INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

UMI®
800-521-0600

IDENTIFICATION OF DNA MARKERS WHICH ARE ASSOCIATED WITH EGG PRODUCTION TRAITS AND MAREK'S DISEASE RESISTANCE IN CHICKENS

Suiyang Li

Department of Animal Science
McGill University, Montreal

April, 1998

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirement for the degree of Doctor of Philosophy

Suggested short title :

DNA Markers Associated with QTLs in Chickens

Acknowledgements

I would like to express my gratitude and appreciation to my supervisors Dr. Urs Kuhnlein and Dr. David Zadworny for their guidance, encouragement and patient advice through the course of the work.

I would like to thank Zhou, Yao, Xiaopeng, Gregoy, Nicola and other colleagues in the laboratory for their help and friendship. I would like also to thank Mrs. Leny Volkov for her technical advice.

My appreciation and gratitude also go to Dr. Paul C. Lague for the time he spent to serve as a member of the supervisory committee and for his valuable advice and suggestions. Meanwhile, the help and friendship from all of the staffs in the Animal Science Department are also gratefully acknowledged.

Special thanks to my dear wife, Qun Lan, for her love and dedication to the family. She is a wonderful wife to me and a wonderful mother to our son. She has ambitious goals for her own future career. However, she has given up many opportunities for herself and decided to support me before taking the time to pursue her graduate study. Needless to say, I attribute much of this thesis to her.

Abstract

Production traits and disease resistance are believed to be under the control of many genes, *i.e.* quantitative trait loci (QTL). The objective of the present study was to establish a methodology for identifying DNA markers which are associated with QTL in chickens using an alternative approach to the traditional linkage analysis. A systematic screening approach was designed to search a chicken liver cDNA library for clones which revealed polymorphisms associated with traits. In the first stage of the experiment, a total of 92 cDNA clones were subjected to restriction fragment length polymorphism (RFLP) analysis. About 33% and 22% of the clones revealed DNA polymorphisms at *Msp*I and *Taq*I restriction sites, respectively. Subsequently, DNA polymorphisms which responded to selection were identified by comparing RFLP frequencies in divergently selected strains of chickens. About 60% of the RFLPs responded to selection for egg production traits and/or Marek's disease (MD) resistance. Trait associations of these RFLPs were then studied by selectively genotyping individuals at the extremes of trait distributions, followed by an analysis of individuals in the entire population and statistical evaluation. Finally, RFLP regions of DNA markers were characterized and PCR assays for rapid RFLP screening were developed. DNA markers in two genes were identified and characterized by this methodology. One was a marker in the chicken mitochondrial genome which arose from a nucleotide substitution (T to C) in the NADH subunit IV gene. Statistical analysis for typing random individual samples from the strains showed that this DNA polymorphism was associated with mature body weight and egg specific gravity which is a strong indicator for egg shell thickness. Other analyzed markers were located in the chicken mitochondrial phospho-enolpyruvate carboxykinase (PEPCK-M). Using the cDNA of this gene as a probe, southern blotting revealed a highly polymorphic band pattern. Statistical analysis for band frequencies between divergently selected strains for susceptibility or resistance to disease showed that some polymorphic bands were associated with Marek's disease resistance. Further, an RFLP in PEPCK-M was found to have significant effects on correlations between traits without affecting the means of individual traits. The latter

observation is a paradigm for a novel way of elucidating the genetic underpinning of quantitative traits.

Résumé

Les traits de production et de résistance aux maladies sembleraient être sous le contrôle de gènes multiples, nommés "traits aux multiples loci" (QTL). L'objectif de cette étude était d'établir une méthode d'identification de marqueurs d'ADN associés avec des QTL chez le poulet en utilisant une approche alternative à la méthode traditionnelle d'analyse par coségrégation. Une méthode systématique de criblage a été mise au point pour chercher, à l'intérieur d'une librairie de cDNA établie à partir du foie, des clones révèlant des polymorphismes associés à des traits de production. En premier lieu, un total de 96 clones ont été analysés par la technique de polymorphismes de longueur de fragments de restriction (RFLP's). Environ 33% et 22% de ces clones ont révélé des polymorphismes à des sites de restriction *Msp*I et *Taq*I respectivement. Subséquemment, les polymorphismes répondant à la sélection pour des traits économiquement importants ont été identifiés en comparant les fréquences RFLP chez des lignées de poulets sélectionnées de façon divergente pour ces traits de production. Environ 60% des RFLP's ont répondu à une sélection pour des traits de production d'oeufs et/ou de résistance à la maladie de Marek (MD). Les associations de ces RFLP's avec des traits de production ont tout d'abord été étudiés en déterminant de façon sélective le génotype des individus appartenant aux extrêmes des distributions phénotypique de ces traits, elles ont ensuite été analysées chez tous les individus des populations concernées, et les données recueillies ont été évaluées statistiquement. Ultérieurement, les régions RFLP des marqueurs identifiés ont été caractérisés et des amorces de PCR ont été élaborées pour permettre un criblage rapide de ces sites RFLP's. Des marqueurs à l'intérieur de deux gènes ont été identifiés et caractérisés de cette façon. L'un était un marqueur à l'intérieur du génome mitochondrial du poulet issu d'une substitution de nucléotide (T pour C) dans la sub-unité IV du gène de la NADH. L'analyse statistique de génotypes d'individus pris au hasard à l'intérieur des lignée a démontré que ce polymorphisme était associé avec le poids à maturité et avec la gravité spécifique des oeufs qui est un important indicateur de l'épaisseur de la coquille. D'autres marqueurs ont été localisés dans le gène mitochondrial de la phospho-enolpyruvate

carboxylase (PEPCK). En utilisant le cDNA de ce gène comme sonde dans des hybridations Southern, un patron de bandes de nature très polymorphique a été observé. L'analyse statistique de la fréquence des bandes entre les lignée sélectionnées de façon divergente pour la susceptibilité ou pour la résistance à la MD a démontré que certaines bandes polymorphiques étaient associées avec la résistance à la MD.

Table of Contents

Acknowledgements i
Abstract iii
Résumé v
Table of Contents vii
List of Tables x
List of Figures xii
Contribution to the Original Knowledge xiii
Contribution of Authors to the Manuscripts xv
Abbreviations xvii

Preface 1

Chapter 1. General Introduction 5

Chapter 2. Literature Review 9
2.1 Identification of quantitative trait genes in chickens 9
2.1.1 QTL mapping in chickens 9
2.1.1.1 The chicken genetic map 9
2.1.1.2 QTL mapping 10
2.1.2 Candidate gene approach 11
2.2 Objectives and overall strategy 12
2.3 Analysis of production traits 13
2.3.1 Origin of strains 13
2.3.2 Egg and egg-related production traits 14
2.4 Analysis of Marek's disease resistance 16
2.4.1 Marek's disease 16
2.4.2 Origin of strains 17
2.5 Experimental design 19

2.5.1 Liver as candidate tissue 19
2.5.2 RFLP as DNA markers 20
2.5.3 The choice of restriction enzymes 21

Connecting Statement I 23

Chapter 3. Genetic Variability in White Leghorns Revealed by Chicken Liver Expressed Sequence Tags (Manuscript #1) 25
Abstract 26
Introduction 27
Materials and Methods 28
Results and Discussion 30
References 33

Connecting Statement II 43

Chapter 4. Rapid Identification of DNA Markers Co-selected for Egg Production Traits and Marek's Disease Resistance in Chickens (Manuscript #2) 45
Abstract 46
Introduction 47
Materials and Methods 49
Results 51
Discussion 54
References 56

Connecting Statement III 69

Chapter 5. Evidence for a Genetic Variation in the Mitochondrial Genome Which Affects Traits in White Leghorn Chickens (Manuscript #3) 71
Summary 72

Introduction 73
Materials and Methods 75
Results 78
Discussion 81
References 84

Connecting Statement IV 93

Chapter 6. Mitochondrial PEP-CK: A Highly Polymorphic Gene with Alleles Co-selected with Marek's Disease Resistance in Chickens (Manuscript #4) 95
Summary 96
Main text 97
References 100

Connecting Statement V 105

Chapter 7. Identification of Quantitative Trait Loci by Analyzing Trait Correlations: PEPCK-M in Chickens as a Paradigm (Manuscript #5) 107
Abstract 109
Introduction 110
Materials and Methods 112
Results 114
Discussion 117
Literature Cited 119

Chapter 8. Conclusions and General Discussion 131

Chapter 9. References 135

Appendix I 151
Appendix II 153

List of Tables

In manuscript #1:

1. Similarity of chicken liver expressed sequence tags with known DNA sequences 36
2. Genetic variability in different classes of chicken liver cDNA clones 39
3. Comparison of the RFLP frequencies in genes coding for metabolic enzymes with RFLP frequencies in other genes 40

In manuscript #2:

1. Origin of strains 59
2. Evaluation of RFLP responsive to selection for egg production 60
3. Ranking RFLPs responsive to selection for egg production traits and identity of clones 62
4. Ranking RFLPs responsive to selection for Marek's disease resistance and identity of clones 64
5. Selective genotyping by bulk analysis for CLEST045 66

In manuscript #3:

1. Frequency of the mitochondrial *Msp*I+ allele in White Leghorn strains of different genetic origin 87
2. Least-square means of body weight, egg weight and egg specific gravity in dependence of the mitochondrial genotype in strain 7 88
3. Significance of the mitochondrial and GH genotype and their interaction in strain 7 89
4. Least-square means for body weight and egg specific gravity in dependence of GH and mitochondrial genotypes in strain 7 90

In manuscript #4:

Frequency of PEPCK-M alleles in strains divergently selected for MD susceptibility 102

In manuscript #5:

1. Segregating haplotypes in exon 9 of PEPCK-M 121
2. Least square means of traits in different *Acc*I RFLP genotype classes 122
3. Influence of the *Acc*I RFLP genotype on trait correlation 123
4. Influence of the *Acc*I RFLP on the correlation of traits with post juvenile weight gain (130-365d) 125

In Appendixes:

I. List of assigned GenBank accession numbers for the chicken liver expressed sequence tags (CLESTs) 151

List of Figures

In manuscript #1:

Bulk analysis of strains for segregating RFLPs by Southern blotting using CLEST058 as a probe 41

In manuscript #2:

A representative Southern blot for study of trait association 67

In manuscript #3:

1. Southern blot of *Msp*I digested DNA from chickens of strain 7 hybridized with the mitochondrial clone CLEST020 91

2. Direct sequencing of the PCR product of the polymorphic region of a *Msp*I^{+} and a *Msp*I^{-} alleles from strain 7 92

In manuscript #4:

Representative Southern blots of *Msp*I-digested DNA probed with the PEPCK-M probe CLEST061 103

In manuscript #5:

1. PEPCK-M genotypes in strain 7 revealed by PCR amplification of parts of exon 9 followed by SSCP analysis 126

2. Proposed regulatory loop that stabilizes egg weight in the breeding population .. 127

3. Best linear fit indicating the relation between EGWT1 and FBWT 128

4. Best linear fit indicating the relation between EGWT1 and HDR1 129

Contributions to Original Knowledge

1. A new methodology for identifying DNA markers that are associated with QTL in chickens was developed. It had several advantages over traditional methods, *e.g.* linkage analysis. First, this method utilized DNA samples and trait information from divergently selected strains of chickens, which have been already established. Therefore, it does not require generation of additional inbred lines or F_2 crosses. Second, the choice of candidate tissues provided a focus on genes of interest rather than anonymous DNA markers. Third, the method may lead to the identification of DNA markers that are very close to genes that are responsible for the traits. Last but not the least, the method can be carried out in a relatively small-scale laboratory and the goals can be achieved in a relatively short period of time.

2. It is the first report of an analysis of a large number of randomly selected cDNA clones from the chicken liver cDNA library by DNA sequencing and an assessment of genetic variability in White Leghorns revealed by these clones. A total of 84 expressed sequence tags (ESTs) from the chicken liver were submitted to the GenBank database (dbEST) with accession number W66528-W66591. The sequences of all these ESTs are available to the public for future QTL mapping or identification of new genes in chickens or in other species.

3. It is the first report in the literature on a DNA marker in the chicken mitochondrial genome, which is associated with egg production traits in chickens. A PCR assay for rapid detection of this DNA marker was established. This is the first strong indication that genes involved in the energy/intermediary metabolism are associated with production traits.

4. It is the first report in the literature that the chicken mitochondrial PEPCK contains a highly polymorphic region, which may be associated with Marek's disease resistance.

This association was inferred from an analysis of 12 divergently selected strains which were derived from 5 different genetic backgrounds.

5. A PCR assay was established for an *Acc*I RFLP in PEPCK-M and it was shown that this RFLP was associated with differences in the correlation between quantitative traits.

Contribution of Authors to the Manuscripts

Manuscript #1

S. Li, Ph.D. candidate, designed all the experiments, performed all of the laboratory work described in the manuscript and wrote the first draft of the manuscript. He conducted bulk analysis of Southern blotting assay for identification of RFLP and analyzed the data of Southern blots. He also carried out the DNA sequencing assay, conducted the homology search of DNA sequences against database, organized the sequence data and submitted them to the EST database.

N. Liu performed a preliminary examination of 10 other cDNA clones from the same chicken liver cDNA library.

D. Zadworny and ***U. Kuhnlein*** provided funding and gave advice and suggestions throughout the project.

Manuscript #2

S. Li, Ph.D. candidate, designed all the experiments, performed all of the laboratory work described in the manuscript and wrote the first draft of the manuscript. He analyzed the band intensities on the Southern blots and evaluated the selection index for each DNA markers. He also conducted the analysis of clones CLEST045 and CLEST020 for trait association by selective genotyping.

D. Zadworny and ***U. Kuhnlein*** provided funding and gave advice and suggestions throughout the project.

Manuscript #3

S. Li, Ph.D. candidate, designed all the experiments, performed all of the laboratory work described in the manuscript and wrote the first draft of the manuscript. He conducted and carried out the experiments for identification of *Msp*I RFLP on the chicken mitochondrial genome. He also designed the PCR primers for amplification of this RFLP region and carried out the experiments of direct sequencing of the PCR

product leading to the discovery of the mutation site on the chicken mitochondrial genome.

S.E. Aggrey, performed the statistical analysis associating the mitochondrial RFLP with traits in strain 7.

D. Zadworny and ***U. Kuhnlein*** provided funding and gave advice and suggestions throughout the project.

Manuscript #4

S. Li, Ph.D. candidate, designed all the experiments, performed all of the laboratory work described in the manuscript and wrote the first draft of the manuscript. He carried out the Southern blotting assays for individuals using PEPCK-M as a probe, analyzed the data from Southern blots and performed the chi-square tests.

S.E. Aggrey, gave some suggestion for the statistical analysis.

D. Zadworny and ***U. Kuhnlein*** provided funding and gave advice and suggestions throughout the project.

Manuscript #5

A. Torkamanzehi, performed the PCR-SSCP analysis for individuals from strain 7, identified the mutation sites by DNA sequencing and wrote the first draft of the manuscript.

S. Li, Ph.D. candidate, participated in the design of experiments and revision of the manuscript. He was responsible for designing the PCR primers, establishing the PCR-SSCP assay and discovering a highly polymorphic region in the 3' end of the coding region of PEPCK-M.

S.E. Aggrey, gave some suggestion for the statistical analysis.

J. Yao, gave some suggestion for the SSCP analysis.

W. Fairfull, provided the blood samples from the strains of chickens and collected the information of trait performance for the individuals that were analyzed in the present study.

D. Zadworny **and** ***U. Kuhnlein*** provided funding and gave advice and suggestions throughout the project.

Abbreviations

1,25-$(OH)_2D_3$	1,25-dihydroxyvitamin D_3
25-OHD_3	25-hydroxyvitamin D_3
A	adenine
AFE	age of first egg
ALV	avian leukosis virus
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
ATPase6	adenosine 5'-triphoshpatase subunit 6
C	cytosine
cDNA	complementary deoxyribonucleic acid
CFAR	Center for Food and Animal Research
CLEST	chicken liver expressed sequence tag
COII	cytochrome oxydase subunit II
CTP	cytidine 5'-triphosphate
d	2'-deoxy-
DNA	deoxyribonucleic acid
EST	expressed sequence tag
EW	egg weight
EWT	egg weight
FBWT	final body weight
G	guanine
GH	growth hormone
GHR	growth hormone receptor
GTP	guanosine 5'-triphosphate
HBW	housing body weight
HBWT	housing body weight
HDR	hen day rate (egg laying rate)
IMP	inosine 5'-monophosphate (inosinate)

LL	lymphoid leukosis
LRP	low-density lipoprotein receptor-related protein
MBW	mature body weight
MD	Marek's disease
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide (reduced form)
NSERC	Natural Sciences and Engineering Research Council
ODC	ornithine decarboxylase
OXPHOS	oxidative phosphorylation
P	probability
PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEPCK	phospho-enolpyruvate carboxykinase
PEPCK-C	cytosolic phospho-enolpyruvate carboxykinase
PEPCK-M	mitochondrial phospho-enolpyruvate carboxykinase
PFK-1	phosphofructokinase-1
PurH	5-aminoimidazole-4-carboxamide-ribonucleotide transformylase-IMP cyclohydrolase
QTL	quantitative trait locus
RAPD	random amplified polymorphic DNA
RFC	residual feed consumption
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulfate
SG	specific gravity
SNP	single nucleotide polymorphism

SSCP	single-stranded conformation polymorphism
T	thymine
tRNA	transfer ribonucleic acid
TTP	thymidine 5'-triphosphate
U	uracil
VNTR	variable number of tandem repeats

Preface

The thesis presented here describes a new method for the rapid identification of DNA markers, which are associated with production traits and disease resistance in chickens. This new method led to the identification of a DNA marker in the chicken mitochondrial genome which affects production traits, and a highly polymorphic region on the chicken mitochondrial phospho-enolpyruvate carboxykinase (PEPCK) gene which is co-selected with resistance to Marek's disease (MD). The thesis resulting from this study contains 5 manuscripts submitted or to be submitted for publication. In accordance with the Guidelines for Thesis Preparation from the Faculty of Graduate Studies and Research, McGill University, the text of paragraphs that apply to the format of this thesis is quoted as following:

> *"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.*
>
> *If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.*
>
> *The thesis must still conform to all other requirements of the Guidelines for Thesis Preparation. The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.*

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers."

The thesis consists of 9 chapters and two Appendixes. Chapter 1 gives a general introduction to the thesis, indicating the objective of this project and the experimental proposal in brief. Chapter 2 gives a review on genetic dissection of complex traits using linkage analysis or alternative approaches, the current status of QTL mapping in chickens and the rationale of this project. Chapters 3 to 7 are five manuscripts resulting from this study. The order of these manuscripts is edited in such a way that they may reflect the different stages of the project in progress. Chapter 3 (manuscript #1) presents the initial screening of a large number of chicken liver cDNA clones for restriction fragment length polymorphisms (RFLP) and the identity of these clones based on a comparison with databases. Chapter 4 (manuscript #2) describes the identification of DNA polymorphisms, which have responded to selection for production traits and disease resistance in chickens. The establishment of a new systematic approach, *e.g.* candidate tissue approach, for rapid identification of DNA markers in chickens is emphasized in this chapter. Chapter 5 (manuscript #3) presents the characterization of a DNA marker in the chicken mitochondrial genome and its trait association. Chapter 6 (manuscript #4) describes that the chicken mitochondrial PEPCK habours RFLP markers at *Msp*I sites that are co-selected with Marek's disease (MD) resistance. In Chapter 7 (manuscript #5), a highly polymorphic region of the chicken mitochondrial PEPCK gene is characterized

by the Single-stranded Conformation Polymorphism (SSCP) and a RFLP in this region is shown to be associated with changes in the correlation between quantitative traits. Conclusions and general discussion of this study are given in Chapter 8. Finally, the references for the whole thesis are listed in Chapter 9.

Chapter 1. General Introduction

Selection for improvement of production traits for farm animals, such as egg weight, egg laying rate and body weight in chickens, has been very successful. However, the genetic basis of selection is not well understood due to the lack of methods to genetically dissect complex traits.

Poultry are an ideal system to study the architecture of complex traits. Their generation time of 20 weeks is relatively short. Well-defined control and selected strains kept at relatively large population sizes are available, and extensive production records have been kept. Besides contributing to basic knowledge, information about the genetic variations that affect complex traits may be applied to breeding at the DNA level. Such selection is different from selection at the phenotypic level. While phenotypic selection affects a whole array of genes that determine the particular phenotype, selection at the DNA level affects an array of phenotypes that are under control of the particular gene. Understanding the genetic architecture of traits may provide knowledge for the development of new breeding strategies, in particular in the design of hybrid breeds and the introgression of genes (Hillel *et al.*, 1992).

Identification of quantitative trait genes, *i.e.* genes that segregate for alleles that differ in their effect on phenotypes, is ultimately achieved by associating phenotypes with nucleotide variations (DNA markers) in genes. Such association studies are confounded by linkage disequilibrium of the quantitative trait loci (QTL) with other markers and by random associations that are expected to occur by chance when a large number of markers are being analyzed. In order to circumvent the latter problem, methods of QTL mapping have been devised which can be used to locate a QTL to chromosome segments, thus greatly reducing the number of genes that have to be tested to detect nucleotide variations associated with traits. QTL mapping has been successfully applied for complex traits of some plant crops. However, it has not yet been extensively applied to farm animals, including chickens, the subject of this research (Tanksley, 1993; Lynch and Walsh, 1998).

An alternative strategy is to restrict association studies to DNA markers in candidate genes. Candidate genes are known genes, which produce proteins involved in determining the trait of interest. Restricting the search to candidate gene markers greatly reduces the

number of markers to be tested and hence increases the resolving power of the statistical analysis. However, it requires that the test population is in linkage equilibrium and does not consist of an admixture of genetically diverse subpopulations (Lander and Schork, 1994).

The candidate gene approach led to the identification of several allelic variations affecting quantitative traits in livestock. In our laboratory, we have provided evidence for genetic variations of the chicken growth hormone (GH) and GH receptor genes, which affected egg production traits and disease resistance. In this thesis we have used an extended version of the candidate gene approach, which we dubbed "candidate tissue approach" or "semi-candidate gene approach". It involves the identification of DNA markers in genes that are expressed in a particular organ whose function is important in relation to the trait of interest. This approach widens the number of genes considered, since unknown but expressed genes are also included.

The liver was chosen as the target tissue, since it plays a major part in the carbohydrate and lipid metabolism of the body as a whole. It provides the interphase between the digestive tract and the blood in the uptake and processing of nutrients and is responsible for the synthesis of most proteins present in the blood. Further, in birds, as well as amphibians, hepatocytes are synthesizing all the proteins present in the egg yolk. Hence it is expected that hepatocytes abundantly express genes which are important in determining production traits. For other traits such as immune responsiveness, the bursa, spleen or thymus may be more promising target tissues. Since abundance of expression may not necessarily entail biological importance, the "candidate tissue approach" may be extended to less frequently expressed genes or to genes specifically expressed in an organ by constructing subtraction libraries (Schraml *et al.*, 1994).

The objective of this thesis was *to establish a screening method for the identification of DNA markers, which are associated with QTL in chickens*. The strategy consisted of five stages: (1) Individual cDNA clones are randomly selected from a chicken liver cDNA library and screened by bulk analysis for those clones that reveal restriction fragment polymorphism (RFLP) in strains of interest. (2) The cDNA which reveal RFLP, whose frequency differs in divergently selected strains of poultry are identified. (3) RFLP that had responded to selection are analyzed for association with traits by selectively genotyping individuals from extremes of the distribution of the trait using bulk analysis. Analyzing

individuals validates the results obtained from bulk screens. (4) The genomic regions harboring DNA markers are characterized and PCR assays for rapid RFLP screening are developed. (5) Based on the sequence information, the relatedness of the gene with known genes is analyzed using sequence data banks.

The thesis is written as a series of papers. The first paper lists the partial sequence of random cDNA clones of a chicken liver cDNA library and their identity, which is established by sequence comparison with known genes from chickens or other species. It allowed us to assess the relative abundance of known expressed genes and the redundancy among randomly isolated liver cDNA clones. Further, for the first time the abundance of DNA polymorphism in expressed genes of egg-laying chickens was assessed, giving an idea about the genetic diversity still present in modern chickens. In a second paper, the change in frequency of marker alleles in liver-expressed genes in selected *vs.* control strains was assessed. The strains compared were strains selected for production traits and their corresponding non-selected strains, as well as strains with contrasting susceptibility for Marek's disease resistance. The magnitude of these changes was used to identify polymorphic genes that were promising for further analysis. This prescreening method was validated in three subsequent papers where markers in two genes, a mitochondrial gene coding for a subunit of NADH dehydrogenase and a nuclear gene coding for the mitochondrially located enzyme phospho-enolpyruvate carboxykinase (PEPCK) were analyzed in more details.

In the literature review proceeding these papers, the status of quantitative trait gene identification in chickens is briefly discussed. It also provides a more detailed rationale of our approach, the development of the strains used in our analysis and the physiological role of the liver with respect to production traits and disease resistance.

Chapter 2. Literature Review

2.1 Identification of quantitative trait genes in chickens

2.1.1 QTL mapping in chickens

As indicated in the General Introduction, the goal of QTL mapping is to identify chromosomal segments that harbor allelic variations responsible for phenotypic differences between individuals. This greatly reduces the number of genes that have to be tested for trait association and hence improves the statistical power of association studies aimed at identifying the responsible allelic variations at the DNA level. Although the validity of this argument has been questioned for traits with low penetrance or traits that explain less than 10% of phenotypic variation (Risch and Merikangas, 1996), QTL mapping is the prominent methodology used in plants and aimals. In plants this is partially due to the relative ease of conducting a large number of crosses, which make high-resolution mapping possible. Further, mapping information can be used to introgress chromosomal DNA segments without knowledge of the molecular basis of particular QTL.

2.1.1.1 The chicken genetic map

The prerequisite for QTL mapping is a genetic map consisting of linked genetic markers evenly distributed along the genome. In chickens, the first genetic linkage map was published in 1936 (Hutt, 1936). This was the first map established in farm animals. As DNA markers such as RFLP, microsatellites, endogenous viral genes, minisatellites and random amplified polymorphic DNAs (RAPD) became available, systematic collaborative efforts to establish a complete genetic map were undertaken by several laboratories (Bumstead and Palyga, 1992). In particular, a genetic map of the chicken Z chromosome (Levin *et al.*, 1993) and an autosomal genetic map (Levin *et al.*, 1994) were generated. Since then, several other genetic maps for chicken have been established (Burt *et al.*, 1995b). Among the maps are two maps solely consisting of microsatellite markers (Cheng *et al.*, 1994, 1995; Crooijmans *et al.*, 1996).

The chicken genome consists of 39 pairs of chromosomes and has an estimated length of 2,600-3,000 cM. Two reference crosses have been used to establish the most

comprehensive linkage map - the East Lansing cross and the Compton cross (Smith *et al.*, 1997). Each of these maps covers 1,800 cM and comprises 282 and 247 markers, respectively (Burt *et al.*, 1995b). A total of 68 markers have been mapped in both crosses, which allowed to coalesce the two maps into a single map containing 461 markers with an average spacing of 10 cM.

A major current effort is to determine the map position of known genes. Examples of known genes that have been mapped are growth hormone (Shaw *et al.*, 1991), nerve growth factor β (NGF-β) (Dominguez-Steglich *et al.*, 1992), transforming growth factor-β3 (TGF-β3) (Burt *et al.*, 1995a), natural resistance-associated macrophage protein 1 (NRAMP1) (Hu *et al.*, 1995), insulin-like growth factor 1 (IGF-1) (Klein *et al.*, 1996) and subgroup A avian sarcoma and leukosis virus receptor (Bates *et al.*, 1998). Furthermore, expressed sequence tags (ESTs) have also been added to the map in recent years (Bumstead *et al.*, 1994; Spike *et al.*, 1996; Ruyter-Spira *et al.*, 1996). In addition to the study of cDNA clones, the genomic sequences of several candidate genes such as aldolase B (ALD-B), vitellogenin 2 (VTG2) and creatine kinase B (CK-B) *etc.*, had also been under investigation for detection of mutations within the introns of these genes to be useful as DNA markers (Smith *et al.*, 1996). With the mapping of known genes it should soon be possible to establish syntetic groups and make inferences from the known map position in other species (Burt *et al.*, 1995b).

2.1.1.2 QTL mapping

QTL mapping has not yet been extensively applied to chickens and only two examples have been described in the literature. One study involved the mapping of the Dominant White locus, determining a qualitative rather than a quantitative trait (Ruyter-Spira *et al.*, 1997). Bulk analysis of segregating populations revealed that 68 of a total of 168 markers were polymorphic. One marker, MCW188, which belongs to the linkage group 22 of the East Lansing reference cross, was found to segregate with the Dominant White locus. A subsequent analysis of individual chickens indicated that this trait locus was localized at a distance of 2 cM from the DNA marker MCW188.

Another example was the mapping of QTL associated with susceptibility to Marek's disease (Vallejo *et al.*, 1998). In this study, inbred lines divergently selected for MD-

resistance were intermated to produce an F_2 cross. A total of 300 individuals were challenged with MD-virus and several endpoints such as viremia, tumor incidence and mortality were measured and used to compute an index for MD susceptibility. Individuals from the two extremes of the distribution of MD susceptibility were genotyped for DNA markers belonging to different linkage groups of the chicken genetic map. Markers that co-segregated with MD-susceptibility were then identified. Further mapping was conducted by analyzing these markers and additional flanking markers in all individuals of the F_2 cross and QTL positions were determined by interval mapping. A total of two significant and two suggestive QTL located in different chromosomal subregions were identified. None of the QTL explained more than 7% of the phenotypic variance, indicating that MD resistance was conferred by multiple genes with relatively small contributions.

2.1.2 Candidate gene approach

In contrast to QTL mapping, numerous successes in identifying quantitative trait genes by the candidate gene approach have been reported. Examples are endogenous viral (*ev*-) genes, which were associated with a reduced egg production rate in a White Leghorn strain (Gavora *et al.*, 1991; Aggrey *et al.*, 1998) and with growth in meat-type chickens (Sabour *et al.*, 1992). Further, *ev*-6 and *ev*-9, both of which expressed endogenous viral envelope proteins, were associated with reduced-immunological tolerance to avian leukosis virus (Kuhnlein *et al.*, 1993). The endogenous viral gene *ev*-21 located on the Z-chromosome, is associated with the slow-feathering trait, used for sexing White Leghorn chickens (Bacon *et al.*, 1988).

Among the genes of the GH-axis, mutants in the GH-receptor (GH-R) gene are known to cause dwarfism, a trait that has been used in the poultry industry since several decades (Agarwal *et al.*, 1994). The same mutation also affects humoral as well as cell-mediated immune responsiveness (Pinard and Monvoisin, 1994). Other GH-R alleles producing less extreme phenotypes have subsequently been characterized and found to affect body weight, rate of egg production and humoral immune responsiveness. Additional genes of the GH axis, such as GH, insulin-like growth factor (IGF-1) and ornithine decarboxylase (ODC) have also been shown to segregate for alleles with differential effects on production and immune traits (Fotouhi *et al.*, 1993; Kuhnlein and Zadworny, 1994;

Aggrey *et al.*, 1996; Feng *et al.*, 1997, Kuhnlein *et al.*, 1997 and unpublished results from this laboratory).

With respect to disease resistance trait, especially Marek's disease, the chicken major histocompatibility complex (B) has been studied extensively (Guillemot *et al.*, 1989; Kean *et al.*, 1994). Chickens with B haplotypes B^{21}/B^{21}, B^{134}/B^{21} and B^{234}/B^{21} were found to be relatively resistant to MD, whereas B^{19}/B^{19} birds were highly susceptible (Hepkema *et al.*, 1993). Certain MHC haplotypes were also found to be associated with production traits, providing evidence for a relationship of metabolic activity and immune defenses (Bacon, 1987; Lamont *et al.*, 1987; Kim *et al.*, 1989; Kean *et al.*, 1994; Cahaner *et al.*, 1996). In addition, two loci at the natural resistance-associated macrophage protein 1 (NRAMP1) and the tumor necrosis factor (TNF) were found to be associated with resistance to *salmonellosis* in chickens (Hu *et al.*, 1997).

2.2 Objectives and overall strategy

The objective of this thesis was to establish and validate a rapid prescreening method for identifying DNA markers that are associated with QTL in chickens by the candidate gene approach. In addition, rather than screening exclusively for DNA markers in known genes, the candidate gene approach was extended to anonymous cDNA clones from cDNA libraries of tissues or organs which play major roles in the trait of interest. The rational was that such organs might express genes involved in a particular trait at a high level. Hence there is the potential to discover new genes whose functions are not yet known. This strategy has been used extensively in humans and the mouse where expressed genes (expressed sequence tags or ESTs) have been systematically catalogued for many different tissues (Adams *et al.*, 1993; Affara *et al.*, 1994; Allikmets *et al.*, 1995; Becker *et al.*, 1995). Most of these expressed sequence tags have been mapped and provide a valuable resource for the identification of candidate genes in chromosomal regions to which QTL have been mapped.

