Systems-wide Chicken DNA Microarrays, Gene Expression Profiling, and Discovery of Functional Genes

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ABSTRACT The goal of our current consortium project is to launch a new era—functional genomics of poultry—by providing genomic resources [expressed sequence tags (EST) and DNA microarrays] and by examining global gene expression in target tissues of chickens. DNA microarray analysis has been a fruitful strategy for the identification of functional genes in several model organisms (i.e., human, rodents, fruit fly, etc.). We have constructed and normalized five tissue-specific or multiple-tissue chicken cDNA libraries [liver, fat, breast, and leg muscle/epiphyseal growth plate, pituitary/hypothalamus/pineal, and reproductive tract (oviduct/ovary/testes)] for high-throughput DNA sequencing of EST. DNA sequence clustering was used to build contigs of overlapping sequence and to identify unique, non-redundant EST clones (unigenes), which permitted printing of systems-wide chicken DNA microarrays. One of the most promising genetic resources for gene exploration and functional gene mapping is provided by two sets of experimental lines of broiler-type chickens developed at INRA, France, by divergent selection for extremes in growth traits (fast-growing versus slow-growing; fatness versus leanness at a similar growth rate). We are using DNA microarrays for global gene expression profiling to identify candidate genes and to map growth, metabolic, and regulatory pathways that control important production traits. Candidate genes will be used for functional gene mapping and QTL analysis of F_2 progeny from intercrosses made between divergent genetic lines (fat × lean lines; fast-growing × slow-growing lines). Using our first chicken liver microarray, we have already identified several interesting differentially expressed genes in commercial broilers and in divergently selected broiler lines. Many of these candidate genes are involved in the lipogenic pathway and are controlled in part by the thyrotropic axis. Thus, genome-wide transcriptional profiling is a powerful tool used to visualize the cascade of genetic circuits that govern complex biological responses. Global gene expression profiling and QTL scans should enable us to functionally map the genetic pathways that control growth, development, and metabolism of chickens. This emerging technology will have broad applications for poultry breeding programs (i.e., use of molecular markers) and for future production systems (i.e., the health and welfare of birds and the quality of poultry products).

(*Key words*: chicken gene index, expressed sequence tag (EST), DNA microarray, functional genomics, gene expression profiling)

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INTRODUCTION

The genetic mechanisms that govern differentiation, growth, and function of metabolic, somatic, and reproductive systems in the chicken are largely unknown. Genome-scale cDNA sequencing and DNA microarray technology, initiated by the Human Genome Project, have enabled exploration and discovery of thousands of genes in several model organisms (i.e., human, mouse, zebra fish, fruit fly, and yeast). The greatest obstacle to implementing similar functional genomics studies in chickens has been the lack of a comprehensive catalog of tissue-specific gene sequences [i.e., expressed sequence tags

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Abbreviation Key: BBSRC = Biotechnology and Biological Sciences Research Council; *C/EBPα* = CCAAT/enhancer binding protein-α; CAP3 = cluster assembly program 3; CFAR = Centre for Food and Animal Research; DBI = Delaware Biotechnology Institute; EST = expressed sequence tags; FGL = fast-growing line; FL = fat line; LL = lean line; M = morgan; PTU = propyl-thiouracil; SGL = slow-growing line; SPARC = secreted protein acidic and rich in cysteine; TIGR = The Institute for Genomic Research; *THIG* = thyroid hormone-inducible gene; *THRG* = thyroid hormone-repressible gene; T_3 = triiodothyronine; UD = University of Delaware.

(EST)] and unavailability of high-density chicken DNA microarrays for gene expression profiling. In response to this international need and through USDA-IFAFS support, we initiated a functional genomics project in 2000 that is focused on development of DNA microarrays from chicken EST, global gene expression profiling, and functional mapping of growth regulating genes in the broiler chicken. The most promising genetic resources for such goals are two populations of broiler chickens divergently selected for extremes in growth rate (Ricard, 1975; Remignon et al., 1994) [fast-growing line (FGL) or slow-growing line, (SGL)] or body composition at a similar growth rate (Leclercq et al., 1980; Leclercq, 1988) [fat line (FL) or lean line (LL)]. In FL and LL chickens, selection for seven generations has resulted in a fourfold difference in abdominal fat content at 9 wk of age. Although the FGL and SGL were originally selected for high or low body weight (at 8 and 32 wk of age), the FGL has 18-fold higher abdominal fat content than the SGL at 9 wk of age.

The original objectives of our consortium project are 1) to construct and normalize broiler chicken cDNA libraries for liver, fat, skeletal muscle, and pituitary/hypothalamus tissue for high-throughput DNA sequencing, 2) to sequence about 5,000 clones from each cDNA library and print tissue-specific cDNA microarrays of approximately 3,000 unique clones/tissue cDNA library, 3) to use DNA microarrays to analyze global gene expression in liver, fat, skeletal muscle, and pituitary/hypothalamus of chickens from these divergent genetic models, and 4) to use global gene expression data to map functional genes in metabolic pathways related to several production traits. In addition, candidate genes (or clusters of genes) identified in key regulatory pathways will be included in QTL analysis of two unique F_2 resource populations (FL $\times$ LL cross; FGL $\times$ SGL cross). In the present paper, we review our approach and progress on this multidisciplinary consortium project on functional genomics of the chicken.

CONSTRUCTION OF cDNA LIBRARIES AND HIGH-THROUGHPUT SEQUENCING

High-throughput sequencing of tissue-specific cDNA libraries provides the most extensive survey available on the transcribed portion of the genome (i.e., the transcriptome). The EST generated are assembled into a genome-wide non-redundant catalog of expressed genes (i.e., unigenes). The unigene clones are then used to construct DNA microarrays, which are an indispensable tool for functional genomics (Cogburn et al., 2003). Microarray analysis requires careful preparation, normalization (i.e., reduction of very abundant genes), and subtraction (i.e., elimination of redundant clones) of cDNA libraries prior to high-throughput sequencing (de Fatima Bonaldo et al., 1996; Martin and Pardee, 2000). These technical refinements enable detection and sequencing of genes that are expressed at low levels (i.e., tissue-specific genes that control cellular differentiation, growth, and development) (de Fatima Bonaldo et al., 1996).

