select
microsatellites for whole population genotyping stage. The overdominance effect can be
detected by the $\chi^2$ test while it can not be detected by allele frequency difference. Therefore,
the $\chi^2$ test could be another criteria for selecting markers. There were two marker-trait
(LEI0043 on equilibrium antibody response to SRBC and ADL0184 on Tmax to BA) that
had very small allele frequency difference between low pool and high pool (0.03 and 0.02,
respectively). However, the $\chi^2$ test with genotype distribution between high pool and low
pool for both marker-trait associations were significant ($P < 0.05$), and significant
associations ($P < 0.05$) were found with whole population genotyping. The mean of the
phenotypic value of three genotypes strongly suggested that there was overdominance for
both traits, which might explain why significant associations still could occur with small
allele frequency differences between pools.

Based on the results, there was general agreement between the $\chi^2$ test with genotype
distribution between high and low pool and whole population genotyping analyses, except
for two of twenty-one marker-trait combinations (LEI0043 and MCW0083 on Tmax to BA).
The individuals from two extreme phenotypic pools generally account for the most variation
for QTL mapping (Dekkers, 2000). For LEI0043, the $P$-value for whole population
genotyping analyses was 0.09 (close to significant level). For MCW0083, the $P$-value for
whole population genotyping analyses was 0.38, which was far from the significant level.
The disagreement among the allele frequency difference, $\chi^2$ test, and whole population
analysis suggests that the rest of individuals outside two the phenotypic extremes did not
support the associations detected in selective genotyping (Type I error). More than 90% of
marker-trait associations were accurately predicted by the criteria of both allele frequency
difference between pools and \( \chi^2 \) test during the selective genotyping stage. On the other
hand, the selection criteria at the selective genotyping stage might be so stringent that some
suggestive markers, which might potentially be associated with some antibody response
parameters, were eliminated before the next stage (Type II error).

The major advantage of interval mapping over individual marker analysis is that QTL
position and effect can be separated. Three candidate genes (TGFβ2, TGFβ3, and ZOV3)
were added to the microsatellites as additional genetic markers for interval mapping analysis
in this study. Comparing interval mapping analysis with individual marker analysis, only
markers with \( P \)-value less than 0.01 in individual marker analysis can be detected in interval
mapping analysis at the chromosome-wise significance level. The variance estimate for the
QTL in the present study ranged from 6.46% to 7.50%. Three out of five QTL had much
greater dominance effects than additive effects. Presence of overdominance effect is not
unexpected in traits of the immune system, because the immune system needs multiple
alleles to code proteins to meet challenges representing the full variety of environmental
antigens. For other biological systems, a single optimal allele often evolves or is selected to
be present in high frequency.

In summary, the combination of allele frequency differences between low and high
phenotypic DNA pools and the \( \chi^2 \) test at the selective genotyping stage was more successful
in selecting markers for the whole-population genotyping stage than was only allele
frequency difference. Individual marker analysis suggested that the regions of GGA3, GGA5,
GGA6, E31, and GGAZ contain QTL affecting antibody response kinetics to SRBC and BA
in adult chickens. There were five significant QTL identified at the 5% chromosome-wise
level based on interval mapping analysis.

Selection for high antibody response to SRBC has resulted in correlated changes in
increased resistance to Marek's and Newcastle diseases, and *Eimeria tenella* (Martin et al.,
1990; Boa-Amponsem et al., 1997). Increased immunity in hens is crucial to protect chicks
from various pathogens through the transmission of maternal antibodies. The results of the
present study lay the foundation for application of marker-assisted selection to improve
passive immunity in chicks. As in all QTL studies, before application of the results in
commercial populations, different resource populations should be examined to verify the linkage and associations identified in the current study. Additional genetic markers in these genomic regions can be used to fine map the QTL affecting antibody response in chickens. In addition, the QTL found in the present study will be beneficial in the positional candidate gene approach to identify causal genes responsible for antibody response in chickens.

Acknowledgments
The authors thank the U.S. Poultry Genome Coordinators for supplying microsatellite primers; Yuandang Zhang and Massoud Malek for interval mapping assistance; Hauke Thomsen for help with the permutation test; Nader Deeb, Jack Dekkers, Max Rothschild, and Chris Haley for helpful discussion. This journal paper of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 2576 and 3521, was supported by Hatch Act and State of Iowa funds.

References


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TABLE 1. Means and standard deviations (SD) of antibody response parameters to SRBC and *Brucella abortus* in F₂ hens

| Variable | SRBC | | | | Brucella abortus |
|----------|------|---|---|---|---|---|---|
|          | Y¹  | Tmax² | Ymax³ | Equilibrium⁴ | Y | Tmax | Ymax | Equilibrium |
| N⁵       | 154 | 141 | 141 | 157 | 153 | 119 | 119 | 157 |
| Mean (log₂) | 5.04 | 11.68 | 5.56 | 3.83 | 10.25 | 15.60 | 8.63 | 7.26 |
| SD       | 1.83 | 9.32 | 1.24 | 0.88 | 1.75 | 10.44 | 1.53 | 1.43 |

¹Primary antibody response.
²Time required to achieve maximum secondary antibody titers.
³Maximum secondary antibody titers.
⁴Equilibrium phase antibody titers.
⁵Number of individuals.
TABLE 2. Suggestive microsatellite markers for primary, secondary, and equilibrium phase immune response in chickens, based on allele frequency difference of more than 0.15 between the low and high pool at the DNA pooling stage

<table>
<thead>
<tr>
<th>Y&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Tmax&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Ymax&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Equilibrium&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
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<td>Location</td>
<td>Marker</td>
<td>Location</td>
</tr>
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<td>Tmax&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Ymax&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Equilibrium&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>Z</td>
</tr>
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<td>MCW0078</td>
<td>5</td>
</tr>
<tr>
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<sup>1</sup>Primary antibody response.
<sup>2</sup>Time required to achieve maximum secondary antibody titers.
<sup>3</sup>Maximum secondary antibody titers.
<sup>4</sup>Equilibrium phase antibody titers.
<table>
<thead>
<tr>
<th></th>
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<tr>
<td>$\chi^2$ (P value)</td>
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<td>0.29</td>
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1 BA = Brucella abortus.
2 Time required to achieve maximum secondary antibody titers.
3 Maximum secondary antibody titers.
4 Primary antibody response.
5 Equilibrium phase antibody titers.
TABLE 4. Main effects ($P$-values) of general linear model test with microsatellite markers on chicken antibody parameters of primary, secondary, and equilibrium phase to SRBC and *Brucella abortus* (BA) in F$_2$ hens

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<th>BA</th>
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$^1$Primary antibody response.
$^2$Time required to achieve maximum secondary antibody titers.
$^3$Maximum secondary antibody titers.
$^4$Equilibrium phase antibody titers.
$^5$NS = $P > 0.20$. 
<table>
<thead>
<tr>
<th>Trait</th>
<th>GGA</th>
<th>Location</th>
<th>F value</th>
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<th>Additive</th>
<th>Dominance</th>
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<tr>
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<td>(cM)</td>
<td></td>
<td></td>
<td>Effect</td>
<td>SE</td>
<td>Effect</td>
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<td>50</td>
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<td>-0.10</td>
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¹Equilibrium phase antibody titers to SRBC.
²Primary antibody response to BA.
³Time to achieve maximum secondary antibody titers.
⁴Equilibrium phase antibody titers to BA.
Figure 1. F-ratio curves following interval mapping analyses of chicken chromosome 3, 5, 6, and Z for antibody response kinetics to SRBC and *Brucella abortus* (BA). a) F-ratio curve of chicken chromosome 3 for primary antibody response to BA (YB) and equilibrium phase antibody titers to SRBC (YES). b) F-ratio curve of chicken chromosome 5 for time to achieve maximum secondary antibody titers to SRBC (TmaxS). c) F-ratio curve of chicken chromosome 6 for equilibrium phase antibody titers to BA (YEB). d) F-ratio curve of chicken chromosome Z for YEB. Arrows on the x-axis indicate the marker position. Lines are provided for 1% chromosome-wise level (—), and the 5% chromosome-wise level (— ) significance.
CHAPTER 7. CHICKEN MHC CLASS I AND H GENE EFFECTS ON ANTIBODY RESPONSE KINETICS IN ADULT CHICKENS

A paper submitted to Immunogenetics

Huaijun Zhou and Susan J. Lamont

Abstract

The major histocompatibility complex (MHC) plays an important role in regulation of immune response. The MHC class I and II genes were selected as candidates to investigate associations with vaccine response to *Salmonella enteritidis* (S. enteritidis) and antibody response kinetics to sheep red blood cell (SRBC) and *Brucella abortus* (B. abortus). Primary antibody response after *S. enteritidis* vaccination at d 10, and antibody response to SRBC and killed *B. abortus* after immunization at 19 and 22 weeks, were measured in a F2 population. The resource population was derived from males of two highly inbred MHC-congenic Fayoumi chicken lines (M5.1 and M15.2) mated with highly inbred G-B1 Leghorn line hens. Secondary phase parameters of minimum titers (Ymin), maximum titers (Ymax), and time needed to achieve Ymin (Tmin) and Ymax (Tmax) were estimated from post-secondary titers by using a non-linear regression model. Associations of single nucleotide polymorphisms (SNP) in MHC class I and II genes with antibody response parameters were determined by a general linear model. Significant associations were found primarily in the M15.2 grandsire lineage. There were significant associations between MHC class I α1 and α2 SNPs and antibody response to *S. enteritidis*, primary antibody response to *B. abortus*, Ymin to SRBC, and Ymax to both SRBC and *B. abortus*. There were significant effects of the MHC class II β1 domain SNP on *S. enteritidis* antibody and Ymax to SRBC. The results suggest that the characterized SNPs might be applied in marker-assisted selection to improve vaccine response and immunocompetence in chickens.

**Key words** Major histocompatibility complex; Class I and class II; Antibody response kinetics; Single nucleotide polymorphism; Inbred line.

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Introduction

The chicken major histocompatibility complex (MHC) or B complex was initially discovered as an erythrocyte antigen or serological group (Briles et al. 1950). Later, the B blood group system was identified to be linked with the MHC (Schieman and Nordskog 1961). The MHC molecules play important roles in the regulation of the immune response by communicating among different cellular components of the immune system: T cells, B cells, and antigen-presenting cells (Lamont 1998a). The crucial function of the MHC in the immune response therefore sets a priority on this promising candidate region for genetic selection to improve avian immunity.

The chicken MHC includes highly polymorphic class I (B-F) and class II (B-L) genes which encode classical MHC molecules (Hunt and Fulton 1998; Kaufman et al. 1992; Zoorob et al. 1993). The class I molecules contain an α chain and noncovalently associated β2m. The α chain genes are composed of the signal region, extracellular domains (α1, α2 and α3), transmembrane region, and cytoplasmic tail, and a peptide-binding cleft formed by folded α1 and α2 domains (Kaufman and Lamont 1996). The class II molecules contain α and β chains (Lamont 1998a). The class II α chain gene in the chicken genome is nonpolymorphic and 5 cM away from the B locus (Kaufman et al. 1995). The class II β chain genes are polymorphic and their copies vary in genetic location (Jacob et al. 2000).

Many studies have demonstrated associations between the chicken MHC and antibody production against a variety of antigens (Dunnington et al. 1992; Lakshmanan et al. 1997; Karaca et al. 1999; Weigend et al. 2001; Lamont 1998b). Antibody titers to sheep red blood cell (SRBC) and Salmonella pullorum bacterin are linked to the MHC in the chicken (Pevzner et al. 1975; Loudovaris et al. 1990). Long-term selection for antibody response to SRBC was accompanied by correlated change of MHC allelic frequencies in the chicken (Dunnington et al. 1984; Pinard et al. 1993). Studies with three MHC-related cDNA probes showed that multiple MHC regions were linked to the antibody response (Yonash et al. 1999).

The α1 and α2 domains of class I genes and class II β chain genes were selected, because of their biological significance and high variability, as candidate regions to investigate the genetic control of the humoral immune response in chickens. A unique F2 resource
population (Zhou et al. 2001), derived from a cross between two genetically distant and highly inbred lines, was used to examine associations between the genetic polymorphisms of MHC class I and class II genes and antibody response kinetics to SRBC and \textit{Brucella abortus} (\textit{B. abortus}) and antibody response to SE vaccine.

\textbf{Materials and methods}

\textit{Experimental animals}

Genetically distinct, highly inbred (> 99%) chicken lines, the Leghorn G-B1 and MHC-congenic Fayoumi M5.1 and M15.2 lines, were used as parental lines (Zhou and Lamont 1999). One sire from each Fayoumi line was mated to nine dams each of the G-B1 line. From \textit{F1} progeny of each Fayoumi sire, four males and 32 females (eight females per male) were mated to produce an \textit{F2} generation of 158 females in one hatch. The two separate branches, therefore, of the \textit{F2} population genetically differ only in the MHC allele contributed by the two MHC congenic Fayoumi sires, M5.1 and M15.2 (Zhou et al. 2001). The number of progeny (\textit{F2}) produced from M5.1 and M15.2 were 71 and 87, respectively.

\textit{Antigen administration and sample collection}

At 10 d of age, \textit{F2} chicks were administered 0.2 ml commercial \textit{S. enteritidis} vaccine (Biommune, Lenexa, KS) subcutaneously in the neck. Blood samples (1.0 ml) were collected at 11 d after vaccination. Prevaccination samples obtained from several chicks showed that pre-vaccination \textit{S. enteritidis} antibody levels were not detectable. At 19 and 23 weeks of age, the same chickens were injected intramuscularly with SRBC and \textit{B. abortus} (Zhou et al. 2001). Blood samples were obtained from the peripheral vein of the wing of each bird preimmunization, at 7 days after primary immunization, and at 4, 7, 10, 18, 32, and 63 d after secondary immunization. Sera were collected after centrifuging the blood samples and stored at \(-20\) °C until all assays were run simultaneously.

