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Functional Genomics of the Chicken—A Model Organism

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ABSTRACT Since the sequencing of the genome and the development of high-throughput tools for the exploration of functional elements of the genome, the chicken has reached model organism status. Functional genomics focuses on understanding the function and regulation of genes and gene products on a global or genome-wide scale. Systems biology attempts to integrate functional information derived from multiple high-content data sets into a holistic view of all biological processes within a cell or organism. Generation of a large collection (~600K) of chicken expressed sequence tags, representing most tissues and developmental stages, has enabled the construction of high-density microarrays for transcriptional profiling. Comprehensive analysis of this large expressed sequence tag collection and a set of ~20K full-length cDNA sequences indicate that the transcriptome of the chicken represents approximately 20,000 genes. Furthermore, comparative analyses of these sequences have facilitated functional annotation of the genome and the creation of several bioinformatic resources for the chicken. Recently, about 20 papers have been published on transcriptional profiling with DNA microarrays in chicken tissues under various conditions. Proteomics is another powerful high-throughput tool currently used for examining the dynamics of protein expression in chicken tissues and fluids. Computational analyses of the chicken genome are providing new insight into the evolution of gene families in birds and other organisms. Abundant functional genomic resources now support large-scale analyses in the chicken and will facilitate identification of transcriptional mechanisms, gene networks, and metabolic or regulatory pathways that will ultimately determine the phenotype of the bird. New technologies such as marker-assisted selection, transgenics, and RNA interference offer the opportunity to modify the phenotype of the chicken to fit defined production goals. This review focuses on functional genomics in the chicken and provides a road map for large-scale exploration of the chicken genome.

Key words: transcriptome, proteome, metabolome, systems biology, gene network

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INTRODUCTION

The completion of sequencing, assembly, and annotation of the chicken genome (International Chicken Genome Sequencing Consortium, 2004) represents a monumental achievement for biologists in basic and applied research (Burt, 2007; Dodgson, 2007). The chicken embryo has been used as a model system for embryology and developmental biology for more than 2 millennia (Stern, 2004, 2005). For more than 8 millennia, the chicken has flourished as a domesticated livestock species deeply integrated into human culture (Dohner, 2001; Price, 2002). Therefore, it is fitting that the chicken would be the first domestic animal chosen for complete genome sequencing. The fact that the chicken genome sequence was completed a century after the birth of classic poultry genetics is another remarkable coincidence (Dodgson, 2003). Functional genomics is a relatively new discipline spawned by the technological revolution (Venter et al., 1996; Rowen et al., 1997), the momentum of inquisitiveness, and the spirited competitiveness (Collins et al., 2003b) that drove early completion of the human genome sequence (International Human Genome Sequencing Consortium, 2001; Venter and et al., 2001). The sequence of the human genome, with its complement of 20,000 to 25,000 protein-encoding genes, was not truly finished until 2004 (Human Genome Sequencing Consortium, 2004).

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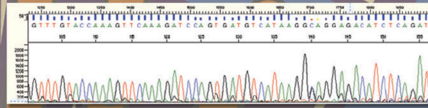
Functional Genomics of the Chicken



High-throughput DNA Sequencing

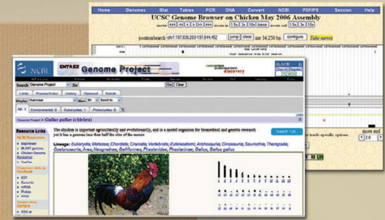
Know Genotype

Genome
(structure/variation)
Genes
(EST/mRNA)

Biological Sample

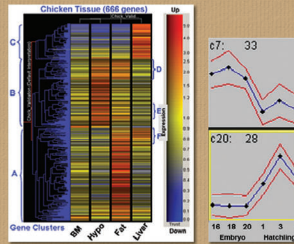
Bioinformatics/ Computational Analyses

EST sequence assembly (TIGR)
gene identification (NCBI/EBI)
genome assembly (*Ensembl*)
polymorphism discovery (ChickVD)



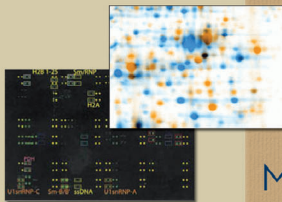
Transcriptome

cDNA arrays
long oligo arrays
GeneChip®
(SAGE, MPSS)



Gene Expression Patterns

Hierarchal clustering, SOMs,
K-means, eQTL

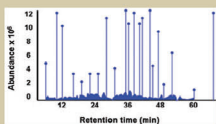


Proteome

protein arrays
2D gels
MALDI-TOF/MS

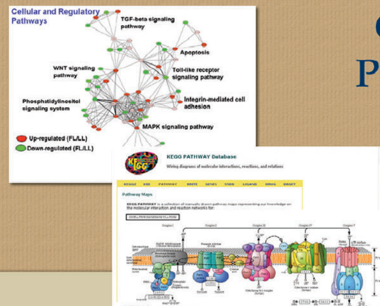
Gene/Protein Annotation

Gene Ontology
UniProt



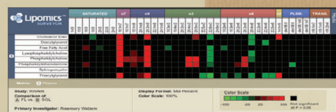
Metabolome

LC/MS
GC/MS



Gene Network/ Pathway Analysis

KEGG, GenMapp,
Pathway Miner,
Cytoscape



Identification of Functional Candidate Genes/Proteins

Change Phenotype

Marker assisted selection
Transgenics
RNA silencing

The chicken genome sequence was completed within a year—a historic accomplishment for avian biologists (International Chicken Genome Sequencing Consortium, 2004; Siegel et al., 2006; Burt, 2007; Dodgson, 2007) empowered by the high-throughput technologies developed in quest of the human genome sequence (Collins et al., 2003a). A critical step toward sequencing of the chicken genome was high-throughput DNA sequencing of expressed sequence tags (**EST**) from dozens of tissue-specific cDNA libraries generated from several international projects (Abdrakhmanov et al., 2000; Tirunagaru et al., 2000; Boardman et al., 2002; Cogburn et al., 2003c; Carré et al., 2006). This feat has advanced the chicken to 14th place (with 599,330 EST) among all model organisms represented in the dbEST division of GenBank. Furthermore, the large international collection of chicken EST, and the subsequent full-length sequencing of 19,626 cDNA (Hubbard et al., 2005) has enabled functional annotation of the assembled chicken genome sequence. The sequencing of the chicken genome and the development of high-throughput screening platforms (microarrays) and bioinformatic tools clearly advanced the chicken to model organism status (Burt, 2005, 2007). As many have recognized, completion of the genome sequence simply marks the "end of the beginning" (Brenner, 2000; Stein, 2004; Dodgson, 2007) of genome exploration in that species. Functional genomics attempts to bridge the gap between the blueprint (genome sequence or genotype) and the living organism (trait or phenotype; see Figure 1).

Functional genomics focuses on understanding the function and regulation of genes and gene products on a genome-wide or global scale. High-throughput screening platforms enable examination of the transcriptome, proteome, or metabolome of an organism. Computational integration of these functional components into a holistic view of the biological processes of an organism has spawned the latest life science discipline—systems biology. Systems biology attempts to provide us with the knowledge of how this genetic blueprint (genome sequence) yields a living organism. This review of functional genomics describes the recent ascent of the chicken to model organism status. Functional genomics and integrated systems biology hold promise for increasing our understanding of the complex biological processes required to complete the avian life cycle and propagate the species.