The chicken cDNA libraries prepared at the University of Delaware (UD; Tables 1 and 2) were deliberately and carefully made by pooling total RNA isolated from tissue samples collected over a wide range of developmental stages (embryonic, pubertal, mature) and distinct genetic backgrounds (commercial and experimental strains). This strategy enabled us to discover unique genes that are expressed at specific developmental stages or genetic background. The liver, abdominal fat, and skeletal muscle RNA pools were made with equal amounts of total RNA from three birds per genetic line [strains 80 or 90 and 21 from the Centre for Food and Animal Research (CFAR), Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada] (Chambers et al., 1989; Sabour et al., 1992) at different ages (1 to 11 wk of age). The growth and endocrine characteristics of these CFAR broiler chicken strains have been described by us earlier (Mao et al., 1998).

The chicken liver cDNA library (*pgl1c*) was constructed from a total RNA pool of d 17 embryos from a commercial broiler cross (20%) and CFAR strains 80 (40%) and 21 (40%). The abdominal fat cDNA library (*pft1c*) was constructed from equal RNA pools from CFAR strains 80 and 21. The skeletal muscle/epiphyseal growth plate cDNA library (*pgm1c*) was constructed from breast (34%) and leg (34%) muscle RNA from CFAR strains 90 and 21 and epiphyseal growth plate RNA from a commercial broiler cross (32%). The neuroendocrine system cDNA library (*pgp1c*) was composed of 40% pituitary, 40% hypothalamic, and 20% pineal total RNA from a commercial broiler cross. The details of RNA pools used to construct these cDNA libraries are available from our website (http://www.chickest.udel.edu/) and our EST entries in GenBank.

To estimate the efficiency of normalization (i.e., the reduction in abundant/redundant clones), we have sequenced one to three 384-well plates of randomly picked clones from each primary library prior to normalization. A list of the 12 most abundant genes sequenced from four primary cDNA libraries is presented in Table 1. This information can be used to generate an electronic Northern blot of the most abundant genes expressed in each single-tissue (or multiple-tissue) library. As expected, albumin is the most abundant gene, representing 13.6% of total clones sequenced, expressed in chicken liver. The most abundant gene expressed in abdominal fat is osteonectin (3.6% of total), which encodes an extracellular matrix protein called SPARC for secreted protein acidic and rich in cysteine. *SPARC* was recently identified as an autocrine/paracrine factor that contributes to development of obesity in mice (Tarte-Deckert et al., 2001). The most abundant genes expressed in skeletal muscle were α-actin (14.1%), GAPDH (9.3%), and cytochrome-C oxidase (7.1% of total). The chicken neuroendocrine system expresses three hormones at high levels: pro-opiomelanocortin (POMC) (5.3%), somatotropin or growth hormone (3.6%), and prolactin (1.3% of total). Some genes (i.e., GAPDH, cytochrome-C oxidase, and β-actin) are highly expressed in most tissues, whereas other genes appear to be tissue specific (i.e., myosin light and heavy chains,

TABLE 1. The 12 most-highly expressed genes in four primary (not normalized) chicken cDNA libraries[1]

Liver library (*pgl1c*)		Fat library (*pft1c*)	
Gene	% of Total	Gene	% of Total
Albumin	13.6	Osteonectin (*SPARC*)	3.6
Ovotransferrin	2.3	Vimentin	1.7
Cytochrome-C oxidase	2.1	β-Actin	1.4
Vitamin-D binding protein	1.9	Ferritin	1.1
Fibrinogen-α	1.8	Fatty acid binding protein	1.1
Hemopexin	1.3	Fatty acid translocase	0.8
Aldolase	1.3	α1-Collagen	0.8
Apolipoprotein-A1	1.2	Class I MHC antigen	0.8
Fetuin	1.2	Polyubiquitin	0.8
Hensin	1.0	Cytochrome-C oxidase	0.8
GAPDH	1.0	Adipophilin	0.6
Vitronectin	1.0	CGI-86 protein	0.6
Muscle library (*pgm1c*)		**Pituitary library (*pgp1c*)**	
α-Actin	14.1	POMC	5.3
GADPH	9.3	Somatotropin	3.6
Cytochrome-C oxidase	7.1	Cytochrome-C oxidase	3.5
Creatine kinase	3.7	Chromogranin A	2.4
Myosin, light-chain	3.3	Cytochrome C reductase	1.4
Elongation factor 1-α	3.2	Prolactin	1.3
Myosin, heavy-chain	2.8	Tubulin-α	1.0
Collagen α-1	2.8	NADH dehydrogenase	0.9
α-Tropomyosin	2.8	GAPDH	0.8
Lactate dehydrogenase	2.4	Tubulin-β	0.8
Ferritin, heavy-chain	2.0	Lipophilin	0.8
Enolase-β	2.0	β-Actin	0.6

[1]The redundancy of expressed sequence tags (EST) was estimated by sequencing of one to three 384-well plates of randomly picked clones from each primary library before the library was normalized (Life Technologies, Inc., Rockville, MD). These values represent the number of clones sequenced for the 12 most abundant genes divided by the total number of clones sequenced from that primary library.

α-tropomyosin and creatine kinase in skeletal muscle, SPARC in abdominal fat). The abundance of these highly expressed genes (Table 1) was dramatically reduced after normalization of each cDNA library.

We have sequenced 30,609 clones (Table 2) from five (primary and normalized) chicken cDNA libraries [liver, abdominal fat, breast and leg muscle/bone growth plate, pituitary/hypothalamus/pineal, and reproductive tract (testis/ovary/oviduct)]. The cDNA clones were sequenced from the 5′-end with an average read of 590 bp. The EST sequences were then entered into GenBank and the original chicken EST database (www.chickest.udel.edu) established by Joan Burnside and Robin Morgan at the Delaware Biotechnology Institute (DBI). Our combined EST sequencing efforts at UD and DBI have provided the avian genomics community and developmental biologists with a large repository of chicken cDNA clones and sequences (almost 41,000 EST).