In chickens well-defined strains are available. Of particular value are pairs of strains that have been divergently selected for the traits of interest, or selected strains where corresponding unselected control strains were maintained. In several cases these strains were maintained at a relatively large effective population size, thus minimizing random

genetic drift and the introduction of linkage disequilibrium between distant genetic markers. Further, in the case of production traits, extensive records are being kept at research institutions as well as by poultry breeding companies.

Based on the availability of this resource, the following strategy for identifying quantitative trait genes was designed. At the first stage, a large number of cDNA clones were screened for those that reveal RFLP in the Southern blots (Southern, 1975). To do so efficiently, a pooling strategy was used (Darvasi and Soller, 1994). It included the comparison of DNA pools from different strains, rather than individuals, probing with 3 different cDNA clones at a time and hybridizing several membranes in one hybridization tube.

The same Southern blots were used to assess the differences in allele frequencies between strains by examining the relative band intensities. Although selection may affect allele frequencies differently in different genetic backgrounds, those alleles that had responded most consistently may be the most valuable for breeding purposes. Consequently a selection index reflecting this consistency was introduced and the most consistent genes were identified.

In the next stage, studies of specific trait association were carried out with individual clones as probes. If possible, RFLP sites within or outside the probing regions were determined. DNA polymerase chain reactions (PCR) were established to amplify the RFLP region (Saiki *et al.*, 1985, 1988; Mullis and Faloona, 1987; Mullis, 1990). Subsequently, the mutation was identified by direct sequencing of the PCR product, and large number of individuals was genotyped.

2.3 Analysis of production traits

2.3.1 Origin of strains

Genetic base I: Strain 7 was established in Agriculture Canada in 1958, by cross-mating of 4 commercial stocks of White Leghorns (H&N Nick Chick, Hy-Line^{R} 934A, Kimber K137, Shaver 288) being sold in North America (Gowe *et al.*, 1993). These 4 stocks were mated in a balanced crossing system to ensure that each stock has the same contribution to the genetic make-up of the strain. Since 1960, this strain was randomly mated and kept as a closed population without any selection.

Strain 8 was selected for high number of eggs produced per hen housed in the laying house from the first egg to 273 days of age. Strain 9 was selected for high egg production rate from the first egg to 273 days of age. In addition, both strains were selected for high fertility, hatchability, viability, egg weight, egg specific gravity, Haugh units as indicators of albumen quality, low incidence of blood spots in eggs, and low body weight (Gavora *et al.*, 1989).

The population size was kept with about 470 hens for the control strain and about 1,100 hens for each selected strain (Gavora *et al.*, 1980). By 1980, the inbreeding coefficients (%) for strain 7, 8 and 9 were 2.4, 9.9 and 11.6, respectively, and the mean increase in inbreeding per year were 0.11, 0.81 and 0.96, respectively (Gowe *et al.*, 1993).

Genetic base II: Strain 5 and 3 were both derived from a common base population in Agriculture Canada in Ottawa in 1950 (Gowe *et al.*, 1959a,b, 1993). Strain 5 was kept as control strain without selection. Strain 3 was selected for high number of eggs produced per hen housed in the laying house from first egg to 273 days of age. Such selection was undertaken from 1951 to 1980, except for 1970 when it was randomly bred without selection, and 1971 when there was reduced selection. In 1953, selection for fertility and hatchability was introduced for strain 3, because it was found that the reproductive performance decreased in selected strain comparing to the control strain. In 1971, strain 1 was derived from strain 3 and selected for high egg production rate from the first egg to 273 days of age. In addition, both strain 3 and 1 were selected for the same traits as the ones for strain 8 and 9 in genetic base I (Gavora *et al.*, 1989).

In order to keep these two strains as close-related populations, either one of each pair of full-sib males was used to reproduce strain 3 and 1, respectively. The population size was kept about 470 hens for control strain and about 1,100 hens for each selected strain (Gavora *et al.*, 1980). By 1980, the inbreeding coefficients (%) for strain 5, 3 and 1 were 6.3, 21.3 and 22.8, respectively, and the mean increase in inbreeding per year were 0.18, 0.68 and 0.73, respectively (Gowe *et al.*, 1993).

2.3.2 Egg and egg-related production traits

A total of 8 egg and egg-related production traits that have been well recorded were available in this study. They are housing body weight (HBW), mature body weight (MBW),

final body weight (FBW), age at first egg (AFE), egg laying rate (HDR), egg weight (EWT), specific gravity (SG) and residual feed consumption (RFC).

Body weight: HBW of pullets were measured to the nearest 10 grams when they were housed at 20 weeks of age in both genetic base I and II (Gavora *et al.*, 1980). MBW analyzed in this experiment was measured when the birds were about 1 year of age. FBW was measured at 497 days of age when the egg production test was ended.

Egg weight: Individual egg weight records were averages of the weights of eggs laid within 5 consecutive days when the birds were about 240 and 450 days of age (Gowe *et al.*, 1993). Egg size (egg weight) was introduced into the selection program in 1953 for strain 1 and 3, while in 1969 for strain 8 and 9.

Egg production: Egg production was measured as a number of eggs or as the rate of egg production. Both measurements are affected by the age at first egg (AFE). The AFE was the age when the first egg (with 5-day trapping) was recorded after day 140. It is a measure for sexual maturity. The hen-day rate (HDR) is a measure of rate of egg production. It is the number of eggs laid divided by the number of days from the age of first egg to 273 days (Gowe *et al.*, 1993).

Egg quality: Once egg production has been improved to a satisfactory level, egg quality becomes one of major concerns for breeders. Egg quality includes egg specific gravity, Haugh units as indicators of albumen quality and low incidence of blood spots in eggs *etc.* Egg specific gravity is a measure of the eggshell thickness (eggshell strength), which may affect the hatchability and damage rate during the long-distance transportation of eggs. It is also an indirect measure of calcium excretion, which accounts for a major metabolic output in the laying hen. Specific gravity was measured for the eggs at 240 days of age (Gowe *et al.*, 1993).

Residual feed consumption: Another important trait in chickens is residual feed consumption, since feed is one of the major expenses in poultry production. RCF is a measure of the metabolic efficiency of feed utilization and reflects the amount of feed consumed after correction by egg mass production, body weight and weight gain (Fairfull and Chambers, 1984; Schulman *et al.*, 1994).

2.4 Analysis of Marek's disease resistance

2.4.1 Marek's disease

Marek's disease (MD) was first described in the literature in 1907, and was named after its discoverer. MD used to be the scourge of the poultry breeder until the discovery of the MD vaccine (Kottaridis, 1969). Although vaccination proved to be efficient, new and more outbreaks have been reported more and more frequently, and currently the world-wide loss due to MD is estimated to be 1 billion dollars.

Marek's disease is a lymphoproliferative disease of chickens and caused by a herpesvirus. The enveloped viral particle is 150-160 nm in diameter. The hexagonal naked viral nucleocapsid is about 85-100 nm in diameter and contains a linear, double-stranded DNA genome, which is about 166-184 kb. MD viruses initially attack chicken B lymphoid cells in Bursa of Fabricius. Subsequently, MD viruses infect T lymphoid cells, where it may induce the transformation of T cells and cause lymphomatosis. Undifferentiated lymphocytes were accumulated in the peripheral nerves, liver, gonads and viscera or in the iris, which cause neural lymphomatosis, leukosis, range paralysis, hepatomas, visceral tumours and iritis (Kottaridis, 1969).

Under field conditions, MD virus may infect chickens as early as at the first day of hatch. Gross lesions can be observed at about 6 to 8 weeks. Mortality rate normally remains very low before 6 weeks, but most of the infected birds die before 18 months after hatching (Hutt and Cole, 1947). Not all infections, however, results in clinical disease. The virus may remain dormant for extended periods of time without observable symptoms.

The transmission of MD occurs from bird to bird by contacting the infected birds or the contaminated materials such as feather, dander, saliva and feces. Mature viral particles are found to be highly concentrated in the feather follicles, which may be the main source for spreading the disease. At 5 months of age the mortality in chickens which had been exposed to MD is still low (Hutt and Cole, 1947). It is therefore important to raise the newly hatched birds up to 5 months of age by completely isolating them from the old birds to avoid heavy losses.

2.4.2 Origin of strains

Genetic bases I and II: Since 1970, every year several females from strains 8 and 9 with the best performance in production traits were selected, and mated to their sons to form inbred lines (Gavora *et al.*, 1989). In subsequent generations, the lines were reproduced by full and half-sib matings with selection for resistance to Marek's disease (MD) in addition to the production traits used in strain 8. After three generations of selection the 10 best lines were intermated to form strain 8R. The last inbred line was incorporated into strain 8R in 1979. Strain 8R was then maintained by selection for high egg production similar to strains 8 and 9. Following the same selection method, strain 3R was derived from strains 3 and 1 in genetic base II.

Major histocompatibility complex plays a role in the MD resistance in chickens (Bacon, 1987). In strains of the genetic base I, the frequencies of haplotypes B^{21} and B^{2} were higher in selected strains 8, 9 and 8R compared to control strain 7, whereas, B^{13}, B^{12} and Y were remained at the same level or decreased in the selected strains (Gavora *et al.*, 1990).

Genetic base III (strains S and K): To study the possibility of establishing strains of chicken with genetic resistance or susceptibility to lymphomatosis by traditional selection approaches, an experiment was carried out beginning with an unselected genetic base population of White Leghorns hatched at Cornell University in 1935 (Hutt and Cole, 1947; Cole and Hutt, 1973; Gavora *et al.*, 1979). Birds under selection were naturally exposed to the "avian leukosis complex". It was later recognized that Marek's disease formed the major portion of the complex (Cole, 1968). Strains S and C were established from the individuals that were resistant and susceptible to lymphomatosis, respectively. In 1936 and 1940, several chickens from a commercial breed Kimber were introduced to the resistant line to form strain K. Strain K was additionally selected for egg production, size of egg and body weight until in 1966, when strains K and S were introduced to the Animal Research Center of Agriculture Canada (Ottawa). Since then, these two strains were maintained by random mating without selection.

The selection was successful. In the chickens hatched in 1951, mortality from the "avian leukosis complex" was 61% and 2.7% for strains S and K, respectively (Hutt and Cole, 1953). In a challenge test conducted by Grunder *et al.* (1972) for 3-week-old chicks

with MD virus strain BC-1 under laboratory conditions, the MD incidence was 85.9% and 31.2% for strains S and K, respectively. In field conditions, the mortality rate was 71.7% and 13.0% for strains S and K, respectively, which correlated well with the studies under the laboratory conditions. By 1971, the inbreeding coefficient accumulated in strain S over a period of 35 years was 39.26% by pedigree analysis (Gavora *et al.*, 1979). The DNA sample used in this study was collected from the populations hatched in 1985. An average annual egg production for strains S and K were 163 and 232 eggs per hen housed, respectively, and average egg weights at 240 days of age were 48.39 g and 52.8 g, respectively (Kuhnlein *et al.*, 1989).

It was reported that the MHC haplotype in strain K was B^{15}/B^{15} homozygous, whereas B^{21} haplotype that was thought to be associated with disease resistance was not present in this strain (Gavora, 1990).

Genetic bases III (strains Rs and Rr), IV and V: In order to study the genetic basis of lymphoid leukosis (LL) infection in chickens, pairs of divergently selected strains were established at the Institute of Poultry and Small Animals in Celle (Germany). The selection was based on tumour formation after inoculating Rous Sarcoma Virus of subgroups A and B into the wing webs of 2 to 3 week-old chickens (Hartmann *et al.*, 1984). The divergently selected sublines were reported to be homozygous resistant and homozygous susceptible to infection of LL virus, respectively.

Sublines were developed from three different genetic base populations. One of these was strain R, which was derived from the strain K (genetic base III). In 1965, hatching eggs of the Cornell strain K were imported to Germany to form strain R. The other two base populations were strain G (genetic base IV) and strain M (genetic base V). Strain G was derived in 1965 from a previously established commercial White Leghorn hybrid maintained in Germany, and strain M was derived from a commercial American White Leghorn line (Cashman) which had been kept as a closed population before importation in 1967.

Selection was made over 3 generations between 1976 to 1979. For each generation, 8 to 12 males were mated to 10 to 12 hens to propagate the individual subline. Subsequently, these six sublines Rs/Rr, Gs/Gr, Ms/Mr were kept as closed populations without selection.

Marek's disease mortality due to natural exposure was monitored in vaccinated as well as unvaccinated chickens. During the rearing and laying period progeny of different sublines were kept in separate pens, but vaccinated and unvaccinated individuals were mixed. All hens that died during the first 1- 8 months of the laying period were examined *post mortem* for the presence of lesion diagnostic for Marek's disease. Both, vaccinated as well as unvaccinated chickens gave the same result. Mortality from MD in strains Rs and Gs was several-fold higher than in their corresponding LL-resistant sublines Rr and Rs. In contrast, the subline Ms had a reduced MD mortality as compared to the subline Mr (Hartmann *et al.*, 1984).

2.5 Experimental design

2.5.1 Liver as candidate tissue

Liver is the largest secretion gland in the body of an animal (Ensminger, 1992). It is the major organ for carbohydrate and lipid metabolism. In particular, it functions as an interphase between the digestive tract and the blood. The majority of the nutrients absorbed from the gut are transported to the liver via the portal vein, where they are further metabolized by the hepatocytes and either stored or again released into the blood stream to support the metabolic needs of the organism as a whole. Further, the liver is directly connected with the lumen of the gut via the canaliculi and larger ducts through which they excrete waste products such as cholesterol and hemoglobin degradation products and bile to facilitate the uptake of fats (Hoe and Wilkinson, 1973). A major function in the clearance of metabolic waste products is carried out by the *Kupffer* cells located in the venous sinusoids of liver. This type of cells are not only capable of phagocytizing metabolic waste products, but also bacteria and other foreign matter present in the blood (Guyton, 1986). Hence, the activity of *Kupffer* cells may influence the disease resistance capacity of animals.

In addition to its role in nutrient up-take, clearance of waste products from the blood and metabolism, the liver is also the major site of production of the proteins present in the blood plasma (Ganong, 1985). This includes the components of the complement system, which play a central role in the immune response. Further, the yolk proteins present in the chicken eggs are produced by liver cells. They are released into the blood stream and absorbed by the oocytes through receptor-mediated endocytosis (Ensminger, 1992). The

synthesis of these proteins is to a large extent controlled by estrogens, indicating interplay between reproduction and liver function.

Based on its metabolic role, the liver is expected to be a site of abundant expression of genes involved in intermediary metabolism. Hence it may be a good candidate tissue when searching for genes segregating for allelic variants which affect production traits. Its choice as a candidate tissue for genetic variations affecting MD resistance may be more tenuous. However, recent results indicate that intermediary metabolism in T-cells may play an important role in the expansion of T-cells and in the maintenance of T memory cells (Field, 1996; Wu, 1996; Huang *et al.*, 1997).

2.5.2 RFLP as DNA markers

DNA markers are markers based on the direct detection of variations in the genetic material, deoxyribonucleic acid (DNA). There are two major types of genetic variations that are currently used as DNA markers. They are the restriction fragment length polymorphism (RFLP) and variable number of tandem repeats (VNTR). RFLP was the first type of DNA marker that was used for study of genetic variation (Botstein *et al.*, 1980). In a Southern blot, the band patterns revealed by a probe may be different between two individuals, as indicated by band shifts. This type of band shift results from the presence or absence of a recognition site of restriction enzyme within or at the flanking region detected by the DNA probe.

Variation in the length of the restriction fragments may also result from the numerous short tandemly repeated sequences within the fragments, which are scattered throughout the genome and called VNTR loci. The number of these tandem repeats at certain gene loci were found to be highly variable among individuals (Capon *et al.*, 1983; Goodbourn *et al.*, 1983). The great advantage of VNTR loci is their high variability. Genetic variations in VNTR regions can be used reliably for parentship and sibship identification (Jeffreys *et al.*, 1985a,b,c). Because molecular size and intensity of bands are unique, no two individuals from an outbred population (except for monozygous twins) would have identical patterns.

Genetic variation also can be detected by other methods, such as PCR-based single-stranded conformation polymorphism (PCR-SSCP) and randomly amplified polymorphic

DNA (RAPD). DNA markers generated from SSCP assay were used for trait association study of bovine growth hormone (Yao *et al.*, 1996a). In addition, DNA markers generated from RAPD were used for constructing the genetic map of the chicken Z chromosome (Levin *et al.*, 1993). More recently, study of genetic variation started to look at single nucleotide polymorphisms (SNPs). The use of SNPs as biallelic genetic markers is promising for rapid, highly automated genotyping (Kruglyak, 1997). Based on SNPs theory, a DNA chip containing up to 135,000 probes complementary to the 16.6-kilobase entire human mitochondrial genome was generated by light-directed chemical synthesis (Chee *et al.*, 1996). In this system, a two-color labeling scheme was developed that allows the detection of every nucleotide difference between two individuals in a single hybridisation.

In our study bulk analysis was used to identify segregating DNA markers in genes which are expressed in the liver, to assess whether the frequency of such markers is affected by selection and to conduct selective genotyping. It entails the analysis of pooled samples and an estimation of allelic frequency from band intensity (Darvasi and Soller, 1994). RFLP markers are ideal for such an approach (Dodgson *et al.*, 1997). However, once genes of interest have been targeted, additional genetic markers may be utilized to document the full range of genetic variability in these genes.

2.5.3 The choice of restriction enzymes

The restriction enzymes chosen in our search for RFLP were *Msp*I and *Taq*I. The recognition DNA sequences for these two enzymes are CCGG and ACGT, respectively. Cytosine in DNA may spontaneously deaminate to form uracil, which, if not repaired, result in a cytosine to thymine transition mutation at the next round of replication. Additionally, both recognition sites are containing CG dimers, which is the consensus site for DNA methylation in higher eukaryotes (Van der Ploeg and Flavell, 1980). Deamination of a 5-methyl cytosine results in the formation of a thymine residue, which has a high probability to remain unrepaired, resulting in a hotspot for a C to T transition (Coulondre *et al.*, 1978; Duncan and Miller, 1980). As a consequence, the average frequency of cytosine to thymidine in CpG sequences may be some 10 fold higher than for other bases.

Both *Msp*I and *Taq*I can cleave at their recognition sequences of the methylation status of the CpG dimer in their recognition sequence. Therefore, tissue specific DNA methylation is not expected to interfere with the detection of RFLP.

Connecting Statement I

As outlined in the introduction, the candidate gene approach can be successfully applied to association studies with quantitative trait loci (QTL) in chickens. This approach, however, requires the availability of genes, which encode rate-limiting proteins in specific pathways. In the chicken, only a relatively small number of such genes have been currently cloned. Therefore, in the first manuscript, the feasibility of utilizing random cDNA clones from a "candidate organ" in association studies was tested. The liver was chosen as a source of clones due to its central role in energy metabolism and in the synthesis of proteins and lipids associated with the production of eggs. The first objective was to randomly select clones from the cDNA library and assess whether or not they revealed any polymorphism using Southern blotting and finally to partially sequence the clones to determine the nature of their gene products.

Chapter 3 (Manuscript #1)

Genetic Variability in White Leghorns Revealed by Chicken Liver Expressed Sequence Tags[¶]

S. LI, N. LIU, D. ZADWORNY and U. KUHNLEIN[1]

Department of Animal Science, McGill University
Sainte Anne de Bellevue, Québec, Canada H9X 3V9

Published in ***Poultry Science*** **77(1): 134-139**

Corresponding Author:
Urs Kuhnlein
Dept. of Animal Science
McGill University
Ste. Anne de Bellevue, QC
Canada H9X 3V9
Tel.: (514) 398 7799
Fax: (514) 398 7964
E-mail: kuhnleinu@adradm.lan.mcgill

[¶]Received for publication March 7, 1997
Accepted for publication July 31, 1997
[1]To whom correspondance should be addressed

Abstract

A total of 92 expressed sequence tags from chicken liver (CLEST) were searched for homology with known genes. Among the CLEST 29% had no sequence similarities with known genes, 34% showed sequence similarity to rRNA, 9% to mitochondrial genes, 23% to known nuclear genes and 5% to human expressed sequence tags. Among the nuclear CLEST (excluding rRNA) clones with sequence similarity to aldolase B were represented 4 times, while all the other clones represented unique genes. The presence of *Msp*I and *Taq*I restriction fragment length polymorphisms (RFLP) associated with CLESTs was analysed by bulk Southern blotting in 16 strains of White Leghorn chickens derived from five different genetic bases. No RFLP were observed with rRNA CLEST and a single *Msp*I RFLP was observed with mitochondrial CLEST. The nuclear CLESTs with sequence similarity to known nuclear genes were grouped into two classes on the basis of their involvement in intermediary metabolism. Among the nine genes coding for metabolic enzymes all but one were polymorphic at *Msp*I and/or *Taq*I sites in at least one of the strains, whereas among the other genes six of nine were polymorphic. The average frequency of clones revealing RFLP per cDNA clone and restriction enzyme for the two classes were 0.7 and 0.3, respectively. The analysis indicated that in White Leghorns, RFLP markers in the vicinity of nuclear CLEST are relatively frequent. Further, RFLP in the vicinity of genes coding for metabolic enzymes were significantly more frequent than near genes coding for other proteins.

Key words: expressed sequence tags, chicken liver, restriction fragment length polymorphism, genetic variation, White Leghorn

Introduction

The identification of genes responsible for trait variations ultimately relies on the analysis of candidate genes either in the vicinity of previously mapped trait loci (Botstein *et al.*, 1980; Paterson *et al.*, 1988) or in a genome-wide search (Risch and Merikangas, 1996). For this purpose, expressed sequence tags (EST) have been characterised from many human tissues (Adams *et al.*, 1993; Affara *et al.*, 1994; Allikmets *et al.*, 1995; Becker *et al.*, 1995). These human EST databases provide a valuable resource for identifying genes in animal livestock on the basis of sequence homology. In addition, tissue and developmental specificity of EST expression provides information about candidate genes to be analysed for trait associations (Schraml *et al.*, 1994).

Our interest is the identification of quantitative trait genes in White Leghorns. We have therefore started to isolate and characterise EST from the liver, the major organ for the synthesis, conversion, redistribution, and storage of metabolites and blood components (see review by Hoe and Wilkinson, 1973). Our purpose was twofold. First we wanted to test the degree of redundancy when isolating random cDNA clones and the success rate in identifying EST on the basis of homology with known genes from chickens and other species. Second, we wanted to test the frequency of genetic markers in or near EST. The identification of such markers is essential for association studies of genes with traits (Risch and Merikangas, 1996).

Materials and Methods

Isolation and characterization of chicken liver cDNA clones

A chicken liver cDNA library (Lamda ZAP) prepared from 7-week-old males of a broiler-breeder strain was purchased from Stratagene[2]. Phagemids containing cDNA inserts were recovered using the *in vivo* excision protocol provided by the manufacturer. The inserts were sequenced using the Sanger chain termination method with the ThermalBase™ sequencing kit (Stratagene[2]). The M13 reverse primer (5'-AACAGCTATGACCATG-3') or T_7 primer (5'-AATACGACTCACTATAG-3') were used in the DNA sequencing reactions according to the instructions of the supplier (Stratagene[2]). The search for sequence similarity was conducted through the BLAST e-mail service (Altschul *et al.*, 1990) using the BLASTN program.

Strains of chickens

Genetic base I: Derived from a common base population established from 4 North American commercial strains in 1956 and consisting of strain 7 (control strain), strains 8 and 9 (selected for egg production traits; Gowe *et al.*, 1993) and strain 8R (selected for Marek's disease resistance; Gavora *et al.*, 1989).

Genetic base II: Derived from a strain of Canadian origin in 1950 and consisting of strain 5 (control), strains 3 and 1 (selected for egg production traits) and strain 3R (selected for Marek's disease resistance; Gavora *et al.*, 1989).

Genetic base III: Developed at Cornell in the late 1930s and consisting of strains S and K divergently selected for resistance to Marek's diseases resistance (Cole and Hutt, 1973) and strains Rs and Rr, derived from strain K and divergently selected for susceptibility to avian leukosis (Hartmann *et al.*, 1984).

Genetic Base IV: Strains Mr and Ms, derived from a North American strain in 1950 and divergently selected for resistance to Rous sarcoma virus (Hartmann *et al.*, 1984).

Genetic Base V: Strains Gr and Gs, derived from a strain of German origin and divergently selected for resistance to Rous sarcoma virus (Hartmann *et al.*, 1984). Blood

[2]Stratagene, 11011 North Torrey Pines Road, La Jolla, CA 92037, USA.

samples (1 to 2 ml) were collected into heparinized tubes and stored at -70°C until DNA was isolated.

Southern blotting

DNA was extracted from 60 µl aliquots of heparinized whole blood of female chickens using the method described by Jeffreys and Morton (1987) and dissolved in TE buffer containing 10 mM Tris-HCl (pH7.5) and 0.1 mM EDTA (pH8.0). The concentration of DNA was estimated using spectrophotometer at a wavelength of 260 nm. Equal amounts of DNA from 20 individuals were pooled for each strain. A 30-µl mixture containing 5 µg of pooled genomic DNA, 15 units of restriction enzyme and 1 × One-Phor-All buffer (Pharmacia[3]) was incubated at 37°C (65°C for *Taq*I) for 3 hours. DNA fragments were separated in a 1% agarose gel at 1.25 V/cm for 20 hours and then transferred to nylon membrane by alkali blotting (Reed and Mann, 1985). The probes used for Southern blot hybridisation were labelled by random primer extension with 4 nucleotides including α-^{32}P-CTP using the ^{T7}QuickPrime^{TM} Kit (Pharmacia). Unincorporated radioactive nucleotides were removed by filtration through a Sephadex G-50 column as described by the supplier (Pharmacia). Prehybridisation, hybridisation and washing were carried as described by Kuhnlein *et al.* (1989). Before rehybridisation the bound probe was removed from the membrane by boiling in 0.1 × SSC, 0.1% SDS for 1 hour. Based on the comparison of blots of pools and of individuals, the resolution is estimated as one to two polymorphic individuals in 20.

[3]Pharmacia Biotech. Inc., 500 Morgan Blvd., Baie d'Urfé, Québec, Canada H9X 3V1.

Results and Discussion

Sequence similarity of chicken cDNA clones with known genes

A total of 112 cDNA clones were randomly selected from a chicken liver cDNA library. Twenty clones contained no detectable inserts. The remaining 92 clones contained inserts with lengths ranging from 50 to 3,000 bp. They were partially sequenced and the sequences analysed for homology to known DNA and protein sequences of chickens and other species (Table 1). Significant DNA sequence homology with known genes or sequence human sequence tags was found for 70% of the inserts.

One third (33%) of the clones contained 28S ribosomal RNA sequences. The 28S rRNA is at the 3'-end of the 45S rRNA transcription unit, which gives rise to the 18S, 5.8S and 28S rRNA species. A single clone corresponded to the 18S ribosomal RNA. Although the ribosomal RNA transcripts do not undergo poly-adenylation, the 3'-end of the 45S precursor contains several conserved sequences (*Sal*I boxes) which are flanked by polyA. It may preclude their complete removal by poly-dT chromatography. No clones corresponding to the 5.8S rRNA were found.

Eight clones (9%) were transcripts of mitochondrial genes. Half of these encoded the mitochondrial 16S rRNA. The 16S rRNA is poly-adenylated and hence expected to be present in a cDNA library. Among the other four mitochondrial clones, two coded for ATPase6, one for $tRNA^{ser}$ and one for cytochrome oxidase II (COII). ATPase6, COII and $tRNA^{ser}$, are contiguous genes located at the mid-point of the mitochondrial genome (Desjardins and Morais, 1990). Why all mitochondrial cDNA clones are from this gene cluster is unknown.

A wide spectrum of nuclear genes other than rRNA was represented, reflecting the many physiological functions of the liver. All of these clones were represented once, with the exception of aldolase B, which was isolated four times (4% of all clones analysed). Although aldolase B is expressed at relatively high levels in liver, kidney, and small intestine (Burgess and Penhoet, 1985), the abundance of this clone in the chicken liver cDNA library was unexpected.

As expected, many sequence tags were from genes involved in intermediary metabolism. Other genes, however, were not expected to be expressed in the liver. These are vigilin that may be involved in the differentiation of chondrocytes and

osteoblasts (Schmidt *et al.*, 1992; Plenz *et al.*, 1993) and in the activation of peripheral lymphocytes (Neu-Yilik *et al.*, 1993) and agrin that induces the aggregation of acetylcoline receptors at neuromuscular junctions (Gesemann *et al.*, 1995).

Four of the clones had matches with human expressed sequence tags from genes with unknown functions, whereas for 28 (30%) no similar nucleotide sequences were found in the data banks (limited matches at the protein level are not reported). Thus, the current success rate in identifying nuclear genes (excluding rRNA) in a random chicken library is about 50% and as cataloguing efforts for EST in other species is progressing, it should soon be possible to identify every randomly isolated cDNA clone. As far as isolation of unique clones from liver cDNA libraries is concerned, care should be taken to identify redundant clones by dot blot hybridisation to rRNA, mitochondrial DNA and aldolase B. Expressed sequence tags in the present study have been submitted to the EST database (dbEST) of GenBank (accession number W66508-W66591).

Frequency of RFLP detected by liver EST in White Leghorns

The sequence variability near EST in White Leghorn chickens was assessed by analysing a total of 16 strains from five different genetic bases for the presence of RFLPs. The restriction enzymes chosen were *Msp*I and *Taq*I. The RFLP at these restriction sites occur relatively frequently, presumably because they contain the dinucleotide CG in their recognition sequence (Barker *et al.*, 1984; Cooper and Schmidtke, 1984). The CG is the consensus sequence for the cytosine methylation site and deamination may lead to C→T transitions (Sved and Bird, 1990).

The presence of segregating RFLP in strains can rapidly be identified by bulk-hybridisation. DNA from individuals is pooled, digested with the restriction enzyme and run on a gel, using one lane per strain, and hybridised with an EST clone. The banding pattern of different strains is then screened for differences in the number or relative intensity of bands. The method can be further economised by hybridising with more than one clone at a time.

The frequency of ESTs that are polymorphic at *Msp*I or *Taq*I sites in at least one of the strains is given in Table 2. None of the ribosomal EST revealed RFLP, in agreement with the known sequence conservation of rRNA. Among the mitochondrial

clones the 16S rRNA EST revealed no RFLP whereas the ATPase6, COII and the $tRNA^{ser}$ EST revealed a single *Msp*I RFLP, which was mapped to the NADH dehydrogenase subunit IV gene (unpublished). Three EST revealed repetitive sequences. Among these three, one clone was not homologous to any of the genes listed in the data banks, while the other two were related to agrin (a basal lamina protein; Gesemann *et al.*, 1995) and 18S rRNA, respectively.

Among the total of 53 nuclear genes represented by EST (including unknown EST, but excluding rRNA), 60% were polymorphic at either a *Msp*I site or a *Taq*I site. There was slight excess of RFLPs at *Msp*I sites (49%) as compared to *Taq*I sites (38%). All RFLP, except five resulted in single band shifts, indicating that the RFLP were located in introns or in flanking regions of the genes.

Among the 18 CLESTs corresponding to known nuclear genes, 9 represented enzymes involved in intermediary metabolism (Table 3). Among these the average frequency of clones revealing RFLP per restriction enzyme was 0.72, whereas the corresponding frequency for the other 9 known nuclear genes was 0.33. Whether this abundance of *Msp*I or *Taq*I RFLP in the vicinity of metabolic enzymes is diagnostic of a higher genetic variability requires more detailed sequence comparisons.

Acknowledgments

This research was made possible by the generous supply of blood samples from various strains of chickens by the Centre for Food and Animal Research (Agriculture Canada, Ottawa) and the Institute for Small Animal Research (Celle/Merbitz, Germany). Research support from the Natural Sciences and Engineering Research Council of Canada (NSERC), Agriculture Canada and Shaver Poultry Breeding Farms Ltd. is gratefully acknowledged.

References

Adams, M. D., M. B. Soares, A. R. Kerlavage, C. Fields, and J. C. Venter, 1993. Rapid cDNA sequencing (expressed sequence tags) from a directionally cloned human infant brain cDNA library. Nature Genet. 4:373-380.

Affara, N. A., E. Bentley, P. Davey, A. Pelmear, and M. H. Jones, 1994. The identification of novel gene sequences of the human adult testis. Genomics 22:205-210.

Allikmets, R., B. Gerrard, D. Glavac, M. Ravnik-Glavac, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, W. Modi, and M. Dean, 1995. Characterization and mapping of three new mammalian ATP-binding transporter genes from an EST database. Mam. Genome 6:114-117.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Barker, D., M. Schafer, and R. White, 1984. Restriction sites containing CpG show a higher frequency of polymorphism in human DNA. Cell 36:131-138.