\textit{S. enteritidis antibody and agglutination assays}

The \textit{S. enteritidis} antibodies were measured by using a commercial enzyme-linked immunosorbent assay (ELISA) with modifications as described by Kaiser et al. (2002). This ELISA is a competitive assay, thus antibody response to \textit{S. enteritidis} was transformed for statistical analysis as follows:
Antibody level = 1 – \( \text{Sample}_{\text{OD630}}/\text{Neg}_{\text{OD630}} \)

where \( \text{Sample}_{\text{OD630}} \) is the sample optical density (OD) measurement at 630 nm, and \( \text{Neg}_{\text{OD630}} \) is the mean of the three negative control’s OD measurements at 630 nm. The transformed data represented the relative \( S. \text{enteritidis} \) antibody level in serum. The SRBC and \( B. \text{abortus} \) antibodies were assayed by agglutination (Zhou et al. 2001).

Genomic sequencing of the MHC class I \( \alpha 1, \alpha 2 \) domains, and class II \( \beta 1 \) and \( \beta 2 \) domains

The primers and PCR condition for the MHC class I \( \alpha 1, \alpha 2 \) domains were followed by Liu et al. (2002). A 304-bp covering the entire exon 3 (\( \alpha 2 \) domain) (Genbank, L28959) and 503-bp fragments covering exon 2 (\( \alpha 1 \) domain) and the intron between exon 2 and exon 3 (Genbank, M31012) were amplified from chicken genomic DNA.

The PCR primers (5'-CCCGCAGCGTTCTTTCTCTTTCT-3'; 5'-CCTTACCCCACGCCTG-3') were designed to amplify a 651-bp fragment covering the entire exon 2 and exon 3 (\( \beta 1 \) and \( \beta 2 \) domains) and the intron between exon 2 and exon 3 (Genbank, AJ248572) (Jacob 2000).

PCR was performed in a total volume of 25 µl, containing 25 ng of genomic DNA, 0.8 µM of each oligonucleotide primer, 2.5 µl of 10 × PCR reaction buffer, 1.5 mM MgCl2, 200 µM of each dNTP, and 1 unit of Taq DNA polymerase (Promega Corporation, Madison, WI). Cycle parameters were: 94°C for 5 min, then 35 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min, eith a final extension step of 15 min at 72°C.

Development of PCR-RFLP assays

Chicken genomic DNA was isolated from venous blood collected in EDTA. PCR was carried out with genomic DNA from one male and one female from each of the pure inbred lines (G-B1, M5.1, and M15.2) to detect potential sequence polymorphisms. The PCR products were purified by MICROCON® centrifugal filters (Millipore Corporation, Bedford, MA). Nucleotide sequencing was performed by the Iowa State University DNA Sequencing and Synthesis Facility. Sequences were analyzed using Sequencher software (Gene Codes Corporation, Ann Arbor, MI). The restriction enzyme sites on these sequences were detected by Webcutter (http://www.firstmarket.com/cutter/cut2.html. Date accessed: July 9, 2002).

Typing the \( F_2 \) population for MHC class I \( \alpha 1 \) and \( \alpha 2 \) domain and class II \( \beta 1 \) and \( \beta 2 \) domain single nucleotide polymorphisms
A PCR of DNA of each individual F2 bird was performed for each polymorphism according to the conditions described above. For the MHC class I α1 domain gene, the PCR product was digested using 1 U BsrBI (New England Biolabs, Inc., Beverly, MA) at 37°C overnight. For the MHC class I α2 domain gene, 1 U Tsp509 I (New England Biolabs, Inc., Beverly, MA) was used to digest at 65°C overnight. For the MHC class II β1 gene, 1 U MseI (New England Biolabs, Inc., Beverly, MA) was used to digest at 37°C overnight. The restriction digests were electrophoresed for 1 h at 100 V on a 2% agarose gel with ethidium bromide. Samples were scored for their individual PCR-RFLP fragment sizes, based on standard DNA molecular weight markers for each gene, by visualizing the bands under UV light.

Statistical analysis

Antibody level to SE vaccination was used as an immune response parameter for statistical analysis. The analyses of antibody response to SRBC and B. abortus were separately conducted by antigen and by phase (primary, secondary, and equilibrium). For the SRBC and B. abortus primary phase, the single time-point measurement taken at 7 d postprimary immunization (Y) was used. Secondary phase parameters of maximum titers (Ymax), time (Tmax) needed to achieve maximum titers, minimum titers (Ymin) and time (Tmin) needed to achieve minimum titers were estimated from seven individual time-point postsecondary titer values by using a nonlinear regression model (Weigend et al. 1997). The titers of the last three sample times were used to calculate the mean of the equilibrium phase (Zhou et al. 2001). This yielded a total of 10 antibody kinetics parameters analyzed in the population.

The selected genes had three levels of geneotypes defined as Leghorn homozygote (LL), heterozygote (LF), and Fayoumi homozygote (FF). The general linear model tests for associations between genotype and antibody response parameters were conducted by using the JMP® program (Sall and Lehman 1996). Values of P < 0.05 were considered significant.

Results

Sequence variation and PCR-RFLP analysis

For the MHC class I α1 domain, the amplified 503-bp fragment contained products of two loci. There were two single nucleotide polymorphisms (SNP) in one locus. The first SNP
showed an A → G, which caused an amino acid change from Gln$^{92}$ in the Leghorn line G-B1 and Fayoumi M5.1 to Arg$^{92}$ in the Fayoumi M15.2 line. The restriction enzyme BsrBI produced fragment sizes of 305 and 198 bp for the Leghorn and M5.1 lines. The second SNP showed a T → G SNP at Ala$^{99}$ from the Fayoumi 15.2 line to the Leghorn line G-B1 and Fayoumi M5.1 without amino acid change. The Fayoumi 15.2 line had fragments of 404 and 99 bp with the same enzyme digestion. The other locus showed no SNP at both sites among three inbred lines; therefore, all lines had a 503 bp fragment (Figure 1a).

For the MHC class I α2 domain, a 304-bp fragment contained products of two loci also. Two SNPs in one locus were used for PCR-RFLP assay. The first SNP showed a C → A SNP which caused an amino acid change from Phe$^{141}$ in the Leghorn G-B1 and Fayoumi M5.1 lines to Leu$^{141}$ in the Fayoumi M15.2 line. The restriction enzyme Tsp509I was used to differentiate between the Leghorn and the M15.2 line at both loci. The digested products had two line-specific fragments (154 and 150 bp) for M15.2 from one locus. The second SNP showed an A → G SNP from Asn$^{178}$ in the Leghorn line G-B1 and Fayoumi M5.1 to Ser$^{178}$ in the Fayoumi 15.2 line. The digested products had two line-specific fragments (264 and 40 bp) for Leghorn. No SNP at the other locus was found between three inbred lines within the two SNP sites. Therefore, there was a 304 bp fragment for the three lines (Fig. 1b).

For the class II β1 and β2 domains, a 651-bp fragment was obtained for all three lines. Three SNPs were uncovered in the β1 domain. Two SNPs in one codon (T → C, T → A) caused an amino acid change from Leu$^{32}$ in the Leghorn G-B1 to Thr$^{32}$ in the Fayoumi M15.2 and M5.1 lines, and one SNP (A → G) caused an amino acid change from Asn$^{81}$ in the Leghorn G-B1 and Fayoumi M15.2 lines to Asp$^{81}$ in the Fayoumi M5.1 line. Both SNPs sites could be used to differentiate between the G-B1 and the two Fayoumi lines by using the restriction enzyme MseI. Digestion of the amplified product using MseI resulted in a 402 bp fragment for both G-B1 and M15.2 lines. The Leghorn line had two additional line-specific fragments (104 and 145 bp). The Fayoumi M15.2 had line-specific fragment 249 bp. The Fayoumi M5.1 line only had a 651 bp fragment. The Fayoumi M15.2 line had a 651 bp fragment also.

*Association of the MHC class I α1, α2 domain and class II β1 domain polymorphisms with antibody response*
The \( P \) values of main effects of MHC class I \( \alpha_1 \), class I \( \alpha_2 \), and class II \( \beta_1 \) on chicken antibody response are shown (Table 1). There were significant associations (\( P < 0.05 \)) between MHC class I \( \alpha_1 \) and \( \alpha_2 \) domain gene polymorphisms and antibody response to SE, primary antibody response to \( B. \) \textit{abortus}, \( \text{Ymin} \) to SRBC, and \( \text{Ymax} \) to both SRBC and \( B. \) \textit{abortus} in F\(_2\) offspring of the M15.2 grandsire. There were significant effects (\( P < 0.05 \)) of the MHC class II polymorphisms on antibody response to SE and on \( \text{Ymax} \) to SRBC in F\(_2\) offspring of the M15.2 grandsire, and on equilibrium phase antibody response to \( B. \) \textit{abortus} in F\(_2\) offspring of the M5.1 grandsires.

Based on the general linear model analysis in F\(_2\) offspring of M15.2 grandsires, 33.1\% of phenotypic variation of antibody titers of SE can be explained by class I \( \alpha_1 \) polymorphism, 28.7\% by class I \( \alpha_2 \) polymorphism, and 24.0\% by class II \( \beta_1 \) polymorphism.

The genotype means of antibody response by MHC class I \( \alpha_1 \), \( \alpha_2 \) domain and class II \( \beta_1 \) domain are presented for the five traits showing significant (\( P < 0.05 \)) main effect of genotype in F\(_2\) offspring of the M15.2 grandsire (Table 2). The antibody response to SE of the Fayoumi homozygous type (FF) was significantly higher than the heterozygous (LF) and Leghorn homozygous type (LL) for all three candidate genes. Hens homozygous for either class I \( \alpha_1 \) or \( \alpha_2 \) alleles from the Leghorn line had a significantly lower primary response to \( B. \) \textit{abortus}. For the \( \text{Ymin} \) and \( \text{Ymax} \) to SRBC, the class I \( \alpha_1 \) and \( \alpha_2 \) Fayoumi homozygous (FF) hens had a response significantly lower than the other two genotypes. The mean of Leghorn hens homozygous (LL) for MHC class I \( \alpha_1 \) or \( \alpha_2 \) was significantly lower than the other two allelic combinations for \( \text{Ymax} \) to \( B. \) \textit{abortus}.

**Discussion**

Gene polymorphism might be utilized to improve antibody production by marker-assisted selection, if allele effects associated with antibody response can be estimated. There was general agreement of MHC allelic effect on antibody response to \( S. \) \textit{enteritidis}, primary antibody response to \( B. \) \textit{abortus} and \( \text{Ymax} \) to \( B. \) \textit{abortus}, in that the hens inheriting both alleles from the M15.2 line had significantly higher antibody response than the other two genotypes (Table 2). In contrast, the Leghorn-allele homozygous hens had significantly higher response than the Fayoumi-allele homozygous hens for \( \text{Ymin} \) and \( \text{Ymax} \) to SRBC.
Allelic effects of MHC polymorphisms on antibody response differ depending upon the type of antigen. The SRBC, a T-cell dependent antigen requiring relatively greater cooperation of T-cell to produce antibody (Munns and Lamont 1991), is a common polyvalent antigen used to stimulate a humoral immune response (Siegel and Gross 1980). The \textit{B. abortus} is a T-independent antigen which contains an intrinsic activity to directly induce the proliferation of B cells for antibody formation (Scott et al. 1994). \textit{S. enteritidis} is a T-independent antigen also (Janeway et al. 1999). Shared T-independence of \textit{B. abortus} and \textit{S. enteritidis} might explain their exhibiting the same allelic effect (Fayoumi homozygotes had significantly higher antibody response than the other two genotypes) of MHC genes on antibody response to both \textit{B. abortus} and \textit{S. enteritidis}. The different immunological pathways induced by SRBC and \textit{B. abortus}, and interactions between MHC class I and class II genes and the pathway components, might explain the different allelic effects of the MHC genes on antibody response by SRBC and \textit{B. abortus}.

There were fourteen significant instances of gene-trait associations ($P < 0.05$) from a total of fifty-two tests between the three candidate genes and 13 antibody response parameters. Thirteen of fourteen instances came from the lineage of the M15.2 grandsire, which suggests that the MHC haplotype of the Fayoumi line might have a profound effect on the ability to detect associations between the MHC alleles from the two breeds forming the cross and antibody response. Difference in the ability to detect gene-trait associations in the same two lineages were also found for interferon-\(\gamma\), interleukin-2, and immunoglobulin light chain genes (Zhou et al. 2001). Especially for antibody response to SE in the present study, there were very significant associations ($P < 0.0001$) in the lineage of M15.2 grandsire only. The SNPs identified between the Leghorn G-B1 line and the two MHC-congenic Fayoumi lines were different, both in position and in nucleotide. Different positions in a gene might have different roles in the regulation of gene expression or function. Some point mutations might change amino acids and, therefore, change the 3D protein structure thus affecting gene function (Weaver 1999).

A large amount of phenotypic variation of \textit{S. enteritidis} antibody response to vaccination was explained by the three MHC genes in the study. The phenotypic variation of antibody titers explained by these three genes is, however, not additive, because of the tight linkage of
class I α and class II β genes on the B-F/B-L region of microchromosome 16 (Kaufman et al. 1999b).

There were two loci amplified at MHC class I α1 or α2 and class II β domains in the current study, based on sequence evidence and PCR-RFLP assay of these three genes (Liu and Lamont, unpublished data). Kaufman et al. (1999a) reported that both of the major and minor loci of MHC class I could be easily amplified at the same time because of their great sequence similarity. Similar findings with the major and minor class II β chain genes were reported (Jacob et al. 2000).