BIOINFORMATICS AND ASSEMBLY OF A CHICKEN GENE INDEX

Prior to 2001, only a few thousand expressed sequence tags (EST), derived mainly from chicken immune tissue (Tirunagaru et al., 2000; Abdrakhmanov et al., 2000), were available for the chicken. A milestone in chicken genomics was reached in 2001 with the completion of several independent chicken EST sequencing projects [mainly at UD, the University of Hamburg, and the Biotechnology and Biological Sciences Research Council

TABLE 2. Number of expressed sequence tags (EST) sequenced from tissue-specific and multiple-tissue cDNA libraries in the University of Delaware collection[1]

Tissue source	System	EST (n)
Abdominal fat	Metabolic	6,739
Liver	Metabolic	5,541
Breast and leg muscle epiphyseal growth plate	Metabolic/somatic	5,764
Pituitary gland/hypothalamus/pineal gland	Neuroendocrine	8,737
Oviduct/ovary/testis	Reproductive	3,828
Total		30,609

[1]The cost of construction, normalization, and sequencing of two cDNA libraries (liver and fat) was equally shared between a USDA-IFAFS grant to L. A. Cogburn and a USDA-NRI grant to J. Burnside and R. Morgan. These EST sequences were entered into the Chicken EST Database (http://www.chickest.udel.edu/) and GenBank. For a complete list of unigenes within each library group or microarray, please see our website: http://udgenome.ags.udel.edu/~cogburn.

TABLE 3. Chicken gene index compiled from University of Delaware (UD), BBSRC, and public collections[1]

	UD EST	BBSRC EST	All public EST
Total EST	40,935	332,920	407,103
EST in Contigs	28,233	255,523	322,230
Contigs	6,106	31,103	33,941
Singlets	12,702	77,397	84,079
Total nonredundant sequences (unigenes)	18,808	108,500	118,020

[1]The CAP3 sequence assembly program (Huang and Madan, 1999) was used to build contigs of expressed sequence tag (EST) sequences using a 40-base overlap and 90% sequence identity. This assembly was made from chicken EST sequences downloaded from all public databases on November 1, 2002. Within the UD collection, a contig must contain at least two EST from the UD sequences, otherwise the UD EST is included in the singlets group, although a single UD EST could be clustered with a contig in the final assembly of all public EST. BBSRC = the British Biotechnology and Biological Sciences Research Council consortium (Boardman et al., 2002).

(BBSRC)]. A large chicken EST database (http://www.chick.umist.ac.uk/) of 299,506 clones, from high-throughput sequencing of 21 normalized tissue-specific cDNA libraries prepared from layer-type chickens, was released on December 17, 2001, by consortium of British institutions (funded by the UK BBSRC). The BBSRC collection now contains 339,314 EST sequences (Boardman et al., 2002).

We have made two sequence assemblies (Table 3) with the cluster assembly program 3 (CAP3) sequence clustering program (Huang and Madan, 1999) at high stringency (40-bp overlap with 90% sequence identity). The CAP3 program was used by the Institute for Genomic Research (TIGR; http://www.TIGR.org) to assemble EST sequences for the TIGR gene indices for cattle (Smith et al., 2001) and pigs (Fahrenkrug et al., 2002). Incidentally, the number of EST sequences now available for the chicken greatly exceeds that of any other species of domestic animal, including pigs and cattle.

The first assembly was made only on 40,935 EST from the UD collection (Table 2). All EST sequences were rigorously screened with the Cross_match program[2] to remove contaminating vector and bacterial sequences. Low-quality sequences [i.e., those containing >5% uncalled (n) bases] and short sequences (<100 bp) were also removed. A total of 28,233 EST formed 6,106 contigs or high fidelity in silico cDNA, whereas the remaining 12,702 EST were classified as singlets that represent unique non-overlapping sequences. Collectively, the non-redundant (contigs + singlets) sequences (i.e., chicken unigenes) represent 18,808 EST clones in the UD collection alone.

To improve gene identification in our collection, we made a second assembly from all chicken EST sequences (403,913) and mRNA sequences (3,190 in GenBank) found in public databases as of November 1, 2002. The CAP3 assembly of all chicken EST present in public databases (Carre et al., 2002b) shows that 322,230 EST form 33,941 high-fidelity contigs, and 84,079 are singlets, which represent known and unknown EST from Blast searches or the noncoding RNA. This large CAP3 database of contigs and singlets was used to assemble a preliminary Chicken Gene Index (Table 3).

The high-fidelity contigs, built with CAP3, were then used for BlastN and BlastX (Altschul et al., 1997) searches against the GenBank database to identify genes that correspond to the in silico cDNA (Cogburn et al., 2003). The Blast searches for the 33,941 contigs (or in silico cDNA) resulted in the identification of 51% as known genes with a high Blast score (>200) and 13% with a lower score (<200; e-value < $1e^{-10}$), whereas 36% remain as unknown genes. The results for the contigs that contain one or more UD EST are slightly better, because 64% were identified as known genes with a high score (>200) and 19% had a low score (<200), whereas 17% remain unknown (<50). CAP3 clustering has allowed us to reduce the number of non-informative sequences in the UD collection. About 24% of our EST without previous identification are now in contigs with a high Blast score, whereas 17% are in contigs with a low Blast score. Although 33% of our EST are still classified as unknown genes, they are now assembled in high-fidelity contigs, which provides a longer in silico cDNA sequence for future gene identification. About 26% of the unknown EST in the UD collection remain as unknown singlets. Alignment details show that most of the unidentified EST represent either 3′- or 5′-untranslated regions of corresponding genes that have not yet been described for the chicken. For the low Blast score EST, they are usually at the junction of the coding region (5′ or 3′ ends) or in some poorly conserved part of the protein, whereas EST with a high score correspond to the coding region of known genes. Furthermore, the Blast searches conducted with the high-fidelity in silico cDNA result in more accurate gene identification, because of a longer assembled sequence.

Sequence clustering of all chicken EST in public databases with the CAP3 program has provided us with a very powerful and comprehensive database that contains a large volume of useful information for functional genomics in chickens. We have condensed 407,103 chicken EST from public databases into 33,941 high-fidelity in silico cDNA (Table 3). This approximates the

[2]Phil Green, Washington University, http://www.phrap.com.

number of genes (35,000) estimated for the chicken's transcriptome (Boardman et al., 2002), although the large singlets group (84,079 EST) contains a significant number of unclustered EST sequences with high Blast scores of known genes. CAP3 clustering has greatly improved gene identification and selection of tissue-specific unigene sets for printing chicken microarrays. For each contig (in silico cDNA), it will be possible from the origin of all EST within the contig to approximate the tissue distribution (i.e., an electronic Northern blot) for the corresponding gene. The construction of these CAP3 clusters also allows, within the detail of individual EST alignments, more information about interesting genes such as polymorphisms, alternative splicing, related gene families or even the complete in silico cDNA sequence of the gene itself. Furthermore, this large collection of chicken in silico cDNA (contigs) and singlet sequences will be critical for development of genome-wide microarrays and the final assembly of the chicken genome sequence in the near future.