Becker, K. G., J. W. Nagle, R. D. Canning, W. E. Biddison, K. Ozato, and P. D. Drew, 1995. Rapid isolation and characterization of 118 novel C_2H_2-type zinc finger cDNAs expressed in human brain. Hum. Mol. Genet. 4:685-691.

Botstein, D., R. L. White, M. Skolnich, and R. Davis, 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Burgess, D. G., and E. E. Penhoet, 1985. Characterization of the chicken aldolase B gene. J. Biol. Chem. 260:4604-4614.

Cole, R. K., and F. B. Hutt, 1973. Selection and heterosis in Cornell White Leghorns: a review with special consideration of inter-strain hybrids. Anim. Breedg. Abstr. 41:103-118

Cooper, D. N., and J. Schmidtke, 1984. DNA restriction fragment length polymorphisms and heterozygosity in the human genome. Hum. Genet. 66:1-16.

Desjardins, P., and R. Morais, 1990. Sequence and gene organization of the chicken mitochondrial genome: A novel gene order in higher vertebrates. J. Mol. Biol. 212: 599-634.

Gavora, J. S., U. Kuhnlein, and J. L. Spencer, 1989. Absence of endogenous viral genes in an inbred line of Leghorn chickens selected for high egg production and Marek's disease resistance. J. Anim. Breed. Genet. 106:217-224.

Gesemann, M., A. J. Denzer, and M. A. Ruegg, 1995. Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. J. Cell Biol. 128: 625-636.

Gowe, R. S., R. W. Fairfull, I. McMillan, and G. S. Schmidt, 1993. A strategy for maintaining high fertility and hatchability in a multiple-trait egg stock selection program. Poultry Sci. 72:1433-1448.

Hartmann, W., D. V.D. Hagen, and G. Heil,1984. Genetic resistance to leukosis virus infection: effects on mortality and egg production in Leghorn hens. Br. Poult. Sci. 106: 379-384.

Hoe, C. M., and J. S. Wilkinson, 1973. Liver function: a review. Australian Vet. J. 49: 163-169.

Jeffreys, A. J., and D. B. Morton, 1987. DNA fingerprints of dogs and cats. Anim. Genet. 18: 1-15.

Kuhnlein, U., J. S. Gavora, J. L. Spencer, D. E. Bernon, and M. Sabour, 1989. Incidence of endogenous viral genes in two strains of White Leghorn Chickens selected for egg production and resistance to Marek's disease. Theor. Appl. Genet. 77:26-32.

Neu-Yilik, G., H. Zorbas, T. R. Gloe, H. M. Raabe, T. A. Hopp-Christensen, and P. K Muller, 1993. Vigilin is a cytoplasmic protein: A study on its expression in primary cells and in established cell lines of different species. Eur. J. Biochem. 213:727-736.

Paterson, A. H., E. S. Lander, J. D. Hewitt, S. Peterson, S. E. Lincoln, and S. D. Tanksley, 1988. Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature 335:721-726.

Plenz, G., Y. Gan, H.M Raabe, and P. K. Muller, 1993. Expression of vigilin in chicken cartilage and bone. Cell Tissue Res. 273: 381-389.

Reed, K. C. and D. A. Mann, 1985. Rapid transfer of DNA from agarose gells to nylon membranes. Nucleic Acids Res. 13:7207-7221.

Risch, N. and K. Merikangas, 1996. The future of genetic studies of complex human diseases. Science 273:1516-1517.

Schmidt, C., B. Henkel, E. Poschl, H. Zorbas, W. G. Purschke, T. R. Gloe, and P. K. Muller, 1992. Complete cDNA sequence of chicken vigilin, a novel protein with amplified and evolutionary conserved domains. Eur. J. Biochem. 206: 625-634.

Schraml, P., R. Shipman, M. Colombi, and C. U. Ludwig, 1994. Identification of genes differentially expressed in normal lung and non-small cell lung carcinoma tissue. Cancer Res. 54:5236-5240.

Smith, E. J., H. H. Cheng and R. L. Vallejo, 1996. Mapping functional chicken genes: an alternative approach. Poultry Sci. 75:642-647.

Spike, C.A., N. Bumstead, L.B. Crittenden, and S. J. Lamont, 1996. RFLP mapping of expressed sequence tags in the chicken. J. Hered. 87:6-9.

Sved, J., and A. Bird, 1990. The expected equilibrium of the CpG dinucleotide in vertebrate genomes under a mutation model. Proc. Natl. Acad. Sci. U.S.A. 87:4692-4696.

Table 1. Similarity of chicken liver expressed sequence tags (CLESTs) with known DNA sequences

CLEST #	Description[1]	Species	Accession #	Length (bp)	Match (%)	Significance (*P*)
Ribosomal RNA:						
002	28S rRNA	Rat	gb/M29181	233	97	$2.0e^{-87}$
012	28S rRNA	Mouse	emb/X00525	111	95	$9.5e^{-35}$
017	28S rRNA	Ape	gb/K03429	122	98	$1.1e^{-41}$
019	28S rRNA	Human	emb/X69372	40	97	$3.0e^{-08}$
022	28S rRNA	Gorilla	gb/M30951	146	91	$2.4e^{-44}$
026	28S rRNA	Gorilla	gb/M30951	70	94	$1.2e^{-18}$
027	28S rRNA	Rat	gb/M29181	213	96	$2.2e^{-78}$
028	28S rRNA	*Xenopus L.*	gb/J01000	212	98	$7.6e^{-80}$
034	28S rRNA	Mouse	emb/X00525	95	94	$4.1e^{-28}$
036	28S rRNA	Human	gb/H49096	80	92	$1.3e^{-41}$
037	28S rRNA	*Xenopus L.*	gb/J01000	159	98	$2.5e^{-59}$
040	28S rRNA	*Xenopus L.*	emb/X59737	101	77	$1.3e^{-20}$
051	28S rRNA	Human	gb/R31405	169	99	$1.9e^{-64}$
054	28S rRNA	Mouse	gb/L36663	91	98	$1.8e^{-29}$
056	28S rRNA	Rat	gb/M29181	213	96	$1.2e^{-77}$
060	28S rRNA	Human	dbj/D51594	164	96	$2.3e^{-59}$
062	28S rRNA	Rat	gb/M29181	165	95	$2.4e^{-56}$
065	28S rRNA	*Amia c.*	emb/Z18726	34	100	$2.1e^{-8}$
068	28S rRNA	Rat	gb/M29181	213	96	$1.3e^{-77}$
070	28S rRNA	Rat	gb/M29181	213	96	$1.3e^{-77}$
071	28S rRNA	Rat	gb/M29181	233	96	$9.7e^{-87}$
083	28S rRNA	Mouse	emb/X00525	173	97	$2.7e^{-61}$
084	28S rRNA	Human	dbj/D51505	210	99	$1.3e^{-81}$
088	28S rRNA	Human	gb/R31255	210	94	$1.9e^{-49}$
089	28S rRNA	Human	dbj/D51586	189	99	$8.0e^{-73}$
093	28S rRNA	Rat	gb/M29181	214	95	$2.8e^{-77}$
094	28S rRNA	Rat	gb/M29181	165	95	$1.5e^{-75}$
097	28S rRNA	Human	gb/H49096	64	92	$9.6e^{-37}$
100	28S rRNA	Human	emb/Z18874	205	99	$1.3e^{-78}$

110	28S rRNA	Human	gb/H49096	80	92	$3.5e^{-42}$
102	28S rRNA	*Xenopus L.*	emb/X07623	61	100	$2.3e^{-17}$
Mitochondrial RNA:						
003	16S rRNA	Chicken	sp/X52392	236	100	$1.8e^{-90}$
031	16S rRNA	Chicken	sp/X52392	236	100	$1.8e^{-90}$
042	16S rRNA	Chicken	sp/X52392	122	98	$1.2e^{-41}$
098	16S rRNA	Chicken	sp/X52392	100	100	$6.7e^{-34}$
020	ATPase6	Chicken	sp/X52392	193	99	$8.0e^{-72}$
049	ATPase6	Chicken	sp/X52392	191	98	$3.0e^{-70}$
047	tRNA-Ser COII	Chicken	sp/X52392	139	100	$1.3e^{-48}$
066	tRNA-Ser COII	Chicken	sp/X52392	180	99	$2.0e^{-66}$
Metabolic enzymes:						
005	aldolase B[2]	Chicken	gb/M10946	241	99	$4.7e^{-91}$
006	aldolase B	Chicken	gb/M10946	127	99	$7.6e^{-85}$
078	aldolase B	Chicken	gb/M10946	140	100	$3.4e^{-91}$
108	aldolase B	Chicken	gb/M10946	151	99	$6.3e^{-66}$
021	creatine kinase B[2,3]	Mouse	gb/M74149	60	71	0.0036
039	lactate dehydrogenase B-4[2]	Duck	gb/J03869	131	96	$6.3e^{-44}$
048	aldehyde dehydrogenase isozyme 3	Human	gb/S61044	66	78	$1.1e^{-10}$
061	mitochondrial PEPCK	Chicken	gb/J05419	137	97	$1.3e^{-82}$
075	cytochrome P450	Human	gb/R36281	114	63	$2.4e^{-06}$
080	ATP synthase	Human	gb/T28033	79	75	$1.7e^{-19}$
081	cytosolic PEPCK	Chicken	gb/M14229	162	100	$9.6e^{-60}$
090	PurH	Chicken	gb/S64492	281	100	$2.4e^{-109}$
Binding proteins (receptors and inhibitors):						
014	PFK-1 inhibitor ($zinc^{2+}$ binding protein)	Rat	emb/X16481	117	77	$6.4e^{-22}$
038	vitamin D-binding protein	Rabbit	emb/D29666	175	61	$4.0e^{-13}$
073	LRP/α-2-macroglobulin receptor	Chicken	emb/X74904	87	98	$9.0e^{-28}$
085	antithrombin 3	Chicken	gb/L07842	75	100	$2.0e^{-36}$
Proteins associated with cell proliferation and metabolism of macromolecules:						
001	agrin	Chicken	gb/M94271	273	98	$7.0e^{-103}$
035	fibrinogen β chain	Chicken	gb/M58514	236	99	$6.9e^{-90}$

072	vigilin	Chicken	emb/X65292	171	97	$1.0e^{-59}$
077	factor X	Human	gb/L29433	137	64	$5.7e^{-11}$
103	elongation factor 1α[2]	Chicken	gb/L00677	147	100	$1.4e^{-53}$
Matches with other EST:						
013	yp72h10.rl	Human	gb/R51543	89	80	$1.2e^{-37}$
024	ym53h11.sl	Human	gb/H22569	81	65	0.0033
104	yh10c02.sl	Human	gb/R61859	33	69	0.0031
112	yf6905.rl	Human	gb/R14118	139	83	$1.7e^{-33}$

[1]Abbreviations: COII = cytochrome oxidase subunit II;

PEPCK = phospho-enolpyruvate carboxykinase;

PurH = 5-aminoimidazole-4-carboxamide-ribonucleotide transformylase-IMP cyclohydrolase;

PFK-1 = phosphofructokinase-1;

LRP = Low-density lipoprotein receptor-related protein.

[2] These genes have been mapped (Chick Gbase, http://www.poultry.mph.msu.edu; Smith *et al.*, 1996; Spike *et al.*, 1996)

[3] Whether sequence similarity with genes of heterologous species indicates gene identity needs confirmation.

Table 2. Genetic variability in different classes of chicken liver clones

cDNA class	Number of clones	RFLP			
		None	*Msp*I[1]	*Taq*I[1]	*Msp*I & *Taq*I
Ribosomal genes	31 (34%)	31	0	0	0
Mitochondrial genes	8 (9%)	4	4	0	0
Known nuclear genes	21 (23%)	4	4	5	8 [2]
Unknown with EST matches	4 (4%)	0	2	0	2
Unknown genes without matches	28 (18%)	17	6	1	4
Total	92 (100%)	56 (61%)	16 (17%)	6 (7%)	14 (15%)

[1]Only *Msp*I or *Taq*I RFLP, but not both.
[2] Includes 4 clones coding for aldolase B.

Table 3. Comparison of the RFLP frequencies in genes coding for metabolic enzymes with RFLP frequencies in other genes [1]

Genes	RFLP	
	*Msp*I	*Taq*I
Metabolic enzymes:		
creatine kinase B	+	-
ATP synthase	-	+
PurH	+	+
aldolase B	+	+
lactate dehydrogenase B-4	+	+
aldehyde dehydrogenase isozyme 3	+	-
mitochondrial PEPCK[2]	+	+
cytosolic PEPCK	-	-
cytochrome P450	+	+
Other genes:		
blood clotting factor X	-	+
vitamin D-binding protein	+	-
vigilin	-	+
elongation factor 1α	-	-
LRP/α-2-macroglobulin receptor	-	-
PFK-1 inhibitor	-	+
antithrombin 3	+	-
agrin	-	-
fibrinogen β chain	-	+

[1]The abundance of RFLP in the two classes of nuclear genes was assessed by averaging the frequency of clones revealing RFLP at *Msp*I sites and the frequency of clones revealing RFLP at *Taq*I sites in at least one of the five genetic bases. It amounted to 0.72 for clones homologous to genes coding for metabolic enzymes and 0.33 for genes coding for proteins not involved in metabolism. The difference is significant ($N = 18$, $\chi^2 = 4.0$, $P < 0.05$).

[2]Revealed three *Msp*I RFLP loci, respectively (unpublished result).

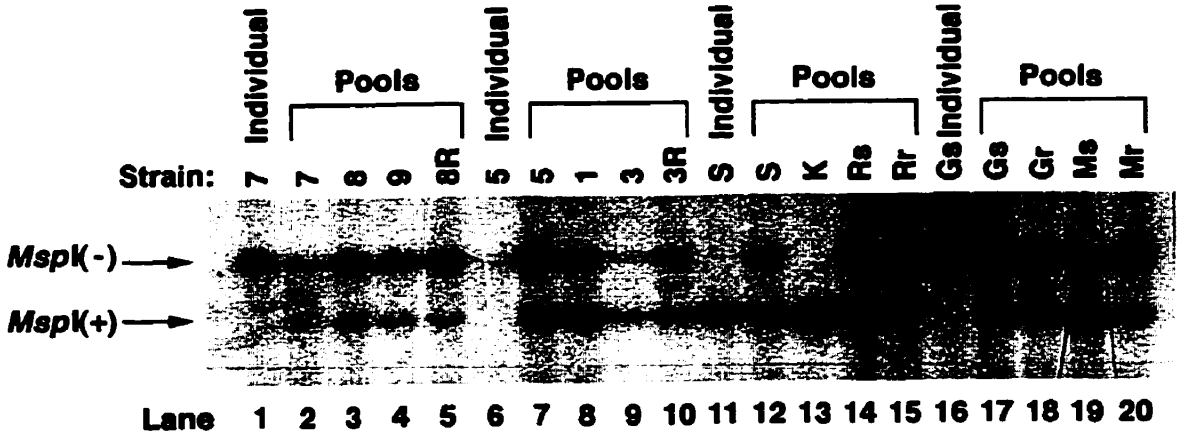

Figure. Bulk analysis of strains for segregating RFLPs by Southern blotting using CLEST058 as a probe. Pooled DNA from 20 individuals each of 16 strains of chickens from 5 different genetic bases were analysed and compared with DNA from an individual of two of the strains. The DNA samples were digested with *Msp*I and probed with CLEST058 (unknown gene). Differences in the banding pattern between strains and individuals indicates the presence of a segregating RFLP in all strains with the exception of strain K. Since only a single band shift is observed, the polymorphic *Msp*I site is presumably located in a region flanking the hybridisation probe.

Connecting Statement II

In the previous chapter, a total of 92 cDNA clones were subjected to Southern blotting analysis for detection of RFLP. About one-third of the clones revealed RFLP at either *Msp*I or *Taq*I sites, indicating that DNA polymorphism detected by the cDNA clones were abundant. This abundance of markers near genes was unexpected. Partial DNA sequencing and comparison to known genes from the nucleotide and protein sequence databases provided information about the identity of these clones. Among these clones, 29% had no sequence similarities with known genes, 34% showed sequence similarity to rRNA, 9% to mitochondrial genes, 23% to known nuclear genes and 5% to human expressed sequence tags. The screening of cDNA clones that revealed RFLP and the identity of these clones comprised the first stage of the research project.

Having established which clones revealed polymorphism, the second stage of the research was to examine whether the frequencies of these RFLP were changed upon selection for traits of interest. To do so, the Southern blots that had been prepared for the detection of RFLP in the first stage were used to screen for differences in band intensities between divergently selected strains. The band intensity reflected the allele frequency in a pool. Changes in band intensities between divergently selected strains indicated the changes of allele frequencies upon selection. To standardize the level of changes of allele frequencies upon selection, a selection index was introduced for the strains compared. A "+1" value was given to a DNA marker when the allele frequency was increased in selected strains for higher production performance or higher resistance to Marek's disease. A "−1" value was given to the same marker allele when the frequency was decreased. As a result, the genes with the most consistent changes amongst the strains in RFLP frequency upon selection had the highest score of selection index. These genes are the most promising for further analysis.

Chapter 4 (Manuscript #2)

Rapid Identification of DNA Markers Co-selected for Egg Production Traits and Marek's Disease Resistance in Chickens

Suiyang Li, David Zadworny and Urs Kuhnlein*

Department of Animal Science, McGill University,
Ste. Anne de Bellevue, Quebec,
Canada H9X 3V9

* Corresponding author:
Tel: 514-398-7799
Fax: 514-398-7964
E-mail: kuhnleinu@agradm.lan.mcgill.ca

Acknowledgement

This research was made possible by the generous supply of blood samples from various strains of chickens by the Centre for Food and Animal Research (CFAR) (Agriculture Canada, Ottawa) and the Institute for Small Animal Research (Celle/Merbitz, Germany). Research support from the Natural Sciences and Engineering Research Council of Canada (NSERC), Agriculture Canada and Shaver Poultry Breeding Farms Ltd. is gratefully acknowledged.

Abstract

Segregation analysis within families or in crosses of inbred lines is the standard approach to QTL mapping. However, an alternative is epidemiological studies within populations, which correlate marker alleles and phenotypes. In randomly bred populations, linkage disequilibrium between QTL and markers is expected to be restricted to closely linked markers and trait-associated markers may be linked to the trait gene or be located in the gene itself. To identify such markers we proposed a three-tiered approach consisting of: (1) analysis of the response to selection, (2) association analysis between marker and traits within strains by selective genotyping and (3) establishment of a polymerase chain reaction for rapid screening of the entire population. In the present study, we screened 27 liver cDNA clones which had a total of 48 RFLPs for association with egg production-related traits and Marek's disease resistance in chickens by comparing divergently selected strains or selected and control strain from 5 different genetic origins. About two-thirds of the RFLPs had responded to selection for egg production traits and/or Marek's disease resistance. Response to selection for egg production measured in duplicate strains was highly reproducible within a genetic base, but not when strains of different origin were compared. This may indicate that the effect of a gene may be highly dependent on the genetic background. Association between marker and trait was analyzed for 7 different production traits for some of the RFLPs. It showed that one of the clones revealed an RFLP, which was consistently co-selected with production traits and associated with juvenile and adult body weight.

Key words: Liver cDNA clone, selection, chicken, genetic variation, DNA markers, quantitative trait loci.

Introduction

The availability of genetic maps based on highly variable markers made it possible to identify genetic disease loci without knowledge about the underlying enzymatic defects (Cooper & Schmidtke, 1992). This methodology has been extended to mapping quantitative trait loci (QTL) in plants and animals. It involves testing for the presence of QTL in marker intervals by analyzing the association of traits in crosses of inbred lines or individuals by least squares or maximum likelihood procedures (Lander & Botstein, 1989; Zeng, 1994). Although less powerful, this method has been extended to the analysis of non-inbred lines and hence is applicable to species for which inbred lines are not available (Haley *et al.*, 1994; Georges *et al.*, 1995).

An alternative approach to mapping QTL in relation to anonymous markers of a gene map is the candidate gene approach. In this approach genetic markers in genes, which code proteins known to have an effect on a particular trait are identified and analyzed for co-segregation with the trait in question. This approach has been successful for many genetic disorders and for several traits in domestic animals (Cowan *et al.*, 1990; Rothschild *et al,* 1996; Yao *et al.*, 1996). The candidate gene approach can also be used in epidemiological studies, where trait associations are analyzed in populations rather than in individual crosses (Morton, 1993). In randomly mated populations linkage disequilibrium is only to be expected for closely linked markers. Significant associations are expected to be only observed for markers which are closely linked to the trait gene or may even be located in the trait gene itself. The latter approach provided evidence for association of alleles of the GH gene with egg production traits (Kuhnlein & Zadworny, 1994).

We previously analyzed random cDNA clones isolated from chicken liver, an organ which is expected to have important roles in intermediary metabolism, storage of nutrients and synthesis of blood serum components (Guyton, 1986). A survey of sequence tags of liver cDNA clones indicated that with the exception of ribosomal clones there was little redundancy and that 90% of all clones corresponded to known genes. The clones were used as probes to screen a series of non-inbred selected and non-selected strains of chickens for restriction fragment length polymorphisms (RFLPs). Among the clones coding for proteins with enzymatic activities, 9 out of 10 clones revealed RFLPs at *Msp*I and/or *Taq*I restriction sites. Genes not coding for enzymes were somewhat less polymorphic.

In this communication we screened the RFLPs revealed by liver sequence tags (CLESTs) for association with egg production traits and Marek's disease resistance in a series of White Leghorn strains of different origin using a two-tiered approach. First, RFLPs were tested for consistent co-selection with Marek's disease resistance or egg production traits. Second, RFLPs that had responded to selection were analyzed for trait associations using bulk segregation analysis. In the latter case, only egg production traits were available for analysis. The results indicate that genes involved in secondary metabolism may segregate with alleles that affect egg production traits as well as Marek's disease resistance.

Materials and Methods

Strains of chickens (Table 1)

Genetic base I: The genetic base I population was established at the Center for Food and Animal Research (CFAR, Agriculture Canada, Ottawa) in 1958 by cross-mating of 4 North American commercial stocks of White Leghorns to form control strain 7. It was then maintained by random mating without selection. In 1969, strains 8 and 9 were derived from strain 7. Both strains were selected for the same array of egg traits, with the exception that strain 8 was selected for high number of eggs produced per hen housed to 273 days of age, while strain 9 was selected for a high rate of egg production per hen day starting from the age at first egg to 273 days of age. In strain 8 there was additional selection pressure on early sexual maturity and low mortality (Gowe *et al.*, 1993). The Marek's disease resistant strain 8R was developed from strains 8 and 9 (Gavora *et al.*, 1989)

Genetic base II: The second genetic base population was established in 1950. Strain 5 and 3 were both derived from a narrow genetic base of stocks. Strain 5 was kept as control strain without selection. Strain 3 was subjected to the same selection criteria as strain 8. Strain 1 was derived from strain 3 and was selected similarly to strain 9 (Gowe *et al.*, 1993). The Marek's disease resistant strain 3R was derived from strains 3 and 1 using the same criteria as for strain 8R.

Genetic base III: Strain S and strain K were developed at Cornell University in the 1930's by Cole and Hutt (1973) and selected on the basis of mortality from the "avian leukosis complex". Strain K was also selected for egg production traits and included some commercial birds in its founder population. They were maintained at CFAR without selection since 1966.

Strains Rr and Rs were derived from a substrain of strain K and divergently selected for tumor formation in response to wing-web injection of Rous sarcoma virus types A and B (Hartmann *et al.*, 1984). Based on mortality, strain Rs is more susceptible to MD than strain Rr.

Genetic base IV and V: Strains Gs and Gr were derived from a European commercial hybrid whereas strains Ms and Mr were derived from a North American commercial strain. Selection was as for Rs and Rr. Compared to their avian leukosis

counterparts, mortality from MD was increased in strain Gs and decreased in strain Ms (Hartmann *et al.*, 1984).

Traits

Body weight was measured at 130 days (HBW) and 365 days (MBW) after hatch. The age at first egg (AFE) is the age when the first egg was recorded. The hen-day rate (HDR) is the number of eggs laid divided by the number of days a hen was alive in the period starting from the age at first egg to 476 days of age. The egg weight was measured at about 450 days of age and was the average of 5 eggs laid consecutively. The residual feed consumption is the amount of feed consumed which cannot be accounted for by body weight, body weight gain and egg mass produced (Fairfull & Chambers, 1984).

Southern blotting

A 30-µl mixture containing 5 µg of genomic DNA, 15 units restriction enzyme and 1 × One-Phor-All buffer (Pharmacia) was incubated at 37°C (65°C for *Taq*I) for 3 hours to overnight. DNA fragments were separated in a 1-% agarose gel at a voltage of 1.25 V/cm for 20 hours and then transferred to a nylon membrane by alkali blotting. The probes used for Southern blot hybridization were labeled by random primer extension with 4 nucleotides including α-^{32}P-CTP using the T_7-QuickPrime Kit (Pharmacia). Prehybridisation, hybridization and washing were carried out according to the method of Vassart *et al.* (1987). The membranes were re-utilized for additional hybridization reactions after boiling in a solution containing 0.1 × SSC and 0.1% SDS for 1 hour.

Results

Identification of genes with RFLPs which respond to selection

In our preliminary search for RFLPs in genes expressed in the liver, which had respond to selection, DNA samples from 20 or more individuals were pooled for each strain, digested with the *Msp*I or *Taq*I and analyzed by Southern blotting. A comparison of the signal strength of bands between strains or shifts in band positions indicated the presence of RFLPs. The comparison of divergently selected strains or control and selected strains also indicated which RFLPs had responded to selection.

The procedure can be extended two ways, DNA samples can be digested with different restriction enzymes and than mixed prior to Southern blotting or hybridization can be carried out with several probes at once. However, the high RFLPs responding to selection were numerous and the efforts required to sort out high individual components made multiplexing inefficient.

The two enzymes used in our analysis where *Msp*I and *Taq*I. Both of these enzymes contain CG, the consensus dinucleotide for methylation, in their recognition sequence. Mutations at these sequences are expected to occur frequently since deamination of methylated cytosine yield thymidine (Barker *et al.*, 1984). In addition, methylation regulates gene expression and mutations affecting methylation sites may affect regulation of expression. However, analysis of other genes revealed also a high frequency of RFLPs at H*ind*III sites (unpublished observations).

For each strain the relative intensity of bands was estimated visually. Strains were then compared and rated as +1, 0 or −1, depending on whether the intensity of a band increased, was unchanged or decreased with selection. A selection index was than computed by adding the responses of all relevant comparisons. Thus for egg production traits, control strain 7 was compared with strains 8 and 9 (genetic base I), and strain 5 with strains 3 and 1 (genetic base II), yielding a maximal score of ±4 for complete concordance and a minimal score of 0 for a random or no response to selection (Table 2). For Marek's disease resistance the score was computed from 6 pairs of strains with contrasting susceptibility belonging to 5 different genetic bases.

RFLPs co-selected with egg production traits

The response to egg production traits was analyzed in sets of strains of two different genetic basis, each set consisting of a control strain and two duplicate selected strains. In strains of genetic base I, 21 of the 27 cDNA clones tested revealed RFLPs which had responded to selection in strain 8, and 20 revealed RFLPs which had responded to selection in strain 9. The selection response of the two strains was highly concordant (Spearman rank correlation, $r = 0.61$, $P < 0.001$). In strains of genetic base II, the frequency of RFLPs responding to selection was similar and the selection response again concordant ($r = 0.53$, $P < 0.001$). The result indicates that selection in these two sets of strains had a reproducibly effect on gene frequencies. Further, the number of genes that affect egg production traits (consisting of an aggregate of 12 different traits; Gowe *et al.*, 1993) is high. These observations are consistent with previous analyses in these strains of endogenous viral genes and minisatellite loci (Kuhnlein *et al.*, 1988, 1991).

Although the response to selection was uniform within each genetic base, there was no concordance between the total selection index of the two genetic bases ($r = 0.1$, $P > 0.5$). This may indicate that the response to selection is dependent on the genetic background. Consequently observations made in one genetic background may be irrelevant in another genetic background.

Among the 27 clones analyzed 8 revealed consistent response in both genetic bases (Table 3). Among these were clones corresponding to mitochondrial phosphoenolpyruvate carboxykinase (PEPCK), aldehyde dehyrogenase 3 and cytochrome P450, respectively. The chicken PEPCK-M gene from chickens has been characterized and the gene could be identified with certainty, while the identity of the other genes is based on sequence homology with human genes. The remaining five clones all represented unknown genes.

RFLPs co-selected with Marek's disease resistance

A total of 9 genes had an average selection index per comparison of ±0.5 or greater (Table 4). Among these, five had no homology to known genes, whereas four clones were homologous to the mitochondrial gene coding for ATPase 6, creatine kinase B, PEPCK-M and adehyde dehydrogenase 3, respectively. The identification of ATPase6 was certain, since the chicken mitochondrial genome has been sequenced (Dejardins & Morais, 1990).

Creatine kinase B was identified by comparison with the human gene, but compared to P450 and aldehyde dehydrogenase, homology was relatively low.

The clones, which scored high in both selection for egg production traits and MD-resistance and could be assigned to known genes were PEPCK-M and aldehyde dehydrogenase isozyme 3. PEPCK-M catalyzes the phosphorylation of oxaloacetate to form phosphoenolpyruvate. There are two such enzymes with this catalytic activity, PEPCK-M located in the mitochondria and PEPCK-C located in the cytoplasm. They are key enzymes in gluconeogenesis and glyceroneogenesis (Hanson and Reshef, 1997). PEPCK-M was found to be highly polymorphic, segregating for at least seven *Msp*I and *Taq*I RFLPs (*i.e.* seven strain dependant bandshifts). Aldehyde dehydrogenase 3 is thought to play a role in oxidation of toxic aldehydes. It is polymorphic in human populations and highly expressed in hepatomas (Yoshida, 1992).

In the case of PEPCK and aldehyde dehydrogenase, the RFLPs co-selected with egg production traits were counter-selected with selection for MD-resistance. However, there was no systematic correlation between selection indexes for egg production and MD resistance (Spearman rank correlation, $r = -0.1$, $P > 0.5$).

Association of RFLPs with production traits within strains

Similar to response to selection, trait associations of genetic markers within strains can be rapidly tested by bulk analysis. In this procedure pools of DNA from individuals ranking at each extreme of a trait distribution are prepared and subjected to Southern blotting using the polymorphic candidate gene as a probe. Thus, in a twenty-lane gel 10 traits can be tested simultaneously. This procedure has been successfully used to screen for associations of growth hormone RFLPs with age at first egg and rate of egg laying (Kuhnlein & Zadworny, 1994) and a GH-receptor RFLP with body weight (Feng *et al.*, 1997).

An example of such an analysis is shown in Figure, where one of the high scoring clones (CLEST045, unknown gene) was tested for trait association in strain 7. An analysis of two generations each of strain 7, 8 and 9 (a total of six comparisons) indicated consistent association with mature body weight and body weight at sexual maturity, but not with other traits (Table 5).

Discussion

Association studies within a population using cDNA clones from candidate tissue provide a complementary approach to segregation analyses using a gene map of anonymous markers. It has the disadvantage that linkage in populations is expected to be low and that therefore only markers tightly linked to the QTL will show association. Therefore many different clones have to be tested for association. Further, while segregation in heterozygotes is 1:1, the trait-associated markers in a breeding population may only occur at a low frequency. The advantages are that association studies can make use of the wealth of knowledge about physiological pathways, which has been accumulated over the past decades. It also provides markers in the vicinity of known genes. Since in randomly bred strains significant linkage disequilibrium is only expected for markers which map closely to each other, the QTL may be in the marker tagged gene itself, or in neighboring genes which may be identified by comparison of map positions in other species (syntenic groups).

Our approach to identifying genes associated with quantitative traits involves three stages. The first stage is to analyze the response to selection by comparing divergently selected strains or control strains with selected strains. The second stage is to screen for trait association within strains. These two stages serve as a pre-screen for the final stage, which consists of a delineation of alleles of the particular gene, the establishment of PCR-assays and a detailed association or segregation analyses.

The response to selection used as a first screen is cumulative over several generations. It is therefore very sensitive to small differences in the phenotypic value of different genotypes. Such small differences may arise from QTL with small effect or QTL whose phenotypic contribution is hidden due to interaction with other QTL. These two classes of QTL may dominate in elite breeding populations since QTL with large additive effects are likely to be fixed rapidly. The strains analyzed in this communication were derived from strains, which had been selected in the past and therefore were likely to contain mainly QTL of the latter two categories.