*Salmonella enteritidis* is a foodborne zoonotic poultry pathogen, which is a public health problem (Nagaraja et al. 1999). One approach to improve disease resistance to *S. enteritidis* is to investigate genes conferring genetic resistance and to utilize associations of the genes with disease resistance to improve immunity by marker assisted selection. Reports on MHC effect on response to *Salmonella* are not consistent. The MHC was associated with chick mortality to *S. enteritidis* challenge in MHC-congenic chicken lines (Cotter et al. 1998). No evidence of MHC association with resistance to *S. typhimurium* was found in another study, however, when challenging partially inbred lines with *S. typhimurium* in chicks (Bumstead and Barrow 1988). A significant effect on antibody response to *S. enteritidis* vaccination of a SNP in the MHC class I α2 was found in a Leghorn by broiler cross (Lamont et al. 2002, Liu et al. 2002). Very significant associations between all three MHC genes and antibody response to *S. enteritidis* vaccination in the present study illustrates the important role of MHC and lays the foundation for its application in genetic selection to improve vaccine response to *S. enteritidis*. Of fifteen candidate genes investigated for associations with antibody response to *S. enteritidis* in the present population (unpublished data, Zhou and Lamont), only the MHC genes showed very significant associations.

The kinetics of antibody production might be of crucial in providing protection against invading pathogens. Parameter estimates, Tmin, Tmax, Ymin, Ymax and equilibrium phase, of the kinetics of the humoral immune response can be used to describe important features of antibody production, such as rapidity and strength (Weigend et al. 1997). Characteristics of antibody response kinetics in adult chickens might be an estimator of immunocompetence during the entire reproduction cycle and also of passive antibody transmitted to chicks.
Kinetic differences of primary and secondary phase antibody production to both SRBC and B. abortus were investigated in chickens of four MHC haplotypes, each on two different background lines, and the speed of reaching maximum response levels to SRBC and B. abortus was positively correlated in both primary and secondary phase, and significant interaction of MHC haplotype and sex on primary response to B. abortus was detected (Karaca et al. 1999). In chicken lines divergently selected for response to SRBC, the high antibody response line showed not only a higher peak of antibody response, but also a greater persistence of antibody level than the low antibody response line (Yang et al. 1999). High antibody lines showed higher transmission of maternal SRBC antibody to their progeny than low antibody lines among responders (Yang et al. 1999).

In conclusion, the region bearing MHC class I α1, α2 and class II β1 domains genes had significant effects on antibody response kinetics to SRBC and B. abortus and, especially, antibody level to S. enteritidis vaccination. These results set the stage for improvement of humoral immunity via genetic selection of optimal MHC genotypes.

Acknowledgements

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References


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PG (eds) *Salmonella enterica* Serovar Enteritidis in Humans and Animals. Iowa State University Press, Ames, IA, pp 397-404


**Table 1** Main effects (P values) of candidate genes polymorphisms on chicken antibody parameters of primary, secondary, and equilibrium phase to sheep red blood cell (SRBC) and *Brucella abortus* and vaccine response to *Salmonella enteritidis* (*S. enteritidis*) in F₂ progeny of M5.1 and M15.2 MHC-congenic grandsires

<table>
<thead>
<tr>
<th>Grandsire MHC</th>
<th>Gene</th>
<th>S. enteritidis</th>
<th>SRBC</th>
<th>Brucella abortus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Y</em></td>
<td><em>Tmin</em></td>
<td><em>Tmax</em></td>
</tr>
<tr>
<td>M5.1</td>
<td>class II β1 domain</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>M15.2</td>
<td>class I α1 domain</td>
<td>0.0001</td>
<td>0.18</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>class I α2 domain</td>
<td>0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>class II β1 domain</td>
<td>0.0001</td>
<td>NS</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*a*Primary antibody response to *Salmonella enteritidis*

*b*Primary antibody response

*c*Time required to achieve minimum secondary antibody titers

*d*Minimum secondary antibody titers

*e*Time required to achieve maximum secondary antibody titers

*f*Maximum secondary antibody titers

*g*Equilibrium phase antibody titers

*h*NS, *P > 0.20*
Table 2 Mean antibody parameters by genotype of MHC class I α1 and α2 domain genes and class II β1 and β2 domain genes in F<sub>2</sub> progeny of the M15.2 grandsire

<table>
<thead>
<tr>
<th>Trait</th>
<th>Class I α1 domain</th>
<th>Class I α2 domain</th>
<th>Class II β1 domain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>LF&lt;sup&gt;b&lt;/sup&gt;</td>
<td>FF&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S. enteritidis&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.65&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.67&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.88&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>YB&lt;sup&gt;e&lt;/sup&gt; (titer log2)</td>
<td>9.46&lt;sup&gt;A&lt;/sup&gt;</td>
<td>10.47&lt;sup&gt;B&lt;/sup&gt;</td>
<td>10.44&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>YminS&lt;sup&gt;f&lt;/sup&gt; (titer log2)</td>
<td>4.06&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.75&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.93&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>YmaxS&lt;sup&gt;g&lt;/sup&gt; (titer log2)</td>
<td>5.71&lt;sup&gt;A&lt;/sup&gt;</td>
<td>5.72&lt;sup&gt;A&lt;/sup&gt;</td>
<td>4.80&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>YmaxB&lt;sup&gt;h&lt;/sup&gt; (titer log2)</td>
<td>7.78&lt;sup&gt;A&lt;/sup&gt;</td>
<td>9.07&lt;sup&gt;B&lt;/sup&gt;</td>
<td>8.78&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>A</sup>-<sup>B</sup>Means in a row within a gene with no common superscript differ significantly (P < 0.05)
<sup>a</sup>Leghorn homozygote
<sup>b</sup>Heterozygote of Leghorn and Fayoumi genotype
<sup>c</sup>Fayoumi homozygote
<sup>d</sup>Antibody response to SE
<sup>e</sup>Primary antibody response to Brucella abortus
<sup>f</sup>Minimum secondary antibody titers to SRBC
<sup>g</sup>Maximum secondary antibody titers to SRBC
<sup>h</sup>Maximum secondary antibody titers to Brucella abortus
**Fig. 1** Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). LL = Leghorn homozygote, LF = heterozygote, FF = Fayoumi homozygote. A MHC class I α1 domain gene with *BsrB* I digestion. B MHC class I α2 domain gene with *Tsp509* I digestion.
CHAPTER 8. ASSOCIATION OF TRANSFORMING GROWTH FACTOR β GENES WITH QUANTITATIVE TRAIT LOCI FOR ANTIBODY RESPONSE KINETICS IN HENS

A paper submitted to Animal Genetics

H. Zhou and S. J. Lamont

Abstract

Transforming growth factor β2 (TGF-β2), 3, and 4 genes, as physiological and positional candidate genes for chicken antibody response, were selected to investigate associations with vaccine response to Salmonella enteritidis (S. enteritidis) and antibody kinetics to sheep red blood cells (SRBC) and Brucella abortus (B. abortus). Primary antibody response after S. enteritidis vaccination at day 10, and antibody response to SRBC and killed B. abortus after immunization at 19 and 22 weeks, were measured in a F₂ population. The resource population was derived from males of two highly inbred major histocompatibility complex (MHC)-congenic Fayoumi chicken lines (named M5.1 and M15.2 ) mated with highly inbred Leghorn G-B1 hens. Secondary phase parameters of maximum titers (Ymax) and time required to achieve Ymax (Tmax) were estimated from post-secondary titers by using a non-linear regression model. Associations of single nucleotide polymorphisms (SNP) in TGF-β2, 3, and 4 genes with antibody response parameters were evaluated. Multiple immune response parameters were significantly associated with the TGF-β2 gene, suggesting that TGF-β2 or linked genes affect antibody response in hens. Significant main effects of the three genes were mostly found in the lineage of the M5.1 grandsire. Significant two-way interactions on antibody response were primarily detected between TGF-β3 and TGF-β4 genes, and in the lineage of the M15.2 grandsire. Effects preferentially detectable in one of the MHC-congenic lineages suggest that there is interaction between the MHC and TGF-β genes. The

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characterized TGF-β SNPs might be applied in marker-assisted selection to improve antibody production.

Keywords transforming growth factor β genes, antibody response kinetics, single nucleotide polymorphism, step-wise elimination procedure, inbred line.

Introduction

Improving health is a major goal in the animal genetics industry, because of direct and indirect economic costs caused by pathogen infection (Dunnington et al. 1992). Various immune mechanisms are involved in defending the organism against infectious diseases (Pinard-van der Laan 2002). In previous studies, selecting for high immune response was thought to indirectly improve general disease resistance (Biozzi et al. 1979; van der Zijpp 1983; Warner et al. 1987). Investigation of genetic control of immune response can be used to genetically improve immunocompetence and, thus, animal health. Most facets of the immune response are likely controlled by polygenes. Both physiological genetic and molecular genetic approaches can be utilized to investigate genetic control of immune response (Lamont 1998b).

The transforming growth factor β (TGF-β) family was discovered two decades ago as a superfamily of regulatory peptides (Wahl 1999). The TGF-β family of proteins is a set of pleiotropic secreted signaling molecules with unique and potent immunoregulatory properties (Letterio & Roberts 1998). The TGF-β were recognized as not only growth factors, but as immune modulatory cytokines also affecting the function and phenotype of lymphocytes, dendritic cells and macrophages (Lebman & Edmiston; Strobl & Knapp 1999; Ashcroft 1999). The TGF-β superfamily genes play an essential role in regulating immune function (Ruscetti & Palladino 1991; McCartney-Francis & Wahl 1994). From their differentiation to their activation and proliferation, T lymphocytes are influenced by TGF-β genes (Letterio & Roberts 1998). The TGF-β can inhibit IgM, IgD, IgA and transferrin receptor, and induce MHC class II expression on both pre-B and mature B cells (Stavnezer 1996).

There are four known TGF-β family genes in the chicken: TGF-β1, TGF-β2, TGF-β3, and TGF-β4 (Jakowlew et al. 1988a, b, c; Jakowlew et al. 1990; Burt & Paton 1992; Burt & Law 1994; Burt & Jakowlew 1992). The TGF-β1, TGF-β2 and TGF-β3 have been mapped on
linkage group E25C31, Chromosome 3, and 5, respectively, (Smith et al. 2000; Moris & Burt 1995; Burt et al. 1995) and TGF-β4 has not been mapped (Groenen et al. 2000). Chicken TGF-β4 mRNA level has been associated with immune response to coccidiosis (Choi et al. 1999; Min et al. 2001). Chicken TGF-β1 may suppress immune responses by maintaining or by promoting the development of suppressor T cells and suppressing the proliferation of B cells (Quere & Thorbecke 1990).

Two basic methods are generally used to identify quantitative trait loci (QTL) responsible for genetic variation: the candidate gene approach and linkage mapping by anonymous markers (Rothschild & Soller 1997). There are two ways to choose candidate genes, either by biological or physiological relation to the trait of interest, or after QTL linkage analyses, called positional candidate gene. Previous linkage mapping studies identified QTLs affecting chicken antibody response kinetics to regions of Chromosome 3 and 5 nearby TGF-β2 and TGF-β3 (Zhou et al. 2002). Therefore, both positional mapping on antibody response and physiological significance for various immune traits in chickens make TGF-β genes logical candidates for investigation of effects on chicken antibody response kinetics.

The objective of the present study was to identify polymorphisms in chicken TGF-β gene, and evaluate associations between TGF-β single nucleotide polymorphisms (SNP) and antibody response.

Materials and methods

Resource population

Genetically distinct, highly inbred (> 99%) chicken lines, the Leghorn G-B1 and MHC-congenic Fayoumi M5.1 and M15.2 lines, were used as parental lines (Zhou & Lamont 1999). One sire from each Fayoumi line was mated to nine dams each of the Leghorn G-B1 line. Four F1 males from each Fayoumi sire was each mated to eight females to produce an F2 generation of 158 females in one hatch. The two separate lineages, therefore, of the F2 population genetically differ only in the MHC allele contributed by the two MHC-congenic Fayoumi grandsires, Fayoumi M5.1 and Fayoumi M15.2 (Zhou et al. 2001). The number of F2 progeny produced from M5.1 and M15.2 were 71 and 87, respectively.
Antigen administration and sample collection

At 10 days of age, F2 chicks were administered 0.2 ml commercial Salmonella enteritidis (S. enteritidis) vaccine (Biomune, Lenexa, KS) subcutaneously in the neck. Blood samples (1.0 ml) were collected at 11 day after vaccination. Prevaccination samples obtained from several chicks showed that pre-vaccination S. enteritidis antibody levels were not detectable. At 19 and 23 weeks of age, the same chickens were injected intramuscularly with sheep red blood cell (SRBC) and Brucella abortus (B. abortus) (Zhou et al. 2001). Blood samples were obtained from the peripheral vein of the wing of each bird preimmunization, at 7 days after primary immunization, and at 4, 7, 10, 18, 32, and 63 days after secondary immunization. Sera were collected after centrifuging the blood samples and stored at -20 °C until all assays were run simultaneously.

S. enteritidis antibody and agglutination assays

The S. enteritidis antibodies were measured by using a commercial enzyme-linked immunosorbent assay (ELISA) with modifications as described by Kaiser et al. (2002). This ELISA is a competitive assay, thus antibody response to S. enteritidis was transformed for statistical analysis as follows:

\[
\text{Antibody level} = 1 - \left( \frac{\text{Sample}_{\text{OD630}}}{\text{Neg}_{\text{OD630}}} \right)
\]

where Sample_{\text{OD630}} is the sample optical density (OD) measurement at 630 nm, and Neg_{\text{OD630}} is the mean of the three negative control’s OD measurements at 630 nm. The transformed data represented the relative S. enteritidis antibody level in serum. The SRBC and B. abortus antibodies were assayed by agglutination (Zhou et al. 2001).