CHICKEN DNA MICROARRAYS AND GENE EXPRESSION PROFILING

Cogburn et al. (2003) have critically reviewed the principles of DNA chip technology and its application for global gene expression profiling in chickens. A strong international interest in chicken immunity and vaccination practices has lead to the earlier creation of an extensive catalog of chicken lymphoid EST from independent high-throughput sequencing projects (Abdrakhmanov et al., 2000; Tirunagaru et al., 2000; Buerstedde et al., 2002). Chicken lymphoid DNA arrays have provided the first opportunities to examine gene expression profiles in normal and challenged lymphoid tissues (Liu et al., 2001; Morgan et al., 2001; Neiman et al., 2001). Toward our goal of developing microarrays for other important chicken tissues, we have recently developed a 3,456 element chicken liver DNA microarray, representing 3,135 unique (non-redundant) genes and a large number of quality control spots, printed on 8 × 12 cm nylon membranes. The liver-specific cDNA microarrays were custom made at DBI with a liver unigene set selected from our annotated chicken EST collection (http://www.chickest.udel.edu). The chicken EST clones were arrayed into 96-well plates for PCR amplification of cDNA inserts. For a complete list of the chicken unigenes printed on the liver array see our website.[3] The original chicken liver cDNA array, printed on nylon membranes, represents a prototype microarray that was used for gene expression profiling in several pilot and validation studies described below.

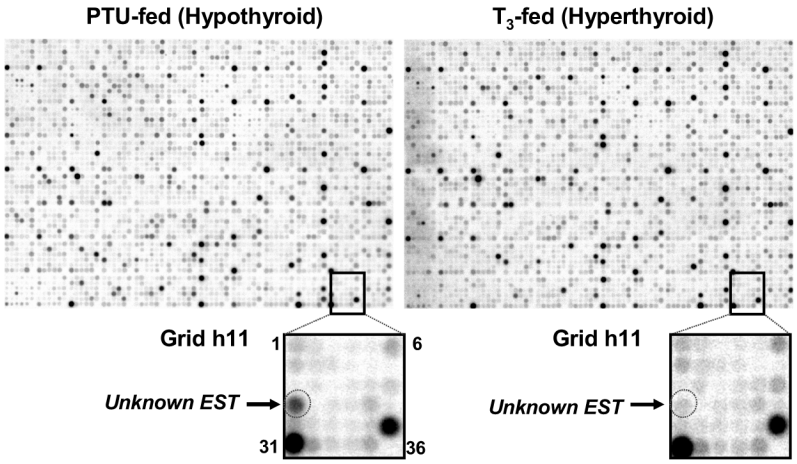
Currently, we are printing two high-density chicken DNA microarrays that represent genes from two major tissue groups (metabolic/somatic and neuroendocrine/reproductive systems). The Chicken Metabolic/Somatic System microarray (an 11K DNA chip) was made with a non-redundant (unigene) set of 11,000 EST clones originally sequenced from liver, abdominal fat, and skeletal muscle/epiphyseal growth plate cDNA libraries (Table 2). The Chicken Neuroendocrine/Reproductive System microarray (an 8K DNA chip) was made from a unigene set of EST clones sequenced from the pituitary/hypothalamus/pineal and reproductive tract (oviduct/ovary/testis) cDNA libraries. A list of the chicken unigenes printed on each systems-wide microarray and a chicken gene expression database are available from our website.[3] These two high-density chicken DNA chips were designed to improve efficiency of array production and to reduce redundancy of common genes expressed in most tissues. The systems-wide DNA microarrays are currently being used for global gene expression profiling in liver, abdominal fat, skeletal muscle (Metabolic/Somatic System Chip), and pituitary/hypothalamus (Neuroendocrine/Reproductive System Chip) tissues collected from our experimental broiler chicken lines (FL and LL; FGL and SGL) in a longitudinal study (1, 3, 5, 7, 9, and 11 wk).

EXPRESSION PROFILING OF LIVER GENES

One of our major goals is to identify functional genes involved in metabolic pathways that modulate body composition of broiler chickens. Candidate genes are found by correlating differences in the expression level of transcripts, represented on the microarrays, with the variation observed in a particular trait. An interesting model system for discovery of metabolic gene networks is manipulation of the thyroid axis (Cogburn et al., 1989; Cogburn, 1991) to produce the lean [triiodothyronine (T_3)-treated] and obese [propyl-thiouracil (PTU)-treated] phenotypes. In the first study, 4-wk-old broiler chickens were fed a diet containing T_3 (1.25 ppm) or 0.6% PTU for 3 wk to experimentally induce the hyperthyroid or hypothyroid state, respectively (Wang et al., 2002). Liver samples were collected at 7 wk and used for isolation of total RNA. The liver cDNA targets from four chickens per treatment group were labeled with ^{33}P-dCTP in an oligo-dT primed reverse transcriptase reaction. The hybridization signals from eight microarrays (four arrays per treatment) were analyzed by Pathways 4 software.[4]

Representative nylon membrane microarrays of hepatic genes in hypothyroid (PTU-fed) versus hyperthyroid (T_3-fed) chickens are presented in Figure 1. The insets show the differential expression of an unknown EST whose expression was higher (2.1-fold) in hypothyroid (PTU-treated and fatter) chickens than in the hyperthyroid (T_3-treated and leaner) chickens. Microarray analysis of liver genes in hypothyroid versus hyperthyroid chickens has identified about 40 differentially expressed genes (Wang et al., 2002). For example, many of these thyroid hormone responsive genes are involved

[3]http://udgenome.ags.udel.edu/~cogburn/
[4]Invitrogen Corporation, Carlsbad, CA.