Random genetic drift and extensive interaction between QTL may lead to spurious responses to selection. However, a previous analysis of endogenous viral markers and DNA fingerprinting bands indicated that the influence of selection for egg production traits on genetic markers in strains of genetic base I and II was reproducible (Kuhnlein *et al.*, 1991).

This reproducibility was also observed for the cDNA clones analyzed here. However, comparison of sets of strains from different genetic bases, revealed that the genetic background might have a strong influence on the response to selection. Hence results obtained in one strain may not be applicable to another strain. For MD-resistance duplicate selected strains were not available and the influence of random genetic drift or genetic background could not be assessed.

The second stage in our search of markers at QTL consisted of an association of markers with traits within a breeding population. This approach is restricted to markers at QTL with large and predominantly additive effects. Such QTL are most responsive to selection and hence are expected to be fixed in selected breeding populations. Association studies may therefore be more fruitfully conducted in non-selected breeding populations.

In summary, we have shown that liver cDNA clones reveal RFLPs at a high frequency. About half of these RFLPs respond reproducibly to selection. However, the response is dependent on the genetic background and selection at the DNA level may require an analysis of the particular strain in question.

References

Barker D., M. Schafer & R. White (1984), Restriction sites containing CpG show a higher frequency of polymorphism in human DNA. Cell 36: 131-138.

Cole R.K. & F.B. Hutt (1973), Selection and Heterosis in Cornell White Leghorns: a review with special consideration of inter-strain hybrids. Animal Breeding Abstracts 41: 103-118.

Cooper D.N. & J. Schmidtcke (1992), Molecular genetic approaches to the analysis and diagnosis of human inherited disease: an overview. Ann. Med. 24: 29-42.

Cowan C.M., M.R. Dentine, R.L. Ax & L.A. Schuler (1990), Structural variation around prolactin gene linked to quantitative traits in a elite Holstein sire family. Theor. Appl. Gen. 79: 577-58.

Dejardins P. & R. Morais (1990), Sequence and gene organization of the chicken mitochondrial genome: A novel gene order in higher vertebrates. Journal of Molecular Biology 212: 599-634

Fairfull R.W. & J.R. Chambers (1984), Breeding for feed efficiency: poultry. Canadian Journal of Animal Science 64: 513-527.

Feng X.P., U. Kuhnlein, S.E. Aggrey, J.S. Gavora & D. Zadworny (1997), Trait association of genetic markers in the growth hormone and the growth hormone receptor gene in a White Leghorn strain. Poultry Science 76: 1770-1775.

Gavora J.S., U. Kuhnlein & J.L. Spencer (1989), Absence of endogenous viral genes in an inbred line of Leghorn chickens selected for high egg production and Marek's disease resistance. J. Anim. Breed. Genet. 106: 217-224.

Georges M., D. Nielson, M. Mackinnon, A. Mishra, R. Okimoto, A.T. Pasquino, L.S. Sargeant, A. Sorensen, M.R. Steele, X. Zhao, J.E. Womack & H. Hoeschele (1995), Mapping quantitative trait loci controlling milk production in dairy cattle by exploiting progeny testing. Genetics 139: 907-920.

Gowe R.S., R.W. Fairfull, I. McMillan & G.S. Schmidt (1993), A strategy for maintaining high fertility and hatchability in a multiple-trait egg stock selection program. Poultry Science 72: 1433-1448.

Guyton A.C. (1986), Textbook of Medical physiology, 7th edition, W.B. Saunders Company, West Washington Square, Philadelphia, PA 19105

Haley C.S., S.A. Knott & J.M. Elsen (1994), Mapping quantitative trait loci in crosses between outbred lines using least squares. Genetics 136: 1195-1207.

Hanson R.W. & L. Reshef (1997), Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annual Review of Biochemistry 66: 581-611.

Hartmann W., V.D. Hagen & G. Heil (1984), Genetic resistance to leucosis virus infection: effects on mortality and egg production in Leghorn hens. Brit. Poultry Science 25: 43-52.

Kuhnlein U. & D. Zadworny (1994), Disease resistance genetics: selection at the DNA level. Proc. 5th World Congr. Gen. Appl. Animal Livestock 21: 249-256.

Kuhnlein U., D. Zadworny, J.S. Gavora & R.W. Fairfull (1991), Identification of markers associated with quantitative trait loci in chickens by DNA fingerprinting. In: DNA Fingerprinting , Approaches and Applications; T.Burke, G. Dolff, A.J. Jeffreys & R. Wolff, eds. Birkhauser Verlag, Basel. pp.274-282.

Kuhnlein U., M. Sabour, J.S. Gavora, R.W. Fairfull & D.E. Bernon (1988), Influence of selection for egg production and Marek's disease resistance on the incidence of endogenous viral genes in White Leghorns. Poultry Science 68:1161-1167.

Lander E.S. & D. Botstein (1989), Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121:185-199.

Morton N.E. (1993), Genetic epidemiology. Annu. Rev. Genet. 27: 523-538.

Rothschild M., C. Jacobson, D. Vaske, C. Tuggle, L. Wang, T. Short, G. Eckhardt, S. Sasaki, A. Vicent, D. McLaren, O. Southwood, H. van der Steen, A. Mileham & G. Plastow (1996), The estrogen receptor locus is associated with a major gene influencing litter size in pigs. Proc. Natl. Acad. Sci. USA 93: 201-205.

Vassart G., M. Georges, R. Monsieur, H. Brocas, A.S. Lequarre & D. Christophe (1987), A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. Science 235: 683-684.

Yao J., S.E. Aggrey, D. Zadworny, J.F. Hayes & U. Kuhnlein (1996), Sequence variations in the bovine growth hormone gene characterized by single-strand conformation polymorphism (SSCP) analysis and their association with milk production traits in Holsteins. Genetics 144:1809-1816

Yoshida A. (1992), Molecular genetics of human aldehyde dehydrogenase. Pharmacogenetics 2: 139-147.

Zeng Z.-B. (1994), Precision mapping of quantitatitive trait loci. Genetics 136: 1437-1468.

Table 1. Origin of strains[a]

Genetic Base	Strains		Resistance to MD[b]	Origin
I	7	control strain	low	
	8	selected for production traits	intermediate	CFAR
	9	selected for production traits	not determined	Agriculture Canada
	8R	selected for MD resistance	high	
II	5	control strain	low	
	3	selected for production traits	intermediate	CFAR
	1	selected for production traits	not determined	Agriculture Canada
	3R	selected for MD resistance	high	
III	S	selected for susceptibility to the "avian leukosis complex"	low	Developed at Cornell,
	K	selected for resistance to the "avian leukosis complex"	high	maintained at CFAR
	Rs	derived from strain K, selected for susceptibility to RSV-induced tumours	low	Institute for Small Animal Research
	Rr	derived from strain K, selected for resistance to RSV-induced tumours	high	Celle/Merbitz
IV	Gs	selected for susceptibility to RSV-induced tumours	low	Inst. for Small Animal
	Gr	selected for resistance to RSV-induced tumours	high	Res. Celle/Merbitz
V	Mr	selected for resistance to RSV-induced tumours	low	Inst. for Small Animal
	Ms	selected for susceptibility to RSV-induced tumours	high	Res. Celle/Merbitz

[a] For details see references in Materials and Methods.

[b] Marek's disease resistance status in strains R, G and M is based on MD mortality records without deliberate challenge (Hatrmann *et al.*, 1984).

Table 2. Evaluation of RFLP responsiveness to selection for egg production

Probe	Allele[a]	Genetic base I		Genetic base II		Total value
		7 *vs.* 8	7 *vs.* 9	5 *vs.*1	5 *vs.* 3	
CLEST005[b]	*Msp*I(+)	0	0	+1	+1	+2
	*Taq*I(+)	0	0	+1	+1	+2
CLEST007	*Msp*I(+)	+1	+1	+1	+1	+4
	*Taq*I(+)	+1	−1	0	0	0
CLEST013	*Msp*I(+)	0	0	+1	+1	+2
	*Taq*I(1)	+1	+1	+1	−1	+2
	(2)	−1	−1	0	+1	−1
	(3)	0	0	−1	−1	−2
	(4)	0	0	−1	−1	−2
CLEST014	*Taq*I(+)	+1	+1	+1	−1	+2
CLEST018	*Taq*I(+)	0	0	0	0	0
CLEST020[c]	*Msp*I(+)	+1	+1	0	0	+2
CLEST021	*Msp*I(+)	+1	+1	−1	−1	0
CLEST024	*Msp*I(+)	−1	−1	0	0	−2
	*Taq*I(+)	+1	0	+1	−1	+1
CLEST025	*Msp*I(+)	0	0	−1	−1	−2
CLEST038	*Msp*I(+)	+1	+1	0	−1	+1
CLEST043	*Msp*I(+)	−1	+1	+1	−1	0
CLEST045	*Msp*I(+)	+1	+1	+1	+1	+4
	*Taq*I(+)	+1	+1	+1	+1	+4
CLEST048	*Msp*I(+)	+1	+1	+1	+1	+4
CLEST055	*Msp*I(1)	+1	+1	−1	−1	0
	(2)	−1	−1	+1	+1	0
	(3)	+1	+1	+1	+1	+4
CLEST058	*Msp*I(+)	+1	+1	+1	+1	+4
CLEST061	*Msp*I(2)	+1	−1	+1	+1	+2
	(3)	0	+1	−1	+1	+1
	(4)	−1	+1	0	0	0
	*Taq*I(1)	+1	0	+1	+1	+3
	(2)	−1	0	−1	−1	−3
	(3)	0	0	0	0	0
CLEST072	*Taq*I(+)	0	+1	0	+1	+2
CLEST075	*Msp*I(1)	0	0	0	0	0
	(2)	−1	−1	−1	+1	−1
	(3)	+1	+1	+1	−1	+2
	*Taq*I(+)	−1	−1	−1	0	−3
CLEST077	*Taq*I(+)	0	0	0	0	0
CLEST085	*Msp*I(+)	0	0	−1	−1	−2
CLEST086	*Msp*I(+)	−1	+1	+1	+1	+2
CLEST090	*Msp*I(+)	−1	−1	+1	+1	0
CLEST095	*Msp*I(1)	0	0	0	0	0
	(2)	+1	+1	−1	0	+1
	(3)	−1	−1	+1	0	−1
	*Taq*I(+)	+1	+1	−1	−1	0
CLEST096	*Msp*I(+)	0	0	0	0	0
CLEST101	*Msp*I(+)	−1	−1	−1	0	−3
CLEST104	*Msp*I(+)	+1	0	−1	0	0
CLEST112	*Msp*I(+)	0	0	+1	+1	+2

Notes:

[a] In the case of intensity shifts in two bands only (*i.e.* single RFLP in a region flanking the probe) the selection of the lower band (presence of a restriction site) is indicated. In cases of intensity shifts in multiple bands (*i.e.* multiple RFLP) all bands were scored.

[b] CLEST 005 contains part of the aldolase B gene. Three other aldolase B clones (CLEST 006, 078 and 108) produced the same hybridization pattern.

Table 3. Ranking RFLP responsiveness to selection for egg production traits and identity of clones

Probe	Gene product	Allele[a]	Selection index
CLEST007	unknown	*Msp*I(+)	+4
CLEST045	unknown	*Msp*I(+)	+4
		*Taq*I(+)	+4
CLEST048	aldehyde dehydrogenase isozyme 3	*Msp*I(+)	+4
CLEST055	unknown	*Msp*I(3)	+4
CLEST058	unknown	*Msp*I(+)	+4
CLEST061	mitochondrial PEPCK	*Taq*I(1)	+3
		*Taq*I(2)	−3
CLEST075	cytochrome P450	*Taq*I(+)	−3
CLEST101	unknown	*Msp*I(+)	−3
CLEST005	aldolase B[b]	*Msp*I(+)	+2
		*Taq*I(+)	+2
CLEST013	unknown	*Msp*I(+)	+2
		*Taq*I(1)	+2
CLEST014	PFK-1 inhibitor	*Taq*I(+)	+2
CLEST020	ATPase6 (mitochondrial genome)[c]	*Msp*I(+)	+2
CLEST061	mitochondrial PEPCK	*Msp*I(2)	+2
CLEST072	vigilin	*Taq*I(+)	+2
CLEST075	cytochrome P450	*Msp*I(3)	+2
CLEST086	unknown	*Msp*I(+)	+2
CLEST112	unknown	*Msp*I(+)	+2
CLEST013	unknown	*Taq*I(3)	−2
		*Taq*I(4)	−2
CLEST024	unknown	*Msp*I(+)	−2
CLEST025	unknown	*Msp*I(+)	−2
CLEST075	cytochrome P450	*Msp*I(2)	−2
CLEST085	antithrombin 3	*Msp*I(+)	−2
CLEST024	unknown	*Taq*I(+)	+1
CLEST038	vitamin D-binding protein	*Msp*I(+)	+1
CLEST061	mitochondrial PEPCK	*Msp*I(3)	+1
CLEST095	unknown	*Msp*I(2)	+1
CLEST013	unknown	*Taq*I(2)	−1
CLEST095	unknown	*Msp*I(3)	−1
CLEST007	unknown	*Taq*I(+)	0
CLEST018	unknown	*Taq*I(+)	0
CLEST021	creatine kinase B	*Msp*I(+)	0
CLEST043	unknown	*Msp*I(+)	0
CLEST055	unknown	*Msp*I(1)	0
		*Msp*I(2)	0
CLEST061	mitochondrial PEPCK	*Msp*I(4)	0
		*Taq*I(3)	0
CLEST075	cytochrome P450	*Msp*I(1)	0
CLEST077	blood clotting factor X	*Taq*I(+)	0
CLEST090	PurH	*Msp*I(+)	0
CLEST095	unknown	*Msp*I(1)	0
		*Taq*I(+)	0
CLEST096	unknown	*Msp*I(+)	0
CLEST104	unknown	*Msp*I(+)	0

Notes:

[a] In the case of that only one restriction site is polymorphic, the (+) and (–) refer to the band allele resulted from the presence and absence of the restriction site, respectively. The selection index was scored according to the (+) allele. In the case of multiple polymorphic restriction sites, the number 1, 2, ... was given to each band allele. The selection index was scored for the most responsive allele of a gene only.

[b] CLEST006, 078 and 108 also encode chicken aldolase B and give rise to the same band pattern of Southern blots.

[c] CLEST047, 049 and 066 also reveal the same RFLP region on the chicken mitochondrial genome.

Table 4. Ranking RFLP responsiveness to selection for Marek's disease resistance and identity of clones

Probe	Gene product	Allele[a]	Selection index
CLEST061	mitochondrial PEPCK	*Msp*I(2)	−6
CLEST061	mitochondrial PEPCK	*Taq*I(2)	+5
CLEST061	mitochondrial PEPCK	*Taq*I(1)	−5
CLEST061	mitochondrial PEPCK	*Msp*I(3)	+4
CLEST086	unknown	*Msp*I(+)	+4
CLEST048	aldehyde dehydrogenase isozyme 3	*Msp*I(+)	−4
CLEST020	ATPase6 (mitochondrial genome)[b]	*Msp*I(+)	+3
CLEST101	unknown	*Msp*I(+)	+3
CLEST104	unknown	*Msp*I(+)	−3
CLEST014	PFK-1 inhibitor	*Taq*I(+)	+2
CLEST018	unknown	*Taq*I(+)	+2
CLEST024	unknown	*Taq*I(+)	+2
CLEST058	unknown	*Msp*I(+)	+2
CLEST085	antithrombin 3	*Msp*I(+)	+2
CLEST090	PurH	*Msp*I(+)	+2
CLEST112	unknown	*Msp*I(+)	+2
CLEST007	unknown	*Taq*I(+)	−2
CLEST021	creatine kinase B	*Msp*I(+)	−2
CLEST045	unknown	*Taq*I(+)	−2
CLEST077	blood clotting factor X	*Taq*I(+)	−2
CLEST005	aldolase B[c]	*Msp*I(+)	+1
		*Taq*I(+)	+1
CLEST013	unknown	*Taq*I(1)	+1
CLEST024	unknown	*Msp*I(+)	+1
CLEST055	unknown	*Msp*I(2)	+1
CLEST061	mitochondrial PEPCK	*Msp*I(4)	+1
CLEST072	vigilin	*Taq*I(+)	+1
CLEST075	cytochrome P450	*Msp*I(1)	+1
CLEST095	unknown	*Taq*I(+)	+1
CLEST013	unknown	*Taq*I(3)	−1
		*Taq*I(4)	−1
CLEST075	cytochrome P450	*Msp*I(3)	−1
CLEST095	unknown	*Msp*I(2)	−1
CLEST007	unknown	*Msp*I(+)	0
CLEST013	unknown	*Msp*I(+)	0
		*Taq*I(2)	0
CLEST025	unknown	*Msp*I(+)	0
CLEST038	vitamin D-binding protein	*Msp*I(+)	0
CLEST043	unknown	*Msp*I(+)	0
CLEST045	unknown	*Msp*I(+)	0
CLEST055	unknown	*Msp*I(1)	0
		*Msp*I(3)	0
CLEST061	mitochondrial PEPCK	*Taq*I(3)	0
CLEST075	cytochrome P450	*Msp*I(2)	0
		*Taq*I(+)	0
CLEST095	unknown	*Msp*I(1)	0
		*Msp*I(3)	0
CLEST096	unknown	*Msp*I(+)	0

Notes:

[a] In the case of that only one restriction site is polymorphic, the (+) and (–) refer to the band allele resulted from the presence and absence of the restriction site, respectively. The selection index was scored according to the (+) allele. In the case of multiple polymorphic restriction sites, the number 1, 2, ... was given to each band allele. The selection index was scored for the most responsive allele of a gene only.

[b] CLEST006, 078 and 108 also encode chicken aldolase B and give rise to the same band pattern of Southern blots.

[c] CLEST047, 049 and 066 also reveal the same RFLP region on the chicken mitochondrial genome.

Table 5. Selective genotyping by bulk analysis for CLEST045[a]

Strain	Sampling	Change in band intensity (high *vs.* low)					
		HBW	MBW	AFE	HDR	EWT	RFC
7	generation 1	+	0	+	−	−	0
	generation 2	+	−	−	0	+	+
8	generation 1	+	−	+	−	+	0
	generation 2	+	−	0	+	0	−
9	generation 1	0	−	0	−	−	0
	generation 2	+	−	+	0	0	−
Total score		+5	−5	+2	−2	0	−1

[a] The *Msp*I RFLP revealed by CLEST045 which ranked +4 in its response to selection for egg production traits (Table 3) was analyzed for association with body weight at sexual maturity (HBW), mature body weight (MBW), age at first egg (AFE), rate of egg production (HDR) and residual feed consumption (RFC) in two successive generations of strains 7, 8 and 9. DNA from twenty individuals which ranking at the extremes of the trait distribution was pooled and the two pools compared. Trait associations were scored as +, 0 or −, depending whether the hybridization signal of the "+" allele (presence of an RFLP) in the top pool (high numerical value of the trait) was higher, equal or lower than the intensity in the bottom pool (low numerical value of the trait).

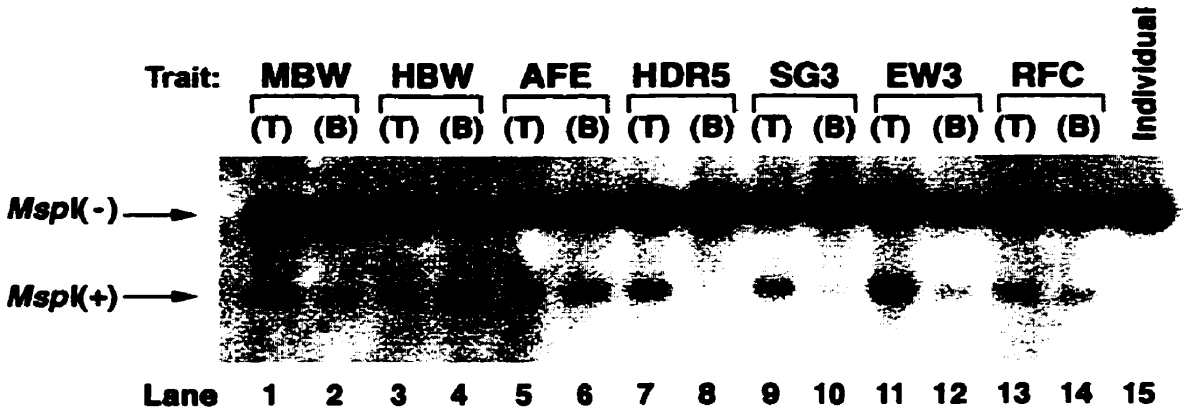

Figure. A representative Southern blot for study of trait association. DNA in lane 1 - 14 were extracted from the mixture of blood samples of 20 individuals from the top and bottom groups of frequency distribution of 7 production traits, respectively. These traits are MBW (mature body weight), HBW (housing body weight), AFE (age of first egg), HDR (egg laying rate), EW (egg weight), SG (specific gravity) and RFC (residual feed consumption). Blood samples were collected in November of 1990. DNA was digested with *Msp*I restriction enzyme. The Southern blot was hybridized with probe of CLEST045. DNA sample of one individual from the strain in lane 15 indicates the presence of polymorphism among the other individuals. Two alleles can be observed. The "+" allele at lower molecular weight level is originated from the presence of a *Msp*I restriction site. The "–" allele at higher molecular weight is resulted from the absence of a *Msp*I restriction site. The relative intensity of an allele represents the frequency of this allele in the pool.

Connecting Statement III

Through stage 1 and 2, a total of 92 randomly selected cDNA clones from a chicken liver cDNA library were screened for those that revealed restriction fragment length polymorphism (RFLP) at *Msp*I and *Taq*I restriction sites. These RFLP were screened for changes in frequency in strains under selection. A selection index was introduced to rank the level of consistency of frequency change upon selection in different genetic bases. DNA markers with the highest rank of selection index were considered the most promising ones for further analysis of association with traits. Among them, one was a DNA marker on the chicken mitochondrial genome.

This mitochondrial DNA marker segregated in strains from 3 different genetic base populations (strains 7, 8, 9, 8R from Genetic base I, strains Gs, Gr from Genetic base IV, strains Ms and Mr from Genetic base V). No segregation was observed in the other 2 genetic base populations (strains 5, 1, 3, 3R from Genetic base II, strains S, K, Rs and Rr from Genetic base III). Since the entire chicken mitochondrial genome had been sequenced and published, it was therefore possible to localize the DNA marker on the chicken mitochondrial genome by Southern blotting assay following digestion with different combination of restriction endonucleases. Subsequently, a DNA polymerase chain reaction (PCR) assay was established, and the exact mutation site was determined by sequencing the PCR product. This allowed for the examination of trait associations within a large number of individuals within strains using a PCR based RFLP assay.

Chapter 5 (Manuscript #3)

Evidence for a Genetic Variation in the Mitochondrial Genome Which Affects Traits in White Leghorn Chickens

S. Li, S. E. Aggrey, D. Zadworny, W. Fairfull and U. Kühnlein*

Dept. of Animal Science, McGill University, Ste. Anne de Bellevue, QC, Canada H9X 3V9 and (*) Centre for Food and Animal Research, Agriculture Canada, Ottawa, ON, Canada K1A 0C6

Published in ***Journal of Heredity*** **89: (in press)**

Corresponding author:

Urs Kuhnlein at the address above

Tel. (514) 398 7799; Fax (514) 398 7964
E-mail: kuhnleinu@agradm.lan.mcgill.ca

Acknowledgments

The study was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Shaver Poultry Farms Ltd. We wish to thank the Centre for Food and Animal Research (CFAR) at Agriculture Canada, Ottawa, for providing us with DNA samples and data on production traits. Special thanks to L. Asselstine and L. Volkov for their help in poultry management, trait measurement and genotyping.

Running title: Trait association of a mitochondrial genetic marker

Summary

A mitochondrial *Msp*I RFLP, which was co-selected with Marek's disease (MD) resistance in White Leghorn chickens was mapped to the NADH subunit IV. The RFLP was due to a transition, resulting in the change of the low usage threonine triplet ACT (*Msp*I$^-$ allele) to the high usage triplet ACC (*Msp*I$^+$ allele). Trait association studies within an unselected strain revealed that the *Msp*I$^-$ allele whose frequency was reduced in MD resistant strains was associated with high body weight and high egg specific gravity (a measure of eggshell thickness). Analysis at three different time points indicated a significant interaction between the mitochondrial genotype and the growth hormone genotype in early but not in late adulthood. The analysis indicates that mitochondrial variants may contribute to phenotypic variation in chickens, and that such contributions may be dependent on the genetic background.

Introduction

Mitochondria play a central role in energy metabolism, amino acid metabolism, fat metabolism and steroidogenesis (Whittaker and Danks, 1978). Although being an autonomously replicating organelle, most of the proteins present in the mitochondrion are encoded by nuclear genes (Anderson *et al.*, 1981). The genes still present in the mitochondrial genome are coding for mitochondrial t-RNA, r-RNA and for genes coding for some of the subunits of the protein complexes responsible for oxidative-phosphorylation (OXPHOS complexes). The mitochondrial genes are devoid of regulatory elements and tissue-specific regulation of mitochondrial genesis and metabolic activity is mediated by cellular genes. Nevertheless, in humans many mutations in mitochondrial genes have been found to affect mitochondrial function. OXPHOS diseases include miss-sense mutations, t-RNA mutations and insertion/deletion mutations. Such mutations affect mainly organs, which are dependent on mitochondrial energy, such as the nervous system, muscle, heart, pancreatic islands, kidney and liver. Further, symptoms in these diseases are mostly progressive, presumably because reduced mitochondrial activity is compounded by the loss of functional mitochondria as part of the aging process (Wallace, 1992).

Inheritance of mitochondria is predominantly maternal, presumably because the number of mitochondria present in the ovum exceeds the number of mitochondria carried by the sperm by several orders of magnitude (Gyllenstein *et al.*, 1991). An additional unique feature of mitochondrial DNA is the lack of recombination and the high frequency of mutations, which is about 10 to 20 times higher than the nuclear DNA (Merriwether *et al.*, 1991). The potential number of mutations that affect traits may therefore be quite high.

In a screen of anonymous chicken liver cDNA library for clones which revealed restriction length polymorphisms (RFLP) at *Msp*I sites in White Leghorns, we found that several clones were of mitochondrial origin and that the associated RFLP was co-selected with egg production and/or disease resistance. All mitochondrial clones revealed the same *Msp*I RFLP (unpublished results). In this communication we identified the molecular nature of this RFLP and tested whether it was associated with body weight and egg quality traits. We further investigated the interaction between the mitochondrial

genotype and a marker in the growth hormone gene, a gene also associated with body weight as well as with egg production traits (Kuhnlein and Zadworny, 1994, Kuhnlein *et al.*, 1997).

Materials and Methods

Strains of chickens and measurement of traits

Strain 7 is a White Leghorn strain, which had been established in 1958 by crossmating 4 North American commercial strains and was propagated by random mating at an effective population size of 457 without selection. The strain analysed here is the generation raised in 1993. Strains 8 and 9 were derived from strain 7 in 1969 and propagated under selection for an array of egg production traits at an effective population size of 184 (Cole and Hutt, 1973; Gowe *et al.*, 1993). All other strains analysed in this study are described in Table 1.

Body weights were measured at 130, 265 and 365 days of age. Egg weights and the egg specific gravities were measured on up to 5 eggs collected from each hen starting from 240, 350 and 450 days of age, respectively. Egg specific gravity, a measure of eggshell thickness, was measured by submerging eggs in NaCl solutions of increasing density (Gavora *et al.*, 1989).

Southern blotting

DNA was extracted from 60 µl of heparinized whole blood as described by Jeffreys and Morton (1987) and dissolved in TE-buffer (10 mM Tris-HCl, 1 mM EDTA; pH7.5). Five µg of DNA was digested with *Msp*I or *Taq*I restriction enzyme and subjected to electrophoresis on a 1-% agarose gel at 1.25 V/cm for 20 hours. The DNA was transferred to a nylon membrane (Bio-Rad) by alkaline blotting (Reed and Mann 1985). Probes were labelled by random primer extension (^{T7}Quick-Prime Kit; Pharmacia) and hybridisation was carried out as described previously (Kuhnlein *et al.*, 1989).

Polymerase chain reaction

The primer sequences used to amplify the mitochondrial segment containing the *Msp*I RFLP locus were 5'-CAGGCCTAGCCATACAAGTAG-3' (forward primer) and 5'-TAAGCTTGTTCAG GAGGCAGG-3' (reverse primer). The reaction (25 µl) contained 2 units of T*th* polymerase, 0.2 µg of DNA, 20 µM of each primer, 125 µM of each nucleotide, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 20mM $MgCl_2$ and 0.01 % gelatin.

After denaturing for 10 min at 95°C, a total of 40 cycles were carried out, each consisting of 1 min at 94°C, 80 sec at 59°C and 90 sec at 72°C. The PCR products were digested with *Msp*I enzyme and analysed by electrophoresis in a 1.5-% agarose gel. The PCR-RFLP assay for the *Sac*I RFLP in intron 4 of the chicken growth hormone has been described (Kuhnlein *et al.*, 1997).

Direct sequencing of PCR products

Twenty-five μl of PCR product was purified by electrophoresis in a 1.5-% low melting agarose gel containing ethidium bromide in 1 × TAE buffer. The band containing the DNA was cut out, placed in 1 volume of distilled water and heated at 95°C for 5 min. Five μl of the preparation was used for direct sequencing using the "dsDNA Cycle Sequencing System" and following the instructions of the supplier (GIBCOBRL).

Statistical analysis

The association of two mitochondrial marker genotypes (haploid) with traits was analysed by single factor analysis of variance. The combined association mitochondrial and GH genotypes were analysed by two factor analysis of variance (type III) using the model $y_{ijk} = mitgeno_i + ghgeno_j + mitgen_i \times ghgeno_j + e_{ijk}$. The deviation of the observed from the expected genotype frequencies, *i.e.* Hardy-Weinberg equilibrium for the GH genotypes and random association with the mitochondrial genotype, were assessed by χ^2 analysis with df = 4.

The discussion on genetic drift vs. selection is based on the following argument. In the case of extreme random genetic drift, an allele may become fixed in some subpopulations, whereas in the remaining subpopulations allele frequencies will have an equal probability to take any value between 0 and 1 (Kimura, 1955). Assuming that in the segregating subpopulations the allele frequencies are μ_1 and μ_2, respectively, and $\mu_1 < \mu_2$, then the probability to obtain a more extreme outcome than observed is $(1 + \mu_1 - \mu_2)^2$ multiplied by a factor 2 to account for the random order of allele designation. If several divergently selected pairs are analyzed, the overall probabilities multiply, but the correction factor of two only applies once, since the order of alleles becomes fixed in the first comparison. The mathematical treatment in the case of moderate random drift is

complex. However, inspection of the allele frequency distributions given by Kimura (1955), indicates that the probability of observing a given allele frequency between subpopulations is even lower (Kuhnlein *et al.*, 1997).

Results

Identification of the mitochondrial RFLP

Bulk screening of a liver cDNA library for clones which revealed *Msp*I or *Taq*I RFLP in a series of White Leghorn strains led to the identification of four clones with sequence homology to the ATPase6 gene and the tRNA-serCOII region (Desjardins and Morais, 1990). Based on the coincidence of RFLP segregation among individuals and restriction fragment size, all four clones revealed the same *Msp*I RFLP, located in the flanking region of the clone. No RFLPs were found at *Taq*I restriction sites.

Southern blotting indicated that the RFLP produced a single band shift from 4,700 bp to 4,100 bp (Figure 1). A fragment of 4,700 bp was expected from the location of two *Msp*I sites located at position 8,901 and 12,673, respectively, indicating that the *Msp*I$^+$ allele (presence of an *Msp*I site) originated from an additional *Msp*I site not present in the published sequence (Desjardin and Morais, 1990). Double digests with other restriction enzymes placed this *Msp*I site close to the 3'-end of this fragment. Appropriate PCR primers were designed, and the location of the RFLP was confirmed by restriction analysis of the amplified DNA fragment.

The identity of the base change was determined by direct sequencing of the PCR product obtained from the DNA of two individuals with different RFLP alleles. Compared to the analysis of subcloned PCR products, direct sequencing is expected to lower the frequency of errors, which may occur during amplification. A nucleotide change from T to C was found at position nt 11,998 resulting in the creation of an additional *Msp*I site (Figure 2). This nucleotide substitution was in the coding region for NADH dehydrogenase subunit IV, but did not alter the amino acid sequence. No other changes between the two RFLP alleles were found in 300 bases in the vicinity of the *Msp*I site.

Homoplasy and incidence of the MspI RFLP in White Leghorn strains

DNA was isolated from red blood cells, which contain an average of 8 mitochondria. Within the limits of resolution of the PCR assay (admixture of the two genotypes with 1:9), nearly all chickens segregated for a single mitochondrial genotype as expected from maternal inheritance of mitochondria. Heteroplasy, the presence of

both mitochondrial alleles, was only observed once among more than 400 individuals tested. Whether heteroplasy in this individual was due to a mixture of the two genotypes in single red blood cells or reflected an admixture of red blood cells of unique genotype was not determined.