Genomic sequencing of chicken TGF-β 2, 3, and 4 genes

TGF-β2. The PCR primers (forward: 5'-GCCATAGGTTCAGTGCAAG-3'; reverse: 5'-TTTATTCCCCCTCCGAGCC-3') were designed to amplify a 620-bp fragment covering the
promoter region by Oligo 5 (National Bioscience, Inc., Plymouth, MN) according to chicken genomic sequence (Genbank, X58071). The PCR was performed in a total volume of 25 μl, containing 25 ng of genomic DNA, 0.8 μM of each oligonucleotide primer, 2.5 μl of 10 x PCR reaction buffer, 1.5 mM MgCl₂, 200 μM of each dNTP, and 1 unit of Taq DNA polymerase (Promega Corporation, Madison, WI). Cycle parameters were: 94°C for 5 min, then 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, eith a final extension step of 15 min at 72°C. To develop a more convenient PCR-RFLP assay for the TGF-β2 gene, a new reverse primer (5'-TGACAGAAGCTCTCTCAAGCC-3') was designed to amplify a 284-bp fragment. PCR conditions were the same as for the above except the annealing temperature was 52°C.

TGF-β3. The primers (Forward: 5'-CGGCCTGGAAATCAGCATAC-3'; Reverse: 5'-GAAGCAGTAGTTGGTATCCAG-3') were designed to amplify a 1078-bp fragment covering part of exon 4, intron 4, and part of exon 5 (Genbank, X60091). The PCR conditions were the same as for the TGF-β2 gene, except the annealing temperature was 56°C.

TGF-β4. Spleen total RNA was isolated using ToTALLY RNA™ Kit (Ambion, Inc., Austin, TX). First-strand cDNA was synthesized using the RETROscript™ First Strand Synthesis Kit for RT-PCR (Ambion, Inc., Austin, TX). The primers (Forward: 5'-CCGACGCGGTCCATCTGTCG-3'; Reverse: 5'-TGAAGGGGTTGCTGGGTC-3') were designed to amplify a 620-bp fragment from cDNA (Genbank, M31160). The PCR condition was the same as for TGF-β2 gene except the annealing temperature was 66°C, 5% dimethyl sulfoxide was added in the reaction. Based on mutation found in the parental lines, new primers (Forward: 5'-GGGGTCTTCAAGCTGAGCGT-3'; Reverse: 5'-TTGGCAATGCTCTGAGCGT-3') were designed to amplify a 240-bp fragment from genomic DNA. The PCR conditions were the same as for the TGF-β3 gene.

Development of PCR-RFLP assays
Chicken genomic DNA was isolated from venous blood collected in EDTA. The PCR was carried out with genomic DNA from one male and one female from each of the highly inbred lines (Leghorn G-B1, Fayoumi M5.1 and M15.2) to detect potential sequence polymorphisms. The PCR products were purified by MICROCON® centrifugal filters (Millipore Corporation, Bedford, MA). Nucleotide sequencing was performed by the Iowa State University DNA Sequencing and Synthesis Facility. Sequences were analyzed using Sequencher\textsuperscript{3.1} software (Gene Codes Corporation, Ann Arbor, MI). The restriction enzyme sites on these sequences were detected by Webcutter\textsuperscript{2.0} (http://www.firstmarket.com/cutter/cut2.html. Date accessed: July 10, 2002)

Typing the F\textsubscript{2} population with TGF-\(\beta\)2, 3, and 4 genes

A PCR of DNA of each individual F\textsubscript{2} bird was performed for each gene according to the conditions described above. The PCR products of TGF-\(\beta\)2, TGF-\(\beta\)3, and TGF-\(\beta\)4 were digested at 37°C overnight with 1 U \textit{Rsa} I, \textit{Bsr} I, and 1 U \textit{Mbo} II (New England Biolabs, Inc., Beverly, MA), respectively. The restriction digests were electrophoresed for 1 h at 100 V on a 2% agarose gel with ethidium bromide. Individual PCR-RFLP fragment sizes in each sample were determined, based on standard DNA molecular weight markers for each gene, by visualizing the banding pattern under UV light.

Statistical analysis

Antibody level to \textit{S. enteritidis} vaccination at 11 days postvaccination was used as an immune response parameter for statistical analysis. The analyses of antibody response to SRBC and \textit{B. abortus} were separately conducted by antigen and by phase (primary, secondary, and equilibrium). For the SRBC and \textit{B. abortus} primary phase, the single time-point measurement taken at 7 day postprimary immunization (Y) was used. Secondary phase parameters of maximum titers (Y\textsubscript{max}), time (T\textsubscript{max}) needed to achieve maximum titers were estimated from seven individual time-point postsecondary titer values by using a nonlinear
regression model (Weigend et al. 1997). The titers of the last three sample times were used to calculate the mean of the equilibrium phase (Zhou et al. 2001). This yielded a total of eight antibody kinetics parameters analyzed.

Data were analyzed separately for F₂ progeny from the two different MHC-congeneric grandsires. The statistical analysis began with a full model as follows:

\[
Y_{ijkl} = \mu + TGF-\beta_2_i + TGF-\beta_3_j + TGF-\beta_4_k + TGF-\beta_2_i \times TGF-\beta_3_j + TGF-\beta_2_i \times TGF-\beta_4_k + TGF-\beta_3_j \times TGF-\beta_4_k + e_{ijkl}
\]

where \( Y_{ijkl} \) is defined as the dependent traits (\( Y, Y_{\text{max}}, \) and \( T_{\text{max}} \) etc). Then, a step-wise elimination procedure was performed to eliminate any interaction effect of \( P > 0.2 \) until \( P \) values of all interaction effects were \( P < 0.2 \). The genes had three levels of genotypes designated as Leghorn homozygote (LL), heterozygote (LF), and Fayoumi homozygote (FF).

The general linear model tests for associations between genotype and antibody response parameters were conducted by using the JMP® program (Sall and Lehman 1996). Values of \( P < 0.05 \) were considered significant.

**Results**

**Sequence variation and PCR-RFLP analysis**

For TGF-\( \beta_2 \), the amplified 284-bp product showed a C → T SNP between the Leghorn line and the Fayoumi lines at base -640 (Genbank X58071). The restriction enzyme Rsa I produced fragment sizes of 184 and 100 bp for the Fayoumi lines, whereas the Leghorn line had no cut site (Fig. 1a).

For TGF-\( \beta_3 \), a 1078-bp product was sequenced. An A → C SNP was identified between the Leghorn and the two Fayoumi lines at Base 2833 (Genbank X60091). The Bsr I digested products had 403, 119, 44, and 12 bp for both lines, two line-specific fragments (296 and 204 bp) for Leghorn, and a line-specific fragment (500 bp) for Fayoumi lines (Fig. 1b).

For TGF-\( \beta_4 \), the amplified 240-bp product showed a C → A SNP, which caused a predicted amino acid change from Glu in the Leghorn line to Asp in the Fayoumi lines. The
restriction enzyme *Mbo* II produced fragment sizes of 173 and 67 bp for the Leghorn lines, whereas the Fayoumi lines had a 240-bp fragment (Fig. 1c).

Associations of the three TGF-β genes polymorphisms with antibody response

The *P* values of main effects and two-way interactions of TGF-β2, 3 and 4 gene SNP on chicken antibody response are shown in Table 1. The TGF-β2 polymorphism had the most frequent associations with antibody response parameters, and these were all in the lineage of the M5.1 grandsire. For F₂ offspring of the M5.1 grandsire, there were significant associations (*P* < 0.05) between the TGF-β2 polymorphism and primary antibody response and Ymax to SRBC and BA and equilibrium antibody response to BA, and between the TGF-β3 polymorphism and primary antibody response and Tmax to SRBC. There were significant (*P* < 0.05) effects of the two-way interaction of TGF-β3 by TGF-β4 on Ymax to *B. abortus*. For F₂ offspring of the M15.2 grandsire, there were significant effects (*P* < 0.05) of the TGF-β2 polymorphism on antibody response to *S. enteritidis*, and between TGF-β4 and Tmax of SRBC, and of the two-way interaction of TGF-β3 and TGF-β4 on Tmax of SRBC and equilibrium antibody response to *B. abortus*, and of TGF-β2 and TGF-β4 on primary antibody response to SRBC.

Allelic effect of TGF-β genes on antibody response

The allelic effects of TGF-β2, 3 and 4 gene polymorphisms on antibody response are presented in Table 2 for the nine instances of significant (*P* < 0.05) main effects of genotype. For comparison purposes, the effects of the candidate gene alleles in both MHC-congenic lineages are presented, even though significant differences usually occurred in one only. Most of the candidate gene main effects (six out of nine) were detected in the M5.1 grandsire lineage. For the primary response to SRBC, the mean of the TGF-β2-FF hens was significantly lower than the other two allelic combinations. For Ymax to SRBC with hens heterozygous for the TGF-β2 polymorphism, had significantly lower than the two homozygotes. For the primary and equilibrium response to *B. abortus*, however, the hens
with heterozygote had a significantly higher response than the Leghorn homozygote. For the secondary response to *B. abortus*, the hens inheriting both TGF-β2 alleles from the Fayoumi line had a significantly higher Ymax. For the primary response to SRBC with TGF-β3 polymorphism, the TGF-β3 heterozygous hens had a response significantly higher than the Leghorn, but not the Fayoumi, homozygote. For the TGF-β3 effect on Tmax of SRBC, the Leghorn homozygous hens required a significantly longer time to achieve maximum secondary antibody response than the other genotypes. Only two main effects were detected in the M15.2 lineage. For TGF-β2 effect on antibody response to *S. enteritidis* vaccination, the heterozygous and the Fayoumi-allele homozygous hens had significantly different responses from each other (heterozygote was higher) but neither differed from the Leghorn-allele homozygous group. For TGF-β4 effect on Tmax of SRBC, the heterozygous hens required a significantly longer time to achieve maximum secondary antibody response than the other two homozygous.

**Effect of candidate gene allelic interactions on antibody response**

To display and test the individual two-way interactions, the four significant gene allelic interactions (Table 1) are presented in graphic form (Figure 2), and the means and standard errors of these traits in each genotype are given (Table 3). For Ymax to BA, the response of hens inheriting two Fayoumi alleles for both TGF-β3 and TGF-β4 genes was higher than hens with heterozygote of TGF-β4 and the Fayoumi homozgote of TGF-β3. Within the TGF-β4-LF genotype, birds from the TGF-β3-LL genotype had a significantly (P < 0.05) higher maximum secondary antibody titer to *B. abortus* than birds from the TGF-β3-FF and TGF-β3-LF genotypes. (Figure 2a).

For F₂ offspring of the M15.2 grandsire, birds of the TGF-β3-LL, TGF-4β-LF genotype combination had greater Tmax to SRBC than any other allelic combination of these two genes (Figure 2b). Birds from the TGF-4β-LL genotype had a significantly (P < 0.05) higher equilibrium antibody titer to *B. abortus* than birds from the TGF-4β-LF genotype, within the TGF-3β-LL genotype. Hens inheriting two Leghorn or two Fayoumi alleles for both TGF-β3 and TGF-β4 genes had higher equilibrium antibody response to *B. abortus* than hens with
combination of TGF-β3-FF and TGF-β4-LL (Figure 2c). Within the TGF-β2-LL genotype, birds from the TGF-4β-LF genotype had significantly ($P < 0.05$) higher primary antibody titer to SRBC than birds from the TGF-β4-LL and TGF-4β-FF genotypes. Within TGF-β4-LF the genotype, hens from the TGF-β2-LL genotype had significantly higher primary antibody response to SRBC than hens from the TGF-β2-LF (Figure 2d).

**Discussion**

Three members of chicken TGF-β superfamily of genes, TGF-β2, TGF-β3, and TGF-β4, were chosen to conduct association studies with antibody response to *S. enteritidis* vaccination and with antibody response kinetics to SRBC and *B. abortus*. The linkage disequilibrium that exists in a F₂ population cross of divergent lines is such that the SNP are expected to serve as markers for the entire gene as well as other nearby linked genes. The TGF-β2 promoter polymorphism does not lie in any of the important regulatory elements identified by sequence analysis (Burt et al. 1991). For the TGF-β3 gene, a neutral mutation was found in the intron region. The neutral polymorphic sites may be in linkage disequilibrium with functional allelic variants in the candidate genes (Rothschild & Soller 1997). The TGF-β4 exon polymorphism caused a predicted amino acid change from the Leghorn line to the Fayoumi line, which might change protein function.

The TGF-β genes play a pivotal role in tissue homeostasis and development of multicellular components in chickens (O'Keefe et al. 1988). The TGF-β genes are involved with the development, growth and fitness traits in chickens (Cogburn et al. 2000; Li et al. 2002). However, there are few studies of TGF-β effects on immune response in chickens. The TGF-β2 and TGF-β3 genes were associated with antibody response to *S. enteritidis* vaccination and bacterial burden to *S. enteritidis* in cecum, respectively, in a broiler by inbred lines cross resource population (Lamont and Malek 2002). In the present study, multiple immune response parameters (six traits) were significantly associated with TGF-β2 genes, which suggests that TGF-β2 or other genes in the region bearing TGF-β2, are associated with antibody response kinetics to SRBC and *B. abortus* in hens. There were fewer instances of significant main effects of TGF-β3 (two) and TGF-β4 (one) in the study,
however, there were four instances of significant two-way interaction effects of TGF-β3 and TGF-β4 genes on antibody response. The TGF-β genes transduct signals by binding to membrane receptors, phosphorylating proteins of the SMAD family, and then SMAD bind DNA and recruit transcription factors to control gene expression (Massague et al. 2000; Miyazono 2000). In mammals, different TGF-β genes may share the same membrane receptors (Massague 2000), which may explain how TGF-β genes interact with each other via signal pathways to affect gene expression. The interaction effects of TGF-β3 and TGF-β4 genes illustrate that certain allelic combinations of the two genes are detrimental or beneficial to antibody production.

More significant associations of TGF-β genes with antibody response were found in the lineage of the Fayoumi M5.1 grandsire than the M15.2 grandsire. The chicken MHC plays an essential role in genetic control of antibody production (Bacon 1987; Lamont 1998a; Rothschild et al. 2000). The TGF-β genes are not linked with the chicken MHC, however, this does not exclude the potential interaction between signal transduction of TGF-β genes and expressed products of the MHC during the process of antibody production. The two grandsires of the present resource population are MHC-congenic, therefore, suggesting that the different effects of TGF-β genes detected in the two lineages reflect the interaction of MHC with TGF-β genes on antibody response.