DEVELOPMENT OF GENOMIC RESOURCES

Assembly of a Comprehensive Catalog of Expressed Genes

The most efficient method for gene discovery, before completion of a genome sequence, is high-throughput sequencing of cDNA (or EST) from normalized cDNA libraries (Soares et al., 1994; Bonaldo et al., 1996). Prior to 1998, only a few hundred chicken EST were present in the dbEST division of GenBank. Figure 2 shows the progression of EST discovery in the chicken during the last decade. The most prolific growth of chicken EST sequences in GenBank occurred during a 4-yr period (1999 to 2002) due to completion of several international EST sequencing efforts (for reviews, see Cogburn et al., 2003c, 2004; Carré et al., 2006). The largest number of chicken EST submitted to GenBank in a single year was 359,674 sequences in 2002 (Figure 2A). The first large-scale chicken EST sequences were derived from primary lymphoid tissues, where 7,409 EST from B-lymphocytes (Abdrakhmanov et al., 2000) and 2,770 unique EST (out of 5,251 clones sequenced) from concanavalin A-activated T cells (Tirunagaru et al., 2000) were entered into Gen-

Figure 1. Road map of functional genomics in the chicken and use of these resources for large-scale exploration of the avian genome. Functional genomics focuses on understanding the function and regulation of genes, proteins, and metabolites on a genome-wide scale. All information derived from the analyses of genome sequence, large-scale analysis of gene and protein expression, and metabolite profiles is integrated into knowledge databases. High-throughput DNA sequencing of cDNA libraries from various tissues has provided a comprehensive catalog of chicken genes and high-density microarrays for transcriptional analyses. High-throughput sequencing of genomic DNA has provided the chicken genomic sequence, with its structure and genetic variation. Bioinformatics and computational analyses allowed assembly and annotation of the genome, whereas various genome browsers (*Ensembl*, Entrez, UCSC, and ChickVD) enable detailed views of the annotated genome and links to other databases (i.e., Gene Ontology, UniProt, KEGG, and TIGR). Biological samples taken from the embryo or chick facilitate high-throughput analysis of the transcriptome, proteome, metabolome, or their combination. Computational analysis of transcriptional data sets provides information on gene expression patterns and transcriptional control over clusters of coexpressed genes in gene networks. Transcriptional profiling coupled with QTL analysis allows identification of expression QTL (eQTL), which encompasses both positional and functional candidate genes. Analysis of the transcriptome and proteome requires access to well-annotated gene (Gene Ontology) and protein (UniProtKB) databases. Systems biology represents the integration of high-content data sets from the transcriptome, proteome, and metabolome into functional maps of biological pathways that determine the phenotype of the chicken. The premise of functional genomics is that if the genotype is known, then we have the knowledge base and tools to change the phenotype of the chicken. Once functional candidate genes, proteins, or both are identified, MAS or other emerging technologies (transgenics and RNA silencing) can be used to modify the phenotype of the bird to fit defined production goals. Recent improvements in avian transgenics enable expression of human biologicals in eggs and provide a vehicle for targeting genes to improve production traits. For example, green fluorescent protein is highly expressed in the beaks, legs, and feet of second-generation transgenic chicks. [This figure of transgenic chicks expressing green fluorescent protein is reprinted by permission from Macmillan Publishers Ltd. (EMBO Reports; McGrew et al., 2004)]. EST = expressed sequence tags; TIGR = The Institute for Genomic Research; NCBI/EBI = National Center for Biotechnology Information/European Bioinformatics Institute; SAGE = serial analysis of gene expression; MPSS = massively parallel signature sequencing; SOMs = self-organizing maps; MALDI-TOF/MS = matrix-assisted laser desorption/ionization time-of-flight/mass spectrometry; LC/MS = liquid chromatography/mass spectrometry; GC/MS = gas chromatography/mass spectrometry; KEGG = Kyoto Encyclopedia of Genes and Genomes.

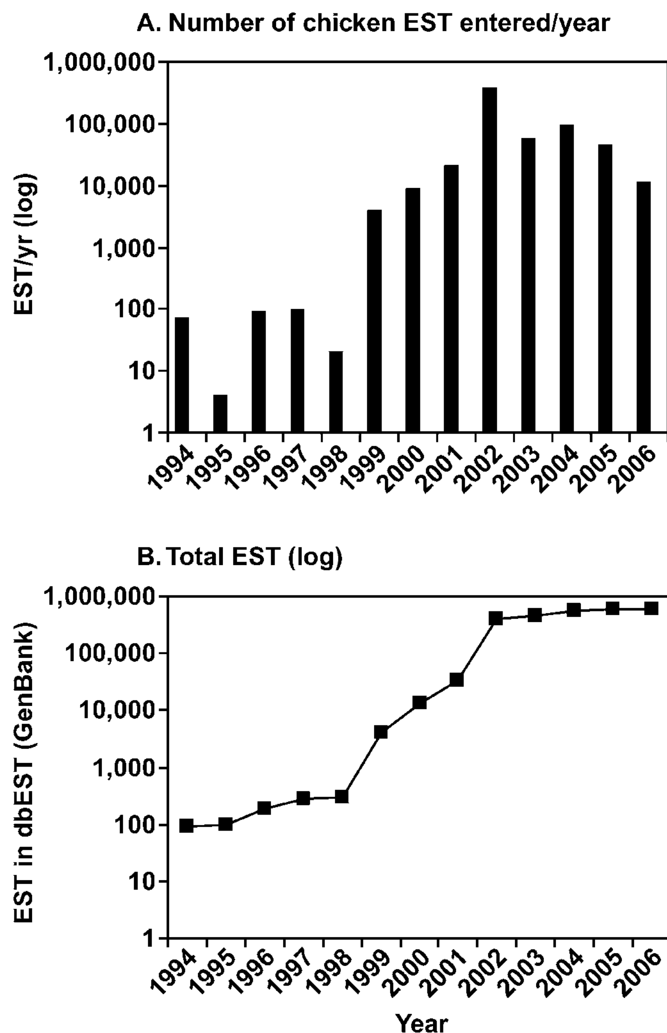


Figure 2. The progression of gene discovery in the chicken during the last decade. This graph shows the number of chicken expressed sequence tags (EST) entered per year (A) and the total accumulation of chicken EST (B) in the dbEST division of GenBank (National Center for Biotechnology Information).

Bank. A large number of EST (35,407) sequenced from single and multitissue cDNA libraries (representing the immune, metabolic-somatic, neuroendocrine, and reproductive systems) were entered into GenBank between 2001 and 2004 (Cogburn et al., 2003c, 2004; Carré et al., 2006). The largest collection of 330,096 chicken EST was sequenced from 22 normalized cDNA libraries constructed from major organs and cell types of egg- and meat-type chickens and embryos across a wide range of developmental stages (Boardman et al., 2002). An additional 4,998 chicken EST were derived from unnormalized cDNA libraries constructed from whole embryos (stage 26), somites, and limb buds (Jorge et al., 2004). Another project yielded 21,285 EST from brain and testis cDNA libraries made from Red Junglefowl and White Leghorn chickens (Savolainen et al., 2005). An additional 13,132 EST came from native Korean chicken testis cDNA libraries (Shin et al., 2005). A set of 14,409 EST was obtained from a cDNA library constructed from intestinal tissue of *Eimeria*-infected chickens (Min et al., 2005). Another group sequenced 8,729 EST from lipopolysaccharide and *Escherichia coli*-stimulated peripheral blood lymphocytes for construction of an avian macrophage microarray (Bliss et al., 2005). Recently, 10,848 EST were sequenced from a cDNA library constructed from chicken primordial germ cells isolated from White Leghorn embryos at d 6.5 (Han et al., 2006). Collectively, nearly 600,000 chicken EST sequences have been deposited in public databases for computational identification of expressed chicken genes.

To obtain an estimate of the number of chicken genes, several assemblies of the chicken EST and mRNA sequences have been made as the EST have accrued in public databases during the last 5 yr (Boardman et al., 2002; Cogburn et al., 2003b,c, 2004; Hubbard et al., 2005; Carré et al., 2006). A periodically updated chicken EST assembly is the *Gallus gallus* Chicken Gene Index (Release 11.0, June 17, 2006) at The Institute for Genome Research (**TIGR**; http://www.tigr.org/tdb/tgi/) or The Gene Index Project (http://compbio.dfci.harvard.edu/tgi/tgipage.html). A computational analysis of an earlier TIGR *G. gallus* Chicken Gene Index (Release 6.0; August 26, 2003) revealed 11,066 provisional chicken orthologs of human genes (Wu et al., 2004). Gene expression patterns, generated from the tissue origin of sequenced EST, suggest that approximately 15% of these putative chicken genes are tissue specific (i.e., expressed in a single tissue or organ), whereas 85% are commonly expressed (i.e., found in 2 or more tissues). A recent analysis of the chicken transcriptome based on completion of 19,626 full-length cDNA sequences and the assembly of 485,337 EST sequences provides evidence for nearly 19,000 genes in the chicken (Hubbard et al., 2005). This analysis of the transcriptome also included a unique set of 2,272 full-length cDNA recently sequenced from chicken bursal lymphocytes (Caldwell et al., 2004). The chicken EST collection and finished cDNA sequences were essential for functional annotation of the chicken genome and construction of a comprehensive catalog of unique chicken genes (UniGenes). These chicken cDNA clone collections enabled the development of several custom microarrays for discovery of functional genes in the chicken. Furthermore, several large repositories provide physical access to chicken cDNA clones for further analysis or expression of most chicken genes (see Table 1).