Analysis of the RFLP in White Leghorn strains originating from different genetic origins revealed segregation in three of the five sets of strains (Table 1). Set one consisted of strains 7, 8, 9 and 8R which had been established from 4 different North American commercial strains in 1956 (Gowe *et al.*, 1993). Set two comprised strains Mr and Ms that had been derived from a North American commercial strain. And set three comprised strains Gr and Gs that had been derived from a strain of German origin. The latter two strains had been divergently selected for the susceptibility to tumour formation upon wing web injection of Rous sarcoma tumour virus (Hartmann *et al.*, 1984). Two other sets of strains, one of Canadian origin and one developed at Cornell University in the late 1930's were fixed for the *MspI*$^-$ allele. Marek's disease (MD) resistant strains had an increased incidence of the *MspI*$^+$ allele as compared to their more susceptible related strains (8 *vs.* 8R, Gs *vs.* Gr and Mr *vs.* Ms). As outlined in the Materials and methods section, the consistent differences are unlikely to be the result of random genetic drift ($P \leq 0.03$). However, in the divergently selected strains G and M, selection for MD resistance was not part of the selection criteria and the origin of the hidden selection, which may have led to differences in MD resistance, is unknown. Nevertheless, a maternally inherited component of MD resistance has been reported (Hartmann *et al.*, 1991).

Trait association in a non-selected White Leghorn population

Comparison of control strain (strain 7) with two substrains selected for a wide array of egg production related traits (strains 8 and 9) indicated co-selection of the *MspI*$^+$ allele (Table 1). Further evidence from typing extreme phenotypes for a series of traits (data not shown) prompted us to analyze trait association of the mitochondrial genotype in more detail.

The association study was carried out in strain 7, a strain of White Leghorns that had been kept non-selected since 1956 at an average effective population size of 457 and

segregated for the mitochondrial *MspI*$^+$ allele at a frequency of 0.30. Analysis of variance was carried out for body weight, egg weight and egg specific gravity at three different ages (Table 2). Egg specific gravity is highly correlated with eggshell thickness.

Significant associations were found for two of the three traits, body weight and specific gravity. For body weight, the association was significant ($P \leq 0.028$) at 365 days of age (mature body weight), where the mean for the *MspI*$^+$ genotype was 66 g lower than for the *MspI*$^-$ genotype. The same trend but of a lesser magnitude was observed at earlier ages. Egg specific gravity was also reduced in chickens with a *MspI*$^+$ mitochondrial genotype, and this difference was significant ($P \leq 0.063$) at 240 days of age.

Both body weight and egg specific gravity is complex multi-genic traits and expected to be influenced by allelic variations in many different genes. In strain 7 we had previously characterized alleles of the GH gene, another candidate gene for growth and production related traits. Among the three alleles segregating in strain 7, the major allele A1 was increased in substrains selected for egg production and related traits and could be distinguished from the other alleles by virtue of a *Sac*I RFLP in intron 4 (Kuhnlein and Zadworny, 1994, Kuhnlein *et al.*, 1997).

The combined effect of the marker genotypes at the two loci is shown in Tables 3 and 4. For body weight at 130 days, the interaction between the mitochondrial and GH genotypes was significant ($P \leq 0.034$). Analysis of individual genotypic classes revealed that the interaction was due to a large effect of the mitochondrial genotype in conjunction with the A1$^-$/A1$^-$ GH genotype, while effects in the other GH genotype classes, although in the same direction, were small. At 265 days of age, none of the genotypes had a significant effect, while at 365 days of age the effect of the mitochondrial genotype was again significant, but not restricted to a particular GH-genotypic class.

A similar effect was observed for egg specific gravity. At an early age, the effect of the mitochondrial genotype was only significant in the GH genotype classes A1$^-$/A1$^-$ and A1$^+$/A1$^-$. At 350 days of age there was no significant effect of the mitochondrial genotype at all, while at 450 days of age the effect of the mitochondrial genotype was significant, but independent of the GH genotype.

Discussion

The mitochondrial genotype was associated with two traits, body weight and egg specific gravity, a trait that is highly associated with eggshell thickness. Association with body weight was significant at a relatively advanced age, despite an increase in variance with age. However a similar trend was observed in younger chickens. The difference in weight gain between the two genotypic classes expressed as percentage of the mean weight gain was 2% between day 0 and 130, 4% between day 130 and 265 and 50% between day 265 and 365. It indicates that the relative influence of the mitochondrial genotype increases with age, suggesting that the effect of mitochondrial genotype on growth may be an age-related phenomenon as has been observed for many human mitochondrial disorders for which the severity of symptoms increases with age (Wallace, 1992). Age-related effects of mitochondrial variants had been attributed to the gradual loss of functional mitochondria throughout adult life. However, this may not be the case in chickens where traits were measured relatively early with respect to the natural life cycle. Rather, the mechanisms by which mitochondrial functions interdigitate with other metabolic enzymes are subject to changes throughout development. This is exemplified by our analysis of the interaction between the mitochondrial genotype and the growth hormone genotype. Interaction between the two genotypes was only significant at an early age.

In contrast to body weight, the average difference of egg specific gravity between the two mitochondrial classes remained similar throughout the three periods of measurements (1.1, 0.8 and 1.2, respectively). The coefficient of variance of this trait also increased with age and only the measurements taken at day 240 reached significance. As with body weight, significant interaction with the GH genotype was only observed at an early age.

The major determinant of specific gravity is eggshell thickness and hence the amount of calcium deposited in the eggshell. The turnover of calcium in laying hens is high, amounting to about 10% of the total body calcium for each egg laid (Soares 1984). Homeostasis of calcium is governed by at least four endocrine systems: calcitonin, parathyroid hormone, 1,25-dihydroxyvitamin D_3 and estrogen. Two key enzymes in the synthetic pathway of 1,25-dihydroxyvitamin D_3, the main hormone for calcium

mobilisation, are enzymes located in renal mitochondria, namely 25-OHD_3-1-α-hydroxylase (Gray *et al.*, 1972) and 25-OHD_3 -24 hydroxylase (Knutson and DeLuca 1974). Divergent selection lines of chickens for thick and thin egg shells has been shown to significantly lower the 1,25-$(OH)_2D_3$ levels in the strain selected for thin shells, raising that the possibility of an involvement of mitochondrial variation (Soares *et al.*, 1980). High plasma estradiol levels, presumably resulting in higher 1,25-$(OH)_2D_3$ levels, have also been associated with better egg shell quality (Grunder *et al.*, 1983). Estradiol level may also be subject to modulation by mitochondrial activity, due to its involvement in steroid biosynthesis.

Although there is ample evidence to implicate mitochondrial genes as candidate genes responsible for genetic variations in eggshell quality as well as body growth, final evidence has to come from measuring mitochondrial functions. The particular marker mutation in the NADH dehydrogenase subunit IV gene occurs in a triplet coding for threonine, with *MspI*$^+$ allele containing the triplet ACC and the *MspI*$^-$ allele the triplet ACT. Both triplets code for threonine. The codon usage of ACC is three times higher than ACT in both, humans and chickens, but the evolutionary significance of this difference is unknown (Anderson *et al.*, 1981; Desjardin and Morais, 1990). Although different codons may affect transcription and/or translation, it is more likely that the particular *Msp*I RFLP is not directly responsible for the trait association, but rather that the association is due to linkage with an unknown mutation in another part of the mitochondrial genome.

One reason for identifying quantitative trait loci (QTL) is their potential usage for selecting at the DNA level. In the case of the mitochondrial marker, selection for the *MspI*$^-$ allele would be expected to result in an increased eggshell thickness, increased body weight and increased MD susceptibility (possibly MD-induced tumor growth). However, the present analysis indicates that the magnitude of the expected selection response may depend on the GH genotype and presumably also on variations in other genes present in the particular strain.

Conventional QTL mapping is conducted by crossing individuals or inbred lines and analysing the co-segregation of phenotypes with marker genotypes in the F2 generation (Lander and Botstein, 1989). Since each offspring inherits a different

admixture of alleles at different loci, such analyses only reveal QTL that have an effect regardless of the particular genetic background. The same is true for an association study of traits and markers in candidate genes within a strain by using analysis of variance. Such QTL may be economically most important, since they can be used for selection at the DNA level in most strains. However the genetic architecture of quantitative traits is likely to be more complex and the effects of alleles in a gene have to be analysed in the context of genetic variations in other genes. It is to be expected that most of the genetic variations for traits under intensive selection are of this type.

References

Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, J Drouin, IC Eperon, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, and Young IG, 1981. Sequence and organization of the human mitochondrial genome. Nature 290: 457-465.

Cole RK, and Hutt FB, 1973. Selection and heterosis in Cornell White Leghorns: a review with special consideration of inter-strain hybrids. Anim Breed Abstr. 41:103-108.

Desjardins P, and Morais R, 1990. Sequence and gene organization of the chicken mitochondrial genome: A novel gene order in higher vertebrates. J Mol Biol 212: 599-634.

Gavora JS, Kuhnlein U, and Spencer JL, 1989. Absence of endogenous viral genes in an inbred line of Leghorn chickens selected for high egg production and Marek's disease resistance. J Anim Breed Genet 106: 217-224.

Gowe RS, Fairfull RW, McMillan I, and Schmidt GS, 1993. A strategy for maintaining high fertility and hatchability in a multiple-trait egg stock selection program. Poult Sci 72: 1433-1448.

Gray RM, Omdahl JL, Ghazarin JG, and DeLuca HF, 1972. 25-hydroxycholecalciferol-1α-hydroxylase. Subcellular location and properties. J Biol Chem 247:7528-7532.

Grunder AA, Hollands KG, and Tsang CPW, 1983. Plasma estrogen, calcium and shell quality in two strains of White Leghorns. Poult Sci 62: 1294-1296.

Gyllensten U, Wharton D, Josefsson A, and Wilson AC, 1991. Parental inheritance of mitochondrial DNA in mice. Nature 352: 255-257.

Hartmann W, V.D. Hagen D, and Heil G, 1984. Genetic resistance to leucosis virus infection: effects on mortality and egg production in Leghorn hens. Brit Poult Sci 106: 379-384.

Hartmann W., 1991. Genetic aspects of resistance to avian leucosis: An overview. Informacion Tecnica Economica Agraria 87A(2-3): 171-189.

Jeffreys AJ, and Morton DB, 1987. DNA fingerprints of dogs and cats. Anim Genet 18:1-15.

Kimura M, 1955. Solution of a process of random genetic drift with a continuous model. Proc. Natl. Acad. Sci. USA 41:144-150.

Knutson JC, and DeLuca HF, 1974. 25-Hydroxy-vitaminD_3-24 hydroxylase. Subcellular location and properties. Biochem 13: 1543-1548.

Kuhnlein U, Gavora JS, Spencer JL, Bernon DE and Sabour M, 1989. Incidence of endogenous viral genes in two strains of White Leghorn chickens selected for egg production and susceptibility or resistance to Marek's disease. Theor and Appl Genet 77: 26-32.

Kuhnlein U and Zadworny D, 1994. Disease resistance genetics: selection at the DNA level. In: Proceedings of the 5th World Congress of Genetics Applied to Lifestock production. Guelph, Ontario, Canada. 21:249-256.

Kuhnlein U, Ni L, Weigend S, Gavora JS, Fairfull W, Zadworny D, 1997. DNA polymorphisms in the chicken growth hormone gene: Response to selection for disease resistance and association with egg production. Anim Genet 28:116-123.

Lander ES, and Botstein D (1989), Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genet 121:185-199.

Merriwether DA, Clark AG, Ballinger SW, Schurr TG, Soodyall H, Jenkins T, Sherry ST, and Wallace DC, 1991. The structure of human mitochondrial DNA variation. J Mol Evol 33(6): 543-555

Reed KC, and DA Mann, 1985. Rapid transfer of DNA from agarose gels to Nylon membranes, Nucl Acids Res 13: 7207-7221.

Soares JH Jr, 1984. Calcium metabolism and its control - A review, Poult Sci 63: 2075-2083.

Soares Jr JH, Ottinger MA, Buss EG, and Kaetzel Jr DM, 1980, Plasma concentration of 1,25-$(OH)_2D_3$, estradiol and calcium in laying hens selected for differential egg shell quality, Poult Sci 58: 1662 (Abstract)

Wallace DC, 1992. Diseases of the mitochondrial DNA. Ann Rev Biochem 61: 1175-1212.

Whittaker PA, and Danks SM, 1978. Mitochondria: structure, function and assembly. New York: Longman Inc.

Table 1. Frequency of the mitochondrial *Msp* I$^+$ allele in White Leghorn strains of different genetic origin[a]

Genetic base	Strain[a]	Selection criteria	MD-resistance	*Msp*I$^+$ allele frequency
I	7	control strain	low	0.30
	8	selected for egg production traits	intermediate	0.42
	9	selected for MD resistance	unknown	0.41
	8R	selected for MD resistance	high	0.56
II	3	selected for egg production traits	intermediate	0.00
	3R	selected for MD resistance	high	0.00
III	S	selected for MD susceptibility	low	0.00
	K	selected for MD resistance	high	0.00
	Rs	selected for ALV susceptibility	low	0.00
	Rr	selected for ALV resistance	high	0.00
IV	Gs	selected for ALV susceptibility	low	0.68
	Gr	selected for ALV resistance	high	0.83
V	Mr	selected for ALV resistance	low	0.43
	Ms	selected for ALV susceptibility	high	0.90

[a] Strains 7, 8, 9, 8R, 3 and 3R have been described (Gowe *et al.*, 1993; Gavora *et al.*, 1989). Strains S and K were developed at Cornell University and were selected for MD resistance (Cole and Hutt, 1973). Strain Rs and Rr were derived from a substrain of strain K under divergent selection for tumour formation in response to wing injection with Rous sarcoma virus (Hartmann *et al.*, 1984). Strains Gs/Gr and Ms/Mr were selected as Rr/Rs, but were of North American and German origin, respectively. MD mortality in Gr and Ms was greater then in Gs and Mr, respectively (Hartmann *et al.*, 1984). Sample sizes were 20, with the exception for strains of genetic base IV and V where 30 individuals were typed.

Table 2. Least-square means of body weight, egg weight and egg specific gravity in dependence of the mitochondrial genotype in strain 7

Trait	Coefficient of variation	Mitochondrial genotype		Significance
		MspI$^+$ (N=104)	*MspI*$^-$ (N=237)	(Pr > F)
Body weight (g) at				
130 days	10.4	1276	1302	0.107
265 days	12.0	1718	1763	0.073
365 days	13.3	1740	1802	0.028
Egg weight (g) at				
240 days	6.70	52.9	52.5	0.310
350 days	7.36	58.5	58.1	0.371
450 days	7.78	60.6	60.7	0.843
Egg specific gravity [a] at				
240 days	5.40	84.7	85.8	0.063
350 days	5.65	81.3	82.1	0.181
450 days	6.86	78.3	79.5	0.095

[a] The units for egg specific gravity are -1×10^3 g.

Table 3. Significance of the mitochondrial and GH genotype and their interaction in strain 7

Trait	Significance (Pr > F) [a]			
	Model	Mitochondrial	GH	Mit. × GH
Body weight at				
130 days	0.059	**0.003**	0.238	**0.034**
265 days	0.291	0.081	0.271	0.722
365 days	0.099	**0.023**	0.153	0.473
Specific gravity at				
140 days	0.077	**0.007**	0.827	**0.064**
250 days	0.311	0.142	0.704	0.452
450 days	0.251	**0.018**	0.501	0.154

[a] Model: $y_{ijk} = mitgeno_i + ghgeno_j + mitgen_i \, x \, ghgeno_j + e_{ijk}$.

Table 4. Least square means for body weight and egg specific gravity in dependence of GH ($A1^{+/-}$) and mitochondrial ($MspI^{+/-}$) genotypes in strain 7

Trait	$A1^+/A1^+$		$A1^+/A1^-$		$A1^-/A1^-$	
	$MspI^+$	$MspI^-$	$MspI^+$	$MspI^-$	$MspI^+$	$MspI^-$
	(N = 58)	(N=134)	(N = 41)	(N = 82)	(N = 5)	(N = 21)
Body weight (g)						
130 days	1270	1304	1272	1287	1148***	1341***
265 days	1736	1777	1705	1743	1626	1752
365 days	1759*	1820*	1733	1777	1584*	1779*
Egg specific gravity						
240 days	85.2	85.3	84.4**	86.2**	82.0**	87.1**
350 days	81.7	81.8	80.9	82.1	81.2	84.0
450 days	79.0	79.3	77.7*	79.6*	74.5*	80.4*

[a] The observed distribution of genotypes did not significantly differ from the distribution expected from Hardy-Weinberg equilibrium for the GH genotypes and random association between GH and mitochondrial genotypes (χ^2 test, df = 4, $P < 0.5$). The significance between the trait means of the two mitochondrial genotypes within each GH genotypic class are indicated by * ($P < 0.1$), ** ($P < 0.05$) and *** ($P < 0.01$).

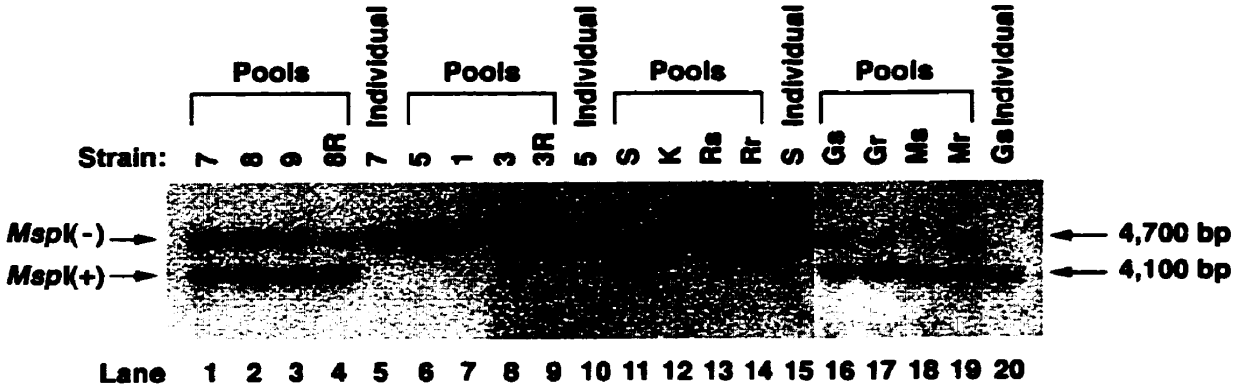

Figure 1. Southern blot of *Msp*I digested DNA from chickens of strain 7 hybridized with the mitochondrial clone CLEST020. The two alleles *Msp*I$^-$ (absence of a *Msp*I restriction site) and *Msp*I$^+$ (presence of a *Msp*I restriction site) are indicated.

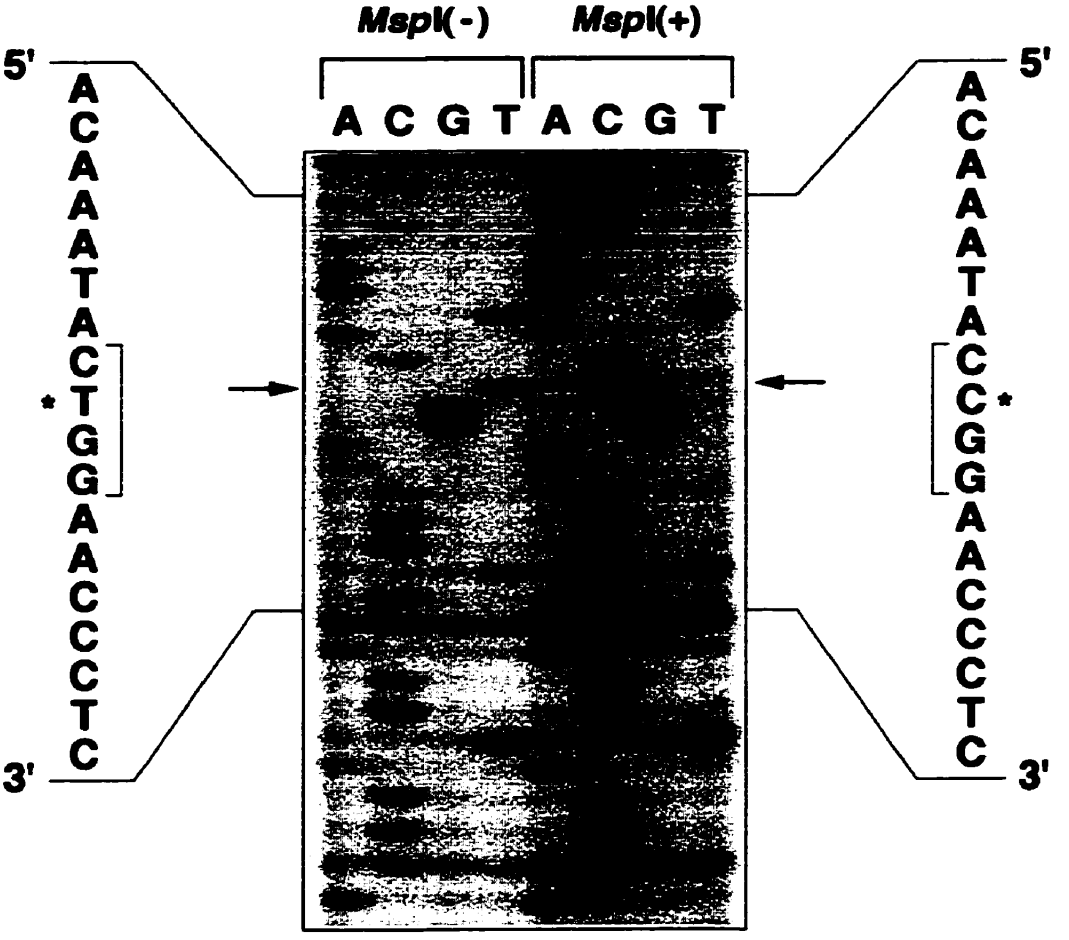

Figure 2. Direct sequencing of the PCR product of the polymorphic region of a *MspI*$^{+}$ and a *MspI*$^{-}$ allele from strain 7. The sequence corresponds to the non-coding strand.

Connecting Statement IV

A new methodology was proposed in this project to identify DNA markers associated with quantitative trait loci (QTL) by using anonymous cDNA clones as probes. Through the multiple screening steps in stage 1 and 2, the most promising DNA markers detected by some of the randomly selected cDNA clones were singled out for further analysis of detailed trait associations. In the previous paper, a *Msp*I RFLP on the chicken mitochondrial genome was characterized. The frequency of the *Msp*I$^-$ allele was consistently lower in Marek's disease (MD)-resistant strains than in susceptible strains. Additionally, this DNA marker was also associated with high body weight and high egg specific gravity (a measure of eggshell thickness). The mitochondrial RFLP was an example of a successful application of the screening method for identification of DNA markers associated with QTL in chickens. In the next chapter, another example is given for a DNA marker on a nuclear gene, the mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M).

In the second stage of the screening procedure, PEPCK-M had the highest selection index for MD resistance, indicating that this cDNA clone was the most promising for further analysis. In addition, PEPCK is known to be a key enzyme in gluconeogenesis. Since there is ample evidence for the involvement of the intermediary metabolism with immune response and tumorigenesis, it was decided to further investigate the association of polymorphisms in this gene with MD in a large number of individuals.

Chapter 6 (Manuscript #4)

Mitochondrial PEP-CK: A Highly Polymorphic Gene with Alleles Co-selected with Marek's Disease Resistance in Chickens[¶]

(Short Communication)

S. Li, D. Zadworny, S. E. Agrrey and U. Kuhnlein*

Department of Animal Science, McGill University, Ste. Anne de Bellevue, Quebec, CANADA H9X 3V9

Submitted to ***Animal Genetics*** **(accepted)**

*** Corresponding author (To whom the reprints should be sent):**

Tele: 514-398-7799

Fax: 514-398-7964

E-mail: kuhnleinu@agradm.lan.mcgill.ca

[¶]Received for publication on March 14, 1998
Accepted for publication on April 9, 1998

Summary

The gene coding for the mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M), a pivotal component with gluconeogenesis from lactate via the Cori cycle was highly polymorphic in strains of egg-type chickens (White Leghorn) of different origins. Based on *Msp*I restriction fragment polymorphisms a total of seven haplotypes could be distinguished. The haplotype frequencies were determined in six pairs of strains that had been divergently selected for susceptibility to Marek's disease, a virus-induced neoplastic disease. The frequency of the most common haplotype (M2) was consistently higher in the susceptible strains than in the corresponding resistant strains ($P < 0.05$, Wilcoxon signed-ranks test). The probability that the observed differences were due to random genetic drift was less than 0.002. The result suggests that PEPCK-M may be a candidate gene that contains genetic variants affecting MD susceptibility. Variations in this glucose metabolism pathway may affect the interaction between proliferation of neoplasia and host metabolism.

Keywords: chicken, mitochondrial phosphoenolpyruvate carboxykinase, Marek's disease susceptibility, genetic marker

Main text

Phosphoenolpyruvate carboxy kinase (PEPCK) catalyzes the formation of phosphoenolpyruvate by decarboxylation of oxaloacetate, a pivotal step in the *de novo* synthesis of glucose (reviewed by Hanson & Patel, 1994). Two enzymes that are encoded by different nuclear genes mediate PEPCK activity, with one enzyme located in the cytosol (PEPCK-C) and the other in the mitochondrial matrix (PEPCK-M) (Weldon *et al.*, 1990). PEPCK-C is required for the net synthesis of glucose from amino acids and pyruvate and in chickens accounts for 60% of the PEPCK activity in the kidney. PEPCK-M, the only detectable enzyme in the liver, is responsible for the recycling of lactate carbon (Cori cycle).

In a previous analysis of anonymous liver cDNA clones by bulk screening we identified PEPCK-M as a gene which was unusually polymorphic at *Msp*I and *Taq*I restriction sites (Li *et al.*, 1998). Further analysis of pooled samples of a series of White Leghorn strains revealed frequency differences between strains selected for egg production traits and their corresponding unselected control strains, suggesting that PEPCK-M alleles have phenotypic effects (A. Torkamanzehi, unpublished). In this communication we analyzed the frequency of *Msp*I restriction fragment polymorphism (RFLP) of PEPCK-M in a series of strains which differed in susceptibility to Marek's disease (MD), a neoplastic disease, caused by a herpesvirus (reviewed by Kottaridis 1969 & Calnek, 1992). PEPCK-M may be considered as a candidate gene for MD susceptibility because tumor growth is intimately associated with host metabolism. In particular, proliferating neoplastic cells derive ATP from anaerobic oxidation of glucose to lactate. Lactate is released into the blood stream and recycled in the liver via the Cori cycle (Dills, 1993; Tayek & Katz, 1997). Hence genetic variants in PEPCK-M may effect tumor growth and/or the wasting of tumour-bearing chickens.

Six pairs of strains, 3/3R, 8/8R, S/K, Rs/Rr, Gs/Gr and Mr/Ms from five different genetic bases were analyzed. Strains 3 and 8 had been selected for egg production traits from two different genetic bases (Gowe *et al.*, 1993). Strains 3R and 8R were subsequently derived from strains 3 and 8, respectively, and selected for MD resistance on the basis of tumor incidence after challenge with MD virus (Gavora *et al.*, 1989; Gavora, 1990). Strains S and K were originally maintained at Cornell University, and

selected for susceptibility and resistance to "avian leukosis complex", respectively (Cole & Hutt, 1973). It was later recognized that Marek's disease was the major portion of this "complex" (Grunder *et al.*, 1972). Strains R (derived from strain K), G and M had been divergently selected for tumor induction upon wing web inoculation with Rous sarcoma virus (RSV) subgroups A and B. Strain Rr and strain Gr (ALV resistance substrains) have a reduced mortality to MD in comparison to their susceptible counterparts Rs and Gs, respectively. The contrary was observed for the strains derived from strain M, where Mr was reported to have a higher susceptibility to MD mortality than Ms (Hartmann *et al.*, 1984; Hartmann, 1991).

Southern blots hybridized with a subclone (CLEST061) comprising the untranslated region of the cDNA (Weldon *et al.*, 1990) revealed the presence of 7 distinct bands (Figure). In each individual either one or two bands were observed, suggesting that the RFLP occurred in flanking regions and that each band represented a distinct allele. Although the genomic structure of the PEPCK-M gene is unknown, the RFLP are most likely located 3' to the transcribed region because the full-length probe yielded almost the same banding pattern as the truncated probe. The absence of additional bands with the full length PEPCK-M cDNA as a probe may be the consequence of an unusual distribution of *Msp*I sites. Based on sequence analysis of chicken PEPCK-M (Weldon *et al.*, 1990), there are 17 *Msp*I restriction sites in the translated segment (1,800 bp) and none in the untranslated 3' region (1,300 bp). The fragment sizes created by these sites would preclude detection of bands under the hybridization conditions used.

The allele M2 was observed in all strains and occurred at an average frequency of 0.62. The allele M4 was less frequent (0.29) and the other haplotypes were rare (0.07 or less). In each of the six pairs of strains, the haplotype M2 occurred at a higher frequency in the MD-susceptible strain, indicating that it may be associated with susceptibility (Table). This consistency is significant (Wilcoxon matched-pairs signed-ranks test; $P < 0.05$). Furthermore, the probability that the observed differences are due to random genetic drift is less than 0.002 (estimated as indicated by Kuhnlein *et al.*, 1997). When the data of all MD-resistant strains are combined and compared to the susceptible strains, the difference of the frequency of M2 was highly significant ($\chi^2 = 30$, df = 1, $P = 3.5 \times 10^{-8}$).

Susceptibility to MD is modulated by variants in numerous genes. In a cross between two inbred lines with contrasting MD susceptibility, seven chromosome regions affecting MD susceptibility were identified (Vallejo *et al.*, 1996). Other strains of chickens may segregate for variants in other genes. The actual number of genes affecting MD susceptibility may be several folds higher. A class of genes, which may affect MD susceptibility but has received little attention, are genes involved in intermediary metabolism. It is indeed known since the early days of cancer research that diet has a profound effect on virally induced neoplasia (Saxton *et al.*, 1944; White & Andervot, 1943). Our results indicate that PEPCK-M may be a promising candidate gene and, together with other genes of the Cori cycle, warrants further analysis.

Acknowledgement

This research was made possible by the generous supply of blood samples of chickens from strains 3, 3R, 8, 8R, S and K by Agriculture Canada (Ottawa) and from strains Rr, Rs, Gr, Gs, Mr and Ms by the Institute for Small Animal Research (Celle/Merbitz, Germany). Further, we thank Dr. Hansen for supplying the full length PEPCK- M cDNA clone. Research support from the Natural Sciences and Engineering Research Council of Canada (NSERC), Agriculture Canada and Shaver Poultry Breeding Farms Ltd. is gratefully acknowledged.

References

Calnek B.W. (1992) Chicken neoplasia - a model for cancer research. *British Poultry Science* **33,** 3-16.

Cole R.F. & Hutt F.B. (1973) Selection and heterosis in Cornell White Leghorns: a review, with special consideration of inter-strain hybrids. *Animal Breeding Abstracts* **41,** 103-18.

Dills W.L. Jr. (1993) Nutritional and physiological consequences of tumour glycolysis. *Parasitology* **107,** S177-86.

Gavora J.S. (1990) Disease genetics. In: *Poultry Breeding and Genetics* (ed. by D. Crawford), pp. 805-46. Elsevier Science Publishers B.V., Amsterdam.

Gavora J.S., Kuhnlein U. & Spencer J.L. (1989) Absence of endogenous viral genes in an inbred line of Leghorn chickens selected for high egg production and Marek's disease resistance. *Journal of Animal Breeding and Genetics* **106,** 217-24.

Gowe R.S., Fairfull R.W., McMillan I. & Schmidt G.S. (1993) A strategy for maintaining high fertility and hatchability in a multiple-trait egg stock selection program. *Poultry Science* **72,** 1433-48.

Grunder A.A., Jeffers T.K., Spencer J.L., Robertson A. & Speckmann G.W. (1972) Resistance of strains of chickens to Marek's disease. *Canadian Journal of Animals Science* **52,** 1-10.

Hanson R.W. & Patel Y.M. (1994) Phosphoenolpyruvate carboxykinase (GTP): The gene and the enzyme. *Advances in Enzymology* **69,** 203-81.

Hartmann W. (1991) Genetic aspects of resistance to Avian Leukosis: An overview. *ITEA* **87,** 171-189.

Hartmann W., v.d. Hagen D. & Heil G. (1984) Genetic resistance to Leukosis virus infection: effects on mortality and egg production in Leghorn hens. *British Poultry Science* **25,** 43-52.

Kottaridis S.D. (1969) Marek's disease: a review. *World's Poultry Science Journal* **25,** 35-45.