The TGF-β2 genes were significantly associated with both primary and maximum secondary antibody response to SRBC and *B. abortus* in the current study. However, allele effects were different between the two antigens. The advantage conferred by the Leghorn alleles appears to be dominant, in that homozygous and heterozygous Leghorn-allele hens outperform the Fayoumi homozygotes in primary antibody production to SRBC, whereas it appears to be overdominant in primary antibody production to *B. abortus*. For maximum secondary antibody response, hens of both homozygotes had higher antibody production to SRBC than that of heterozygotes, whereas hens with the Fayoumi-derived alleles had significant higher antibody production to *B. abortus* than that of other two TGF-β2 genotypes. Different allele effects between SRBC and *B. abortus* antigens were previously reported for the interferon γ gene (Zhou et al. 2001) and MHC class I and class II genes.
(Zhou and Lamont 2002, unpublished data) in the same resource population. SRBC and *B. abortus* are two distinct types of antigens. SRBC, a T-cell dependent antigen, requires relatively greater cooperation of T-cells with B-cells to produce antibody (Munns & Lamont 1991), whereas *B. abortus*, a T-cell independent antigen (Toivanen et al. 1972), stimulates B cells with little assistance from T helper cells (Scott et al. 1994). The different pathways used for induction of antibody formation to SRBC and *B. abortus* might explain the different allele effect of TGF-β2 genes detected for antibody response to SRBC and *B. abortus*.

Disease outbreaks and expenses involved in vaccination and biosecurity program have a great economic impact on commercial animal production (Dunnington et al. 1992). Genetic resistance to disease could be an effective tool to control diseases for livestock (Warner et al. 1987). Phenotypic data for pathogen challenge is very difficult to collect practically and commercial breeding populations cannot be exposed to infectious pathogens for the purpose of selection for disease resistance. Furthermore, selection for resistance to a specific disease tends to be specific, and has little effect on general disease resistance (Gavora 1990). Genetic selection to improve immune response provides an alternative to direct disease challenge to select animals that resist a wider range of diseases (Boa-Amponsem et al. 1997). Cross et al. (1980) demonstrated higher antibody response to Newcastle disease, and greater resistance to *Mycoplasma gallisepticum* and *Eimeria necatrix* in a line selected for high antibody response to SRBC, than in the low-antibody selected line. Subsequent studies suggested that the high line had greater resistance to *Eimeria tenella* (Martin et al. 1986). The SRBC are complex, multideterminant natural antigens, which are similar in structure to most common pathogens (van der Zijpp et al. 1983). Discovery of significant associations of TGF-β genes with antibody response to SRBC and *B. abortus* illustrates their promising value for application in marker-assisted selection to improve chicken health.

**Acknowledgements**

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References


genes with response to *Salmonella enteritidis* in poultry. *Genetics, Selection and Evolution* Submitted.


Table 1. Main effects (P-values) and two-way interaction effects of transforming growth factor-β (TGF-β) polymorphisms on chicken antibody response to SRBC, *Brucella abortus* and *Salmonella enteritidis (S. enteritidis)* antibody response in F₂ progeny of M5.1 and M15.2 grandsires

<table>
<thead>
<tr>
<th>Grandsire MHC</th>
<th>Gene(s)</th>
<th>S. enteritidis¹</th>
<th>SRBC</th>
<th>Brucella abortus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Y²</td>
<td>Tmax³</td>
<td>Ymax⁴</td>
</tr>
<tr>
<td>M5.1</td>
<td>TGF-β2</td>
<td>NS⁶</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TGF-β3</td>
<td>NS</td>
<td>0.03</td>
<td>0.002</td>
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<tr>
<td></td>
<td>TGF-β4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TGF-β2* TGF-β3</td>
<td>EIE⁷</td>
<td>EIE</td>
<td>EIE</td>
</tr>
<tr>
<td></td>
<td>TGF-β2* TGF-β4</td>
<td>EIE</td>
<td>EIE</td>
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</tr>
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<td>TGF-β3* TGF-β4</td>
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<td>EIE</td>
<td>EIE</td>
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<td>TGF-β2</td>
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<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
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<tr>
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<td>EIE</td>
<td>EIE</td>
<td>EIE</td>
</tr>
<tr>
<td></td>
<td>TGF-β2* TGF-β4</td>
<td>EIE</td>
<td>0.04</td>
<td>EIE</td>
</tr>
<tr>
<td></td>
<td>TGF-β3* TGF-β4</td>
<td>0.09</td>
<td>EIE</td>
<td>0.04</td>
</tr>
</tbody>
</table>

¹Antibody response to *Salmonella enteritidis*
²Primary antibody response
³Time required to achieve maximum secondary antibody titer
⁴Maximum secondary antibody titer
⁵Equilibrium phase antibody titer
⁶NS, P > 0.20
⁷EIE, Eliminated interaction effect during step-wise statistical procedure, P > 0.20.
Table 2. Mean antibody parameters, by genotype, of transforming growth factor-β (TGF-β) genes in F₂ offspring of M5.1 and M15.2 grandsire.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Trait</th>
<th>M5.1</th>
<th>M15.2</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>LL</td>
<td>LF</td>
</tr>
<tr>
<td>TGF-β2</td>
<td><em>S. enteritidis</em>⁶</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YS* (titer log2)</td>
<td>0.35ᵇ</td>
<td>0.37ᵃ</td>
</tr>
<tr>
<td></td>
<td>YmaxS₈ (titer log2)</td>
<td>5.41ᵇ</td>
<td>5.49ᵃ</td>
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<td></td>
<td>YB₉ (titer log2)</td>
<td>5.82ᵇ</td>
<td>5.00ᵇ</td>
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<tr>
<td></td>
<td>YmaxB¹⁰ (titer log2)</td>
<td>9.14ᵇ</td>
<td>10.74ᵇ</td>
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<tr>
<td></td>
<td>Equil.B¹¹ (titer log2)</td>
<td>8.19ᵇ</td>
<td>8.40ᵇ</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>YS (titer log2)</td>
<td>7.53ᵇ</td>
<td>6.64ᵇ</td>
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<tr>
<td></td>
<td>TmaxS¹² (day)</td>
<td>3.75ᵇ</td>
<td>5.76ᵇ</td>
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<tr>
<td></td>
<td>TmaxS¹² (day)</td>
<td>18.49ᵇ</td>
<td>9.58ᵇ</td>
</tr>
<tr>
<td>TGF-β4</td>
<td>TmaxS (day)</td>
<td>14.80ᵇ</td>
<td>11.03ᵃ</td>
</tr>
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</table>

ᵃᵇMeans in a row within a grandsire MHC type with no common superscript differ significantly (P < 0.05).
¹F₂ offspring of M5.1 grandsire.
²F₂ offspring of M15.2 grandsire.
³Leghorn homozygote.
⁴Heterozygote of Leghorn and Fayoumi genotype.
⁵Fayoumi homozygote.
⁶Antibody response to *Salmonella enteritidis*.
⁷Primary antibody response to SRBC.
⁸Maximum secondary antibody titers to SRBC.
⁹Primary antibody response to *Brucella abortus*.
¹⁰Maximum secondary antibody titers to *Brucella abortus*.
¹¹Equilibrium phase antibody titers to *Brucella abortus*.
¹²Time required to achieve maximum secondary antibody titers to SRBC.
Table 3. Mean and standard error of interaction effects of transforming growth factor-β (TGF-β) 3 and TGF-β 4 on Ymax antibody response to Brucella abortus (BA) in F2 offspring of M5.1 grandsire and on Tmax to SRBC and equilibrium antibody response to BA, and interaction effect of TGF-β2 and TGF-β4 on primary antibody response to SRBC in F2 offspring of M15.2 grandsire.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>YmaxB (titer log2)</th>
<th>to SRBC</th>
<th>TmaxS (d)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>LL</td>
<td>LF</td>
<td>FF</td>
</tr>
<tr>
<td>TGF-β3</td>
<td></td>
<td>10.85 ± 1.56</td>
<td>8.67 ± 0.52</td>
<td>9.52 ± 0.65</td>
</tr>
<tr>
<td>TGF-β4</td>
<td>LL</td>
<td>9.99 ± 0.62</td>
<td>7.44 ± 0.55</td>
<td>7.37 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>LF</td>
<td>7.93 ± 0.96</td>
<td>9.13 ± 0.83</td>
<td>9.39 ± 0.63</td>
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</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Equil.B (titer log2)</th>
<th>Primary antibody response to SRBC</th>
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<td></td>
<td></td>
<td>LL</td>
<td>LF</td>
</tr>
<tr>
<td>TGF-β3</td>
<td></td>
<td>8.68 ± 0.69</td>
<td>7.60 ± 0.48</td>
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<tr>
<td>TGF-β4</td>
<td>LL</td>
<td>6.88 ± 0.49</td>
<td>7.90 ± 0.30</td>
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<tr>
<td></td>
<td>LF</td>
<td>7.35 ± 0.62</td>
<td>7.30 ± 0.41</td>
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1. LL = Leghorn homozygote; LF = heterozygote of Leghorn and Fayoumi genotype; FF = Fayoumi homozygote.
2. Effect ± SEM.
Figure 1. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) pattern for transforming growth factor-β (TGF-β) genes. (a) TGF-β2 promoter region with \textit{Rsa} I digestion. (b) TGF-β3 intron 4 with \textit{Bsr} I digestion. (c) TGF-β4 exon region with \textit{Mbo} II digestion. \textbf{L} = Granddam (Leghorn) allele; \textbf{F} = Grandsire (Fayoumi) allele. The numbers listed on the right of the figure are fragment sizes (bp).
TGFB4-FF
TGFB4-LF
TGFB4-LL

(a)

(b)

Genotype

TGFB4-FF
TGFB4-LF
TGFB4-LL

Genotype

(c)

(d)

TGFB4-FF
TGFB4-LF
TGFB4-LL

Genotype

TGFB4-FF
TGFB4-LF
TGFB4-LL

Genotype

TGFB4-FF
TGFB4-LF
TGFB4-LL

Genotype
Figure 2. Mean antibody response parameters to SRBC and *Brucella abortus* (*B. abortus*) by pairwise combination of transforming growth factor-β (TGF-β) genes. Tmax is time required to achieve the maximum secondary antibody titers; Y_max is the maximum secondary antibody titers; (a) Effect of TGF-β3 by TGF-β4 genotype on Y_max to *B. abortus* in F_2 offspring of M5.1 grandsire. (b) Effect of TGF-β3 by TGF-β4 genotype on Tmax to SRBC in F_2 offspring of M15.2 grandsire. (c) Effect of TGF-β3 by TGF-β4 genotype on equilibrium antibody response to *B. abortus* in F_2 offspring of M15.2 grandsire. (d) Effect of TGF-β2 by TGF-β4 genotype on primary antibody response to SRBC in F_2 offspring of M15.2 grandsire. Two statistical contrasts are presented. The first letter above each bar represents the comparison of antibody response means of TGF-β4 genotypes within TGF-β2 or TGF-β3 genotype. The second letter above each bar represents the comparison of antibody response means of TGF-β2 or TGF-β3 genotype with TGF-β4 genotypes. *a,b* Means bearing no common superscript (comparing first letter and second letter separately) are significantly different (*P* < 0.05). LL = Leghorn homozygote; FF = Fayoumi homozygote; LF = heterozygote.
CHAPTER 9. ASSOCIATIONS OF SIX CANDIDATE GENES WITH ANTIBODY RESPONSE KINETICS IN HENS

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Huajun Zhou and Susan J. Lamont

ABSTRACT

The chicken B cell marker (ChB6), caspase-1, inhibitor of apoptosis protein-1 (IAP-1), interleukin-15 receptor α chain (IL-15Rα), interleukin-2 receptor γ chain (IL-2Rγ) and immunoglobulin supfamily gene (ZOV3), as physiological candidate genes for chicken immune response, were selected to investigate associations with antibody kinetics to sheep blood cells (SRBC) and killed Brucella abortus (B. abortus). An F2 population was derived from mating highly inbred (> 99%) males of two MHC-congenic Fayoumi lines (named M5.1 and M15.2) with G-B1 Leghorn hens. Antibody response to SRBC and B. abortus after immunization at 19 and 22 weeks were measured. Secondary phase parameters of maximum titers (Ymax) and time required to achieve Ymax (Tmax) were estimated from post-secondary titers by using a non-linear regression model. The DNA polymorphisms of six genes were identified, and associations of single nucleotide polymorphisms (SNP) in the six genes with antibody response parameters were analyzed. Significant main effects of the gene polymorphisms were mostly found in the lineage of M5.1 grandsire and primarily on antibody response to B. abortus. There was general agreement of allelic effect within antibody parameters among genes. These results suggest that the SNPs characterized in the study may serve as markers for genetic enhancement of humoral immune capacity in the chicken.

(Key words: candidate gene, antibody response kinetics, inbred line, antibody parameters, single nucleotide polymorphism)

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Abbreviation Key: B. abortus = Brucella abortus; chB6 = chicken B-cell marker; FDR = false discovery rate; FF = homozygous Fayoumi phenotype; IAP1 = inhibitor of apoptosis protein 1; IL-15Rα = interleukin-15 receptor α chain; IL-2Rγ = interleukin-2 receptor γ chain; LF = heterozygous Leghorn-Fayoumi phenotype; LL = homozygous Leghorn phenotype; MAS = marker-assisted selection; SNP = single nucleotide polymorphism; Tmax = time to achieve maximum secondary antibody titers; Y = primary antibody response; Ymax = maximum secondary antibody titers; ZOV3 = immunoglobulin superfamily protein.

INTRODUCTION

Improving animal health is a major goal in the current animal breeding industry (Soller and Andersson 1998). Disease-related costs and losses are expected to be reduced by genetic control of resistance to pathogens and improvement of the immune capacity of animals. The complex immune system of poultry provides an opportunity for investigating polygenic regulation of immune response in chickens (Lamont 1998a). Candidate gene analysis is a powerful approach to detect genes controlling traits of economic importance in farm animals, such as immune response (Rothschild and Soller 1997). Candidate genes chosen for studying immune response traits may have known physiological function with immune response or be in regulatory or biochemical pathways affecting immune response.