Development of Chicken Microarrays. More than a dozen chicken microarrays have been developed within the last 7 yr (see Table 1). Only a few papers on gene expression profiling with chicken DNA microarrays were published between 2000 and 2003. A primer on the principles of microarray analysis and analytical requirements for the chicken was published earlier (Cogburn et al., 2003b). The initial chicken lymphoid cDNA microarrays provided the first glimpse of global gene expression in the immune system of the chicken during normal development (Neiman et al., 2001; Cui et al., 2004) or provoked immune responses in isolated cells (Liu et al., 2001a; Morgan et al., 2001; Karaca et al., 2004). Tissue-specific DNA microarrays were developed for transcriptional profiling in the liver (Cogburn et al., 2003c), pineal gland (Bailey

et al., 2003), retina (Hackam et al., 2003), intestine (Min et al., 2003; van Hemert et al., 2003), and bursa of Fabricius (Neiman et al., 2003). The Del-Mar 14K Chicken Integrated Systems microarray [Gene Expression Omnibus (**GEO**) Accession No. GPL1731; Cogburn et al., 2004] was constructed from 2 earlier arrays representing the metabolic-somatic (GEO Accession No. GPL1737) and neuroendocrine-reproductive systems (GEO Accession No. GPL1744; Ellestad et al., 2006). A high-density (13K) multitissue chicken cDNA array (Burnside et al., 2005) was recently developed from a set of 11,447 nonredundant EST from the Biotechnology and Biological Sciences Research Council collection (Boardman et al., 2002) and a collection of lymphoid cDNA representing 4,162 EST. A focused and well-annotated 5K immune array was produced by the Roslin Institute for examining host defense (Smith et al., 2006). The chicken intestinal intraepithelial lymphocyte microarray and the avian macrophage microarray provide robust transcriptional platforms for characterizing host responses against invasion of mucosal pathogens (Lillehoj et al., 2007). The Chicken Genome GeneChip, containing probes for 33,457 chicken and viral pathogen transcripts, is commercially available from Affymetrics (http://www.affymetrix.com). A long-oligo (70-mer) array [*Gallus gallus* (chicken) Roslin/ARK CoRe Array V1.0] representing 20,673 transcripts is available from Operon (https://www.operon.com/) in ready-to-spot 384-well plates. Printed 20.7K chicken long-oligo arrays are also available from the University of Arizona (http://www.grl.steelecenter.arizona.edu/) and ARK Genomics (http://www.ark-genomics.org/). It is likely that commercial long-oligo arrays and the chicken genome array (GeneChip) will eventually replace custom microarrays as standardized high-quality platforms for transcriptional analysis. Some additional chicken microarrays are introduced in the gene expression section below.

Several reviews have described the availability of chicken genomics resources, including EST collections and microarrays (Burt, 2004; Cogburn et al., 2004; Antin and Konieczka, 2005; Fadiel et al., 2005). Initial overviews of functional genomics in the chicken have been published (Brown et al., 2003; Cogburn et al., 2003b,c; Burt, 2005; Moore et al., 2005). A list of functional genomics resources for the chicken and links to useful Web sites are provided in Table 1.

Functional Annotation of the Genome Sequence

The next critical step after genome sequencing is the rigorous functional annotation of the genome sequence. Assignment of the major functional units (genes) to the chicken genome sequence was enabled by the comprehensive catalog of chicken EST from chicken tissues (Abdrakhmanov et al., 2000; Tirunagaru et al., 2000; Boardman et al., 2002; Carré et al., 2006) and by the finished or complete cDNA sequencing of most chicken genes (Hubbard et al., 2005). The most common functional annotation of a genome uses the unified Gene Ontology (**GO**; http://www.geneontology.org/) assignment of gene and protein function to 3 broad categories: *cellular component*, *molecular function*, and *biological process* (Ashburner et al., 2000). Currently, the GO Annotation (**GOA**) database at the European Bioinformatics Institute (http://www.ebi.ac.uk/GOA/) contains 33,796 distinct human proteins, whereas only 16,146 distinct proteins have been identified in the chicken. Both transcriptomic and proteomic data are excellent sources for structural and functional annotation of chicken genes. At present, the majority of "known" genes in the chicken genome are annotated electronically by sequence homology or by ab initio gene prediction algorithms (Eyras et al., 2005). Experimental evidence of expression from transcriptomic and proteomic studies allows rapid verification of predicted gene transcripts and proteins to support functional annotation of the chicken genome sequence.

HIGH-THROUGHPUT GENOME-WIDE SCREENING

Gene Expression Profiling with Microarrays

Through the use of cDNA microarrays, investigators can measure mRNA levels for thousands of genes simultaneously, rather than one gene at a time. This high-throughput approach has been widely adopted in biological research. Various chicken tissues have been used for large-scale transcriptional analysis during normal development or after specific perturbations. This section reviews these studies to illustrate the power of DNA microarrays for transcriptional profiling and the discovery of functional genes in the chicken.

Neuroendocrine and Reproductive Systems. The pituitary gland and hypothalamus of the brain constitute the central components of the neuroendocrine system. This system plays a dominant role in controlling growth, metabolism, and reproduction. Nutrient availability and peripheral neuroendocrine signals from peripheral receptors and glands are integrated in the central nervous system; reciprocally, the hypothalamus communicates signals by regulating the release of hypothalamic-releasing hormones and release-inhibiting hormones, which reach the anterior pituitary gland via hypophyseal portal circulation. These hypothalamic-releasing and release-inhibiting hormones control secretion of trophic hormones from the anterior pituitary gland. In addition, magnocellular neurons originating in the hypothalamus and terminating in the posterior pituitary gland release stored neurohormones directly into systemic circulation. The hormones secreted from the anterior and posterior pituitary glands include those regulating growth and metabolism [growth hormone (**GH**) and thyroid-stimulating hormone (**TSH**), reproduction [luteinizing hormone (**LH**), follicle-stimulating hormone (**FSH**), and prolactin (**PRL**)], stress responses (adrenocorticotropic hormone), and renal function (Arg vasotocin). The hypothalamus also plays a critical role in controlling feed intake via the function of sev-

Table 1. Functional genomics resources for the chicken

Name[1]	URL	Title	Organization	Platform no. or array no.
Chicken genome browsers				
NCBI	http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9031			
UCSC	http://genome.ucsc.edu/cgi-bin/hgGateway			
European Bioinformatics Institute	http://www.ensembl.org/Gallus_gallus/index.html			
Gallus Genome GBrowser	http://birdbase.net/cgi-bin/gbrowse/gallus/			
Chicken cDNA clone repositories				
University of Delaware ChickEST Database	http://www.chickest.udel.edu/			
BBSRC ChickEST Database	http://www.chick.umist.ac.uk/			
ARK-Genomics	http://www.ark-genomics.org/microarrays/			
Chicken EST assemblies				
University of Delaware (UD) ChickEST Database	http://www.chickest.udel.edu/			
UD CAP3 Chicken EST Assembly	http://cogburn.dbi.udel.edu/			
BBSRC ChickEST Database	http://www.chick.umist.ac.uk/			
The Institute for Genome Research (TIGR)	http://www.tigr.org/db.shtml			
The Gene Index Project (formally at TIGR)	http://compbio.dfci.harvard.edu/tgi/tgipage.html			
Chicken Germ Cell EST Database	http://chickgce.snu.ac.kr/			
Chicken microarrays				
NCBI Gene Expression Omnibus (GEO)	http://www.ncbi.nlm.nih.gov/geo/	Chick Pineal	Texas A&M University (USA)	GPL1289
		Avian Macrophage Microarray (AMM)	University of Delaware (USA)	GPL1461
		DEL-MAR 14K Integrated Systems	University of Delaware (USA)	GPL1731
		UD 7.4K Metabolic/Somatic Systems	University of Delaware (USA)	GPL1737
		UD_Liver_3.2K	University of Delaware (USA)	GPL1742
		Chicken_Neuroendocrine_System_5K	University of Maryland (USA)	GPL1744
		FHCRC Chicken 13K v2.0	Fred Hutchinson Cancer Res. Ctr. (USA)	GPL1836
		Intestine-Spleen Array	Inst. Anim. Sci. Health (The Netherlands)	GPL2719
		GeneChip Chicken Genome Array	Affymetrix Inc. (USA)	GPL3213

Continued

Table 1 (continued). Functional genomics resources for the chicken

Name[1]	URL	Title	Organization	Platform no. or array no.
Zebrafinch microarray		NIH	Duke Zebrafinch 18K v1.0, Duke University Med. Ctr.	GPL3621
European Bioinformatics Institute ArrayExpress	http://www.ebi.ac.uk/arrayexpress/	KTH UniChicken 15k Uppsala UTG	Uppsala University (Sweden)	A-MEXP-154
		UTU Chicken BursaEST 37K array	University of Turku (Finland)	A-MEXP-155
		KTH UniChicken 2×14k cDNAv1	Uppsala University (Sweden)	A-MEXP-266
		ARK-Genomics Immune Array 5K v3.1	Roslin Institute (UK)	A-MEXP-307
		ChickenIMAGE *G. gallus* 3.8K v1	CNRS (France)	A-MEXP-509
		ChickenIMAGE *G. gallus* 3.8K v2	CNRS (France)	A-MEXP-526
Chick Embryo In Situ Hybridization Database	http://geisha.arizona.edu/geisha/			
Chicken gene ontologies				
Gene Ontology Annotation (GOA)	http://www.ebi.ac.uk/GOA/CHICKEN_release.html			
ChickGO at AgBase (Mississippi State University)	http://www.agbase.msstate.edu/			

[1]NCBI = National Center for Biotechnology Information; UCSC = University of California, Santa Cruz; BBSRC = Biotechnology and Biological Sciences Research Council; EST = expressed sequence tag.

eral neuropeptides. Stimulation of feed intake involves neuropeptide Y and agouti-related protein, whereas inhibition of intake involves α-melanocyte-stimulating hormone, cocaine- and amphetamine-regulated transcript, and corticotrophin-releasing hormone.