Higher Expression in Hypothyroid State: (PTU/T_3 ratio)

Gene	Fold-difference	Function
Glutathione S-transferase	2.81	detoxification
Steroid 21-hydroxylase	2.57	drug metabolism
Unknown gene, *THRG*	2.11	unknown
Acetyl-CoA acetyltransferase	2.02	lipid metabolism
Adipophilin	1.94	lipid storage
Prostaglandin synthase	1.92	PGD2 synthesis
Orphan phosphatase	1.84	ATPase (catalytic)
S-cyclophilin	1.80	protein folding
Heat shock protein-90	1.76	molecular chaperone
Zinc finger protein 198	1.73	hematopoiesis
Tumor metastasis-suppressor	1.70	cell growth
Polyamine oxidase (*PAO*)	1.70	detoxification

Higher Expression in Hyperthyroid State: (T_3/PTU ratio)

Gene	Fold-difference	Function
Spot 14 (*THIG*)	2.56	transcription factor
Tectorin-like protein	1.95	extracellular matrix
β-tubulin cofactor A	1.90	molecular chaperone
Lactate dehydrogenase-β	1.89	anaerobic glycolysis
C/EBPα	1.82	transcription factor
Hexokinase 2	1.70	glycolysis
EGF receptor substrate 8	1.66	mitogenic signaling
LFABP	1.66	lipid transport
Phospholipase A2	1.62	lipid metabolism
Thioredoxin (*PDIP5*)	1.61	redox reactions
Lanosterol synthase	1.60	sterol synthesis
Leptin receptor	1.51	lipid metabolism

FIGURE 1. Microarray analysis of liver genes in hypothyroid versus hyperthyroid broiler chickens. The chicken liver DNA microarrays were printed on 8 × 12 cm nylon membranes in grids of 6 × 6 spots in a 96-well plate format. Each liver-specific microarray contains 3,456 elements printed as single spots. The cDNA targets from each liver sample were labeled with ^{33}P-dCTP in an oligo-dT-primed reverse transcriptase reaction and hybridized to a single microarray. This analysis included four liver microarrays (one array/bird) for each hormone treatment group triiodothyronine (T_3) and propyl-thiouracil (PTU). The insets show differential expression of an unknown candidate gene we have named thyroid hormone-repressible gene (*THRG*). Examples of differentially expressed genes are presented for hypothyroid and hyperthyroid conditions. The differential expression of 14 genes under each condition was verified by real-time quantitative PCR (TaqMan). The ratio represents the average normalized hybridization signal per gene of four birds per condition. EST = expressed sequence tag.

in the lipogenic pathway [i.e., *Spot 14*, CCAAT/enhancer binding protein-α (*C/EBP*α), *adipophilin*, *liver fatty acid binding protein* (*LFABP*), *phospholipase A2*, *lanosterol synthase*, *leptin receptor*, and *acetyl-Co-A acetyltransferase*).

Another interesting model that we have used to identify genes involved in lipid metabolism is the perihatch chicken. About 90% of the total energy required by the embryo for growth comes from β-oxidation of fatty acids that are derived from yolk lipids (Speake et al., 1998). The last phase of embryonic development is marked by a dramatic accumulation (>30% of its dry mass) of cholesterol esters in the liver (Feast et al., 1998). The major metabolic adjustment that a hatchling chick must undergo is the switch between energy sources from lipids (mainly stored yolk) to utilization of its ingested food, mainly carbohydrates and protein (Noy and Sklan, 2001). Early hepatic expression of several lipogenic enzymes provides the chick with the ability to convert dietary carbohydrate into fat stores (Speake et al., 1998). An understanding of the choreography of hepatic gene expression in the liver of embryos and hatchlings during this critical period will be important for unraveling the genetic circuits that regulate lipid metabolism and contribute to fat accretion in broiler chickens.

Liver samples were collected from four embryos (commercial broiler cross) at 16, 18, and 20 d and four chicks at 1, 3, and 9 d posthatching. The ^{33}P-labeled cDNA targets, prepared from each liver sample, were hybridized to a single liver DNA microarray (n = 24). Global gene expression was examined in the liver during the perihatch period, or the metabolic jump from chorioallantoic (embryo) to pulmonary respiration (hatchling) (Glass et al., 2002). In this study, our major comparison was the differential expression of hepatic genes between embryos (12 arrays) and hatchling chicks (12 arrays).

The major issues involved with computational and statistical analyses of gene expression generated from microarrays have recently been discussed (Cogburn et al., 2003). Cluster analysis using self-organizing maps (SOM) (Tamayo et al., 1999; Toronen et al., 1999) reveals

A. Cluster 11 in 5 x 5 SOMs Analysis

Genes with Higher Expression in Embryos
Malic enzyme
Aldolase A
Thyroid receptor induced protein -7 (*TRIP7*)
Vitamin D binding protein
Acetyl-CoA acetyl-transferase 2
B-cell translocation gene-1 *(BTG1)*
Adenylosuccinate lyase
Phosphoenolpyruvate (PEP) carboxykinase

B. Cluster 3 in 5 x 5 SOMs Analysis

Genes with Higher Expression in Hatchlings

Spot 14 [Thyroid hormone-inducible gene (*THIG*)]
Fatty acid synthase (*FAS*)
Liver fatty acid (*LFABP*)
Neuregulin 1 (*NRG1*)
C/EBPα
Hemopexin
CYP1A5 (Uroporphyrinogen oxidase)
Arg:Gly amidinotransferase (AT)
Chromogranin B (*Chgb*)

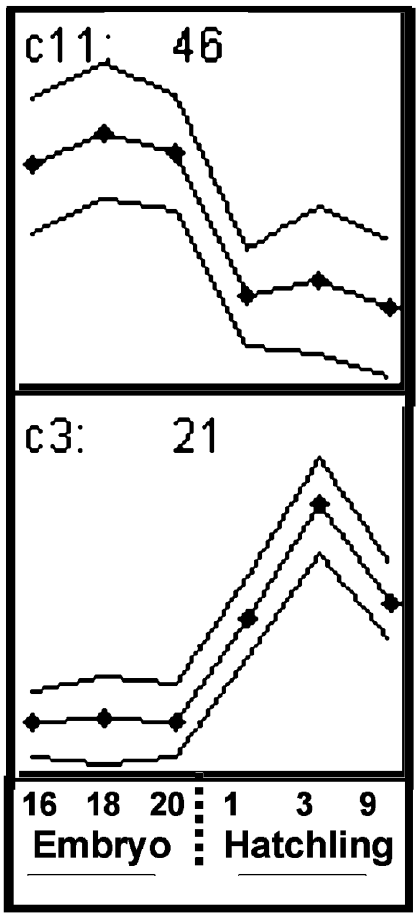


FIGURE 2. Self-organizing maps (SOMs) analysis reveals basic patterns of gene expression in liver during the perihatch period (16 d embryo to 9 d posthatching). A 5 × 5 SOMs analysis shows two distinct clusters of genes with higher expression in the embryo (A) or higher expression in the hatchling (B). Cluster 11 contains 46 hepatic genes that are expressed at higher levels in 16-, 18-, and 20-d embryos. Cluster 3 contains 21 hepatic genes that are expressed at higher levels in 1-, 3-, and 9-d-old chicks (some genes in each cluster are presented in the left panels). This microarray analysis included 24 liver arrays (4 arrays/age × 6 ages). The dotted vertical line represents the relative time of hatching.