The chicken B-cell marker (chB6) gene is expressed on the B-cell precursor in the chicken embryo and some macrophages (Houssaint et al., 1987, 1991). The chB6 gene plays a role in regulating B-cell development (Tregaskes et al., 1996). ChB6 alleles are associated with expression level of major histocompatibility complex (MHC) class II, regression of Rous sarcoma and resistance to Marek’s disease (Frederickson and Gilmour 1985; Gilmour et al., 1986; Tregaskes et al., 1996). Caspase-1 (IL-1β-converting enzyme) is mainly responsible for cytokine maturation, such as processing IL-1β and IL-18 (Wang et al., 2000). Caspase-1 activation induces apoptosis, as well as processing the proinflammatory cytokines, pro-IL-1β and pro-IL18, into their mature bioactive forms. The inhibitor of apoptosis protein-1 (IAP-1) gene is a member of the IAP family, which is involved in host antiapoptotic mechanisms (Deveraux et al., 1999). IAPs have multiple biological activities that include
binding and inhibiting caspases, regulating cell cycle progression, and modulating receptor-mediated signal transduction (Yang and Li 2000). The IAPs prevent apoptosis by binding and inhibiting caspase's activity at various steps of the apoptotic signaling pathway (Deveraux et al., 1997; Roy et al., 1997).

Interleukin-15 (IL-15) is a proinflammatory cytokine that has similar biological functions as IL-2, another gene that has previously been identified as a candidate in immune response (Estess et al., 1999 and Trentin et al., 1997). Like IL-2, IL-15 is involved in recruiting T-cells to tissues during immune response, proliferation of T cells and Natural Killer cells, growth stimulation of B cells and immunoglobulin synthesis (Kumaki et al., 1996). Interaction between IL-15 and the IL-15 receptor α (IL-15Rα) chain compete with tumor necrosis factor and the tumor necrosis factor receptor1, a complex that stimulates an apoptotic pathway (Bulfone-Paus et al., 1999). Cytokine IL-15 and its receptor have been implicated in the pathways of several disease processes such as leukemia, cancer, and inflammatory bowel disease (Bulfone-Paus et al., 1999; Yamada et al., 1998). The interleukin-2 receptor γ chain (IL-2Rγ) is shared by receptor complexes IL-2, IL-4, IL-7, IL-9 and IL-15, all of which are cytokines involved in lymphocyte development and/or activation. IL-2Rγ is physically and functionally associated with the JAK3 tyrosine kinase (Demoulin and Renaud 1998). The IL-2Rγ plays a pivotal role in formation of the complete IL-2 receptor, and mutations of the gamma chain gene cause human X-linked severe combined immunodeficiency, resulted in a complete or profound T cell defects (Ohbo et al., 1995). The immunoglobulin superfamily protein (ZOV3) is related to the immune response (Saitoh et al., 1993).

The significance of roles of chB6, caspase-1, IAP1, IL-15Rα, IL-2Rγ, and ZOV3 in physiological or regulatory immune function make them logical candidates for evaluation of effects on chicken antibody response kinetics. The goals of this study were to identify polymorphisms of the six genes in chickens and examine associations of their single nucleotide polymorphisms (SNP) with antibody response kinetics.
MATERIALS AND METHODS

Resource population

Genetically distinct, highly inbred (> 99%) chicken lines, the Leghorn G-B1 and MHC-congenic Fayoumi M5.1 and M15.2 lines, were used as parental lines (Zhou and Lamont 1999). One sire from each Fayoumi line was mated to nine dams each of the Leghorn G-B1 line. Four F1 males from each Fayoumi sire was each mated to eight females to produce an F2 generation of 158 females in one hatch. The two separate lineages, therefore, of the F2 population genetically differ only in the MHC allele contributed by the two MHC-congenic Fayoumi grandsires, Fayoumi M5.1 and Fayoumi M15.2 (Zhou et al., 2001a). The number of F2 progeny produced from M5.1 and M15.2 were 71 and 87, respectively.

Antigen Administration, Sample Collection and Agglutination Assays

At 19 and 23 weeks of age, F2 hens were intramuscularly injected with whole SRBC and Brucella abortus (B. abortus) antigens (Zhou et al., 2001a). Blood samples were obtained from the peripheral wing vein of each bird preimmunization, at 7 days after primary immunization, and at 4, 7, 10, 18, 32, and 63 days after secondary immunization. Sera were collected after centrifuging the blood samples and stored at -20°C until all assays were run simultaneously. The SRBC and B. abortus antibodies were assayed by micro-agglutination (Zhou et al., 2001a).

Genomic Sequencing of Chicken chB6, Caspase-1, IAP1, IL-15 Receptor α, IL-2 Receptor γ and ZOV3 Genes

Primer sequences, Genbank accession number from which primer design was based, and annealing temperature of PCR amplification for all six genes are presented in Table 1. The PCR primers were designed by Oligo 5.

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**ChB6.** Primers were designed to amplify a 215-bp fragment covering exon 3 from genomic DNA. The PCR was performed in a total volume of 25 μl, containing 25 ng of genomic DNA, 0.8 μM of each oligonucleotide primer, 2.5 μl of 10 × PCR reaction buffer, 1.5 mM MgCl₂, 200 μM of each dNTP, and 1 unit of *Taq* DNA polymerase³. Cycle parameters were: 94°C for 5 min, then 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension step of 15 min at 72°C.

**Caspase-1.** Primers were designed to amplify from chicken genomic DNA, a 1070-bp fragment covering from the 5' flanking region to partial exon 2. The PCR conditions were the same as for the ChB6 gene, except the annealing temperature was 60°C.

**IAPl.** Primers were designed to amplify a 394-bp fragment including a previously identified DNA polymorphism between the parental lines (Zhou et al., 2001b). The PCR conditions were the same as for the ChB6 gene, except the annealing temperature was 62°C.

**IL-15Ra.** Spleen total RNA was isolated using ToTALLY RNA™ Kit⁴. First-strand cDNA was synthesized using the RETROscript™ First Strand Synthesis Kit⁴ for RT-PCR. Primers (Table 1) were designed to amplify a 430-bp fragment from cDNA. The PCR condition was the same as for ChB6 gene except the annealing temperature was 58°C. Based on a mutation found in the parental lines, new primers (Table 1) were designed to amplify a 123-bp fragment from genomic DNA. The PCR conditions were the same as for the ChB6 gene.

**IL-2Rα.** Primers were designed to amplify a 600-bp fragment from chicken genomic DNA. The PCR condition was the same as for the ChB6 gene, except the annealing temperature was 52°C.

**ZOV3.** Primers were designed to amplify a 320-bp fragment covering a previously identified DNA polymorphism between the parental lines (Zhou et al., 2001b). The PCR condition was the same as for the ChB6 gene, except the annealing temperature was 52°C.

**Development of PCR-RFLP Assays**

Chicken genomic DNA was isolated from venous blood collected in EDTA. The PCR was carried out with genomic DNA from two birds (one male and one female) from each of the

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³ Promega Corporation, Madison, WI 53711.
⁴ Ambion, Inc., Austin, TX
highly inbred lines (Leghorn G-B1, Fayoumi M5.1 and M15.2) to detect potential sequence polymorphisms. The PCR products were purified by MICROCON® centrifugal filters⁵. Nucleotide sequencing was performed by the Iowa State University DNA Sequencing and Synthesis Facility. Sequences were analyzed using Sequencher³ software. The restriction enzyme sites on these sequences were detected by Webcutter² (http://www.firstmarket.com/cutter/cut2.html. Date accessed: July 12, 2002)

Typing the F₂ Population with ChB6, Caspase-1, IAP-1, IL-15Rα, IL-2Rγ and ZOV3 Genes

A PCR of DNA of each individual F₂ bird was performed for each gene according to the conditions described above. The PCR products of ChB6, caspase-1, IAP-1, IL-15Rα, IL-2Rγ and ZOV3 genes were digested at 37°C overnight with 1 U Pvu II, Hsp 92 II, Bgl I , Alu I, Hph I, and SnaB I⁷, MA, respectively. The restriction digests were electrophoresed for 1 h at 100 V on a 2% agarose gel with ethidium bromide. Individual PCR-RFLP fragment sizes in each sample were determined, based on standard DNA molecular weight markers for each gene, by visualizing the banding pattern under UV light.

Statistical Analysis

The analyses of antibody response to SRBC and B. abortus were separately conducted by antigen and by phase (primary, secondary, and equilibrium). For the SRBC and B. abortus primary phase, the single time-point measurement taken at 7 day postprimary immunization (Y) was used. Secondary phase parameters of maximum titers (Ymax), time (Tmax) needed to achieve maximum titers were estimated from seven individual time-point postsecondary titer values by using a nonlinear regression model (Weigend et al., 1997). The titers of the last three sample times were used to calculate the mean of the equilibrium phase (Zhou et al., 2001a). This yielded a total of eight antibody kinetics parameters analyzed.

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³ Millipore Corporation, Bedford, MA 01730.
⁴ Gene Codes Corporation, Ann Arbor, MI 48108.
Data were analyzed separately for F₂ progeny from the two different MHC-congenic grandsires, because MHC haplotype has been found in many previous studies to impact antibody production. The genes had three levels of genotypes designated as Leghorn homozygote (LL), heterozygote (LF), and Fayoumi homozygote (FF). General linear model tests for associations between genotype and antibody response parameters were conducted by using the JMP® program (Sall and Lehman 1996).

RESULTS

Sequence Variation and PCR-RFLP Analysis

The digested fragments with each enzyme of the six gene polymorphisms for the Leghorn and Fayoumi homozygotes are presented in Table 1. An amplified 215-bp fragment of chB6 gene showed a C → A substitution at base 470, which caused a predicted amino acid change from Gln (Leghorn line) to Lys (Fayoumi line). The digested patterns with Pvu II are presented in Figure 1a. For the caspase-1 gene, a 1070-bp amplified fragment showed a T → C substitution at the -368 bp between the Leghorn and Fayoumi lines. A PCR-RFLP assay was developed with Hsp92 II (Figure 1b). For the IAP1 gene, a 394-bp fragment showed a T → A substitution from the Leghorn to the Fayoumi lines, and a PCR-RFLP assay was developed to identify a Bgl I SNP to characterize the polymorphism at Ala¹⁵⁷ (Genbank AAF35319.1) (Figure 1c). For IL-15Rα, the amplified 123-bp product showed a G → A SNP between the Leghorn and the Fayoumi lines. The restriction enzyme Alu I produced fragment sizes of 80 and 43 bp for the Fayoumi lines, whereas the Leghorn line had no cut site (Fig. 1d). For IL-2Rγ, a 600-bp product was sequenced. A T → C SNP was identified between the Leghorn and the Fayoumi M5.1 lines, but no polymorphism was found between the Leghorn and the Fayoumi M15.2 lines. The Hph I digested products are shown (Figure 1d). For ZOV3, the amplified 320-bp product showed a T → G SNP, which caused a predicted amino acid change from Cys¹⁵⁷ (Genbank D16151) in the Leghorn line to Phe¹⁵⁷ in the Fayoumi lines. The SnaB I produced fragment sizes of 270 and 50 bp for the Leghorn lines, whereas

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the Fayoumi lines had a 320-bp fragment (Figure 1e). Because ZOV3 maps to the Z chromosome, no heterozygous pattern was generated from the hens.

**Associations of the Polymorphisms of Six Genes with Antibody Response**

The $P$ values of main effects of the chB6, caspase-1, IAP-1, IL-15R$\alpha$, IL-2R$\gamma$ and ZOV3 gene SNPs on chicken antibody response are shown in Table 2. The IL-15R$\alpha$ polymorphism had the most frequent associations with antibody response parameters. For $F_2$ offspring of the M5.1 grandsire, there were significant associations ($P < 0.05$) between the chB6 and IAP-1 polymorphisms and primary antibody response to *B. abortus*, and between the caspase-1, IL-15R$\alpha$ and ZOV3 polymorphisms and Tmax to *B. abortus*. For $F_2$ offspring of the M15.2 grandsire, there were significant effects ($P < 0.05$) of the IAP1 polymorphism on equilibrium antibody response to *B. abortus*, and between IL-15R$\alpha$ and primary antibody response to *B. abortus*.

**Allelic Effect of Six Genes on Antibody Response**

The allelic effects of six gene polymorphisms on antibody response are presented in Table 3 for the nine instances of significant ($P < 0.05$) main effects of genotype. For comparison purposes, the effect of the candidate gene alleles in both MHC-congenic lineages is presented, even though significant differences usually occurred in one lineage only. Most of the candidate gene main effects (seven out of nine) were detected in the M5.1 grandsire lineage. For primary response to *B. abortus*, the mean of the hens homozygous for the Fayoumi allele of chB6 or IL-15R$\alpha$ was significantly higher than the other two allelic combinations in the M5.1 and M15.2 grandsire lineage, respectively, while the IAP-1 Leghorn allele homozygous hens were significantly lower than the other two genotypes. For Tmax to *B. abortus*, the hens homozygous for the Fayoumi allele for the caspase-1 and ZOV3 polymorphism required a significantly longer time to achieve secondary maximum antibody response than the Leghorn homozygotes. The Fayoumi homozygous hens for the IL-15R$\alpha$ polymorphism required a significantly longer time to achieve secondary maximum.
response than the heterozygous hens. For the primary response to SRBC, the hens inheriting either IL-15Rα or ZOV3 homozygous alleles from the Fayoumi line were significantly higher than the other genotype hens. For equilibrium antibody response to *B. abortus* with IAP1 polymorphism, the IAP1 Leghorn homozygous hens had a response significantly higher than the Fayoumi homozygotes in the M15.2 grandsire lineage.