Traditionally, regulation of gene expression within the neuroendocrine system has been studied one gene or several select genes at a time. This focused approach has been very effective in defining multiple integrated pathways involved in neuroendocrine regulation of feed intake, metabolism, and somatic growth (Richards and Proszkowiec-Weglarz, 2007). For example, anterior pituitary levels of mRNA for PRL, GH, TSH, and LH in poultry have been analyzed by Northern blotting, ribonuclease protection assays, PCR, and in situ hybridization (Talbot et al., 1991; Kansaku et al., 1994; Tong et al., 1997; Ramesh et al., 1998; Bossis and Porter, 2000; Fu and Porter, 2004; Muchow et al., 2005). Similar studies have evaluated expression of mRNA for hypothalamic neuropeptides, including neuropeptide Y, vasoactive intestinal polypeptide, corticotrophin-releasing hormone, and gonadotropin-releasing hormone in poultry (Talbot et al., 1995; Boswell et al., 1999; Sun et al., 2001; Chaiseha et al., 2004; Saito et al., 2005; Vandenborne et al., 2005). The development of chicken cDNA microarrays has enabled analysis of gene expression profiles for thousands of genes simultaneously in individual samples of the pituitary gland or hypothalamus.

Microarray analysis of RNA from small tissue samples (i.e., individual pituitary glands) is problematic because less than 25 μg of total RNA (needed for a standard microarray analysis) can be recovered from small tissue samples. To overcome this obstacle, RNA amplification protocols have been developed for use with microarrays. Ribonucleic acid amplification procedures, originally published by Eberwine and colleagues (Phillips and Eberwine, 1996), have recently been adapted for use with chicken pituitary samples (Porter and Ellestad, 2005; Ellestad et al., 2006). These procedures transcribe RNA in vitro by using T7 RNA polymerase and cDNA samples produced with an oligo(dT) primer containing the T7 promoter. This procedure typically yields 5 to 10 μg of amplified RNA (equivalent to mRNA) from 500 ng of starting total RNA, an approximately 300-fold amplification. Performing a second round of amplification allows for analysis of RNA from single cells collected by laser capture microscopy. Readers interested in microarray analysis of small samples, (i.e., pituitary glands or cultured cells) are directed to previous reports for detailed descriptions of this procedure (Phillips and Eberwine, 1996; Porter and Ellestad, 2005; Ellestad et al., 2006).

In the first study to examine global gene expression patterns in the chicken neuroendocrine system, Cassone and colleagues (Bailey et al., 2003) developed a cDNA microarray specific to another component of the neuroendocrine system, the pineal gland. This gland secretes melatonin (**MT**) and functions in synchronizing daily rhythms of activity and reproductive timing in birds. In that study, RNA samples were analyzed from pineal

glands of animals exposed to light-dark cycles or to total darkness. A number of genes were identified whose mRNA levels fluctuated in a rhythmic pattern, corresponding to the prevailing light-dark cycle. These included genes involved in MT synthesis and orthologs of mammalian clock genes, as expected. Genes involved in other processes (i.e., transduction of light, immune and endocrine signaling) were also found to fluctuate rhythmically. In a second study, this group extended their transcriptional analyses to the retina of chicks exposed to light-dark cycles or constant darkness (Bailey et al., 2004). Again, expression of chicken orthologs of mammalian clock genes and genes involved in MT synthesis fluctuated with the prevailing photoperiod. More important, application of cDNA microarray technology to this system allowed identification of a number of novel candidate genes with rhythmic expression in both the pineal gland and the retina. Furthermore, several genes involved in intermediary metabolism and protein degradation exhibited rhythmicity, pointing out the extent and complexity of such coordination. None of these genes had previously been implicated in the regulation of daily rhythms.

A second and more extensive cDNA microarray for the chicken neuroendocrine system was developed with clones sequenced from a cDNA library constructed with RNA pooled from the hypothalamus, pituitary gland, and pineal gland. The EST sequencing from this library and development of the Chicken Neuroendocrine System 5K microarray (GEO Accession No. GPL1744) was described earlier (Cogburn et al., 2003c, 2004; Porter and Ellestad, 2005; Ellestad et al., 2006). Porter and colleagues used the Chicken Neuroendocrine System 5K microarrays to examine the ontogeny of hypothalamic gene expression during the perihatch period. Ribonucleic acid was isolated from hypothalami before [embryonic day (**e**)17 and e19] and after hatching [posthatch day (**d**)1 and d3] and analyzed with the microarrays. Expression levels of 105 genes changed substantially during this period of development. Transcription profiles for myelin basic protein (*MBP*), stathmin (***STMN1***), dopamine, and cAMP-regulated neuronal phosphoprotein (*DARPP*), 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (*CNP2*), receptor-type protein Tyr-protein phosphatase N2 precursor (*PTPN2*), bone morphogenic protein 7 (*BMP7*), and glyceraldehyde phospho-dehydrogenase (*GAPDH*) were confirmed by quantitative real-time PCR (**qRT-PCR**). The *STMN1* gene was overexpressed in undifferentiated neurons, and hypothalamic *STMN1* levels decreased from e17 to d3. In contrast, *MBP* levels increased from e17 to d3, which agrees with the observation that myelination of the central nervous system occurs primarily after hatch. In addition to these predicted changes, the abundance of *DARPP* and *CNP2* increased dramatically between d1 and d3, indicating a substantial increase in neuronal signaling within the hypothalamus after hatching. In addition, levels of *PTPN2* and *BMP7* transcripts decreased dramatically but transiently on e19 and d1, respectively, indicating changes in signaling events within the hypothalamus specific to the perihatch period.

The 5K Chicken Neuroendocrine System microarray was recently used to profile developmental changes in gene expression in the pituitary gland from e10 to e17 (Ellestad et al., 2006). This period of embryonic development is characterized by the differentiation of 3 distinct anterior pituitary cell types that produce specific trophic hormones (TSH, GH, and PRL). In that study, 393 genes were differentially expressed during this period of embryonic development (e10 to e17). Self-organizing map (**SOM**) analysis (Tamayo et al., 1999) enabled clustering of these differentially expressed genes based on their transcriptional profiles during development. The *TSHβ* mRNA levels increased steadily during embryonic development, whereas *PRL* expression was nearly absent through e14, and then increased dramatically on e17. In contrast, expression of *β-actin* decreased in the pituitary during embryonic development, whereas the abundance of *GH* was low on e10 and e12, but increased dramatically by e17. A previous report indicated that glucocorticoid treatment increased *GH* mRNA indirectly through induction of another unidentified gene in the chicken (Bossis and Porter, 2003). Interestingly, 2 genes that respond to glucocorticoid treatment in mammals, the glucocorticoid-induced Leu zipper (*GILZ*) and dexamethasone-induced Ras 1 (*DEXRAS1*), exhibit expression profiles that are similar to *GH*. The expression profiles of 33 differentially expressed genes identified by microarray analysis were confirmed by using an independent method, qRT-PCR. These findings demonstrate that microarray analysis can be performed on amplified RNA from individual pituitary glands from chicken embryos as early as e10. Moreover, a number of unique genes were identified that could play a role in regulating the differentiation of anterior pituitary cells.

Microarray technology has also been used to study global gene responses in cultured pituitary cells. In the first study of this type, Porter and colleagues aimed to identify genes directly and rapidly regulated by the adrenal glucocorticoid corticosterone (**CS**) within the embryonic pituitary gland. Pituitary cells from e11 embryos were treated with CS (for 1.5, 3, 6, 12, or 24 h) in the absence or presence of cycloheximide, a protein synthesis inhibitor. Amplified pituitary RNA was then analyzed with the Del-Mar 14K Integrated Systems microarrays. Expression of 27 genes was affected by CS at 1.5 or 3 h both in the absence and in the presence of cycloheximide; 13 of these genes were induced at least 2-fold by CS within 3 h. None of these direct targets of CS had previously been demonstrated in the anterior pituitary gland.