a unique group (cluster 11) of 46 genes whose expression is higher in embryos and a different group (cluster 3) of 21 genes whose expression is higher in hatchlings (Figure 2). For example, cluster 11 contains a group of metabolic enzymes with higher expression in embryos (d 16 to 20) [i.e., *malic enzyme*, *aldolase A*, *acetyl-CoA acetyltransferase*, *adenylosuccinate lyase* (*ADL*) and *phosphoenol-pyruvate carboxykinase* (*PEPCK*)]. One gene, called *TRIP7* (*thyroid receptor induced protein-7*), is implicated in postembryonic tissue remodeling (Amano et al., 2002), and it enhances transcription from chromatin templates (West et al., 2001). On the other hand, cluster 3 contains several genes that have higher expression after hatching (1 to 9 d of age). These up-regulated genes are involved in lipogenesis [*Spot 14*, *fatty acid synthase* (*FAS*), *liver fatty acid binding protein* (*LFABP*), *CYP1A5*, *C/EBPα*], amino acid metabolism [*Arg/Gly amidinotransferase* (*AT*)], and heme biosynthesis [*hemopexin and cytochrome P450 1A5* (*CYP1A5*)]. A complete list of differentially expressed genes from these studies is available on our website.[3] Thus, DNA microarray analysis of chicken liver during the perihatch period has revealed distinct patterns of gene expression, in which a number of up-regulated genes found in the newly hatched chick are involved in lipid and energy metabolism (Glass et al., 2002), which extends the pioneering work on genetic control of intermediary metabolism in the chick by Goodridge (Goodridge et al., 1974, 1991; Morris et al., 1984; Wilson et al., 1986; Stapleton et al., 1990; Swierczynski et al., 1991; Chung et al., 1999). In future studies, global gene expression profiling in multiple chicken tissues will permit development of a detailed genetic blueprint that illustrates the developmental events and hierarchy of genes that govern adaptation to posthatch growth and development in chickens.

IDENTIFICATION OF FUNCTIONAL GENES

One differentially expressed candidate gene identified by DNA microarray analysis is an unknown chicken EST (see insets, Figure 1) whose hepatic expression is regulated by thyroid status (hypo- versus hyperthyroid). Our CAP3 database shows that this unknown gene is represented by a contig (or in silico cDNA of 810 bp) assembled from 17 liver EST. Two single nucleotide polymorphisms (SNP) were found in the putative cod-

ing region of this unknown gene. One EST (GAR33-G5-5B) clustered in this unknown gene contig was independently identified by differential mRNA display in liver of the FL and LL chickens (Carre et al., 2001). Single-strand conformation polymorphism (SSCP) analysis has revealed a fat-specific mRNA and a lean-specific mRNA in liver of FL and LL chickens, respectively. Northern blot analysis of cultured hepatocytes and Leghorn male hepatoma (LMH) cells clearly shows that the expression of this unknown gene is down-regulated by T_3 and a fatty acid analog (ETYA) and up-regulated by dexamethasone (Carre et al., 2002a). Collectively, these functional studies indicate that this unique gene should be called thyroid hormone-repressible gene (*THRG*). The differential expression of *THRG* in liver of hypothyroid versus hyperthyroid chickens and the two single nucleotide polymorphisms, potentially related to fatness, make *THRG* worthy of further characterization as a candidate gene.

Another differentially expressed gene revealed by microarray analysis of hypothyroid versus hyperthyroid chickens is *Spot 14*. The expression of *Spot 14* was 2.6-fold higher in the liver of T_3-fed chickens when compared to PTU-fed chickens (Figure 1). Several years ago, we first identified *Spot 14* as a differentially expressed gene in the liver of divergently selected FGL versus SGL chickens (Cogburn et al., 2000, 2003). *Spot 14* was originally named as an ultrarapid T_3-inducible protein (i.e., spot #14) discovered on a two-dimensional electrophoresis gel (Seelig et al., 1982). *Spot 14* is a thyroid hormone-induced hepatic transcription factor (Brown et al., 1997) that regulates expression of a cascade of enzymes in the lipogenic pathway (see Figure 3 and discussion below). The in silico cDNA (904 bp) or contig representing chicken *Spot 14* was assembled by CAP3 clustering from 59 EST derived from liver and abdominal fat. Close examination of the EST sequences in the Spot 14 contig reveals a polymorphism (a 9-bp insertion/deletion) in the coding region just upstream of a putative DNA binding domain (X. Wang, W. Carre and L. A. Cogburn, unpublished data). The 9-bp insertion is prevalent in EST from Leghorn chickens, whereas the deletion is more prevalent in EST derived from broiler chickens. Furthermore, the *Spot 14* insertion/deletion is polymorphic in FL and LL chickens from which a differentially displayed mRNA (EST) corresponding to *Spot 14* was identified earlier (Carre et al., 2001, 2002a). Cytogenetic mapping shows that *Spot 14* is localized on chicken chromosome 1 q4.1–4.4. Thus, *Spot 14* (or *THIG*) is a very interesting candidate gene because of its regulation by thyroid hormone, its control over transcription of major lipogenic enzymes, and the insertion/deletion polymorphism found in different populations of chickens.