**DISCUSSION**

Immune response traits in chickens are controlled by the immune system, which is a complex system involving many different cell types and soluble factors. These cells and factors must act in concert to produce an effective response to pathogens (Pinard-van der Laan et al., 1998). Genetics has a great influence on modulation of immune response (Lamont 1998a). Genetic selection to improve immune response provides an alternative to enhance disease resistance (Boa-Amponsem et al., 1997). Therefore, understanding the genetic basis of polygenic control of immune response can be utilized to improve chicken health.

Six immunity-related genes were selected to determine associations of DNA polymorphisms of these genes with antibody response kinetics to SRBC and *B. abortus* in the F2 population in the present study. Gene polymorphisms might be used to enhance antibody production by genetic selection, if the allelic effects associated with antibody response can be determined. There were generally consistent allelic effects within antibody response parameters among the studied genes. For primary antibody response to *B. abortus*, the hens inheriting both alleles of chB6, IAP1 and IL-15Rα from the Fayoumi line had significantly higher antibody level. For effects of caspase-1, IL-15Rα, and ZOV3 polymorphisms on Tmax to *B. abortus*, the Fayoumi homozygous hens required a significantly longer time to achieve maximum secondary antibody response than other genotypes. The results of this study lay the foundation to improve immune capacity in chickens via marker-assisted selection. The data also show the value of exploring the effect of specific alleles in lines unselected for commercial production traits (eg. Fayoumi) and, therefore, reservoirs of genetic diversity to meet potential future needs in poultry production.
More significant associations of the genes with antibody response were detected in the lineage of the Fayoumi M5.1 grandsire than the M15.2 grandsire. The two grandsires of the present resource population are MHC-congenic, therefore, suggesting that the different apparent effects of the six genes as detected in the two lineages reflect the interaction of the MHC with these six genes on antibody production. The MHC plays a critical role in regulation of immune response through its cell-surface molecules interacting with both the foreign antigens and with complementary structures of other immune cells (Lamont 1998b). Associations primarily in one grandsire lineage of interferon-γ, immunoglobulin light chain, interleukin-2, MHC class I α and II β, and transforming growth factors β genes with antibody response to SRBC and *B. abortus* in the F2 population have been previously reported (Zhou et al., 2001, Zhou and Lamont 2002, unpublished data).

Significant associations of the genes on immune trait parameters in the study were primarily detected in antibody response to *B. abortus* (7 instances out of 9). The SRBC and *B. abortus* are T-cell dependent and T-cell independent antigens, respectively, which require different degrees of cooperation of T-cells to produce antibody (Munns and Lamont 1991; Scott et al., 1994). For *B. abortus*, B-cells can be activated by the antigen itself to produce antibody, whereas helper T cells and MHC class II molecules are needed to activate B-cells to antibody formation for SRBC (Janeway et al., 1999). The signal pathway to produce antibody for *B. abortus* is relatively simple compared to that for SRBC. Physiological interaction of the studied genes with any of the components in the pathway of antibody formation with *B. abortus* may not occur with SRBC, which might cause the different effects detected for the two antigens.

There were nine significant associations (*P* < 0.05) found between the six genes and immune response traits. Most significant associations were clustered with IAP1 (2), IL-15Rα (3), and ZOV3 (2) genes. Only one significant association was found with chB6 and caspase-1, and none with IL-2Rγ. The false discovery rate (FDR) approach was proposed to establish statistical significance in a multiple-test situation (Benjamini and Hochberg 1995). The FDR is the proportion of false-positive tests among the individual comparison-wise tests that are declared significant. For F2 offspring of the M5.1 grandsire, 24 statistical analysis were conducted for each antigen. At an α = 0.05, one of twenty tests would be expected to be false
positive. Seven significant associations were detected in the study, however, and five of these associations were in response to *B. abortus* (Table 2). Additionally, clusters of significance were detected as associated with specific immune parameters and a specific antigen: three for both primary antibody response and Tmax to *B. abortus*. The clustering of associations gives confidence in the true significance of most of the detected associations. In contrast, in the M15.2 lineage, only two significant associations of forty statistical tests were detected, which is interpreted as likely being false discovery.

There were few significant associations of caspase-1 and IAP1 with antibody response to SRBC and *B. abortus* detected in the current study. However, the same DNA polymorphism of the caspase-1 gene was previously associated with spleen and cecum *Salmonella enteritidis* (*S. enteritidis*) bacterial load and antibody response to *S. enteritidis* vaccination, and of that of the IAP1 gene with spleen *S. enteritidis* bacterial load, in a broiler by inbred line cross F1 resource population (Liu 2002). In another study in populations of modern outbred broiler lines, the caspase-1 and IAP1 polymorphisms were associated with cecum and liver *S. enteritidis* bacterial load, and cecum *S. enteritidis* bacterial load, respectively (Kramer, Malek and Lamont 2002, unpublished data).

Selection for high antibody level tends to produce populations that are more disease resistant (Pinard et al., 1993). A line selected for high antibody response to SRBC demonstrated higher antibody response to Newcastle disease, and greater resistance to *Mycoplasma gallisepticum* and *Eimeria necatrix* than that of the low-antibody selected line (Cross et al., 1980). Subsequent studies suggested that the high line had greater resistance to *Eimera tenella* (Martin et al., 1986). Selection for immune response to nonpathogenic antigens such as SRBC and killed *B. abortus* has been considered as an attractive supplement to genetic control of disease in poultry (Parmentier et al., 2001).

In summary, the identified significant effects of the gene polymorphisms on antibody response kinetics to SRBC and *B. abortus* pave the way for potential application of the findings in improvement of poultry health by marker-assisted selection.
ACKNOWLEDGMENTS

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white Leghorn chickens selected for high and low antibody responsiveness to sheep


chB6 (Bu-1) is a highly glycosylated protein of unique structure. Immunogenetics. 44:212-
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TABLE 1. Primer design and restriction enzyme digestions for polymorphism identification of chicken B cell marker (ChB6), caspase-1, inhibitor of apoptosis protein-1 (IAP-1), interleukin-15 receptor α chain (IL-15Rα), interleukin-2 receptor γ chain (IL-2Rγ) and immunoglobulin superfamily gene (ZOV3).

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession number</th>
<th>Primer Sequence</th>
<th>PCR product (bp)</th>
<th>Annealing Tm</th>
<th>Restriction enzyme</th>
<th>Fragments after digestion (bp)</th>
</tr>
</thead>
</table>
| ChB6        | X92865                   | Forward 5'-GCTTCCCAATGGAACTG-3'  
Reverse 5'-GAGCACAATGGGCTAGTC-3' | 215              | 55 °C          | Pvu II          | 147, 68 (LL), 215 (FF) |
| Caspase-1   | AF031351 (FF)            | Forward 5'-CCATGCTTGGGCTCAGTG-3'  
Reverse 5'-GGTCCCGCAGATCCAGTG-3' | 1070             | 60 °C          | Hsp 92 II       | 421, 244, 227, 122, 56 (LL), 312, 244, 227, 122, 109, 56 |
| IAP-1       | AF221083                 | Forward 5'-TCACCATCTCTACGTTCCAT-3'  
Reverse 5'-CATTGAAACTTGGTGGTCT-3' | 394              | 62 °C          | Bgl I           | 254, 140 (LL), 394 (FF) |
| IL-15Rα     | AI980376                 | Forward 5'-CCTTGGTAGTTCCTAGGGCT-3'  
Reverse 5'-TGGAACCTGGTATACACCT-3' | 430 (cDNA)       | 58 °C          | Alu I           | 123 (LL), 80, 43 (FF) |
| IL-2Rγ      | BQ037061                 | Forward 5'-CCACGCGTGGACTATAGGAA-3'  
Reverse 5'-CATCTTTAGGACTCCGACCCA-3' | 600              | 52 °C          | Hph I           | 465, 64, 40, 31 (LL), 454, 64, 40, 31, 11 (FF) |
| ZOV3        | AF221566                 | Forward 5'-GGCTTGGGACCTGATATGAC-3'  
Reverse 5'-GGCTAAGTAGGTCAAGTGAC-3' | 320              | 52 °C          | SnaB I          | 270, 50 (L), 320 (F) |

1LL = Leghorn G-B1 homozygote.  
2FF = Fayoumi homozygote.  
3L- = Leghorn G-B1 hemizygote.  
4F- = Fayoumi hemizygote.
TABLE 2. Main effects ($P$-values) of chicken B cell marker (ChB6), caspase-1, inhibitor of apoptosis protein-1 (IAP-1), interleukin-15 receptor $\alpha$ chain (IL-15R$\alpha$), interleukin-2 receptor $\gamma$ chain (IL-2R$\gamma$) and immunoglobulin-superfamily protein gene (ZOV3) on SRBC and Brucella abortus antibody response in F$_2$ progeny of M5.1 and M15.2 grandsires.

<table>
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<th>Grandsire MHC</th>
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<th>$Y^1$</th>
<th>$T_{max}^2$</th>
<th>$Y_{max}^3$</th>
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<th>$T_{max}$</th>
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<td>0.02</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>IL-15R$\alpha$</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.004</td>
<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td></td>
<td>ZOV3</td>
<td>NS</td>
<td>NS</td>
<td>0.19</td>
<td>NS</td>
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</tr>
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</table>

$^1$Primary antibody response.
$^2$Time required to achieve maximum secondary antibody titers.
$^3$Maximum secondary antibody titers.
$^4$Equilibrium phase antibody titers.
$^5$NS, $P > 0.20$.
$^6$All $P$ values are rounded to two significant digits. Actual value is $P = 0.0478$.
$^7$All $P$ values are rounded to two significant digits. Actual value is $P = 0.0469$. 

Primary antibody response.
Time required to achieve maximum secondary antibody titers.
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NS, $P > 0.20$.
All $P$ values are rounded to two significant digits. Actual value is $P = 0.0478$.
All $P$ values are rounded to two significant digits. Actual value is $P = 0.0469$. 

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TABLE 3. Mean antibody parameters, by genotype, of chicken B cell marker (ChB6), caspase-1, inhibitor of apoptosis protein-1 (IAP-1), interleukin-15 receptor α chain (IL-15Rα), interleukin-2 receptor γ chain (IL-2Rγ) and immunoglobulin-superfamily protein gene (ZOV3) to SRBC and *Brucella abortus* antibody response in F2 progeny of M5.1 and M15.2 grandsires.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Trait</th>
<th>M5.11</th>
<th>M15.22</th>
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<tr>
<td></td>
<td></td>
<td>LL3 LF4 FF5</td>
<td>LL LF FF</td>
</tr>
<tr>
<td>ChB6</td>
<td>YB (titer log2)</td>
<td>9.93a 9.86a 11.5b</td>
<td>10.07a 9.97a 10.64a</td>
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<tr>
<td></td>
<td>Caspase-1</td>
<td>9.77a 15.92ab 20.84b</td>
<td>11.23a 15.76a 18.18a</td>
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<tr>
<td>IAP-1</td>
<td>YB (titer log2)</td>
<td>8.92a 10.40a 10.65b</td>
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<tr>
<td></td>
<td>Equil.B (titer log2)</td>
<td>7.43a 7.04a 6.70a</td>
<td>7.93a 7.36ab 6.93b</td>
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<tr>
<td>IL-15Rα</td>
<td>YS (titer log2)</td>
<td>5.40ab 4.21a 5.72b</td>
<td>4.74a 4.58a 5.15a</td>
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<tr>
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<td>YB (titer log2)</td>
<td>10.80a 9.97a 9.93a</td>
<td>10.19a 9.48a 11.15b</td>
</tr>
<tr>
<td></td>
<td>TmaxB (day)</td>
<td>16.96ab 13.18a 19.85b</td>
<td>13.17a 15.80a 18.61a</td>
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<tr>
<td>ZOV3</td>
<td>YS (titer log2)</td>
<td>5.81a 4.75b</td>
<td>4.67a 4.97a</td>
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<td></td>
<td>TmaxB (day)</td>
<td>12.91a 19.06b</td>
<td>15.25a 15.76a</td>
</tr>
</tbody>
</table>

a-b Means in a row within a grandsire MHC type with no common superscript differ significantly (*P* < 0.05).
1F2 offspring of M5.1 grandsire.
2F2 offspring of M15.2 grandsire.
3Leghorn homozygote.
4Heterozygote of Leghorn and Fayoumi genotype.
5Fayoumi homozygote.
6Primary antibody response to *Brucella abortus*.
7Time required to achieve maximum secondary antibody titers to *Brucella abortus*.
8Equilibrium phase antibody titers to *Brucella abortus*.
9Primary antibody response to SRBC.
CHAPTER 10. GENERAL CONCLUSION AND DISCUSSION

General Conclusions

The genome scan and the candidate gene approach for identification of QTL affecting antibody response to *Salmonella enteritidis* (SE) vaccination and antibody response kinetics to sheep red blood cell (SRBC) and *Brucella abortus* (BA) were effective in identifying QTL in the current study. For the genome scan approach, QTL affecting immune response to SRBC and BA were detected by a stepwise process that culminated in interval mapping. A total of five significant QTL were detected at the 5% chromosome-wise level on chicken chromosomes 3, 5, 6 and Z. The results, therefore, suggest that regions on chromosomes 3, 5, 6 and Z contain QTL affecting antibody kinetics to SRBC and BA. For the candidate gene approach, twelve candidate genes, [caspase-1, MHC class I α1, α2, class II β1, chicken B cell marker, inhibitor of apoptosis protein 1 (IAP1), interferon-γ, immunoglobulin light chain, interleukin-15 α chain, interleukin-2, interleukin-2 receptor γ chain, and immunoglobulin superfamily gene (ZOV3)], were selected based on their biological or physiological functions on immune response. Transforming growth factor β (TGF-β) 2, 3 and 4, as positional candidate genes, following up the previous genome scan approach, were used to investigate associations between DNA polymorphisms and antibody response to SE vaccination and antibody response kinetics to SRBC and BA. Single nucleotide polymorphisms of interferon-γ, immunoglobulin light chain, interleukin-2, MHC class I α1, α2, class II β1, and TGF-β 2, 3 and 4 were significantly associated with multiple traits of antibody response to SE vaccination or antibody response kinetics to SRBC and BA or both. Only one or two significant associations with antibody response traits were detected in other candidate genes. For some candidate genes, significant associations were primarily detected in the lineage of the MHC-congenic M5.1 grandsire, but in that of the M15.2 grandsire for the others. Strong interactions of MHC with candidate genes on antibody response were indicated from the study. All these genes characterized in the present study are, therefore, strong potential candidates for application in marker-assisted selection to improve vaccine response and immunocompetence in chickens.
General Discussion

Justification of genome scan and candidate gene approach  There are many advantages to use the genome scan approach. No prior information on gene function is required and it is relatively assumption free (Rothschild and Soller 1997). In theory, the genome scan can potentially detect all QTL with appreciable effect by using anonymous genetic markers spread across the genome, given appropriate experimental design and population size (Soller and Andersson 1998). The linkage mapping approach will be more desirable if there exists an extensive degree of linkage disequilibrium (LD) in a population design such as F2 or backcross of a cross between inbred lines (Rothschild and Soller 1997). However, linkage mapping still has some disadvantages. The size of the regions affecting the trait of interest identified by a genome scan is typically 10-20 cM, and the exact position and number of QTL is unknown (Dekkers and Hospital 2002). The principle underlying the genome scan is the existence of LD between the marker and the QTL. Marker-QTL LD can exist at the population level, but always exists within families, even between loosely linked loci. The LD will erode in the course of the selection program after successive generations due to recombination between the marker and QTL (Dekkers and Hospital 2002). However, this phenomenon can be used to advantage in advanced intercross lines (AIL) to more closely identify the position of a QTL.