Transcriptional profiling with cDNA microarrays has also been used to identify differentially expressed genes in the neuroendocrine system in a set of divergently selected chickens. Hallböök and colleagues (Ka et al., 2005) compared hypothalamic gene expression at hatching between 2 chicken lines genetically selected for high or low BW at 8 wk of age (Dunnington and Siegel, 1996). This microarray analysis indicated that 41 genes, including endogenous avian leukosis virus (***ALV***), were differen-

tially expressed between the 2 lines, although no details were provided.

Another functional genomics project (Cogburn et al., 2003c, 2005) has focused on a different population of broiler chickens genetically selected for either high-growth (**HG**) or low-growth (**LG**) BW (Ricard, 1975). The idea was that global transcript profiling in multiple tissues of divergent lines could identify genes that contribute to such large differences in production traits. Body weight in these experimental broiler lines diverges after 3 wk of age, with a greater than 2-fold difference at 11 wk. Gene expression profiles in the anterior pituitary gland were recently compared at 1, 3, 5, and 7 wk of age by using the Del-Mar 14K Integrated Systems microarrays (Porter et al., 2007). The microarray analysis identified 263 genes that were differentially expressed in the pituitary gland between the HG and LG lines in at least one age. These included 4 of the 6 trophic hormones produced by the anterior pituitary gland. Three pituitary hormones (*TSHβ*, *LHβ*, and *FSHβ*) were more abundant in the HG line, whereas the fourth gene (*GH*) was expressed at higher levels in the LG line. The expression patterns of *TSHβ*, *LHβ*, *GH*, and 6 other genes were confirmed by qRT-PCR analysis.

An additional study (Porter et al., 2007) examined gene expression profiles in the anterior pituitary gland and hypothalamus of genetically selected fat (**FL**) and lean (**LL**) lines of chickens (Leclercq, 1988) during juvenile development. Anterior pituitary glands and hypothalami were collected before (1 and 3 wk) and after (5 to 11 wk) the divergence in weight of the abdominal fat pad. The Del-Mar 14K Chicken Integrated Systems microarrays were used for transcriptional profiling of these samples. Interestingly, differences in gene expression profiles were found in both the anterior pituitary and the hypothalamus between FL and LL chickens at 1 and 3 wk. This indicates early divergence in the expression of genes in the neuroendocrine system between the FL and LL chickens. This system, which regulates feed intake, growth, and metabolism, could be programmed differently between these genetic lines, resulting in large (2- to 3-fold) differences in accumulation of body fat. For the pituitary gland, microarray analysis identified 386 differentially expressed genes between the FL and LL birds by using stringent criteria. For the hypothalamus, 206 genes were differentially expressed between the FL and LL. Several of the differentially expressed genes identified by microarray analysis in the anterior pituitary gland were confirmed by qRT-PCR; one of these genes was a member of the aldo-keto reductase family (*AKR*), which catalyzes the reduction of the aldehydes to ketones. Expression of *AKR* was greater in the pituitaries of LL birds compared with FL birds.

Thus, microarray analysis has enabled examination of the ontogeny of gene expression in the chicken neuroendocrine system during late embryonic and early posthatch development and the effects of drug or hormonal treatments on global gene expression in cultured pituitary cells. Rhythmic patterns of gene expression have been described in the pineal gland and retina of birds exposed to dark-light cycles. Finally, numerous differentially expressed genes have been discovered in the neuroendocrine system of chickens divergently selected for body composition (high vs. low body fat) or growth rate (high vs. low BW). Gene expression profiling with cDNA microarrays has already identified numerous candidate genes in the neuroendocrine system that could control the growth and metabolism of the chicken, and most likely other vertebrates.

Metabolic and Somatic Systems. To date, there have been only a few reports of gene expression profiling in metabolic (liver, fat, muscle) and somatic (skeletal muscle and bone growth plate) tissue of the chicken. Most studies have focused on the liver because this organ regulates whole-body metabolism of major nutrients (i.e., glucose, amino acids, and lipids). This is particularly evident in avian species, in which the liver is the main site of de novo lipogenesis (Goodridge and Ball, 1967). Several different models have been used for initial transcriptional profiling in metabolic and somatic tissues of the chicken and analysis of gene networks (Cogburn et al., 2003b,c, 2004; Wang et al., 2007). These experimental models include divergent selection (FL vs. LL and HG vs. LG; Cogburn et al., 2003c), metabolic perturbation [the fasting and refeeding response (Duclos et al., 2004), the abrupt embryo-to-hatchling transition (Glass et al., 2002)], and hormonal perturbation (Wang et al., 2007). Some observations from these original gene expression studies are presented below.

The first microarray analysis of chicken liver used a nylon membrane-based array of 1,200 (1.2K) cDNA derived from activated T cells (Morgan et al., 2001) to examine developmental differences (3 to 9 wk) between broiler lines divergently selected for either HG or LG (Cogburn et al., 2003b). Hierarchical clustering with SOM analysis (Tamayo et al., 1999) identified 59 differentially expressed genes in the liver of HG birds that belonged to 4 distinct clusters, and 6 distinct clusters containing 76 genes in the LG. Thyroid hormone-responsive Spot 14 (***THRSP***) and superoxide dismutase 3 (*SOD3*) were among the first differentially expressed genes discovered in the liver of HG and LG chickens (Cogburn et al., 2003b) with this early chicken cDNA microarray.

A prototype 3.2K liver-specific microarray (GEO Accession No. GPL1742) was developed and first used to examine hepatic gene expression during the abrupt embryo-to-hatchling transition period (Cogburn et al., 2004). Gene cluster analysis, using a spanning tree clustering method (Rejto and Tusnady, 2006), revealed 756 differentially expressed genes that formed 32 distinct expression patterns. These clusters of coexpressed genes are involved in the metabolic switch from embryonic to terrestrial life in the perihatch chick. For example, one group of 49 genes [Cluster (**C**) 6, C25, and C32] showed higher levels of expression in embryos, whereas 3 other clusters (C15, C22, and C29) showed higher expression after hatching (Figure 3). Expression of genes in other clusters increased sharply just before (C28) or just after (C14) hatching, whereas the

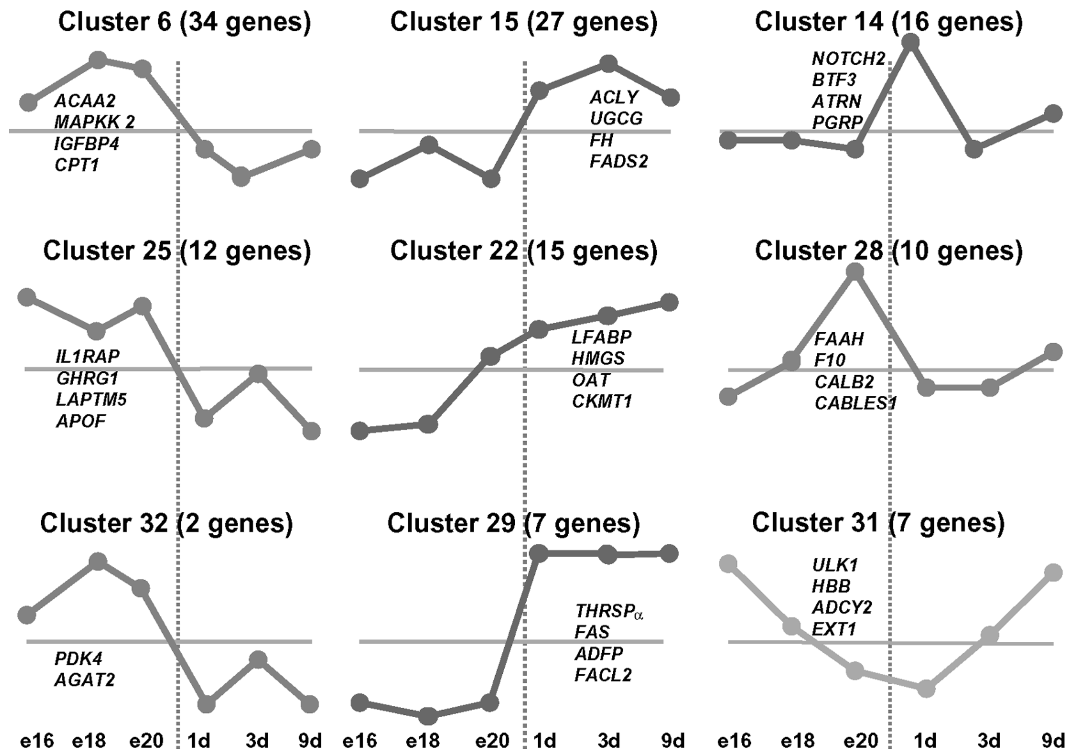


Figure 3. Clusters of coregulated hepatic genes during the embryo-to-hatchling transition with distinct expression patterns. A spanning tree clustering method (Rejto and Tusnady, 2006) revealed 32 distinct expression patterns from 756 differentially expressed genes. Transcriptional profiles were determined with individual liver-specific 3.2K microarrays (GEO Platform No. GPL1742) for 4 embryos at embryonic day (e)16, e18, and e20 and 4 chicks at posthatch day (d)1, d3, and d9. The dotted vertical line indicates hatching, and the horizontal line in each graph denotes the mean expression level (centroid) of that cluster. The number of genes within the cluster is shown, and some examples of cluster members are given. For inclusion in a cluster, a gene must be differentially expressed at one or more ages.