Kuhnlein U., Liu N., Weigend S., Gavora J.S., Fairfull W. & Zadworny D. (1997) DNA Polymorphisms in the chicken growth hormone gene: response to selection for

disease resistance and association with egg production. *Animal Genetics* **28,** 116-23.

Li S., Liu N., Zadworny D. & Kuhnlein U. (1998) Genetic variability in White Leghorns revealed by chicken liver expressed sequence tags. *Poultry Science* **77,** 134-9.

Saxton J.A., Boon M.C. & Furth J. (1944) Observations on the inhibition of development of spontaneous leukemia in mice by underfeeding. *Cancer Research* **4,** 401-9.

Tayek J.A. & Katz J. (1997) Glucose production, recycling, Cori cycle, and gluconeogenesis in humans: relationship to serum cortisol. American Journal of *Physiology* **272,** E476-84.

Vallejo R.L., Bacon, L.D., Witter, R.L. & Cheng H.H. (1996) Genetic mapping of quantitative trait loci affecting susceptibility to Marek's disease in the chicken. In: *Current Research in Marek's Disease. Proceedings of the 5th International Meeting on Marek's Disease* (ed. by R.F. Silva, H.H. Cheng, L.F. Lee and L.F. Velicer. American Association of Avian Pathologists, pp8-14

Weldon S.L., Rando A., Matathias A.S., Hod Y., Kalonick P.A., Savon S., Cook J.S. & Hanson R.W. (1990) Mitochondrial phospho*enol*pyruvate carboxykinase from the chicken: Comparison of the cDNA and protein sequences with the cytosolic isozyme. *Journal of Biological Chemistry* **265,** 7308-17.

White J. & Andervot H.B. (1943) Effect of diet relatively low in cysteine on the production of spontaneous mammary-gland tumors in C3H female mice. *Journal of the National Cancer Institute* **3,** 449-51.

Table: Frequency of PEPCK-M alleles in strains divergently selected for MD resistance

Genetic Base	Strain	2N	MD Resistance	Frequency of Alleles						
				M1	M2	M3	M4	M5	M6	M7
I	8	40	Low	0	0.78	0	0.22	0	0	0
	8R	36	High	0	0.64	0	0.36	0	0	0
II	3	46	Low	0	0.93*	0	0.04	0	0.04	0
	3R	26	High	0	0.73	0.04	0	0.04	0.08	0.19
III	S	72	Low	0	0.76*	0	0.24*	0	0	0
	K	76	High	0.01	0.55	0	0.43	0	0	0
	Rs	40	Low	0	0.52^{+}	0.02	0.38**	0	0.08	0
	Rr	40	High	0	0.30	0	0.70	0	0	0
IV	Gs	38	Low	0	0.53^{+}	0	0.21	0	0.26	0
	Gr	38	High	0.08	0.29	0	0.26	0	0.37	0
V	Mr	40	Low	0	0.85**	0	0.15**	0	0	0
	Ms	40	High	0	0.55	0	0.45	0	0	0

Significance of differences between divergently selected strains (χ^2 -test): $^{+}$ P < 0.1; * P<0.05; ** P<0.01.

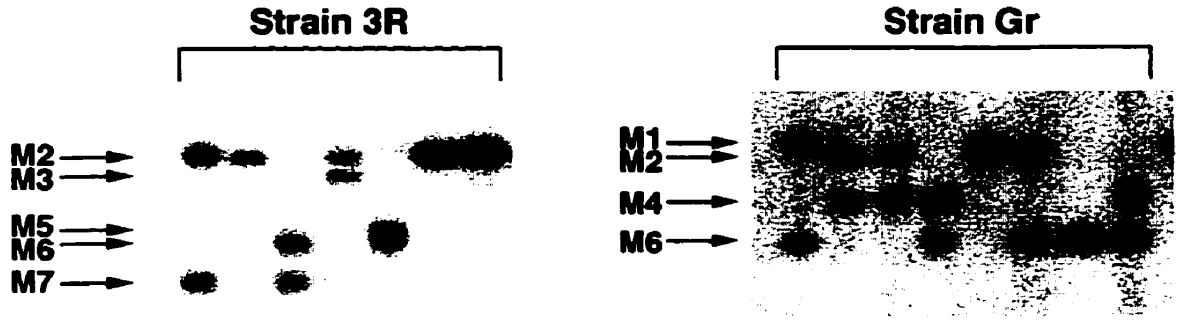

Figure. Representative Southern blots of *Msp*I-digested DNA hybridized with the PEPCK-M probe CLEST061. The probe encompasses the 3'-end of the cDNA (nt. 1,907-3,571, Weldon *et al.*, 1990). DNA samples were from strains Gr and 3R. A total of seven different bands were observed with approsimate mocular weights between 2-6 kb. Only three bands, M2, M4 and M6 occurred at frequencies above 10% in any of the 12 strains analyzed. Southern blotting was carried out as described by Li *et al.*, (1998).

Connecting Statement V

In the previous manuscript, the segregation of the PEPCK-M marker alleles was analyzed by Southern blotting assay in a total of 532 individuals from 6 pairs of strains divergently selected for MD resistance. A total of 7 bands were observed in these Southern blots, indicating that this gene is unusually polymorphic. The frequency of the M2 allele was consistently lower in the strains resistant to MD, suggesting the co-selection of this allele with susceptibility to MD. In addition to the RFLP on the *Msp*I restriction sites that were revealed by Southern blotting assay with mitochondrial phospho-enolpyruvate carboxykinase (PEPCK-M) as probes, polymorphism in the chicken PEPCK-M gene was also observed by a PCR-SSCP assay. In a region corresponding to the exon 9 of PEPCK-C, sequencing analysis of different SSCP haplotypes revealed the presence of an *Acc*I RFLP. A PCR based RFLP assay was developed and a large number of chickens were analyzed for trait association of this RFLP. A highlight of this analysis was that genetic variants might affect the correlation between traits while having no effect on the mean of each trait individually.

Chapter 7 (Manuscript #5)

Identification of Quantitative Trait Loci by Analyzing Trait Correlations: PEPCK-M in Chickens as a Paradigm

Adam Torkamanzehi[*], Suiyang Li[†], Samuel E. Aggrey[†], Jianbo Yao[†], W. Fairfull[‡], David Zadworny[†] and Urs Kuhnlein[†]

Submitted to ***Genetics***

[*]Faculty of Agriculture, University of Sistan and Beluchistan, Zahedan, Iran

[†]Department of Animal Science, Macdonald Campus of McGill University, Ste. Anne de Bellevue, QC, Canada H9X 3V9

[‡]Agriculture Canada, Ottawa, Canada K1A 0C6.

Running Head

Trait correlates of a PEPCK-M RFLP

Key Words

DNA marker, Mitochondrial PEPCK, Quantitative trait locus, Chicken

Corresponding Author

Urs Kuhnlein

Department of Animal Science

Macdonald Campus of McGill University

Ste. Anne de Bellevue, Quebec

Canada H9X 3V9

Tel.: 514-398-7799

Fax: 514-398-7964

E-mail: kuhnleinu@agradm.lan.mcgill.ca

Abstract

Egg-laying chickens were analyzed for phenotypic correlates of an *Acc*I restriction fragment length polymorphism located in exon 9 of the mitochondrial form of phospho-enolpyruvate carboxykinase (PEPCK-M). PEPCK-M is a pivotal regulatory gene in gluconeogenesis and glyceroneogenesis. The traits analyzed were the age at first egg (AFE), juvenile body weight (HBWT), adult body weight (FBWT), rate of egg laying (HDR) and egg weight (EGWT). The only significant effect of the marker genotype on single traits was observed for AFE ($P < 0.03$). In contrast, significant genotype dependencies were found for the correlations HBWT *vs.* EGWT ($P = 0.04$), FBWT *vs.* EGWT ($P = 0.015$) and HDR *vs.* EGWT ($P = 0.009$) and HWT *vs.* FBWT ($P = 0.005$). Correlation analyses between these and additional traits indicate the presence of a regulatory loop which stabilizes EGWT and which - during the peak period of egg laying - appears to be affected by variants of PEPCK-M. The analysis of biologically meaningful trait correlations rather than of single traits may be a powerful tool for identifying genetic variations that affect complex traits.

Introduction

In a previous communication we documented that the association between the rate of egg laying (HDR) and juvenile body weight (HBWT) in chickens was dependent on the GHR genotype (FENG *et al.* 1997). Two GHR genotypes, characterized by a restriction fragment length polymorphism (RFLP), were found to have opposite effects on the rate of laying in chickens with low body weight and in chickens with high body weight, while there was no significant effect of the GHR genotype on the rate of egg laying averaged over the entire population. Similar, but less extreme effects were found for the effects of the *GH* genotype on the association between the rate of egg laying and the on-set of sexual maturity. The results indicated that some genes might segregate for variants that affect the correlation between traits, but may have little or no effect on the means of single traits.

In this study we analysed the effect of variants of mitochondrial phosphoenolpyruate carboxykinase (PEPCK-M) on parameters of egg production in chickens. PEPCK, which catalyses the phosphorylation of oxaloacetate to form phosphoenolpyruvate, is a key regulatory enzyme in the *de novo* synthesis of glucose and 3-glycerophosphate and is present in a variety of tissues (UTTER and KURAHASHI 1953; RESHEF *et al.* 1970; HANSON and PATEL 1994). In vertebrates there are two isozyme forms of the enzyme encoded by separate genes (NORDLIE and LARDY 1963): one located in the cytosol (PEPCK-C) and one in the mitochondria (PEPCK-M). PEPCK-C is mainly involved in net synthesis of glucose from amino acids and pyruvate, while PEPCK-M recycles lactate carbon (Cori cycle). In adult chickens, PEPCK-M is the sole form in the liver and constitutes 60% of the PEPCK in the kidney (SHEN and MISTRY 1978; WEISE *et al.* 1991). The isozymes of PEPCK have nearly identical catalytic properties; however, PEPCK-C is under intensive metabolic control whereas PEPCK-M appears to be constitutively expressed The structure of the PEPCK-M gene is still unknown, but it may be larger than 30 kb long. It encodes two cDNA species that are 4.2 and 3.4kb in length (WELDON *et al.* 1990; HANSON and PATEL 1994).

PEPCK-M was singled out as an unusually polymorphic gene among chicken liver expressed sequence tags (LI *et al.* 1998). In this communication we analyzed the exon 9 of PEPCK-M in more detail, established a PCR assay for a genetic marker in this exon and analyzed its association with traits related to egg production. As observed previously for

GHR, the marker genotype had little influence on the means of individual traits, but a significant effect on trait correlations. It may reflect that the phenotypic effect of many variants in genes that affect metabolic flow may only be evident in individuals that deviate from normal homeostatic set points. Such quantitative gene loci (QTL) would escape detection by currently used methodologies for gene mapping.

Materials and Methods

Origins of chickens and traits measurements

Strain 7 had been established by crossing 4 commercial North American White Leghorn stocks at the Centre for Food and Animal Research (CFAR, Agriculture Canada) in 1958, and kept by random mating without selection with an effective population size of 457 (GOWE *et al.* 1993). Chickens analysed in this report were from the generation hatched in 1993. The traits analysed were age at first egg (AFE), body weight at an age of 130 d (HBWT) and 365 d (FWT), the average egg production rates (percentage of eggs laid per hen per day) from AFE to 273 d (HDR1), 274-385 d (HDR2) and 386-457 d (HDR3) and egg weight at 240 d (EGWT1), 350 d (EGWT2) and 450 d (EGWT3). To prevent confoundment with AFE, the rates of egg laying were adjusted for differences in AFE. The egg weights are the average from eggs laid in five consecutive days. Blood samples from these chickens were collected from the wing vein, stored at -70°C and DNA extracted as described by JEFFREYS and MORTON (1987).

Primers and polymerase chain reaction (PCR) conditions

Primers used to amplify the 300-bp fragment from position nt. 1,524 to nt. 1,823 were 5'CATGAGCCCCTTTTTCGGCTA3' (forward primer) and 5'TCCATAGGGAACAGTTGGGAG3' (reverse primer). Primers used to amplify the 401 bp fragments from nt. 1,517 to nt. 1,917 were 5'CCTTCGCCATGAGCCCCTTTTTC3' (forward primer) and 5'CAGCTCCGCCATGACATCCCT 3' (reverse primer). Both fragments are located in the exon 9. PCR reactions were carried out in a volume of 25 μl containing 100 ng of genomic DNA, 0.5 μM of each primer, 1 × PCR buffer [10 mM Tris-HCl (pH9.0), 1.5 mM $MgCl_2$, 50 mM KCl], 200 μM dNTP and 0.625 units of T*th* DNA Polymerase (Pharmacia, Uppsala, Sweden). Thirty-five cycles of amplification were performed at 94°C for 60 sec, 62°C for 80 sec and 72°C for 90 sec after an initial denaturation at 95°C for 3 min.

Single strand conformation polymorphism (SSCP) and RFLP analysis

One microliter of PCR product was mixed with 15 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05 xylene cyanol), denatured at

100°C for 5 min and cooled on ice for 5 min. Samples were then loaded on 15% non-denaturing acrylamide gels [acrylamide:bisacrylamide (49:1); 12 . 10 . 0.75 mm], and subjected to electrophoresis in 1 × Tris-borate (pH 8.3)-EDTA buffer using a vertical minigel apparatus (Bio-Rad) for 24 hr at 10 volts/cm. After electrophoresis at room temperature, gels were separated from the glass plates and silver stained.

For RFLP analysis, 10 μl of the PCR products were digested with 1.7 units of *Acc*I at 37°C overnight, and analysed on 2% agarose gel. Genotypes were identified by comparison of bands from digested samples with undigested and/or marker controls.

Subcloning of RFLP fragments and DNA sequencing

One microlitre of PCR product was ligated and cloned using the TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the supplier's instructions. Transformed colonies were cultured and recombinant plasmid DNA was extracted using standard methods (SAMBROOK *et al.* 1989). Presence of the inserts was confirmed by restriction enzyme digestion and agarose gel electrophoresis. Sequencing was carried out by the dideoxy-chain termination method using the T7 sequencing kit (Pharmacia, Uppsala, Sweden).

Statistical analysis

Chi-square analysis was used to test for significant deviation of allele frequencies from Hardy-Weinberg equilibrium. The frequency of haplotypes was determined by linkage disequilibrium analysis (HILL 1974; KUHNLEIN *et al.* 1997). Associations between the marker genotypes and production traits were analysed using one way analysis of variance. Trait correlations were analysed by standard procedures (ZAR 1984). In particular, differences among correlation coefficients were analysed for significance by a χ^2-test of the z-transformed correlation coefficients and pair-wise comparisons by a Tukey-type test of the z-transformed correlation coefficients. Only individuals with complete data sets were used in the analysis.

Results

Sequence variability in exon 9

The PEPCK-M sequence corresponding to the largest translated exon of PEPCK-C (exon 9, 450 bp) was identified by sequence comparison and primers were designed to amplify a 300 bp fragment. SSCP analysis of the amplified fragment in of 35 chickens from strain 7 revealed the presence of 6 different single strand conformers, indicative of 3 alleles (Figure1a). Two alleles (*A1* and *A2*) were present in both homozygote and heterozygote states, whereas the third allele (*A3*) was less frequent and occurred only as heterozygotes (*A1/A3*). Chi-square analysis did not reveal any deviation from Hardy-Weinberg equilibrium.

The amplified fragment from two individuals homozygous for the two predominant SSCP alleles *A1* and *A2*, respectively, were cloned and sequenced. Allele *A1* was identical to the published sequence of WELDON *et al.* (1990) with the exception of a T to C transition at position nt. 1,700, resulting in an amino acid change from isoleucine to threonine. Allele *A2* differed from *A1* by three transitions, a T to C at position nt. 1,578, A to G at position nt. 1,647 and T to C at position nt. 1,650. These three transitions did not result in an amino acid change. The transition of T to C at position nt. 1,578 of the *A2* allele results in the loss of an *Acc*I site (change of GTCTAC to GTCCAC) at this position, hence creating a readily detectable RFLP (Figure 1b). Linkage disequilibrium analysis between the *A1* genotype and the *Acc*I genotype in a subsample of 35 individuals indicated that 80% of the *A1* alleles were *Acc*I $^{+}$, while all *A2* and *A3* alleles were *Acc*I $^{-}$ (Table 1).

The 300 bp fragment from exon 9 of mitochondrial PEPCK was extended at both ends to cover 401 bp of the region, using the second set of primers in order to facilitate *Acc*I RFLP analysis by agarose gel electrophoresis. A total of 358 female individuals from strain 7 were typed. As with the SSCP polymorphisms, the frequency of the *Acc*I genotypes did not significantly deviate from Hardy-Weinberg equilibrium.

Genotypic effects on traits and trait correlations

The association of the *Acc*I genotypes with traits was tested for housing body weight (HBWT), mature body weight (FBWT), age at first egg (AFE) as well as the egg production rate (HDR) and egg weight (EGWT) measured in three consecutive time periods. There

was no significant association between traits and genotypes with the exception of AFE (Table 2).

A different picture emerged when trait correlations were analysed (Table 3). HBWT, the earliest trait measured, was positively associated with FBWT and EGWT1, EGWT2 and EGWT3 and negatively associated with AFE (*i.e.* birds with a high HBWT tended to start ovulation early). AFE, the next trait recorded following HBWT, was negatively associated with HDR1 indicating chickens which ovulate late tend to have a reduced initial rate of egg-laying. HDR1 was positively associated with HDR2 and HDR2 positively associated with HDR3. Finally, the egg weights, which had been found to be positively associated with body weight, were negatively associated with the rates of egg laying. These dependencies may reflect the presence of a regulatory loop, which stabilizes egg weight (Figure 2).

Significant differences between PEPCK-M genotypes were observed for 5 different trait correlations; HBWT *vs.* FBWT and EGWT1, FBWT *vs.* EGWT1, HDR1 *vs.* EGWT1 and EGWT3 (χ^2-test for comparing multiple correlation coefficients; ZAR 1984; *P*-values are indicated above the diagonal in Table 3). Correlation coefficients between body weight at 130 d (HBWT) and at 365 d (FBWT) were positive for all three genotypes (Table 3), but significantly higher for the heterozygotes *Acc*I$^-$/*Acc*I$^+$ than for the homozygotes *Acc*I$^+$/*Acc*I$^+$ (Tukey-type test using Fisher's z transformation; Levy, 1976; $P < 0.005$). The correlation coefficient for the homozygotes *Acc*I$^-$/*Acc*I$^-$ was not significantly different from either one of the other genotypic classes. Similarly, the correlation coefficients between the egg weight measured in the peak period of egg laying (EGWT1) and either HBWT or FBWT were higher for the heterozygotes *Acc*I$^-$/*Acc*I$^+$ than for the homozygotes *Acc*I$^+$/*Acc*I$^+$ ($P < 0.1$ and $P < 0.025$, respectively), while the AccI$^-$/AccI$^-$ homozygotes did not differ significantly from the other genotypes. Similar patterns were observed for egg weights measured in the declining phase of egg laying (EGWT2 and EGWT3), but the variation among the correlation coefficients was not significant (Table 3). The regression lines of EGWT1 on FBWT for the three genotypic classes are shown in Figure 3. They indicate that chickens below the median body weights laid eggs of similar weight, while among chickens with high body weights those of the *Acc*I$^-$/*Acc*I$^+$ genotype tended to lay heavier eggs.

The rate of egg laying during the peak period (HDR1) was negatively correlated with egg weight (EGWT1) for the *AccI*$^{-}$/*AccI*$^{-}$ homozygotes but not for the other two genotypes (Table 3). Pair-wise comparisons indicated that the correlation coefficient for the *AccI*$^{-}$/*AccI*$^{-}$ homozygotes differed significantly from the correlation coefficent for heterozygotes as well as for the *AccI*$^{+}$/*AccI*$^{+}$ homozygotes ($P < 0.025$ and $P < 0.05$, respectively). Similar, but non significant tendencies for HDR/EGWT correlations were observed in later age periods, when the rate of egg laying was declining. Linear regression of EGWT1 on HDR1 indicates that as the rate of egg-laying is increasing, chickens of the *AccI*$^{-}$ /*AccI*$^{-}$ genotype lay increasingly smaller eggs than the chickens of the other two genotypes (Figure 4).

The correlation between HBWT and FBWT may be trivial and simply reflect that chickens that had a high juvenile body weight remained heavy throughout adult life. The correlation between the late weight gain (130 d - 365 d) and body weight at 130 d was therefore also analyzed (Table 4). In the entire population, the weight gain after 130 d was not significantly associated with HBWT. Hence late growth appeared independent of the rate of growth between hatch and 130 d. However, correlation coefficients of the three PEPCK-M genotype classes varied ($P < 0.01$). The correlation was positive for the genotype *AccI*$^{-}$/*AccI*$^{+}$ indicating that in this class, a low growth rate before sexual maturity was compensated by an increased weight gain after sexual maturity. For the other two genotypic classes, the correlation was negative, indicating that chickens with a low early growth rate also had a low late growth rate. The late growth rate was positively correlated with egg weight and EGWT1, differed significantly among the genotypic classes ($P < 0.03$).

Discussion

Correlation analyses between traits indicate the presence of trait dependencies, which stabilize average egg weight in the breeding population (Figure 4). High body weight (or growth rate) is associated with the production of large eggs. However, high body weight is also associated with an earlier AFE, which in turn is associated with an increase in the rate of egg-laying and a lower egg weight. Such interdependence between traits may reflect past evolution for an optimal relationship between egg size, body weight and rate of egg laying. The PEPCK-M genotypes had a significant effect on the correlation between several traits of this regulatory loop, indicating that PEPCK-M may have a role in regulating homeostasis between traits.

The effect of the PEPCK-M genotype on correlations followed two distinct patterns. In all correlations involving body weight or growth, the highest correlation coefficients were found for the heterozygous genotype, while the two homozygotes were identical. Hence, in heterozygotes small differences in body weight were associated with relatively large differences in egg weight. Further, for body weights above the median value of the population, the PEPCK-M marker genotype was over-dominant for high egg weight as well as for high adult growth rate, while for body weights below the median the differences in egg weight between different genotypes were small (Figure 3).

In contrast, the most extreme correlation coefficients between the rate of egg laying and egg weight were found for the *AccI /AccI* homozygote (Figure 3). In chickens with rates of laying above the median of the population, the *AccI /AccI* genotype was associated with a low egg weight, while the average egg weight for the other two genotypes were similar. Again, the PEPCK-M genotype had only a small effect in chickens below the median HDR of the population.

PEPCK is pivotal in gluconeogenesis and glyceroneogenesis, since it bypasses the irreversible step of glycolysis, which leads to the formation of oxaloacetate from pyruvate (HANSON and PATEL 1994). Genetic variants that affect PEPCK activity may therefore influence the balance between gluconeogenesis and glycolysis. Chickens with a high demand on biosynthetic pathways (high growth rate, HDR or EGWT) may therefore be particularly sensitive to variations in PEPCK activity. However, the basis of over dominance

in trait correlations involving body weight and dominance in trait correlations involving HDR remains to be elucidated.

The current analysis is based on the analysis of a single marker in the PEPCK-M gene. However, SSCP analysis and sequencing indicate segregation for at least one additional allele in exon 9 and we have observed 5 additional *Msp*I RFLPs elsewhere in this gene (LI *et al.* 1998). Hence PEPCK-M is likely to segregate for a multitude of haplotype, which have potentially differential effects on single traits and trait correlations. Furtherly, interaction between PEPCK-M haplotype and variants of its cytosolic counterpart as well as with variants in other pivotal genes in the gluconeogenesis pathway have to be elucidated in order to implement selection at the DNA level.

Similar to PEPCK-M, variants in genes of the GH-axis were also found to affect trait correlations, perhaps reflecting their influence on major metabolic pathways (FENG *et al.* 1997). An increased susceptibility of traits to genetic variation in metabolically stressed animals may be the norm, while in metabolically "normal" animals, epistasis will cancel the effects of variants in a particular gene. Hence, when searching for variants in candidate genes, which affect a particular trait resolution may be improved by restricting the analysis of a particular trait to individuals, which are at the extreme with respect to a second trait.

We thank L. VOLKOV for her technical assistance. The research was supported by a grant through the Partnership Program of the Natural Sciences and Engineering Research Council of Canada (NSERC), Agriculture Canada and Shaver Poultry Breeding Farms Ltd.

Literature Cited

FENG, X. P., U. KUHNLEIN, S. E. AGGREY, J. S. GAVORA and D. ZADWORNY, 1997 Trait association of genetic markers in the growth hormone and the growth hormone receptor gene in a White Leghorn strain. Poultry Sci. **76**: 1770-1775.

GOWE, R. S., R. W. FAIRFULL, I. MCMILLAN and G. S. SCHMIDT, 1993 A strategy for maintaining high fertility and hatchability in a multiple-trait egg stock selection program. Poultry Sci. **72**: 1433-1448.

HANSON, R. W. and Y. M. PATEL, 1994 Phosphoenolpyruvate carboxykinase (GTP): The gene and the enzyme. Adv. Enzymol. **69**: 203-281.

HILL, W. G., 1974 Estimation of linkage disequilibrium in randomly mating populations. Heredity **33**: 229-239.

JEFFREYS, A. J. and D. B. MORTON, 1987 DNA fingerprinting in dogs and cats. Anim. Genet. **18**: 1-15.

KUHNLEIN, U., L. NI, S. WEIGEND, J. S. GAVORA, W. FAIRFULL and D. ZADWORNY, 1997 DNA polymorphisms in the chicken growth hormone gene: response to selection for disease resistance and association with egg production. Animal Genetics **28**: 116-123.

LI, S., N. LIU, D. ZADWORNY and U. KUHNLEIN, 1998 Genetic variability in White Leghorns revealed by chicken liver expressed sequence tags. Poultry Sci. **77**: 134-139.

NORDLIE, R. C. and H. A. LARDIE, 1963 Mammalian liver P-enolpyruvate carboxykinase. J. Biol. Chem. **238**: 2259-2263.

RESHEF, L., F. J. BALLARD and R. W. HANSON, 1970 A possible role for glyceroneogenesis in rat adipose tissue. J. Biol. Chem. **271**: 8068-8074.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.

SHEN C. S. and S. P. MISTRY, 1978 Development of gluconeogenic, glycolytic and pentose-shunt enzymes in chicken kidney. Poultry Sci. **58**: 663-667.

UTTER, M. F. and K. KURAHASHI, 1953 Mechanism of action of oxalacetate carboxylase. J. Amer. Soc. **75**: 785-787.

WEISE, T. J., D. O. LAMBETH and P. D. RAY, 1991 The intracellular distribution and activities of PEPCK isozymes in the various tissues of several mammals and birds. Comp. Biochem. Physiol. B. **100**: 297-302.

WELDON, S. L., A. RANDO, A. S. MATATH, Y. HOD, P. A. KALOVIC, S. SAVON, and J. S. COOK, 1990 Mitochondrial P-enolpyruvate carboxykinase from the chicken. Nucleotide sequence of the cDNA and the deduced amino acid sequence. J. Biol. Chem. **165**: 7308-7317.

ZAR, J. H., 1984 *Biostatistical Analysis* (2nd Ed.). Prentice Hall, Englewood Cliffs, N.J.

Table 1

Segregating haplotypes in exon 9 of PEPCK-M

SSCP alleles	RFLP alleles	
	AccI$^+$	*AccI*$^-$
A1	0.54	0.13
A2	0.00	0.28
A3	0.00	0.03

The frequencies of SSCP genotypes in this sample of strain 7 (N = 35) and in an additional sample of a substrain of strain 7 (N = 37) did not significantly deviate from a Hardy-Weinberg equilibrium (N = 72, $\chi^2 = 7.1$, df = 5, $P = 0.22$). Similarly no deviation from Hardy-Weinberg equilibrium was found for the *Acc*I genotypes (N = 358, $\chi^2 = 0.77$, df = 2, $P = 0.68$). A subsample of 35 individuals were typed for both SCCP and *Acc*I RFLP and the frequency of segregating haplotypes determined by linkage disequilibrium analysis (HILL *et al.* 1964; KUHNLEIN *et al.* 1997)

Table 2
Least square means of traits in different *Acc*I RFLP genotype classes

Trait	*Acc*I RFLP genotype			Significance(P)[a]
	+/+	+/-	-/-	
Age at first egg (AFE), (days)	165.5	169.1	165.0	0.03
Body weight (kg)				
130 days	1.30	1.29	1.30	0.75
365 days	1.74	1.75	1.75	0.97
Hen day rate of lay (%)				
AFE to 273 days	83.1	83.2	85.0	0.42
274 to 385 days	68.6	69.5	68.8	0.84
386 to 457 days	55.0	54.6	56.3	0.83
Egg weight (g)				
240 days	52.2	52.9	52.2	0.16
350 days	57.4	58.5	58.0	0.20
450 days	59.9	60.9	60.7	0.50

[a] Single trait analysis of variance

Table 3

Influence of the *Acc*I RFLP genotype on trait correlation

Trait	Genotype	HBWT	FBWT	AFE	HDR1	HDR2	HDR3	EGWT1	EGWT2	EGWT3
	all									
HBWT	-/-		**0.005**	0.62	0.86	0.76	0.56	**0.040**	0.098	0.12
	+/-									
	+/+									
	all	0.65*								
FBWT	-/-	**0.59***		0.27	0.46	0.51	0.25	**0.015**	0.24	0.58
	+/-	**0.75***								
	+/+	**0.50***								
	all	-0.26*	0.01							
AFE	-/-	-0.34*	-0.14		0.11	0.42	0.52	1.00	0.75	0.33
	+/-	-0.21*	-0.04							
	+/+	-0.31*	0.13							
	all	0.03	-0.03	-0.14*						
HDR1	-/-	0.10	-0.01	-0.27		0.33	0.74	**0.009**	0.095	**0.022**
	+/-	0.02	-0.09	-0.21*						
	+/+	-0.01	0.07	0.04						
	all	0.06	0.01	-0.02	0.43*					
HDR2	-/-	0.16	0.17	0.18	0.20		0.17	0.44	0.96	0.88
	+/-	0.03	-0.04	-0.07	0.45*					
	+/+	0.09	0.05	0.00	0.45*					
	all	0.01	-0.01	-0.06	0.29*	0.70*				
HDR3	-/-	-0.05	-0.12	0.13	0.22	0.22		0.08	0.43	0.12
	+/-	-0.04	-0.08	-0.09	0.26*	0.68*				
	+/+	0.09	0.12	-0.04	0.33*	0.77*				

	all	0.31*	0.41*	0.09	-0.15*	-0.11	-0.08			
	-/-	**0.16**	**0.24**	0.08	**-0.57***	-0.28	-0.42*		0.055	0.15
EGWT1	+/-	**0.45***	**0.54***	0.07	**-0.06**	-0.12	-0.05			
	+/+	**0.18**	**0.26***	0.08	**-0.11**	-0.03	-0.01			
	all	0.28*	0.37*	0.09	-0.16*	-0.16*	-0.10	0.74*		
EGWT2	-/-	0.12	0.22	0.16	-0.46*	-0.19	-0.30	0.81*		0.81
	+/-	0.39*	0.44*	0.04	-0.08	-0.15	-0.08	0.78*		
	+/+	0.16	0.29*	0.11	-0.16	-0.14	-0.05	0.64*		
EGWT3	all	0.28*	0.36*	0.09	-0.12	-0.13*	-0.09	0.68*	0.82*	
	-/-	0.08	0.22	0.29	-0.50*	-0.20	-0.37*	0.77*	0.85*	
	+/-	0.38*	0.40*	0.01	-0.01	-0.13	-0.11	0.72*	0.81*	
	+/+	0.19*	0.33*	0.10	-0.13	-0.10	0.03	0.60*	0.82*	

The values below the diagonal are the correlation coefficients between traits for all individuals combined (N= 273) and for each *Acc*I RFLP genotype (-/-, N=35; +/-, N=148; +/+, N= 96). The values above the diagonal are the P-values for significance of differences among the correlation coefficients obtained for the three genotypes (z-transformation and chi-square test; ZAR 1984). Correlation coefficients that significantly differed among genotypes are marked in bold. Abbreviations are: AFE, age at first egg; HBWT, body weight at 130 days; FBWT, body weight at 365 days; HDR1, hen day rate of lay AFE to 273 days; HDR2, hen day rate of lay 274 to385 days; HDR3, hen day rate of lay 386 to 457 days; EGWT1, egg weight at 240 days; EGWT2, egg weight at 350 days; EGWT3, egg weight at 457 days. Significant correlation coefficients (two-tailed, $P<0.05$) are marked by *.