There are many strengths of the candidate gene approach. Candidate gene analysis can be a single-generation study, rather than requiring establishment of a dedicated two or three-generation resource population for the genome-scan approach. Therefore, candidate gene analysis can be easily extended to commercial breeding populations and once the effect of a candidate gene is verified, it can be directly applied for MAS in the breeding population (Rothschild and Soller 1997). In addition, positive results can be readily verified in other populations. For the candidate gene, DNA polymorphisms are investigated in a gene that is hypothesized to affect the trait and therefore are generally tightly linked to the QTL (Dekkers and Hospital 2002).

In this manner, except in the rare instance in which the studied polymorphism is also the causative mutation, identifying polymorphisms in candidate gene, may be considered an
example of extremely tight linkage disequilibrium of markers and causative mutation. However, the candidate gene approach has its limitations. Most genes in chickens have not been identified, mapped or sequenced. With the development of comparative mapping between human and chicken, an abundant source of new positional candidate genes will be available in the near future (Burt et al. 1999). Significant associations detected by candidate gene analysis are often results from non-trait genes affecting the trait phenotype through pleiotropy (Rothschild and Soller 1997). The effect, in most cases, will be mapped in a very broad region (15-25 cM), which contains hundreds of genes. It will be very difficult to find the causative gene responsible for the phenotype of the trait (Rothschild and Soller 1997). For a within-population study, the extent of linkage disequilibrium will be less than 1 cM; however, in the instance of a recent cross between two populations, the extent of LD will be more than 20 cM (Soller and Andersson 1998). The identified association needs to be confirmed with additional independent populations. In cross-family population-wide studies, false positive results may be obtained if the DNA polymorphism analyzed is in linkage equilibrium with the causative mutation. Multiple-site intragenic haplotypes analysis can help avoid false positive associations, because at least one of the intragenic haplotype will necessarily be co-extensively linked with the causative mutation (Templeton et al. 1987).

In summary, both the genome scan approach and the candidate gene approach are very efficient to identify QTL affecting a trait of interest. The genome scan approach is complementary to the candidate gene approach. A positional candidate gene analysis can effectively follow a linkage analysis study.

In the current study, the genome scan and candidate gene approaches were conducted in the unique F₂ population. It is a powerful design for the linkage mapping QTL affecting immune response parameters, due to a large extent of linkage disequilibrium. However, this design has a shortfall for the candidate gene approach. Based on the existing linkage disequilibrium in an F₂ population, a candidate gene with significant association with phenotype variation may be up to 20 cM away from the causative variation responsible for phenotypic variation. The candidate gene associations found in this study, therefore, still needed to be validated in other populations before utilizing them in a breeding program by marker-assisted selection.
Gene network. Most complex traits are controlled by a network of genes that function in concert. Gene networks can be characterized by evaluating their LD, to hypothesize the interaction level between pairs of genes. Physiologic LD is distinct from physical LD. Physiological LD depends on the biological properties of the gene network, including forces such as interaction of genes and their products, in contrast with physical LD, which depends on gene location on the chromosome. A large discrepancy between physical LD and physiological LD is suggestive of strong biological interaction between the genes on their products. Fifteen candidate genes with proven or potential relation to immune response in the present study were characterized for allelic inheritance of all 15 genes by PCR-restriction fragment length polymorphism or PCR-single strand conformation polymorphism. Genotypic data of all genes in the F2 population provided an opportunity to evaluate the physiological LD between genes. Linkage disequilibrium analysis of all pairs of genes was conducted based on genotypic data by using the Arlequin software (http://anthro.unige.ch/arlequlin. Date accessed: July 1, 2002). A proposed gene network was constructed based on the probability of linkage disequilibrium between each gene pair (Figure 1). Significant linkage disequilibrium (P < 0.05) was detected for some pairs of genes that are known, based on known map position, to not be physically linked, such as MHC class I α1 (chromosome 16) and TGF-β3 (chromosome 5), MHC class II β1 (chromosome 16) and IL-2 (chromosome 4), and IL-2 (chromosome 4) and TGF-β3 (chromosome 5). Some pairs of genes (Caspase-1 and MHC class II β1, IAP1 and TGF-β2, ZOV3 and IL-15 α chain) showed significant linkage disequilibrium, but at least one of each pair of genes is of unknown map position. The physiological LD detected between these pairs of genes helps to set priorities to map these unmapped genes or investigate gene interactions. The gene network derived here proposes specific genes and specific gene interactions as a starting point to efficiently target future investigations of genetic control of complex biological traits by additional methods, such as protein-protein interaction and gene expression studies. Given that this is an F2 population, not a long-term selected population, the biological forces determining the physiological LD are likely related to the survival of each of the founder lines through an intense inbreeding process, that is, allelic combinations related to successful reproduction, development and
survival. The general concept of gene network detection through physiological LD could also be applied to long-term selected lines, in which certain traits of interest were selected.

Future Directions and Recommendations

Comparative genomics approach The genome scan and candidate gene approach in the present study were conducted to identify QTL affecting antibody response in chickens. Several significant associations of QTL with antibody response parameters were detected based on both approaches. For the QTL detected from the linkage mapping analysis, the chromosome region of the QTL may contain hundreds of genes. For the candidate gene analysis, genetic polymorphisms with significant effect may not be the causative mutation responsible for phenotype, and other candidate genes flanking the region still need be investigated. There is a lack of information on gene function or sequences in chickens. The rapid development of the human genome project and the highly conserved synteny between chicken and human provide an opportunity to utilize information from the human genome to find more candidate genes for analysis in chickens (Burt et al. 1999).

New technology to find QTL affecting complex traits For the purpose of fine mapping of QTL, several approaches can be taken, such as increasing the size and number of families for genotyping, using advanced intercross lines to increase recombinations, and haplotype analysis (Vignal et al. 2002). Whatever the approach will take, high densities of markers will be needed. Microsatellite markers are the most popular markers used in linkage mapping, however, the density of microsatellites is low, particularly in chickens (Primmer et al. 1997). Single nucleotide polymorphisms is emerging as a new class of markers with two important properties: high density in the genome and high polymorphisms. There are nearly three million SNPs in human deposited in a public database (Marth et al. 2001). A map of 1.42 million SNPs has been constructed with an average density of one SNP every 1.91 kb (Sachidanandam et al. 2001). SNP often occur in exons, promoters, splice sites or other regulatory regions that will be the most common polymorphism and the more likely responsible for phenotypic variation (Vignal et al. 2002). With the development of high
throughput of SNP genotype, SNP will become a good complementary to microsatellite markers in gene mapping study.

The effort to identify SNPs in chickens was initiated in University of Delaware. Chicken SNPs were extracted from trace data of 18 cDNA libraries of University of Delaware chicken EST database using a phred/phrap/polyphred/consed pipeline. The detected 1210 chicken SNPs have been submitted to the dbSNP (http://chicksnps.afs.udel.edu/. Date accessed: October 10, 2002).

In the present study, 23 immunity related genes were investigated. Of the 23 genes studied, 21 genes had a total of 12 kb sequenced with an average density of one SNP every 440 bp (27/12000). The MHC genes, as expected because of their role in recognizing diverse epitopes, were much more variable. The MHC class I α1 and α2 domain genes had an average density of one SNP every 9 bp (65/537) (Liu et al. 2002), and class II β1 and β2 genes had an average density of one SNP every 16 bp (42/651).

Quantitative trait variation is controlled by many genetic and nongenetic factors. A global approach is needed to apply to genetic dissection of complex traits. Gene expression studies using microarray technology provide an opportunity to evaluate transcriptional activities of all genes simultaneously (Gu et al. 2002). Instead of a gene-by-gene approach, the qualitative or quantitative measurement of transcription levels of thousands of genes can be analyzed. Those genes that have significant gene expression differences between different stages or states by microarray analysis can be followed by the candidate gene approach.

Microarray DNA technology has been widely applied to analyze gene expression changes on a genome-wide scale. Oligonucleotide microarray analysis was used to identified highly expressed genes in cancerous and noncancerous tissues (Hippo et al. 2002). A bovine-specific cDNA microarray was developed to analyze the gene expression profile of peripheral blood mononuclear cells from cattle infected with mycobacterium paratuberculosis (Coussens et al. 2002). In chickens, an immune system cDNA microarray was developed to compare gene expression profiling during normal embryonic B-cell development and during myc-induced neoplastic transformation in the bursa (Neiman et al. 2001). Microarray technology can be integrated with linkage mapping to identify positional
candidate genes. Liu et al. (2001) conducted a study using a DNA microarray containing 1200 genes, in which were selected based on previous genetic mapping data. Fifteen identified genes were significantly associated with Marek’s disease resistance in chickens, and twelve of them had mapped human orthologues. The combination of the DNA microassay and linkage mapping shows the power in developing a more dense linkage map and improved comparative map.

Recently, the chicken genome has been rated as a "high priority" for full genome sequencing, along with those of the chimpanzee, honeybee, sea urchin, *T. thermophila*, and several fungi by National Human Genome Research Institute (NHGRI). The Washington University Genome Center has recently been granted permission to begin work on the chicken genome sequence (along with the chimp) (http://poultry.mph.msu.edu/newsltrs/Newsltrs.asp. Date accessed: June 12, 2002).

The human genome has been fully sequenced, and it was found that the old paradigm of one gene equals one protein is incorrect (Godovac-Zimmermann 2002). One gene could generate 6 to 8 proteins, because of splice variants and post-transcriptional modifications (Godovac-Zimmermann and Brown 2001). It will be very essential to evaluate the quantitative, temporal levels of these proteins, and the highly complex networks of interactions, in order to understand biological processes and functions such as signal pathways. Complex networks of interactions can be divergent in differentiated cells of the same organism. Proteomics as a new technology is able to analyze the quantitative level, locations, temporal changes, and interactions of many thousands of proteins simultaneously (Hochstrasser et al. 2002). Proteome chips technology has been applied in yeast with 5800 proteins and many new calmodulin and phospholipid interacting proteins were identified (Zhu et al. 2001). Proteomics is still far away from application in farm animals, compared to human, and mouse, however, the analytical power of this technique in functional genomics sets it up as a promising research tool for livestock in the future.


Figure 1. Gene network among fifteen immunity genes in an F2 Population
APPENDIX ABBREVIATIONS

APC = antigen-presenting cell; BA = Brucella abortus; B2M = Beta-2-Microglobulin; BS = band-sharing values; chB6 = chicken B-cell marker; DAD1 = Defender Against Cell Death 1; Dps = the proportion of shared alleles distances; ELISA = enzyme-linked immunosorbent antibody assay; Equil. = equilibrium phase; FDR = false discovery rate; FF = homozygote from Fayoumi allele; IAM = infinite allele model; IAP1 = Inhibitor of Apoptosis 1; IFN-γ = interferon-γ; IgL = immunoglobulin light chain; IL2 = Interleukin 2 Precursor; IL-15Rα = interleukin-15 receptor α chain; IL-2Rγ = interleukin-2 receptor γ chain; IREB1 = IRE-Binding Protein 1; LAP18 = Leukemia-Associated Phosphoprotein p18; LF = heterozygote from Leghorn and Fayoumi alleles; LL = homozygote from Leghorn allele; MAFL = L-MAF, bZIP Transcription Factor; MAS = marker-assisted selection; MHC = major histocompatibility complex; OD = optical density; PBS = phosphate buffered saline; POU1F1 = Pou Domain, Class I Transcription Factor 1; RAPD = randomly amplified polymorphic DNA; RFLP = restriction fragment length polymorphism; RREB1 = Ras-Responsive Element Binding Protein 1; S. enteritidis = Salmonella enteritidis; SMM = stepwise mutation model; SNP = single nucleotide polymorphism; SRBC = sheep red blood cells; TAD = Thymocyte Activation and Developmental Protein; Tmax = time needed to achieve secondary maximum titers; Tmin = time to achieve secondary minimum titers; TBPl = TA-TA Binding Protein 1; TCRG = T-cell Receptor Gamma Chain Vgl-Jg2; TGFβ2 = transforming growth factor β 2 gene; TGFβ3 = transforming growth factor β 3 gene; QTL = quantitative trait loci; Y = primary phase response; Ymax = maximum secondary titers; Ymin = minimum secondary titers; ZOV3 = immunoglobulin superfamily gene.
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