genes in cluster C31 progressively declined in the late embryo (e16 to e20) and newly hatched (d1) chick and then sharply increased thereafter. Several genes, expressed at higher levels in embryos, are directly involved in the fat catabolism, transcription, or signal transduction pathways. In contrast, the transcriptional pattern of several other clusters of hepatic genes increases sharply after hatching. These gene clusters encode numerous metabolic enzymes, transcription factors, inflammatory factors, transporters, and signaling proteins. Many of the up-regulated genes in the newly hatched chick reflect enhanced lipogenesis [*THRSP*, fatty acid synthase (***FASN***), stearoyl-coenzyme A (**CoA**) desaturase 1 or Δ^9-desaturase (***SCD1***), cytosolic malic enzyme 1 (*ME1*), adipose differentiation-related protein (*ADFP*), and fatty acid-binding protein 1 (*FABP1*)] after the initial ingestion of carbohydrate- and protein-enriched feed. Furthermore, qRT-PCR analysis has confirmed similar patterns of gene expression first revealed by microarray analysis. Some of these functional genes have been integrated into a working model of transcriptional control of the citric acid and fat biosynthesis pathways in the liver of the chicken (see Figure 4 in Cogburn et al., 2004). Thus, microarray analysis and clustering of coexpressed genes have provided the first global view of the transcriptional control over metabolism during the abrupt embryo-to-hatchling transition.

Recently, gene expression profiling in the liver of chickens with altered thyroid status (hyper- vs. hypothyroidism), chronic GH injection, or both facilitated the discovery of numerous thyroid hormone- and GH-responsive genes (Wang et al., 2007). Several genes were identified that had not been previously ascribed as either T_3-responsive [e.g., avian β-defensin 9 (***AvBD9***), epidermal growth factor receptor pathway substrate 8-like protein 2 (*EPS8L2*), Rho GTPase activating protein 1 (*RhoGAP*), longevity assurance homolog 2 (*LASS2*), and ovotransferrin (*OTF*)] or GH-responsive [glycogen synthase 2 (*GYS2*), hexokinase 1 (*HK1*), squalene epoxidase (*SQLE*), and uridine diphosphoglucose pyrophosphorylase 2 (*UPG2*)] genes. Although T_3 and GH are strong hormonal antagonists in the chicken, this study showed a remarkable degree of cooperation between the somatotropic and thyrotropic axes in transcriptional control of multiple pathways. This original descriptive study on hormonal perturbation also provided the first functional annotation for a large number of chicken genes that are either homologs or orthologs of genes characterized in other organisms.

The influence of nutritional state (fasting vs. refeeding) on the liver transcriptome was examined in the divergently selected HG and LG chickens (Ricard, 1975) by using the 3.2K liver-specific arrays (Duclos et al., 2004). In total, 429 differentially expressed genes that form 21 unique gene clusters were identified in the HG genotype, compared with 346 differentially expressed genes that form 16 clusters in the LG genotype. A number of func-

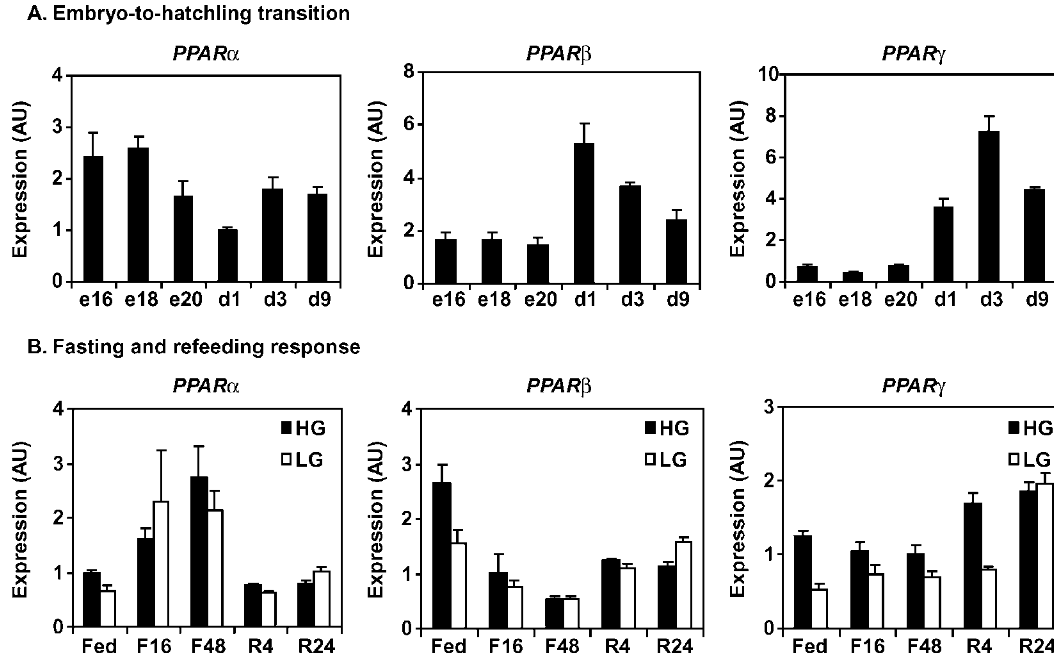


Figure 4. Expression of peroxisome proliferator-activated receptor (*PPAR*) genes in liver during the embryo-to-hatchling transition (A) and the fasting-refeeding response (B). The abundance of *PPARα*, *PPARβ*, and *PPARγ* transcripts were determined by quantitative reverse transcription-PCR (qRT-PCR) analysis by using gene-specific primers and SYBR Green chemistry. Gene expression levels, calculated by the delta-delta threshold crossing point method, are presented in arbitrary units (AU). Each bar represents the average of 4 birds. For the embryo-to-hatchling transition study, liver samples were collected from embryos at embryonic day (e)16, e18, and e20 and hatchlings at posthatch day (d)1, d3, and d9. For the fasting and refeeding experiment (B), 6-wk-old high-growth (HG) or low-growth (LG) cockerels were assigned to 5 different nutritional states: fully fed (fed), fasted for 16 h (F16), fasted for 48 h (F48), refed for 4 h (R4), or refed for 24 h (R24). Four birds from each genotype were killed under each nutritional state for collection of liver samples. Total RNA was isolated using Qiagen Midi-RNeasy kits for microarray and qRT-PCR analyses.

tional gene clusters were down-regulated with fasting, whereas after refeeding, the expression of these hepatic genes was increased. One cluster contained *FASN*, which was depressed by prolonged fasting and sharply up-regulated during refeeding. The transcriptional response of *FASN* to abrupt changes in feed intake was confirmed by independent qRT-PCR analysis and is consistent with an earlier report on transcriptional control of *FASN* (Back et al., 1986). In contrast, some genes followed an opposite pattern, with an increase during starvation and a decrease during refeeding; L-lactate dehydrogenase-*β* (*LDHβ*) illustrates this pattern of feeding-induced repression. Adipophilin or *ADRP* is a marker of fat accumulation that belongs to a gene cluster induced by refeeding. The peroxisome proliferator-activated receptors (**PPAR**) belong to a family of ligand-activated transcription factors that control key metabolic pathways (i.e., adipogenesis, fat metabolism, and insulin signaling; Lee et al., 2003a). Transcriptional profiling in the liver of chickens clearly shows that the PPAR respond to abrupt changes in metabolism during the embryo-hatchling transition and the fasting-refeeding response (Figure 4). In the perihatch chick, expression of *PPARβ* and *PPARγ* transcripts was highest in the liver after hatching, whereas *PPARα* levels were lowest in day-old hatchlings (Figure 4A). Hepatic *PPARα* mRNA levels increased sharply in fasting chickens, which reflects an increase in catabolism of stored fat (Figure 4B). In contrast, hepatic expression of *PPARγ* increases after hatching and refeeding, which suggests that this transcription factor supports lipogenesis in the chicken. When compared with the HG chickens, hepatic expression of *PPARγ* was lower in the LG line, except at 24 h after refeeding. The abundance of *PPARβ* declined in 6-wk-old HG and LG chickens during prolonged fasting but returned to normal following refeeding. The PPAR play a central role in transcriptional control of energy balance via their activation by lipid ligands, subsequent interaction with coactivators or corepressors, and binding of these heterodimers to PPAR response elements in the promoter of numerous metabolic enzymes (Feige and Auwerx, 2007).