Table 4

Influence of the *Acc*I RFLP genotype on the correlation of traits with post juvenile weight gain (130-365d)

Trait	*Acc*I Genotypes				Comparison of correlation coefficients (*P*)[b]
	All[a] (N=273)	-/- (N=35)	+/- (N=148)	+/+ (N=96)	
HBWT	-0.02	-0.22	0.15	-0.20	0.01
EGWT1	0.16*	0.14	0.32*	-0.01	0.03
EGWT2	0.16*	0.12	0.39*	0.16	0.09
EGWT3	0.14*	0.08	0.39*	0.18	0.10

[a] Significant correlations ($P<0.05$) are marked with *.

[b] Significance of deviations among the correlation coefficients for different genotypes (z-transformation and χ-square analysis; ZAR 1984).

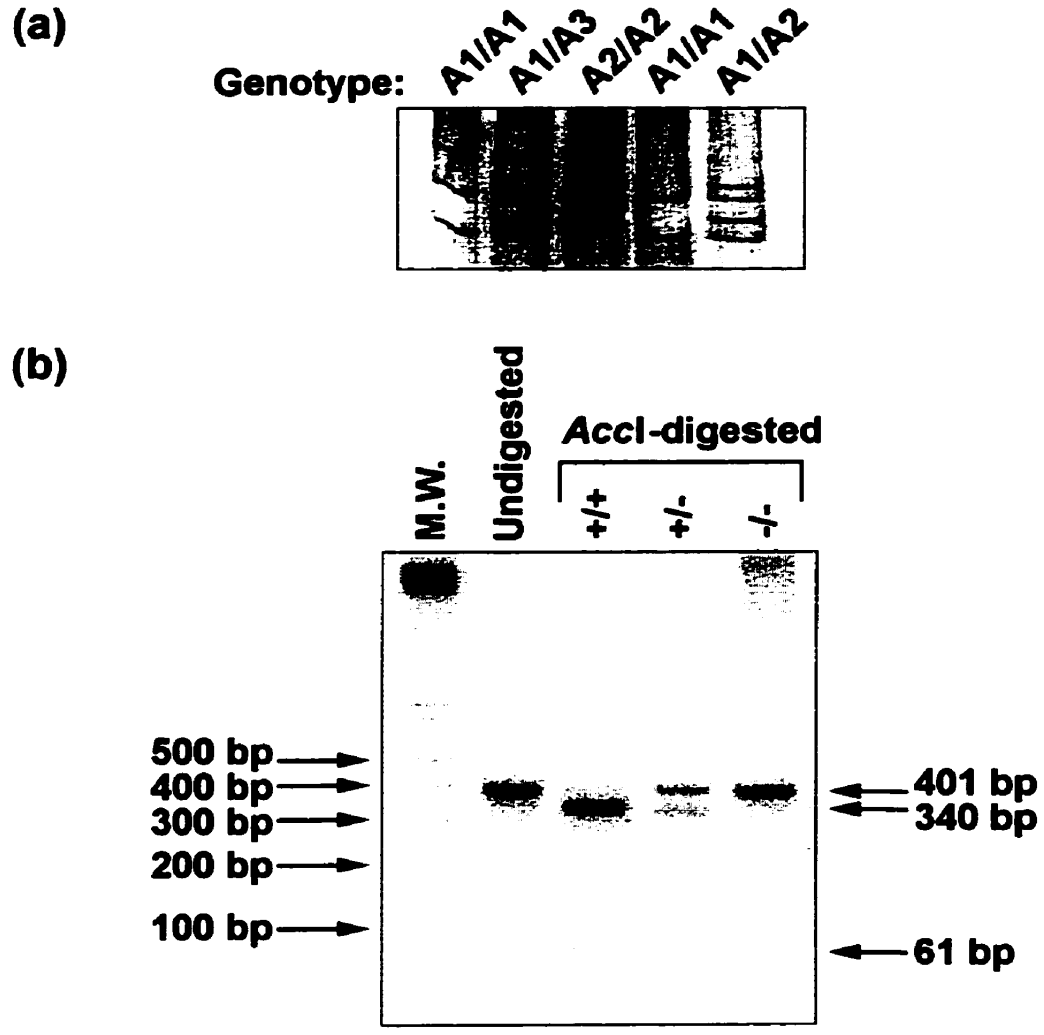

FIGURE 1. PEPCK-M genotypes in strain 7 revealed by PCR amplification of parts of exon 9 followed by SSCP analysis (**a**) and *Acc*I restriction analysis (**b**). Primer positions and sequences are indicated in Materials and Methods.

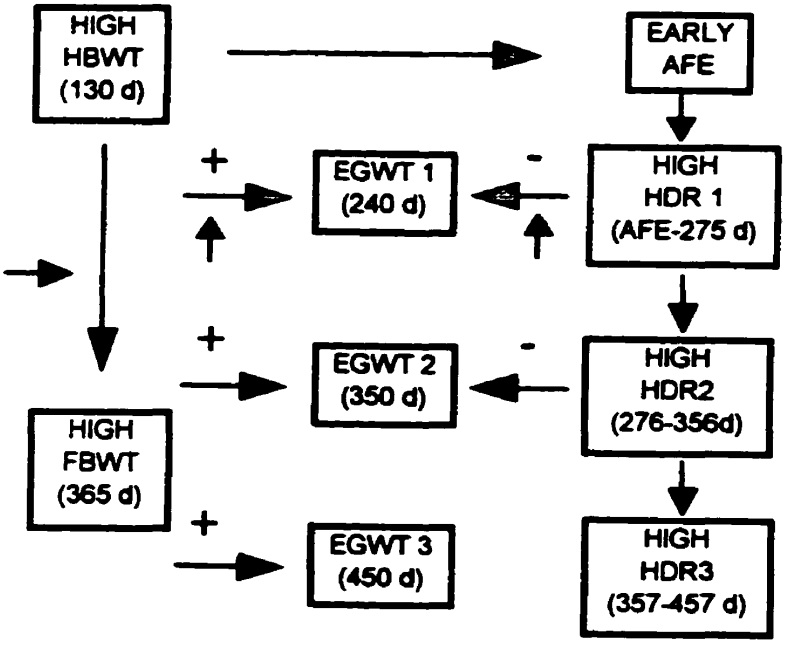

FIGURE 2. Proposed regulatory loop which stabilizes egg weight in the breeding population. The open arrows designate correlations that were significant for all genotypes combined. The black arrows mark the correlations, which significantly differed among PEPCK-M genotype classes. Significant effects of the PEPCK-M genotype on egg weights were observed during the peak period of egg laying (AFE-275 d). For correlations involving body weight (HBWT *vs.* FBWT, HBWT *vs.* EGWT1 and FBWT *vs.* EGWT1) the correlation coefficients were positive and significantly higher for the *AccI*$^{-}$/*AccI*$^{+}$ heterozygotes than for the *AccI*$^{+}$/*AccI*$^{+}$ homozygotes. The correlation between HDR1 and EGWT1 was negative and significantly lower for the *AccI*$^{-}$/*AccI*$^{-}$ homozygotes than the other two genotypes (see Figure 3 and Figure 4).

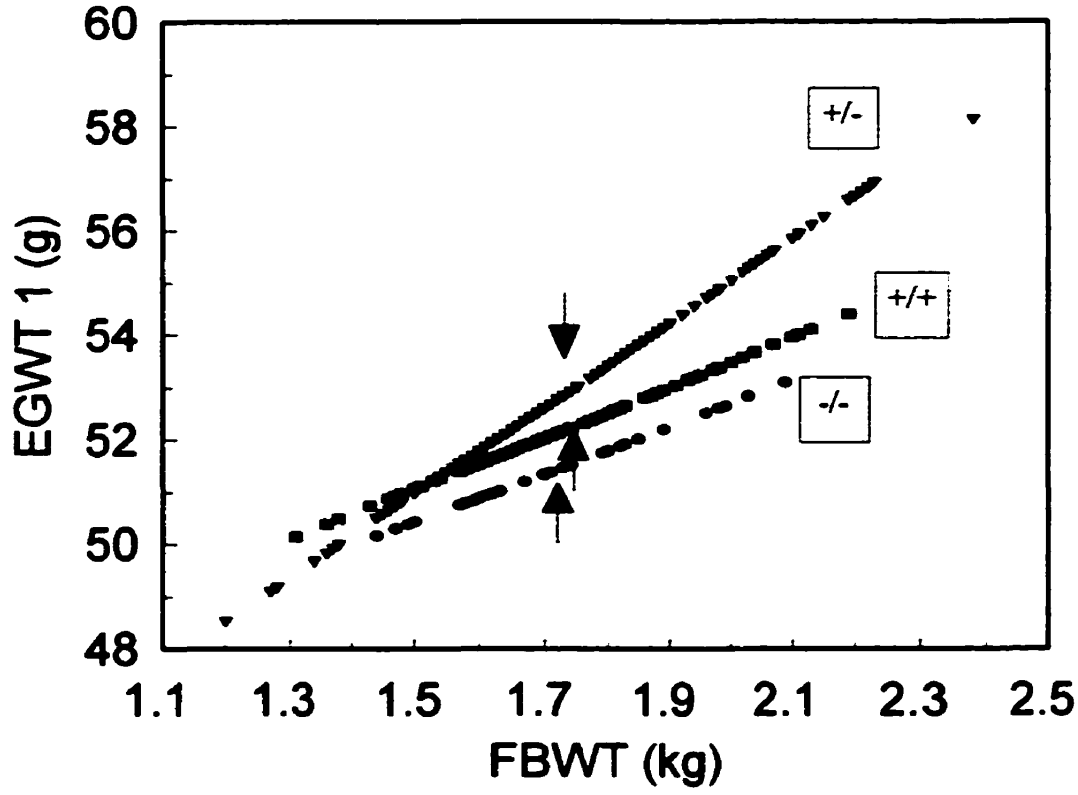

FIGURE 3. Best linear fit indicating the relation between EGWT1 and FBWT. The correlation coefficients differed significantly for the three genotypes ($P = 0.015$, Table 2). Pair-wise comparisons indicate that the correlation coefficient for the *Acc*I$^-$/*Acc*I$^+$ genotypes was significantly different from the correlation coefficient for the *Acc*I$^+$/*Acc*I$^+$ genotypes ($P < 0.025$). Other contrasts were not significant. The median FBWT for each genotype class is marked with an arrow. It was 1.71 kg for the *Acc*I$^-$/*Acc*I$^-$ genotypes, 1.72 kg for the *Acc*I$^-$ /*Acc*I$^+$ genotype and 1.77 kg for the *Acc*I$^+$/*Acc*I$^+$ genotype.

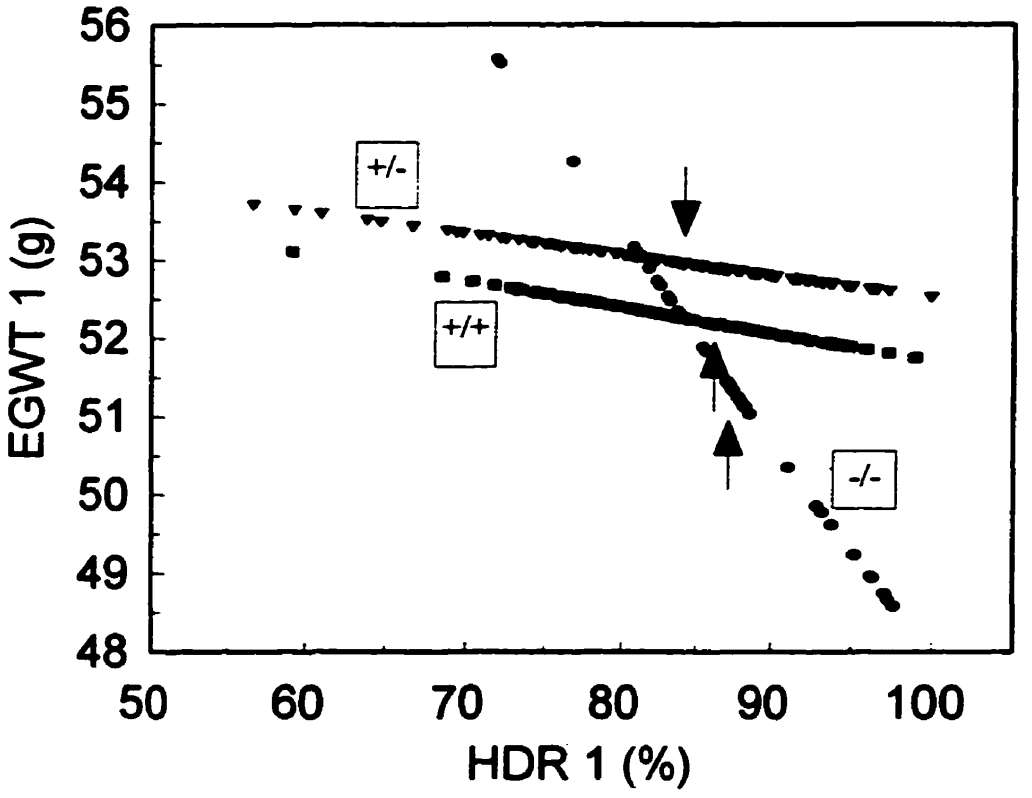

FIGURE 4. Best linear fit indicating the relation between EGWT1 and HDR1. The correlation coefficients differed significantly for the three genotypes ($P = 0.009$, Table 2). Pair-wise comparisons indicate that the correlation coefficient for the *AccI*$^-$/*AccI*$^-$ genotypes was significantly different from the coefficient for the *AccI*$^-$/*AccI*$^+$ genotype ($P < 0.025$) and the -/- genotypes ($P < 0.025$), whereas the *AccI*$^-$/*AccI*$^+$ and -/- coefficients were similar. An arrow marks the median HDR1 for each genotype. They are 87.3 % for the *AccI*$^-$/*AccI*$^-$ genotype, 84.2% for the *AccI*$^-$/*AccI*$^+$ genotype and 85.9% for the *AccI*$^-$/*AccI*$^+$ genotype.

Chapter 8. Conclusions and General Discussion

A rapid method for the identification of DNA markers, which are associated with egg production traits and Marek's disease (MD) resistance in chickens, was established. RFLP analysis showed that chicken liver expressed sequence tags (CLESTs) revealed an abundance of DNA polymorphisms at *Msp*I and *Taq*I restriction sites (Chapter 3). A significant proportion of these DNA polymorphisms was responsive to selection for egg production traits and Marek's disease resistance (Chapter 4). Using this methodology, a DNA marker in the chicken mitochondrial genome was identified and characterized, and found to be associated with the housing body weight and egg specific gravity (Chapter 5). Since the mitochondrial genome contains genes that are critically involved in energy/intermediary metabolism, it was not surprising to find that a genetic variant in the mitochondrial genome had effects on quite disparate production traits. In addition, this mitochondrial RFLP was also co-selected with MD resistance in chickens (Chapter 5). DNA markers in the chicken mitochondrial PEPCK gene had responded to selection for MD resistance (Chapter 6) and affected the correlation between production traits (Chapter 7). The first evidence suggests that mitochondrial components play a role in the immune responsiveness of birds to viral infection. However, the exact mechanism can not be elucidated just based on the observation in this study. The present study also suggests that egg production traits and MD resistance in chickens may have common genetic determinants. Such knowledge is important for the understanding of the genetic basis of selection and for future selection at the DNA level (Lande and Thompson, 1989).

The success of using this methodology for rapid DNA marker identification is based on a careful design of the experiment. The choice of candidate tissue for the cDNA library, the choice of restriction enzymes to reveal the RFLPs and the choice of pooling strategy for screening large number of anonymous cDNA clones for RFLP analysis affect the efficiency of this method. As far as disease resistance is concerned, thymus or spleen cDNA libraries may be the better choice of tissue than the liver cDNA library.

Pooling strategies: The bulk analysis for screening a large number of cDNA clones takes advantages of bulk processing, including pooling of the DNA samples of individuals for restriction digestion and carrying out Southern blotting with several membranes and

probes simultaneously. In addition, pooling blood samples from individuals prior to DNA extraction rather than pooling the DNA may save the work of extracting DNA from a large number of individuals. The optimal number of probes, which should be mixed for Southern blot hybridization at one time depends on the number of polymorphisms revealed per candidate clones. In this experiment, about one out of every three or four clones was polymorphic. A mixture of three or four clones will therefore reveal at least one clone, which may be polymorphic. On the other hand, ribosomal RNA clones represented about 32% of the total number of clones in our cDNA library and should be identified by dot-blotting prior to RFLP analysis. In this case, the frequency of polymorphisms among the candidate clones would be expected to increase. In consequence, the optimal number of probes for a mix should be re-considered.

Selective genotyping: The experimental strategy originally proposed for this project contained five stages (see Chapter 1). Once the DNA polymorphisms that had responded to selection were identified, trait association studies were performed by selective genotyping for the individuals from the trait distribution extremes. This strategy is effective only when the QTLs have clear additive effects on the trait of interest. However, in many cases interactions among QTL exist and changes in the allele frequencies throughout the trait distribution may occur. It is therefore more informative to sample the individuals throughout the trait distribution in order to have a better picture of QTL effects (Chapter 5). In this case, a PCR assay for rapid RFLP analysis has to be established.

Establishment of PCR assays: PCR assay is the choice for rapid screening of a large number of individuals. Establishment of PCR assay requires the knowledge of the DNA sequences in the flanking region of the DNA polymorphism. Partial sequencing for candidate clones followed by homology search with known genes from the databases may help to characterize and localize the DNA polymorphism. If a RFLP region is located in a known gene whose DNA sequence is known, the DNA polymerase chain reaction (PCR) can be established. Then the trait association studies for large number of individuals using PCR assay are much faster than using the traditional Southern blotting assay. Finally, once the RFLP regions are amplified with PCR, the genetic variation of interest can be readily determined by direct sequencing of the PCR product.

In summary, a new method for rapid identification of DNA markers associated with QTL in chickens was established in this thesis. It is easily performed and led to the successful identifying a mitochondrial DNA marker associated with traits. Markers in a series of other clones co-selected with egg production traits and Marek's disease resistance were also identified and await further analysis. The method can be extended to other tissue cDNA libraries and traits not only in chickens, but also in other species. Such knowledge should contribute to the understanding of the genetic architecture of complex traits and may have uses in future selection at the DNA level.

Chapter 9. References

Adams, M.D., M.B. Soares, A.R. Kerlavage, C. Fields and J.C. Venter, (1993), Rapid cDNA sequencing (expressed sequence tags) from a directionally cloned human infant brain cDNA library. *Nature Genetics* **4:** 373-380

Affara, N.A., E. Bentley, P. Davey, A. Pelmear and M.H. Jones (1994), The identification of novel gene sequences of the human adult testis. *Genomics* **22:** 205-210

Agarwal, S.K., L.A. Cogborn and J. Burnside (1994), Dysfunctional growth hormone receptor in a strain of sex-linked dwarf chicken evidence for a mutation in the intracellular domain. *Journal of Endocrinology* **142:** 427-434

Aggrey, S.E., M. Lessard, D. Hutchings, S. Joseph, X.P. Feng, D. Zadworny and U. Kuhnlein (1996), Association of genetic markers with immune traits. In: *Current Research on Marek's disease*, Proceedings of the 5th International Symposium on Marek's disease, American Association of Avian Pathologists, Inc., Kennett Square, Pennsylvania, pp80-85

Aggrey, S.E., U. Kuhnlein, J.S. Gavora and D. Zadworny (1998), Association of endogenous viral genes with quantitative traits in chickens selected for high egg production and susceptibility or resistance to Marek's disease. *British Poultry Science* **39:** 39-41

Allikmets, R., B. Gerrard, D. Glavac, M. Ravnik-Glavac, N.A. Jenkins, D.J. Gilbert, N.G. Copeland, W. Modi and M. Dean (1995), Characterization and mapping of three new mammalian ATP-binding transporter genes from an EST database. *Mammalian Genome* **6:** 114-117

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman (1990), Basic local alignment search tool. *Journal of Molecular Biology* **215:** 403-410

Anderson S, A.T. Bankier, B.G. Barrell, M.H.L. de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J.H. Smith, R. Staden and I.G. Young (1981), Sequence and organization of the human mitochondrial genome. *Nature* **290:** 457-465

Bacon, L.D. (1987), Influence of the major histocompatibility complex on disease resistance and productivity. *Poultry Science* **66:** 802-811

Bacon, L.D., E. Smith and L.B. Crittenden (1988), Association of the slow feathering (*K*) and an endogenous viral (*ev 21*) gene on the Z chromosome of chickens. *Poultry Science* **67:** 191-197

Barker, D., M. Schafer and R. White (1984), Restriction sites containing CpG show a higher frequency of polymorphism in human DNA. *Cell* **36:** 131-138

Bates, P., L. Rong, H.E. Varmus, J.A. Young and L.B. Crittenden (1998), Genetic mapping of the cloned subgroup A avian sarcoma and leukosis virus receptor gene to the TVA locus. *Journal of Virology* **72:** 2505-2508

Becker, K.G., J.W. Nagle, R.D. Canning, W.E. Biddison, K. Ozato and P.D. Drew (1995), Rapid isolation and characterization of 118 novel C_2H_2-type zinc finger cDNAs expressed in human brain. *Human Molecular Genetics* **4:** 685-691

Botstein, D., R.L. White, M. Skolnick and R.W. Davis (1980), Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American *Journal of Human Genetics* **32:** 314-331

Bumstead, N. and J. Palyga (1992), A preliminary linkage map of the chicken genome. *Genomics* **13:** 690-697

Bumstead, N., J.R. Young, C. Tregaskes, J. Palyga and P.P. Dunn (1994), Linkage mapping and partial sequencing of 10 cDNA loci in the chicken. *Animal Genetics* **25:** 337-341

Burgess, D.G., and E.E. Penhoet (1985) Characterization of the chicken aldolase B gene. *Journal of Biological Chemistry* **260:** 4604-4614

Burmester, B.R. and R.L. Witter (1970), Marek's disease, the dynamics of infection. *World's Poultry Congree*, 14th, Madrid, Vol. **3**, p297-301

Burt, D.W., B.R. Dey, I.R. Paton, D.R. Morrice and A.S. Law (1995a), The chicken transforming growth factor-beta 3 gene: genomic structure, transcriptional analysis and chromosomal location. *DNA and Cell Biology* **14:** 111-123

Burt, D.W., N. Bumstead, J.J. Bitgood, F.A. Ponce de Leon and L.B. Crittenden (1995b), Chicken genome mapping: a new era in avian genetics. *Trends in Genetics* **11:** 190-194

Cahaner, A., E.J. Smith, S. Swenson and S.J. Lamont (1996), Associations of individual genomic heterozygosity, estimated by molecular fingerprinting, and of dam major histocompatibility complex with growth and egg production traits in layer chickens. *Poultry Science* **75:** 1463-1467

Calnek, B.W. (1992), Chicken neoplasia - a model for cancer research. *British Poultry Science* **33:** 3-16

Capon, D.J., E.Y. Chen, A.D. Levinson, P.H. Seeburg and D.V. Goeddel (1983), Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature* **302:** 33-37

Chee, M., R. Yang, E. Hubbell, A. Berno, X.C. Huang, D. Stern, J. Winkler, D.J. Lockhart, M.S. Morris and S.P.A. Fodor (1996), Accessing genetic information with high-density DNA arrays. *Science* **274:** 610-613

Cheng, H.H. and L.B. Crittenden (1994), Microsatellite markers for genetic mapping in the chicken. *Poultry Science* **73:** 539-546

Cheng, H.H., I. Levin, R.L. Vallejo, H. Khatib, J.B. Dodgson, L.B. Crittenden and J. Hillel (1995), Development of a genetic map of the chicken with markers of high utility. *Poultry Science* **74:** 1855-1874

Cole, R.K. (1968), Studies on genetic resistance to Marek's disease. *Avian Diseases* **12:** 9-28

Cole, R.K. and F.B. Hutt (1973), Selection and heterosis in Cornell White Leghorns: a review, with special consideration of inter-strain hybrids. *Animal Breeding Abstracts* **41:** 103-118

Cooper, D.N. and J. Schmidtke (1984), DNA restriction fragment length polymorphisms and heterozygosity in the human genome. *Human Genetics* **66:** 1-16

Cooper, D.N. and J. Schmidtke (1992), Molecular genetic approaches to the analysis and diagnosis of human inherited disease: an overview. *Annals of Medicine* **24:** 29-42

Coulondre, C., J.H. Miller, P.J. Farabaugh and W. Gilbert (1978), Molecular basis of base substitution hotspots in Escherichia coli. *Nature* **274:** 775-780

Cowan, C.M., M.R. Dentine, R.L. Ax and L.A. Schuler (1990), Structural variation around prolactin gene linked to quantitative traits in an elite Holstein sire family. *Theoretical and Applied Genetics* **79:** 577-558

Crittenden, L.B. (1991), Retroviral elements in the genome of the chicken: Implications for poultry genetics and breeding. *Critical Review in Poultry Biology* **3:** 73-109

Crooijmans, R.P., P.A. van Oers, J.A. Strijk, J.J. van der Poel and M.A. Groenen (1996), Preliminary linkage map of the chicken (*Gallus domesticus*) genome based on microsatellite markers: 77 new markers mapped. *Poultry Science* **75:** 746-754

Darvasi, A. and M. Soller (1994), Selective DNA pooling for determination of linkage between a molecular marker and a quantitative trait locus. *Genetics* **138:** 1365-1373

Dejardins, P. and R. Morais (1990), Sequence and gene organization of the chicken mitochondrial genome: A novel gene order in higher vertebrates. *Journal of Molecular Biology* **212:** 599-634

Dills, W.L. Jr. (1993), Nutritional and physiological consequences of tumour glycolysis. *Parasitology* **107:** S177-86

Dodgson, J.B., H.H. Cheng and R. Okimoto (1997), DNA marker technoloty: a revolution in animal genetics. *Poultry Science* **76:** 1108-1114

Dominguez-Steglich, M., P. Lichter, A. Carrier, C. Auffray and M. Schmid (1992), Mapping the beta NGF gene in situ to a microchromosome in chicken, *Genomics* **12:** 829-832

Duncan, B.K. and J.H. Miller (1980), Mutagenic deamination of cytosine residues in DNA. *Nature* **287:** 560-561

Ensminger, M.E. (1992), *Poultry Science*, 3rd edition, Interstate Publishers, Inc., Danville, Illinois, p30-31

Fairfull, R.W. and J.R. Chambers (1984), Breeding for feed efficiency in poultry. *Canadian Journal of Animal Science* **64:** 513-527

Feng, X.P., U. Kuhnlein, S.E. Aggrey, J.S. Gavora and D. Zadworny (1997), Trait association of genetic markers in the growth hormone and the growth hormone receptor gene in a White Leghorn strain. *Poultry Science* **76:** 1770-1775

Field, C.J. (1996), Using immunological techniques to determine the effect of nutrition on T-cell function. *Canadian Journal of Physiology and Pharmacology* **74:** 769-777

Fotouhi, N., C.N. Karatzas, U. Kuhnlein and D. Zadworny (1993), Identification of growth hormone DNA polymorphisms which respond to divergent selection for abdominal fat content in chickens. *Theoretical and Applied Genetics* **85:** 931-936

Ganong, W.F. (1985), *Review of Medical Physiology*, 12th edition, Lange Medical Publications, Los Altos, California 94023, p408-409

Gavora, J.S. (1990), Disease genetics. In: *Poultry Breeding and Genetics*, edited by D. Crawford, Elsevier Science Publishers B.V., Amsterdam, pp805-846

Gavora, J.S., A. Emsley and R.K. Cole (1979), Inbreeding in 36 generations of development of Cornell S strain of Leghorns. *Poultry Science* **58:** 1133-1136

Gavora, J.S., J.L. Spencer, R.S. Gowe and D.L. Harris (1980), Lymphoid Leukosis virus infection: Effects on production and mortality and consequences in selection for high egg production. *Poultry Science* **59:** 2165-2178

Gavora, J.S., U. Kuhnlein and J.L. Spencer (1989), Absence of endogenous viral genes in an inbred line of Leghorn chickens selected for high egg production and Marek's disease resistance. *Journal of Animal Breeding and Genetics* **106:** 217-224

Gavora, J.S., U. Kuhnlein, L.B. Crittenden, J.L. Spencer and M.P. Sabour (1991), Endogenous viral genes: association with reduced egg production rate and egg size in White Leghorns, *Poultry Science* **70:** 618-623

Georges, M., D. Nielson, M. Mackinnon, A. Mishra, R. Okimoto, A.T. Pasquino, L.S. Sargeant, A. Sorensen, M.R. Steele, X. Zhao, J.E. Womack and H. Hoeschele (1995), Mapping quantitative trait loci controlling milk production in dairy cattle by exploiting progeny testing. *Genetics* **139:** 907-920

Gesemann, M., A.J. Denzer and M.A. Ruegg (1995), Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. *Journal of Cell Biology* **128:** 625-636

Goodbourn, S.E.Y., D.R. Higgs, J.B. Clegg and D.J. Weatherall (1983), Molecular basis of length polymorphism in the human xi-globin gene complex. *Proceedings of National Academy of Science in U.S.A.* **80:** 5022-5026

Gowe, R.S. and R.W. Fairfull (1984), Effect of selection for part record number of eggs from bousing vs. selection for hen-day rate of production from age at first egg. *Annales Agriculturae Fenniae* **23:** 196-203

Gowe, R.S., A. Robertson and B.D.H. Latter (1959a), Environment and poultry breeding problems, 5. The design of poultry control strains. *Poultry Science* **38:** 462-471

Gowe, R.S., A.S. Johnson, J.H. Downs, R. Gibson, W.F. Mountain, J.H. Strain and B.F. Tinney (1959b), Environment and poultry breeding problems, 4. The value of a random-bred control strain in a selection study. *Poultry Science* **38:** 443-462

Gowe, R.S., R.W. Fairfull, I. McMillan and G.S. Schmidt (1993), A strategy for maintaining high fertility and hatchability in a multiple-trait egg stock selection program. *Poultry Science* **72:** 1433-1448

Gray, R.M., J.L. Omdahl, J.G. Ghazarin and H.F. DeLuca (1972), 25-hydroxycholecalciferol-1α-hydroxylase subcellular location and properties. *Journal of Biological Chemistry* **247:** 7528-7532

Grunder, A.A., K.G. Hollands and C.P.W. Tsang (1983), Plasma estrogen, calcium and shell quality in two strains of White Leghorns. *Poultry Science* **62:** 1294-1296

Grunder, A.A., T.K. Jeffers, J.L. Spencer, A. Robertson and G.W. Speckmann (1972), Resistance of strains of chickens to Marek's disease. *Canadian Journal of Animals Science* **52:** 1-10

Guillemot, F., J.F. Kaufman, K. Skjoedt and C. Auffray (1989), The major histocompatibility complex in the chicken. *Trends in Genetics* **5:** 300-304

Guyton, A.C. (1986), *Textbook of Medical Physiology*, 7th edition, W.B. Saunders Company, West Washington Square, Philadelphia, PA 19105

Gyllensten, U., D. Wharton, A. Josefsson and A.C. Wilson (1991), Parental inheritance of mitochondrial DNA in mice. *Nature* **352:** 255-257

Haley, C.S., S.A. Knott and J.M. Edson (1994), Mapping quantitative trait loci in crosses between outbred lines using least squares. *Genetics* **136:** 1195-1207

Hanson, R.W. and L. Reshef (1997), Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annual Review of Biochemistry* **66:** 581-611

Hanson, R.W. and Y.M. Patel (1994), Phosphoenolpyruvate carboxykinase (GTP): The gene and the enzyme. *Advances in Enzymology* **69:** 203-281

Hartmann, W. (1991), Genetic aspects of resistance to avian leucosis: An overview. *Informacion Tecnica Economica Agraria* **87A(2-3):** 171-189

Hartmann, W., D. v.d. Hagen and G. Heil (1984), Genetic resistance to Leukosis virus infection: effects on mortality and egg production in Leghorn hens. *British Poultry Science* **25:** 43-52

Hepkema, B.G., J.J. Blankert, G.A. Albers, M.G. Tilanus, E. Egberts, A.J. van der Zijpp and E.J. Hensen (1993), Mapping of susceptibility to Marek's disease within the major histocompatibility (B) complex by refined typing of White Leghorn chickens. *Animal Genetics* **24:** 283-287

Hill, W.G. (1974), Estimation of linkage disequilibrium in randomly mating populations. *Journal of Heredity* **33:** 229-239

Hillel, J., E.A. Dunnington and P.B. Siegel (1992), DNA markers in poultry breeding and genetic analysis. *Poultry Science Review* **4:** 169-186

Hoe, C.M., and J.S. Wilkinson (1973), Liver function: a review. *Australian Veterinary Journal* **49:** 163-169

Hu, J., N. Bumstead, D. Burke, F.A. Ponce de Leon, E. Skamens, P. Gros and D. Malo (1995), Genetic and physical mapping of the natural resistance-associated macrophage protein 1 (NRAMP1) in chicken. *Mammalian Genome* **6:** 809-815