MAPPING OF FUNCTIONAL GENES IN METABOLIC PATHWAYS

We have developed a metabolic pathway map in chicken liver, which shows the importance of *Spot 14*—an hepatic protein that is up-regulated by T_3 and post-hatch development—in the lipogenic pathway (Figure 3). More than 20 yr ago, *Spot 14* was first identified as a thyroid hormone-inducible protein from two-dimensional gel electrophoresis of liver lysates (Seelig et al., 1981, 1982). This hepatic transcription factor (Brown et al., 1997) activates the lipogenic pathway in mammals. A similar mechanism has been suggested in chicken liver since the expression of several enzymes in the lipogenic pathway (Figure 3) is increased concomitantly with that of *Spot 14* (Figures 1 to 2). The transcription of malic enzyme and phosphoenol-pyruvate carboxykinase (*PEPCK*) in chicken liver is also controlled by *C/EBP*α (Chung et al., 1999); an hepatic transcription factor that is up-regulated by in T_3-fed (Figure 1) and posthatch (Figure 2) chickens. *Spot 14* (Liaw and Towle, 1984) is found primarily in liver and adipose tissue of the rat (Jump et al., 1990; Kinlaw et al., 1995), human and mouse (Grillasca et al., 1996), where it is transcriptionally up-regulated by glucose, T_3, and insulin (Jump et al., 1990). *Spot 14* also regulates expression of type I deiodinase (Brown et al., 1997), which converts the prohormone thyroxine (T_4) to metabolically-active T_3 in most tissues, and induces expression of the thyroid hormone receptor-β (TR-β). Microarray analysis has shown that *Spot 14* is the most abundantly induced gene in hyperthyroid (T_3-fed) mice (Feng et al., 2000) and chickens (see Figure 1; Wang et al., 2002). However, the relationship between *Spot 14* and adiposity could be more complex, especially because T_3-fed chickens express *Spot 14* mRNA transcripts at higher levels, but they are leaner than PTU-fed chickens. Surprisingly, the targeted disruption of the *Spot 14* gene in mice leads to an increase in de novo lipogenesis (Zhu et al., 2001). Furthermore, heightened *Spot 14* gene expression has become an important diagnostic link between two important prognostic indicators of human breast cancer (i.e., enhanced lipogenesis and chromosome 11q13 amplification) (Moncur et al., 1998). A QTL scan of the F_2 progeny from a cross between genetically lean and fat mice revealed an obesity-related QTL (F*ob3*, on chromosome 15) near a cluster of thyroid-related genes [thyrotropin-releasing hormone (*Trhrf*), congenital goiter (*Cog*), thyroglobulin (*Tgn*), somatostatin receptor-3 (*Smstr3*), and thyrotroph embryonic factor (*Tef*)] (Horvat et al., 2000). Our studies have shown that *Spot 14* is polymorphic, up-regulated by T_3, and expressed at higher levels in liver of FGL than SGL chickens (Cogburn et al., 2003). The expression of *Spot 14* dramatically increases in the liver of chicks after hatching as they begin to deposit abdominal fat (Figure 2). These observations indicate that *Spot 14* should be called a thyroid hormone-inducible gene (*THIG*). Thus, *Spot 14* (or *THIG*) is a very interesting, differentially expressed, candidate gene for further study and mapping of functional genes that control lipid metabolism (Figure 3). Clearly, the lipogenic pathway in broiler chickens is thyroid-hormone sensitive, overly active, and potentially a key target for genomic manipulation.

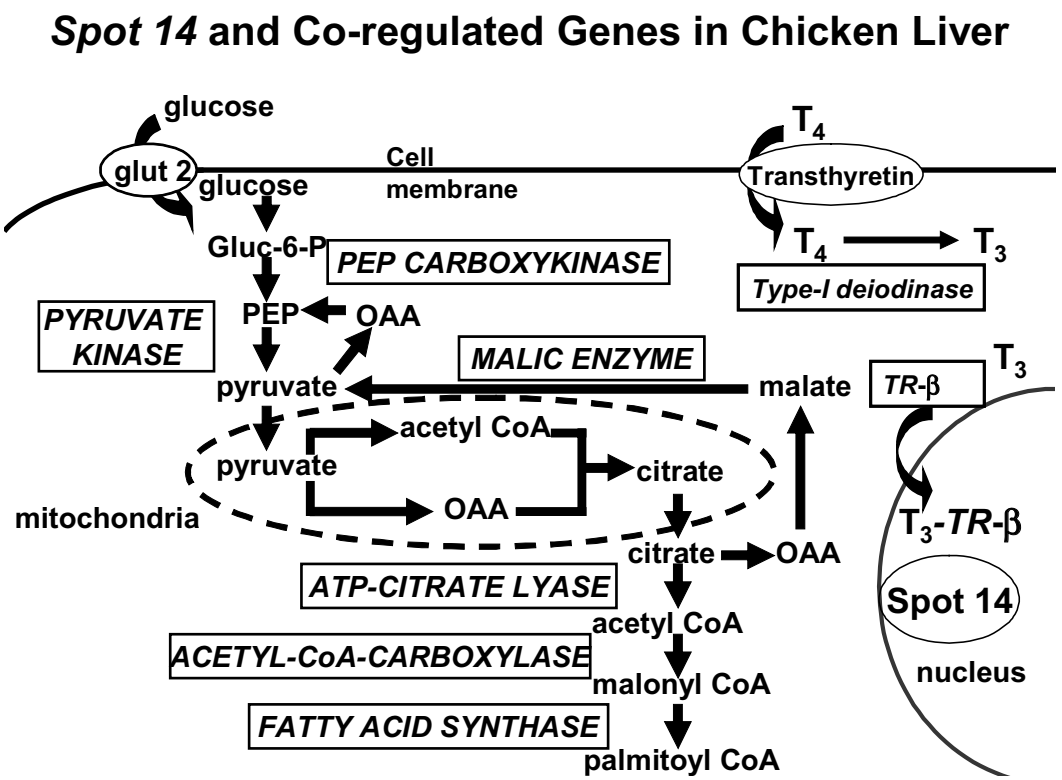


FIGURE 3. Spot 14 and co-regulated genes in the lipogenic pathway in chicken liver. The differentially expressed nuclear protein, Spot 14, regulates transcription of a cascade of six lipogenic enzymes and some thyroid-related genes in liver, as indicated by the white boxes. For convenience in presentation, PEPCK is depicted as a cytosolic enzyme. However, the major—if not the exclusive—form of PEPCK in chicken liver is the mitochondral form (Weldon et al., 1990). The cytosolic form of PEPCK represents a regulatory component of gluconeogenesis. Phosphoenolpyruvate (PEP) carboxykinase (PEPCK); oxalacetate (OAA); thyroid hormone receptor-β (TR-β); glucose tranasporter-2 (glut 2).