A large number of hepatic genes showed differences in mRNA levels between the 2 genotypes (HG vs. LG) in at least one of the metabolic states. Most of the differences between genotypes were apparent in the fed (44 genes) or refed state at 24 h (308 genes). However, the genes that showed a consistent difference between the 2 genotypes were less numerous than those that responded to nutritional state. In the HG genotype, only 3 genes [transketolase (*TKT*), methionyl-tRNA formyltranferase (*MTFMT*), and aminopeptidase (*ANPEP*)] were consistently expressed at higher levels, whereas 2 genes [XAP-5 (*XAP5*) and ribosomal protein S27 (*RPS27*)] were consistently expressed at lower levels. Hepatic insulin-like growth factor-1 (*IGF-I*) mRNA levels were higher in HG than in LG, as previously reported (Beccavin et al., 2001). Three genes involved in the control of lipid metabolism [*ADRP*, glutathione S-transferase A1 (*GSTA1*), and

Table 2. Differentially expressed genes in liver of 4 divergent broiler lines[1]

Line	Age (wk)					
	1	3	5	7[2]	9	11
↑ HG	31	5	12	199	36	12
↑ LG	22	10	32	176	17	13
↑ FL	11	20	21	137	107	86
↑ LL	9	14	26	263	104	107

[1]These genes showed a significant line or line × age interaction and a false discovery rate (FDR) of ≤0.20. The values represent up-regulated genes. Abbreviations: HG = high growth; LG = low growth; FL = fat line; LL = lean line.

[2]Indicates the large number of differentially expressed genes at 7 wk of age.

PPARγ] were higher in the HG than in the LG genotype in at least one nutritional condition. This is consistent with the higher percentage of abdominal fat observed in the HG compared with the LG chickens. In particular, the relative abdominal fat weight (percentage of BW) of HG birds was about 6-fold greater than that in the LG at 6 wk.

In a more extensive study, hepatic gene expression profiles were examined in the HG and LG chickens during juvenile development (1 to 11 wk) with the Del-Mar 14K Integrated Systems cDNA microarray (Cogburn et al., 2004). Surprisingly, the most highly expressed gene in the liver of the LG line, relative to the HG line, across all ages was an endogenous retrovirus related to the *ALV* envelope protein (*PR57*). This time-course study revealed 532 hepatic genes that showed a significant interaction between genotype and age (Table 2). The largest number of differentially expressed genes was found at 7 wk of age, when 199 hepatic genes were up-regulated in the HG line and 176 genes were up-regulated in the LG line. A large number of up-regulated genes found in the liver of HG birds at 7 wk are involved in the synthesis, transport, and metabolism of lipids, which supports their phenotype of higher abdominal fat content (Table 2). The differential expression (higher in HG birds) of an original candidate gene, *THRSP*, was confirmed by qRT-PCR analysis. Thyroid hormone-responsive Spot 14-α is an important transcription factor that controls expression of several lipogenic genes (Towle et al., 1997). Furthermore, Cogburn and colleagues have identified insertion-deletion polymorphisms in chicken *THRSPα* that are associated with QTL for abdominal fatness on chicken chromosome 1 (*GGA1*; Wang et al., 2004). The "leaner" LG line shows higher expression of several genes involved in energy metabolism, signal transduction, and hematopoiesis.

A large number of differentially expressed genes were also found in breast muscle (pectoralis major) between the HG and LG lines (Jenkins et al., 2006), although the differences were of low amplitude. Among 3,000 genes that were differentially expressed between the 2 genotypes at 1 of 6 stages between 1 and 11 wk of age, only approximately 80 showed a consistent difference (>1.2-fold) across at least 2 ages. The gene with the largest difference encoded a retroviral envelope protein (*PR57*) already observed in liver samples, which was largely overexpressed in LG and likely results from the activity of an endogenous retroviral locus. Several genes encoding enzymes involved in the glycolytic pathway and in the oxidative phosphorylation pathway were also within this list, together with genes of yet unknown function.

Some differentially expressed genes in breast muscle are involved in muscle hypertrophy [integrin-β1 (*ITGB1*) and glycogen synthase kinase-3β (*GSK3β*)]. Other genes associated with metabolic pathways involved in muscle growth include protein phosphatase 2A-α (*PPP2R1A*), TRAF4-associated factor 2 (*TRAF4AF2*), and prohibitin (*PHB*). Additional differentially expressed genes include regulators of protein catabolism such as cullin 2 (*CUL2*) and ubiquitin-conjugating enzyme E2D 3 isoform 1 (*UBE2D3*). Another differentially expressed gene, annexin-V (*ANXA5*), is a marker of muscle growth and is regulated by a selection for growth potential in cattle (Sudre et al., 2003). Additionally, several unknown genes are differentially expressed in breast muscle between the HG and LG lines.

The FL and LL chickens, introduced earlier, represent unique models available to identify genes in metabolic pathways that contribute to excessive fatness or leanness. In agreement with the fat phenotype, genes coding for several lipogenic enzymes [adenosine triphosphate citrate lyase (*ACLY*), acetyl-CoA carboxylase (*ACC*), *FASN*, *ME1*, and *SCD1*] were also found to be more abundant in the liver of FL chickens (Assaf et al., 2004). Surprisingly, sterol response element-binding protein 1 (*SREBP1*), a transcription factor that governs the expression of these lipogenic enzymes, was expressed at similar levels in the liver of FL and LL (Assaf et al., 2003).

Gene expression in the liver of FL and LL chickens was recently examined with a low-density "focused" microarray of 323 cDNA (Bourneuf et al., 2006). The spotted chicken cDNA represent genes involved in or related to carbohydrate and lipid metabolism, including some signaling and transcription factors and 195 cDNA previously identified by differential mRNA display analysis in the liver of FL and LL birds (Carré et al., 2001, 2002). Hepatic expression of several enzymes involved in lipogenesis [*ACC*, *FASN*, *SCD1*, apolipoprotein A1 (*APOA1*), *SREBP1*, and mitochondrial malate dehydrogenase 2 (*MDH2*)] were overexpressed in the genetically fat chickens. In contrast, 10 genes were down-regulated in the liver of FL chickens [peroxisomal 2,4-dienoyl CoA reductase

(*DCER2*), activating transcription factor 4 (*ATF4*), cyclic adenosine monophosphate-response element-binding protein 2 (*C/EBP2*), pyruvate carboxylase (*PC*), α-amylase (*AMY1A*), cytochrome B (*CYTB*), ras homolog gene family member F (*RHOF*), Ran GTPase-activating protein 1 (*RANGAP1*)], as were other transcripts that belong to the cytochrome P450 family. The differential expression between the FL and LL lines of most genes identified by the low-density arrays was confirmed by qRT-PCR analysis.

Recently, the developmental profiles (1 to 11 wk) of hepatic gene expression in the FL and LL chickens were examined with the high-density Del-Mar 14K Integrated Systems microarray. Across 6 ages, 1,805 hepatic genes were differentially expressed in the FL and LL chickens (Table 2). Similar to the HG and LG lines, most of the differentially expressed hepatic genes were found at 7 wk of age, well after the phenotypic differences are established. Sixteen genes involved in mitogen-activated protein kinase signaling were differentially expressed between the FL and LL at 7 wk, when 12 genes were upregulated in the liver of FL birds and only 4 genes were expressed at higher levels in the LL. Gene network analysis with Pathway Miner software (Pandey et al., 2004) showed that 9 genes in the wingless signaling pathway and 7 genes in the phosphatidyl inositol signaling pathway were differentially expressed in the liver of FL and LL birds. This gene association network of cellular and regulatory pathways showed a high representation of genes in several important signaling systems [mitogen-activated protein kinase (16 members), inositol trisphosphate (7 members), wingless (9 members), transforming growth factor-β (9 members), and Toll-like receptor (**TLR**; 6 members)], in addition to apoptosis and integrin-mediated cell adhesion pathways (see the gene network in Figure 1).