Hu, J., N. Bumstead, P. Barrow, G. Sebastiani, L. Olien, K. Morgan and D. Malo (1997), Resistance to salmonellosis in the chicken is linked to NRAMP1 and TNC. *Genome Research* **7:** 693-704

Huang, S., S. Apasov, M. Koshiba and M. Sitkovsky (1997), Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. *Blood* **90:** 1600-1610

Hutt, F.B. (1936), *Genetics of the Fowl*, Vol. VI, Neue Forschungen in Tierzucht und Abstammungslebre (Duerst Festschrif), pp105-112

Hutt, F.B. and R.K. Cole (1947), Genetic control of lymphomatosis in the fowl. *Science* **106:** 379-384

Hutt, F.B. and R.K. Cole (1953), The interaction of genetic and enviromental influences affecting the incidence of Avian Leucosis. *Science* **117:** 659-697

Jeffreys, A. J., and D. B. Morton (1987), DNA fingerprints in dogs and cats. *Animal Genetics* **18:** 1-15

Jeffreys, A.J., J.F.Y. Brookfield and R. Semeonoff (1985a), Positive identification of an immigration test-case using human DNA fingerprints. *Nature* **317:** 818-819

Jeffreys, A.J., V. Wilson and S.L. Thein (1985b), Individual-specific 'fingerprints' of human DNA. *Nature* **316:** 76-79

Jeffreys, A.J., V. Wilson and S.L. Thein (1985c), Hypervariable 'minisatellite' region in human DNA. *Nature* **314:** 67-73

Kean, R.P., W.E. Briles, A. Cahaner, A.E. Freeman and S.J. Lamont (1994), Differences in major histocompatibility complex frequencies after multitrait, divergent selection for immunocompetence. *Poultry Science* **73:** 7-17

Kim, C.D., S.J. Lamont and M.F. Rothschild (1989), Associations of major histocompatibility complex haplotypes with body weight and egg production traits in S1 White Leghorn chickens. *Poultry Science* **68:** 464-469

Kimura, M. (1955), Solution of a process of random genetic drift with a continuous model. *Proceedings of the National Academy of Sciences in U.S.A.* **41:**144-150

Klein, S., D.R. Morrice, H. Sang, L.B. Crittenden and D.W. Burt (1996), Genetic and physical mapping of the chicken IGF-1 gene to chromosome 1 and conservation of synteny with other vertebrate genome. *Journal of Heredity* **87:** 10-14

Knutson, J.C. and H.F. DeLuca (1974), 25-Hydroxy-vitaminD$_3$-24 hydroxylase, subcellular location and properties. *Biochemistry* **13:** 1543-1548

Kottaridis, S.D. (1969), Marek's disease: a review. *World's Poultry Science Journal* **25:** 35-45

Kruglyak, L. (1997), The use of a genetic map of biallelic markers in linkage studies. *Nature Genetics* **17:** 21-24

Kuhnlein, U. and D. Zadworny (1994), Disease resistance genetics: selection at the DNA level. In: *Proceedings of the 5th World Congress of Genetics Applied to Lifestock Production*. Guelph, Ontario, Canada. **21:** 249-256

Kuhnlein U., D. Zadworny, J.S. Gavora and R.W. Fairfull (1991), Identification of markers associated with quantitative trait loci in chickens by DNA fingerprinting. In: *DNA Fingerprinting , Approaches and Applications*; T. Burke, G. Dolff, A.J. Jeffreys & R. Wolff, eds. Birkhauser Verlag, Basel. pp274-282

Kuhnlein, U., J.S. Gavora, J.L. Spencer, D.E. Bernon and M. Sabour (1989), Incidence of endogenous viral genes in two strains of White Leghorn chickens selected for egg production and susceptibility or resistance to Marek's disease. *Theoretical and Applied Genetics* **77:** 26-32

Kuhnlein, U., L. Ni, S. Weigend, J.S. Gavora, W. Fairfull and D. Zadworny (1997), DNA Polymorphisms in the chicken growth hormone gene: response to selection for disease resistance and association with egg production. *Animal Genetics* **28:** 116-23

Kuhnlein, U., M. Sabour, J.S. Gavora, R.W. Fairfull and D.E. Bernon (1988), Influence of selection for egg production and Marek's disease resistance on the incidence of endogenous viral genes in White Leghorns. *Poultry Science* **68:** 1161-1167

Kuhnlein, U., R.W. Fairfull, R. Gowe, A. Kulenkamp, L. Mou and D. Zadworny (1993), Synergism between the endogenous viral loci ev6 and ev9 in inducing immunological tolerance to avian leukosis virus. *British Poultry Science* **34:** 93-104

Lamont, S.J., N. Lakshmanan, Y. Plotsky, M.G. Kaiser, M. Kuhn, J.A. Arthur, N.J. Beck and N.P. O'Sullivan (1996), Genetic markers linked to quantitative traits in poultry. *Animal Genetics* **27:** 1-8

Lamont, S.J., Y.H. Hou, B.M. Young and A.W. Nordskog (1987), Research note: differences in major histocompatibility comple gene frequencies associated with feed efficiency and laying performance. *Poultry Science* **66:** 1064-1066

Lande, R. and R. Thompson (1989), Efficiency of Marker-assisted selection in the improvement of quantitative traits. *Genetics* **124:** 743-756

Lander, E.S. and D. Botstein (1989), Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics* **121:** 185-199

Lander, E.S. and N.J. Schork (1994), Genetic dissection of complex traits. *Science* **265:** 2037-2048

Levin, I., L. Santangelo, H. Cheng, L.B. Crittenden and J.B. Dodgson (1994), An autosomal genetic linkage map of the chicken. *Journal of Heredity* **85:** 79-85

Levin, I., L.B. Crittenden and J.B. Dodgson (1993), Genetic map of the chicken Z chromosome using random amplified polymorphic DNA (RAPD) markers. *Genomics* **16:** 224-230

Li, S., N. Liu, D. Zadworny and U. Kuhnlein (1998), Genetic variability in White Leghorns revealed by chicken liver expressed sequence tags. *Poultry Science* **77:** 134-139

Lynch, M. and B. Walsh (1998), *Genetics and Analysis of Quantitative Traits*, Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts

Maxam, A.M. and W. Gilbert (1977), A new method for sequencing DNA, *Proceedings of the National Academy of Sciences in U.S.A.* **74:** 560-564

McCouch, S.R. and R.W. Doerge (1995), QTL mapping in rice. *Trends in Genetics* **11:** 482-487

Merriwether, D.A., A.G. Clark, S.W. Ballinger, T.G. Schurr, H. Soodyall, T. Jenkins, S.T. Sherry and D.C. Wallace (1991), The structure of human mitochondrial DNA variation. *Journal of Molecular Evolution* **33:** 543-555

Morton, N.E. (1993), Genetic epidemiology. *Annual Reviews in Genetics* **27:** 523-538

Mullis, K.B. (1990), The unusual origin of the polymerase chain reaction. *Scienctific American* **262:** 56-61

Mullis, K.B. and F.A. Faloona (1987), Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology* **155:** 335-350

Neu-Yilik, G., H. Zorbas, T. R. Gloe, H. M. Raabe, T. A. Hopp-Christensen, and P. K Muller (1993), Vigilin is a cytoplasmic protein: A study on its expression in primary cells and in established cell lines of different species. *European Journal of Biochemistry* **213:** 727-736

Nordlie, R.C. and H.A. Lardie (1963), Mammalian liver P-enolpyruvate carboxykinase. *Journal of Biological Chemistry* **238:** 2259-2263

O'Malley, B.W., M.A. Kirscher and C.W. Barden (1968), *Proeedings of the Society for Experimental Biology and Medicine* **127:** 521

Paterson, A.H., E.S. Lander, J.D. Hewitt, S. Peterson, S.E. Lincoln, and S.D. Tanksley (1988), Resolution of quantitative traits into Mendelian factors by using a

complete linkage map of restriction fragment length polymorphisms. *Nature* **335:** 721-726

Pinard, M.H. and J.L. (1994), Effects of sex-linked dwarf gene and of major histocompatibility complex (MHC) types on immune response in White Leghorn chickens. *Proceedings of the 5th World Congress in Genetics and Applied Liverstock Production* **20:** 238-241

Plenz, G., Y. Gan, H.M Raabe and P.K. Muller (1993), Expression of vigilin in chicken cartilage and bone. *Cell and Tissue Research* **273:** 381-389

Reed, K.C. and D.A. Mann (1985), Rapid transfer of DNA from agarose gels to Nylon membranes. *Nucleic Acids Research* **13:** 7207-7221

Reshef, L., F.J. Ballard and R.W. Hanson (1970), A possible role for glyceroneogenesis in rat adipose tissue. *Journal of Biological Chemistry* **271:** 8068-8074

Risch, N. and K. Merikangas (1996). The future of genetic studies of complex human diseases. *Science* **273:** 1516-1517

Rothschild, M., C. Jacobson, D. Vaske, C. Tuggle, L. Wang, T. Short, G. Eckhardt, S. Sasaki, A. Vicent, D. McLaren, O. Southwood, H. van der Steen, A. Mileham and G. Plastow (1996), The estrogen receptor locus is associated with a major gene influencing litter size in pigs. *Proceedings of the National Academy of Sciences in U.S.A.* **93:** 201-205

Ruyter-Spira, C.P., R.P. Crooijmans, R.J. Dijkhof, P.A. van Oers, J.A. Strijk, J.J. van der Poel and M.A. Groenen (1996), Development and mapping of polymorhpic microsatellite markers derived from a chicken brain cDNA library. *Animal Genetics* **27:** 229-234

Ruyter-Spira, C.P., Z.L. Gu, J.J. van der Poel and M.A. Groenen (1997), Bulked segregant analysis using microsatellites: mapping of the Dominant White locus in the chicken. *Poultry Science* **76:** 386-391

Sabour, M.P., J.R. Chambers, A.A. Grunder, U. Kuhnlein and J.S. Gavora (1992), Endogenous viral gene distribution in populations of meat-type chickens. *Poultry Science* **71:** 1259-1270

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis and H.A. Erlich (1988), Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487-491

Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich and N. Arnheim (1985), Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230:** 1350-1354

Sambrook, J., E.F. Fritsch and T. Maniatis (1989), *Molecular Cloning*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.

Sanger, F., S. Nicklen and A.R. Coulson (1977), DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences in U.S.A.* **74:** 5463-5467

Saxton, J.A., M.C. Boon and J. Furth (1944), Observations on the inhibition of development of spontaneous leukemia in mice by underfeeding. *Cancer Research* **4:** 401-409

Schmidt, C., B. Henkel, E. Poschl, H. Zorbas, W. G. Purschke, T. R. Gloe, and P. K. Muller (1992), Complete cDNA sequence of chicken vigilin, a novel protein with amplified and evolutionary conserved domains. *European Journal of Biochemistry* **206:** 625-634

Schraml, P., R. Shipman, M. Colombi, and C.U. Ludwig (1994), Identification of genes differentially expressed in normal lung and non-small cell lung carcinoma tissue. *Cancer Research* **54:** 5236-5240

Schulman, N., M. Tuiskula-Haavisto, L. Siitonen and E.A. mantysaari (1994), Genetic variation of residual feed consumption in a selected finnish egg-layer population. *Poultry Science* **73:** 1479-1484

Shaw, E.M., R.N. Shoffner, D.N. Foster and K.S. Guise (1991), Mapping of the growth hormone gene by in situ hybridiztion to chicken chromosome 1. *Journal of Heredity* **82:** 505-508

Shen, C.S. and S.P. Mistry (1978), Development of gluconeogenic, glycolytic and pentose-shunt enzymes in chicken kidney. *Poultry Science* **58:** 663-667

Smith, E.J., H.H. Cheng and R.L. Vallejo (1996), Mapping functional chicken genes: an alternative approach. *Poultry Science* **75:** 642-647

Smith, E.J., L.A. Lyons, H.H. Cheng and S.P. Suchyta (1997), Comparative mapping of the chicken genome using the East Lansing reference population. *Poultry Science* **76:** 743-747

Soares, J.H. Jr (1984), Calcium metabolism and its control - A review. *Poultry Science* **63:** 2075-2083

Soares, J.H. Jr, M.A. Ottinge, E.G. Buss and Jr. D.M. Kaetzel (1980), Plasma concentration of 1,25-$(OH)_2D_3$, estradiol and calcium in laying hens selected for differential egg shell quality. *Poultry Science* **58:** 1662 (Abstract)

Southern, E.M. (1975), Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98:** 503-517

Spike, C.A., N. Bumstead, L.B. Crittenden and S.J. Lamont (1996), RFLP mapping of expressed sequence tags in the chickens. *Journal of Heredity* **87:** 6-9

Sved, J. and A. Bird (1990), The expected equilibrium of the CpG dinucleotide in vertebrate genomes under a mutation model. *Proceedings of the National Academy of Sciences in U.S.A.* **87:** 4692-4696

Tanksley, S.D. (1993), Mapping polygenes. *Annual Review of Genetics* **27:** 205-233

Tayek, J.A. and J. Katz (1997), Glucose production, recycling, Cori cycle, and gluconeogenesis in humans: relationship to serum cortisol. *American Journal of Physiology* **272:** 476-484

Utter, M.F. and K. Kurahashi (1953), Mechanism of action of oxalacetate carboxylase. *Journal of American Society* **75:** 785-787

Vallejo, R.L., L.D. Bacon, H-C Liu, R.L. Witter, M.A.M. Groenen, J. Hillel and H.H. Cheng (1998), Genetic mapping of quantitative trait loci affecting susceptibility to Marek's disease virus induced tumors in F_2 intercross chickens. *Genetics* **148:** 349-360

Vallejo, R.L., L.D. Bacon, R.L. Witter and H.H. Cheng (1996), Genetic mapping of quantitative trait loci affecting susceptibility to Marek's disease in the chicken. In: *Current Research in Marek's Disease. Proceedings of the 5th International Meeting on Marek's Disease* (ed. by R.F. Silva, H.H. Cheng, L.F. Lee and L.F. Velicer. American Association of Avian Pathologists, pp8-14

Van der ploeg, L.H. and R.A. Flavell (1980), DNA methylation in the human gamma delta beta-globin locus in erythroid and nonerythroid tissues. *Cell* **19:** 947-958

Vassart, G., M. Georges, R. Monsieur, H. Brocas, A.S. Lequarre and D. Christophe (1987), A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. *Science* **235:** 683-684

Wallace, D.C. (1992), Diseases of the mitochondrial DNA. *Annual Reviews in Biochemistry* **61:** 1175-1212

Weise, T.J., D.O. Lambeth and P.D. Ray (1991), The intracellular distribution and activities of PEPCK isozymes in the various tissues of several mammals and birds. *Comparative Biochemistry and Physiology-Part B, Biochemistry and Molecular Biology* **100:** 297-302

Weldon, S.L., A. Rando, A.S. Matathias, Y. Hod, P.A. Kalonick, S. Savon, J.S. Cook and R.W. Hanson (1990), Mitochondrial phospho-enolpyruvate carboxykinase from the chicken: Comparison of the cDNA and protein sequences with the cytosolic isozyme. *Journal of Biological Chemistry* **265:** 7308-7317

White, J. and H.B. Andervot (1943), Effect of diet relatively low in cysteine on the production of spontaneous mammary-gland tumors in C3H female mice. *Journal of the National Cancer Institute* **3:** 449-451

Whittaker, P.A. and S.M. Danks (1978), *Mitochondria: Structure, Function and Assembly.* New York, Longman Inc.

Wu, G. (1996), Effects of concanavalin A and phorbol myristate acetate on glutamine metabolism and proliferation of porcine intestinal intraepithelial lymphocytes. *Comparative Biochemistry and Physiology* **114:** 363-368

Yao, J., D. Zadworny, U. Kuhnlein, S.E. Aggrey and J.F. Hayes (1996a), A MapI polymorphism in the bovine ornithine decarboxylase gene and its possible association with selection for milk production in Holstein bulls. *Animal Genetics* **27:** 283-284

Yao, J., S.E. Aggrey, D. Zadworny, J.F. Hayes and U. Kuhnlein (1996b), Sequence variations in the bovine growth hormone gene characterized by single-strand conformation polymorphism (SSCP) analysis and their association with milk production traits in Holsteins. *Genetics* **144:** 1809-1816

Yoshida, A. (1992), Molecular genetics of human aldehyde dehydrogenase. *Pharmacogenetics* **2:** 139-147

Zar, J.H. (1984), *Biostatistical Analysis*, 2nd edition, Prentice Hall, Englewood Cliffs, N.J.

Zeng, Z.-B. (1994), Precision mapping of quantitatitive trait loci. *Genetics* **136:** 1437-1468

Appendix I. A list of assigned GenBank accession numbers for the chicken liver expressed sequence tags (CLESTs) which have been submitted to the database (dbEST)

User ID	Clone #	dbEST ID	Accession #	User ID	Clone #	dbEST ID	Accession #
EST001	CLEST004	574596	W66528	EST023	CLEST035	574618	W66560
EST002	CLEST011	574597	W66529	EST024	CLEST038	574619	W66561
EST003	CLEST023	574598	W66530	EST025	CLEST043	574620	W66562
EST004	CLEST029	574599	W66531	EST026	CLEST048	574621	W66563
EST005	CLEST030	574600	W66532	EST027	CLEST048	574622	W66564
EST006	CLEST033	574601	W66533	EST028	CLEST055	574623	W66565
EST007	CLEST044	574602	W66534	EST030	CLEST058	574624	W66566
EST008	CLEST052	574603	W66535	EST031	CLEST072	574625	W66567
EST009	CLEST053	574604	W66536	EST032	CLEST077	574626	W66568
EST010	CLEST059	574605	W66547	EST033	CLEST080	574627	W66569
EST011	CLEST063	574606	W66548	EST034	CLEST065	574628	W66570
EST012	CLEST073	574607	W66549	EST035	CLEST025	574629	W66571
EST013	CLEST074	574608	W66550	EST036	CLEST086	574630	W66572
EST014	CLEST081	574609	W66551	EST037	CLEST096	574631	W66573
EST015	CLEST099	574610	W66552	EST038	CLEST104	574632	W66574
EST016	CLEST103	574611	W66553	EST039	CLEST112	574633	W66575
EST017	CLEST105	574612	W66554	EST040	CLEST005	574634	W66576
EST018	CLEST111	574613	W66555	EST041	CLEST005	574635	W66577
EST019	CLEST111	574614	W66556	EST042	CLEST006	574636	W66578
EST020	CLEST014	574615	W66557	EST043	CLEST007	574637	W66579
EST021	CLEST018	574616	W66558	EST044	CLEST013	574638	W66580
EST022	CLEST102	574617	W66559	EST045	CLEST013	574639	W66581

(Continued)

User ID	Clone #	dbEST ID	Accession #	User ID	Clone #	dbEST ID	Accession #
EST046	CLEST024	574640	W66582	EST066	CLEST003	574660	W66508
EST047	CLEST024	574641	W66583	EST067	CLEST031	574661	W66509
EST048	CLEST039	574642	W66584	EST068	CLEST036	574662	W66510
EST049	CLEST039	574643	W66585	EST069	CLEST042	574663	W66511
EST050	CLEST045	574644	W66586	EST070	CLEST042	574664	W66512
EST051	CLEST045	574645	W66587	EST071	CLEST042	574665	W66513
EST052	CLEST061	574646	W66588	EST072	CLEST098	574666	W66514
EST053	CLEST061	574647	W66589	EST073	CLEST020	574667	W66515
EST054	CLEST075	574648	W66590	EST074	CLEST020	574668	W66516
EST055	CLEST075	574649	W66591	EST075	CLEST020	574669	W66517
EST056	CLEST078	574650	W66537	EST076	CLEST020	574670	W66518
EST057	CLEST078	574651	W66538	EST077	CLEST047	574671	W66519
EST058	CLEST090	574652	W66539	EST078	CLEST049	574672	W66520
EST059	CLEST090	574653	W66540	EST079	CLEST049	574673	W66521
EST060	CLEST095	574654	W66541	EST080	CLEST066	574674	W66522
EST061	CLEST101	574655	W66542	EST081	CLEST085	574675	W66523
EST062	CLEST108	574656	W66543	EST082	CLEST085	574676	W66524
EST063	CLEST001	574657	W66544	EST083	CLEST085	574677	W66525
EST064	CLEST001	574658	W66545	EST084	CLEST021	574678	W66526
EST065	CLEST041	574659	W66546	EST085	CLEST021	574679	W66527

Appendix II. Laboratory Techniques

A2.1 Preparation of chicken liver cDNA clones

Chicken liver cDNA library: The chicken liver cDNA library was commercially available from biological company (Strategene). Individuals are selected from the males of broiler-breeders, which are at about 7 weeks old. Messenger RNA was isolated with oligo-dT methods. The cDNA clone are packaged in Uni-ZAP XR lambda vectors at *EcoR*I polycloning site. In order to facilitate DNA isolation and preparation, DNA clones in the lambda-ZAP vectors are *in vivo* excised to become pBluescript DNA clones.

Titers: XL1-Blue cells was prepared in LB liquid medium containing 0.2% maltose and 10 mM $MgSO_4$ and incubated in a waterbath at 37°C with shaking overnight until $OD_{600} = 1.0$. One ul of an aliquot from the chicken liver cDNA library was re-suspended in 200 µl of SM buffer and further diluted at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} times, respectively. Five µl of each dilution was then mixed with 3 ml LB top agar, which was containing 0.7% agarose and prewarmed to 42°C, and poured onto a LB agar plate. The concentration of the library therefore can be estimated by scoring the plaques in the plates of different dilutions.

In vivo excision: A mixture of 200 µl XL1-Blue cells with $OD_{600} = 1.0$, 200 µl Uni-ZAP XR phage stock containing more than 1×10^5 phage particles and 1 µl R408 helper phage containing more than 1×10^6 pfu/ml was prepared in a 15-ml conical tube. Then, 5 ml 2 × YT liquid media was added and incubated in waterbath at 37°C with constant shaking for 3 hours. Subsequently, the tube was heated at 70°C for 20 minutes and centrifuged at 4,000 g for 5 minutes. The supernatant was transferred to another sterile tube. This stock contains the pBluescript phagemid packaged as filamentous phage particle and can be stored at 4°C for 1 - 2 months.

Recovery of phagemid DNA clones: An appropriate amount (20 - 200 µl) of phage stock was mixed with 200 µl XL1-Blue host cells in a 15 ml conical tube, and incubated at 37°C for 15 minutes. One to 100 µl of mixture was plated on LB agar plates containing 100 µg/µl ampicillin, and incubated at 37°C overnight. Colonies appearing on the plate contain the pBluescript double stranded phagemid with the cloned DNA insert. Meanwhile, the bacteria infected with helper phage will not grow because they do not contain ampicillin

resistance genes. The bacteria containing pBluescript plasmid can be therefore stored in 15% glycerol and kept at -70°C.

Minipreparation of plasmid DNA: Fifty μl of blood cells was first suspended in 500 μl 1 × SSC and centrifuged at 13,000 × g for 1 minute. Supernatant was removed and the pellet was resuspended in 100 μl of solution I, mixed well with vortexing and kept in ice water for 5 minutes. Then 200 μl of solution II was added to hydrolyse the membranes of the cell and its organelles. The solution was chilled in ice for 5 minutes, then 150 μl of solution III was added to precipitate proteins. The solution was mixed gently and chilled in ice for 5 minutes, then centrifuged at 13,000 × g for 3 minutes. The supernatant was subsequently transferred to another Eppendorf tube, followed by phenol-chloroform (25:24:1 phenol/chloroform/isoamyl alcohol) extraction. Two volume of 100% ethanol was added and the solution was kept at -20°C for at least 30 minutes. Then, the DNA was precipitated by centrifuged at 13,000 × g for 10 minutes. Finally, the DNA was dissolved in a proper amount of TE buffer.

A2.2 Southern blotting assay

DNA preparation: The whole blood sample of chickens was primarily collected into a heparized tube and stored at -70°C. After thawed out, a 60-μl aliquot of the whole blood was mixed with 5 ml 1 × SSC in a 15-ml polypropylene tube and spined down for 10 minutes at 3,000 × g. The supernatant was completely removed. The pellet was re-suspended in 2 ml 0.2 M sodium acetate (pH7.0) and dispersed well by vortexing. Then the cells were partially degraded by adding 100 ul of 20% SDS, gently vortexed and incubated in a waterbath at 50°C for 2 hours. Subsequently, the samples were extracted with 2-ml phenol-chloroform (25:24:1 phenol/chloroform/isoamyl alcohol). The tubes are shaked quite vigorously for 10 minutes and spined down at 1,700 × g for 10 minutes. The upper aqueous phase was removed to another tube, and re-extracted with 2-ml phenol-chloroform. Genomic DNA was precipitated with 2 volumes of 100% ice-cold ethanol. The DNA was then fished out with a glass rod, washed once with 75% ethanol and dried by air for about 30 minutes. DNA was dissolved in TE buffer containing 10 mM Tris.HCl (pH7.5) and 1 mM EDTA (pH8.0). The concentration of DNA was estimated using spectrophotometry

(Beckman, Model DU-20) at a wavelength of 260 nm. Subsequently, DNA samples were stored at -20°C until they were needed.

Restriction digestion: A 30 µl mixture containing 5 µg genomic DNA, 15 units restriction enzyme and 1 × One-Phor-All buffer was incubated at 37°C (65°C for *TaqI*) for 3 hours to overnight according to the recommendations of the supplier (Phamacia). Reactions were placed into a waterbath at 65°C for 10 minutes to inactive the enzymes prior to the addition of 5 µl 6 × gel-loading buffer (15% Ficoll, 0.25% Xylene cyanol and 0.25% Bromophenol blue, w/v).

Electrophoresis: 2.5 g agarose powder (electrophoresis grade, ICN) was melted and dissolved in 250 ml 1 × TPE buffer containing 90 mM Tris-phosphate and 2 mM EDTA (pH8.0) in a microwave oven. Subsequently, the agarose solution was cooled to 60°C and then poured carefully into a casting tray (20 × 15 cm). When the agarose had gelled, the slab gel was placed into the electrophoresis tank (Bio-Rad) supplied with sufficient 1 × TPE buffer to cover the gel to a depth of about 1 - 2 mm. DNA samples digested by restriction enzymes were loaded into the wells of the submerged gel, and electrophoresis was carried out at a voltage of 1.25 V/cm for 20 hours. Completeness of digestion and migration of DNA samples was visualized using ultraviolet light following staining of the gel for 15 minutes in a solution of 0.5-µg/ml ethidium bromide. Excess gel was trimmed to the size of 18 × 15 cm, and then flipped upside down into a tray containing 200 ml of distilled water.

Alkali blotting: The distilled water was replaced with 200 ml 0.25 M HCl. The tray was therefore gently rocked on a shaking platform for 25 minutes, and followed by rinsing with distilled water. A sheet of Zeta-probe membrane was cut to the size of the gel and marked with permanent ink marker in top right corner. Subsequently, the membrane was thoroughly wetted in 400 ml 0.1% SDS preheated to 100°C, and then wash twice with boiling distilled water followed by cold water. Four sheets of 3MM paper were added up on a bridge of tray containing 0.4 M NaOH and served as wicks which were about 5 mm wider than the gel and long enough to reach into the 0.4 M NaOH transfer solution. The tower was then built up subsequently one by one with the gel, the Zeta-probe membrane and 4 sheets of 3MM paper with the size of the gel. The surface of each layer was wetted thoroughly with 0.4 M NaOH and air bubbles were removed with pipet. Finally, 10-cm

stack of paper towels was built up for absorbtion of 0.4 M NaOH buffer from the tray. The whole tower can be properly suppressed with a weight of about 200-g. After sitting at room temperature overnight, the Zeta-probe membrane was carefully removed from the gel, and rinsed with 200 ml solution containing 2 × SSC and 0.1% SDS.

Radioactive labelling of probes: The probes used for southern Blotting were labelled by random primer extension with four nucleotides including alpha-^{32}P-CTP using ^{T7}QuickPrime^{TM} Kit (Pharmacia). A 34-μl solution containing 200 ng DNA was boiled in water for 10 minutes to denature the double stranded DNA clones, followed by chilled on ice for 5 minutes. Ten-μl 5 × reaction was added, mixed well by pipetting. Five-μl alpha-^{32}P-CTP (ICN) and 1 μl DNA polymerase (10 units/μl) was then added. Reactions were kept in a small lead container and incubated at 37°C for 3 hours. Two hundred μl of column buffer containing 10 mM Tris.HCl (pH8.0), 1 mM EDTA (pH8.0) and 100 mM NaCl was used to stop the reaction, and the 250 μl reaction solution was then applied to the Sephadex G-50 column and the instructions of the supplier (Pharmacia) were then followed. The radioactivity of the probe was measured by liquid scintillation counting (LKB, 1209 Rackabeta).

Prehybridization: The membrane was rolled and inserted into the hybridization tube. Fifteen ml prehybridization solution containing 150 mg skim milk powder (Carnation), 6 ml 20 × SSPE, 1.5 ml Herring sperm DNA, 15 ml deionized formamide, 1.5 ml 20% SDS and 6 ml distilled water was added. With the cap closed tightly, the tube was then placed in the rotisserie at 42°C for 2 hours to overnight.

Hybridization: The radioactive probe was first denatured by boiling for 10 minutes and chilled on ice. And then the probe was added to the hybridization buffer, which was containing 6 ml 20 × SSPE, 7.5 ml 40% Dextransulfate, 15 ml deionized formamide and 1.5 ml 20% SDS and prewarmed to 80°C. The prehybridization buffer was removed and replaced with the hybridization buffer. The hybridization tube was then incubated at 42°C overnight.

Washes and autoradiography: After incubation, the hybridization tube was rinsed with 50 ml solution containing 2 × SSC and 0.1% SDS. Another 50 ml of the same solution was then replaced and the tube was incubated at 42°C for 15 minutes. Subsequently, the

tube was washed in solutions containing 0.5 × SSC and 0.1% SDS, 0.1 × SSC and 0.1% SDS, respectively, following the same procedure. Once again, the membrane was washed at 52°C with 0.1 × SSC and 0.1% SDS for 30 minutes. The membrane was then taken out from the tube, and blotted dry on 3MM paper and wrapped in plastic wrap. Autoradiography was carried out by exposure to Kodak XAR-5 films for 16 hours at -70°C with two screens. The efficiency of the wash steps was monitored using a Geiger counter. In order to minimize background additional washes were performed until background radioactivity was equal or less to 200 cpm.

X-ray film was developed manually as following: the film was bathed in X-ray developer (Kodak) for 5 minutes, rinsed with deionized water for 1 minute, bathed in rapid fixer for 5 minutes, and then washed in running water for 15 minutes. The temperature of all solutions were 18 - 20°C. The autoradiography was air-dried.

Rehybridization: The membrane was suitable for repeated rehybridizations with the same or different probes. The probes can be easily removed from the membrane by boiling in the solution containing 0.1 × SSC and 0.1% SDS for 1 hour. Complete removal of the probe was monitored by Geiger counting and/or autoradiography. Subsequently, the membrane was stored at 4°C until required for rehybridization.

A2.3 Polymorase chain reaction (PCR)

Twenty-five ul reactions were prepared using 25 units of T*th* polymerase, 200 ng chicken genomic DNA, 20 μM each of primers, 125 μM each of dATP, dCTP, dGTP and dTTP (Pharmacia) in 1 × buffer, which consists of 10 mM Tris.HCl (pH8.3), 50 mM KCl, 20 mM $MgCl_2$ and 0.01% gelatin. After the tubes containing the reactions were placed in the rack of the Thermomachine, one drop of mineral oil was applied to each tube to prevent evaporation. The reaction was denatured at 95°C for 10 minutes prior to start of cycles. A total of 40 cycles of the reaction were carried out. Each cycle consists of denaturation of template at 94°C for 1 minute, annealing of primers to the templates at 59°C for 80 seconds and extension of primers at 72°C for 90 seconds. The PCR product was analyzed by either polyacrylamide gel (PAG) or agarose gel electrophoresis using DNA molecular weight marker.

A2.4 DNA sequencing

The cDNA clones were packaged in the vectors which had been designed ready for DNA sequencing with Sanger chain termination method using ThermalBase^{TM} sequencing kit (Strategene) (Sanger *et al.*, 1977). One of the two primers, the M13 reverse primer of sequence 5'-AACAGCTATGACCATG-3' and the T_7 primer of sequence 5'-AATACGACTCACTATAG-3' were used in the DNA sequencing reactions. Sequencing reactions were carried out according to the instruction of supplier (Strategene).

A2.5 Single-stranded conformation polymorphism (SSCP) assay

A 0.5-μl PCR product was added in 10 μl of loading buffer containing 95% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue, and boiled for 10 minutes. Polyacrylamide gel electrophoresis (PAGE) was carried out in 12% PAG with 1 × TBE at 100 V for 8 hours. Then the gel was stained with silver staining method according to Maniatis' book (Sambrook *et al.*, 1989).