DEVELOPMENT OF UNIQUE RESOURCE POPULATIONS FOR QTL SCANNING

The fourth goal of our project was to create two new genetic resource populations for generating linkage maps of genetic markers and segments of the chicken genome that segregate QTL that affect growth and body composition traits. Recent advances in QTL mapping technology have produced comprehensive linkage maps with the hope of providing marker-assisted selection to improve animal production. Two earlier studies have reported on QTL analysis of limited production traits (BW, carcass weight, and carcass percentage at 7 wk) in a broiler resource population (Van Kaam et al., 1998, 1999); however, these studies were not designed to examine the genetic architecture of growth rate and fat deposition. Two QTL affecting BW (at 13 and 16 wk) were identified at 2.2 Morgans (M) on chromosome 1 and at 0.6 M on chromosome 2 in an F_2 cross between Satsumadori (slow-growing native breed) and White Plymouth Rock (fast-growing broilers) chickens (Tatsuda and Fujinaka, 2001). These QTL were mapped to chromosomes 1 and 2, and their positions are in agreement with earlier studies (Van Kaam et al., 1998, 1999). A QTL analysis of the F_2 population from a commercial broiler line × White Leghorn cross has revealed additional QTL for BW on chromosomes 4, 7, 8, and 13 (Sewalem et al., 2002). An additional QTL scan for fatness in the same broiler-layer F_2 resource population has revealed a number of interesting QTL for abdominal fat weight on chromosomes 3, 7, 15, and 28; abdominal fatness (adjusted for eviscerated carcass weight) on chromosomes 1, 5, 7, and 28; skin and subcutaneous fat weight on chromosomes 3, 7, and 13; skin fat weight (adjusted for eviscerated carcass weight) on chromosomes 3 and 28; and skin fat weight (adjusted for abdominal fat weight) on chromosomes 5, 7, and 15 (Ikeobi et al., 2002).

We have generated two new unique F_2 populations by crossing divergently selected chicken lines (FL × LL and FGL × SGL). This design has some advantages: alleles with large effects could be segregating, increased power of statistical analysis due to high heterozygosity in the F_1 when there are fixed differences between lines, and increased power of statistical analysis as the linkage phase between markers and expressed genes or QTL are expected to be more consistent among F_1 birds, in contrast to the situation for outbred populations in which the linkage phase may vary between families (Patterson, 1998). Another major advantage is that these divergent lines were originally established with commercial lines, and the likelihood that expressed genes or QTL found are still segregating in commercial lines could be high.

Five sires from the FL were mated to 10 dams from the LL (the FL × LL cross). Similarly, five sires from the FGL were mated to 10 dams from the SGL (the FGL × SGL cross). The two F_2 populations were generated from five to 10 sires and 15 to 40 dams from each of the F_1 populations. Several phenotypic measurements were made on 500 individuals from the two F_2 populations at 1 to 9 wk (BW), at 7 wk (fasted BW, body temperature, plasma levels of glucose, and IGF-I), and finally at 9 wk [length and diameter of the shank, initial and ultimate pH of breast meat, meat color index, meat drippings (an estimate of retained moisture), abdominal fat weight, and weights of breast and leg muscles].

An initial QTL scan for fatness and breast muscle weight at 8 wk has been performed on the F_2 population from an earlier FL × LL cross in collaboration with several INRA groups (Lagarrigue et al., 2003) (see http://www.intl-pag.org/11/abstracts/P5l_P595_XI.html). In each of the five families, the 40 most extreme individuals (20 for each extreme) of each distribution (abdominal fat and breast muscle weights) were selected for genotyping. These phenotypic traits were corrected for the mother and hatching lot effects by two-way analysis of variance, which included 8 wk BW as a covariable, using the GLM procedure from the SAS package.[5] A total of 130 markers, well distributed on the genome and heterozygous for most of the F_1 sires, were selected for genotyping. Fifty-eight percent of the markers were polymorphic by sire.

The analysis of the data from 309 animals (F_0, F_1, and F_2) revealed seven QTL regions controlling the variability of abdominal fat weight adjusted for 8 wk BW. Two QTL regions with a high threshold of significance ($P < 0.05$) for abdominal fatness (% BW at 8 wk) were found on chromosome 5 at 1.35 and 1.64 M positions. Another putative QTL region affecting abdominal fatness was observed on chromosome 5 around the 0.60 M position, although with a lower threshold of significance ($P < 0.10$). Four additional putative QTL regions for abdominal fatness were segregated within our F_2 population on chromosomes 1, 3, 7, and 13 around the 4.49, 0.84, 0.32, and 0.22 M positions, respectively. The QTL found on chromosomes 3, 5, and 7 are in agreement with the fatness QTL described by Ikeobi et al. (2002), with the exception that we have identified two additional QTL for abdominal fatness on chromosome 5 at 1.35 and 1.64 M. We also found a single QTL region ($P < 0.05$) for the breast muscle weight (% BW at 8 wk) on chromosome 1 at position 4.39 M. This QTL for breast muscle yield could be similar to a QTL for carcass percentage (% BW at 7 wk) that was identified on chromosome 1 at 4.8 M (Van Kaam et al., 1999). Therefore, several QTL regions implicated in control of abdominal fatness and a single region associated with breast muscle weight have been identified in the F_2 population from an earlier FL × LL cross (Lagarrigue et al., 2003). Complementary analyses will be done to determine the additive or dominant effects of the various QTL identified in the first study. This part of our current project continues with the study of QTL regions for abdominal fatness, breast muscle weight, and several other phenotypic parameters in the two new F_2 populations recently created from FL × LL and FGL × SGL crosses.

We anticipate that these experimental chickens lines, divergently selected for these extremes in growth rate or body composition, will prove to be precious genetic resources for unraveling the basic molecular mechanisms that govern growth, development, and body composition of chickens. We have embarked on gaining insight into these cellular and metabolic processes by using DNA microarray analysis to examine expression of genes that could be responsible for the extremes in growth rate and body composition observed in divergently selected broiler lines. Development of genetic markers from differentially expressed genes identified in liver, fat, skeletal muscle, pituitary, and hypothalamus will be combined with classical QTL scans to improve our understanding of the genetic architecture of growth-related traits and the underlying biological pathways.

In summary, we have constructed and normalized five tissue-specific chicken cDNA libraries. High-throughput sequencing from these libraries has yielded 30,609 EST for chickens. We have assembled a preliminary Chicken Gene Index from the world's public collection of chicken EST (>407,602). The 33,941 high-fidelity contigs (in silico cDNA) assembled with CAP3 clustering could represent the major portion of the chicken's transcriptome (~35,000 genes). Currently, we are producing two high-density chicken DNA microarrays (a Chicken Metabolic/Somatic System Gene Chip and a Chicken Neuroendocrine/Reproductive System Gene Chip) to establish basic gene expression profiles in tissues collected from our divergently selected broiler lines. A number of candidate genes have already been identified in liver that could contribute to determination of important phenotypic traits in broiler chickens. The current bonanza of chicken gene sequences and chicken DNA microarrays for global gene expression profiling now provide us with powerful molecular tools to launch the new era of functional genomics in chickens.

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[5]SAS Institute Inc., Cary, NC.

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