An analysis of adipose tissue from an egg-laying breed and a "fat" grandsire broiler breed at 10 wk of age with a 9K chicken cDNA microarray has identified 67 differentially expressed transcripts, although only 42 EST correspond to known genes (Wang et al., 2006). This undefined chicken 9K cDNA microarray was obtained from the Beijing Genomics Institute. Surprisingly, only 3 of the 42 differentially expressed genes are directly related to lipid metabolism [*APOA1*, lipoprotein lipase (*LPL*), and leptin receptor gene-related protein (*LEPR-GRP*)]. Nonetheless, this is the first paper published on a microarray analysis of abdominal fat tissue in the chicken. Perhaps transcriptional profiling of adipose tissue in the FL and LL chickens during juvenile development would afford a higher resolution of the genes and metabolic pathways controlling excessive fattening in the broiler chicken.

Thus, divergently selected chickens represent a unique model for identification of genes that control important production traits. As a whole, transcriptome studies should provide a better understanding of the genes and their regulatory networks that control growth, tissue development, and ultimately body composition. The intensive genetic selection (almost exclusively for growth) applied to broiler chickens for many years could have isolated a relatively limited number of general mechanisms or pathways. In contrast, 176 cases of obesity in humans result from single mutations in only 11 genes. In genome-wide scans, the number of QTL for obesity-related phenotypes in humans continues to increase. In the 2006 Obesity Map update, 253 QTL involved in the development of obesity in humans were identified from 61 scans (Rankinen et al., 2006). It is of particular interest that these putative obesity loci are found on all human chromosomes except chromosome Y (*HSAY*).

Immune System. Neiman and collaborators (2001) were the first to develop a chicken immune system array. This immune array, containing 2,200 elements, was used to analyze *myc*-oncogene-induced lymphomagenesis in the chicken bursa of Fabricius. Genes whose expression levels correlated with *myc* expression in transformed follicles and metastatic tumors were identified, including genes involved in nucleolar function, ribosome biogenesis, and protein synthesis. Subsequently, this immune array was expanded to 3,451 cDNA and used to compare the transcriptional signature of chick bursal lymphomas resulting from ALV insertional mutation of c-*myb* vs. transformation by v-*Rel* (Neiman et al., 2003). These arrays were also used to identify genes regulated by the v-*jun* oncogene in chick embryo fibroblasts (Black et al., 2004), and a pattern of expression was observed that is strikingly similar to the one produced by the Marek's disease virus (**MDV**) *meq* oncogene (Levy et al., 2005). Another immune system array was used to study the host response to infection with MDV (Morgan et al., 2001) and herpes virus of turkeys infection (Karaca et al., 2004), and to catalog gene expression in the developing chick thymus (Cui et al., 2004). As expected, many of the genes identified in the viral studies responded to interferon. A number of differences worthy of additional study were also detected; these could contribute to the pathology of MDV or the vaccination response to herpes virus of turkeys. Collectively, these studies have established common mechanisms for the transformation of chicken cells and point to differences that are characteristic of individual pathogens.

In a follow-up study on *c-myc*-induced tumorigenesis, Neiman and colleagues used the 13K chicken cDNA microarray (Burnside et al., 2005), which is enriched for chicken immune system EST, for comparative genomic hybridization (Neiman et al., 2006). Gene amplification and chromosomal instability were detected in *myc*-transformed bursal follicles and lymphomas and were mapped by using the arrays. These data established the relationship between a copy number change and RNA expression patterns. The study showed that cDNA microarrays are useful for determining both gene expression and gene copy number.

An avian macrophage-specific array containing nearly 5,000 genes expressed in peripheral blood lymphocytes has been used to examine the transcriptional response of macrophages to gram-negative bacteria in comparison with the response to lipopolysaccharide (Bliss et al., 2005).

Bacteria elicit a more complex response, and there is common signaling through TLR 4, although additional pathways are activated by whole bacteria. This avian innate immune microarray has been used in experiments with several avian cell or tissue types as well as a variety of pathogens (Bliss et al., 2005; Dalloul et al., 2007). Changes in expression of innate immune genes have been observed in vivo (with intestinal epithelial and splenic tissues) and in vitro [with peripheral blood monocytes, heterophils, nonadherent blood lymphocytes, and avian macrophage cell lines (HD11, HTC)]. In addition, innate immune responses have been elucidated after stimulation with bacteria (*Salmonella*, *E. coli*, and *Mycoplasma*), viruses (avian influenza), intestinal parasites (*Eimeria*), bacterial components (lipopolysaccharide), and immune modulators (interferon-γ). Another avian immune system array has been used to evaluate the host response to different respiratory pathogens (Munir and Kapur, 2003; Dar et al., 2005). As would be expected, infection of chickens with respiratory viruses leads to a marked increase in expression of genes related to interferon activation and inflammatory and protein trafficking. A microarray analysis of chicken intestinal lymphocyte genes induced or repressed in response to infection with *Eimeria* has also been reported (Min et al., 2003). Infection of chickens with *Eimeria* parasites stimulates transcription of interferon-γ, interleukin-15, and several cytokines in intestinal intraepithelial lymphocytes. The transcriptional responses of chickens to challenge with 2 important enteric pathogens (*Eimeria* and *Salmonella*) have been reviewed in detail (Lillehoj et al., 2007).

Microarrays have a potential use in identifying candidate genes for desired traits. Liu et al. (2001a) used microarrays to identify differentially expressed genes in MDV-resistant lines of birds, and they successfully integrated gene expression with genetic mapping data to identify functional candidate genes for disease resistance (see the expression QTL or "genetical genomics" section below). In another application of microarray technology, Degen et al. (2003) used microarrays to identify host-derived natural adjuvants. Enhancement of the immune response is a major issue in vaccine development, and the use of natural adjuvants is more desirable than commonly used chemical adjuvants. Global gene expression profiling can be used not only to identify candidate genes, but also to evaluate their effectiveness.

An immune system array has been used to study the response to infection with infectious bursal disease virus and also to identify differences in gene expression between resistant and susceptible lines (Ruby et al., 2006). Genes involved in the inflammatory response were induced in both lines; however, differences between the 2 lines were observed and a model for resistance was established in which a more rapid and robust inflammatory response serves to limit infection and pathology. Using microarrays, van Hemert et al. (2006) found differences in gene expression in *Salmonella*-resistant and *Salmonella*-susceptible lines as well. In response to *Salmonella* infection, the resistant, fast-growing chicken broiler line induced genes that affected T-cell activation, whereas in the more susceptible, slow-growing broiler line, genes involved in macrophage activation seemed to be more affected at d 1 postinfection. These studies point to the value of microarrays in identifying genes associated with disease-resistance phenotypes. Once verified, the candidate gene(s) could be the goal of marker-assisted selection.

A 5K chicken immuno-microarray developed by ARK Genomics (Roslin Institute) has been used to study the immune response to vaccination with avian influenza and provides insight into virus-host interaction (Degen et al., 2006). As expected, genes associated with a strong immune response were highly elevated in infected, naive birds compared with immunized birds. This study also identified genes affected by an immune adjuvant, demonstrating another utility of microarrays in evaluating adjuvant influence.

Cellular and Gene Networks

The advent of cDNA microarrays for global gene expression analysis (Schena et al., 1995, 1996) created the need to interpret vast data sets and to organize genes by their temporal expression patterns. Hierarchical clustering (Eisen et al., 1998) and SOM (Tamayo et al., 1999; Toronen et al., 1999) were developed to visualize and understand gene expression patterns. The assumption is that genes with a similar function cluster together, presumably due to common transcriptional regulation, and they usually belong to similar metabolic or regulatory pathways. The features and limitations of these early, unsupervised clustering methods have been reevaluated to provide more informed choices for potential users (Yin et al., 2006).

Perhaps the greatest challenge of functional genomics has been to extract useful information on genetic interactions from large data sets (van Someren et al., 2002). Detection of the genetic interactions that determine phenotype, which themselves are related to protein and metabolite interactions, requires a mixture of computational and experimental approaches (Carter, 2005). Several early papers (Wagner, 2001; Brazhnik et al., 2002; de la Fuente et al., 2002) introduced the concepts and mathematical methods for reconstructing gene networks from gene expression profiles. Some biologists believe that reconstructing gene networks from genetic perturbation experiments represents the "holy grail of functional genomics" (Wagner, 2001). The major requirement for gene network analysis is the systematic perturbation of each gene in a network or pathway to determine the interaction of each gene with other members (Brazhnik et al., 2002; de la Fuente et al., 2002). The strengths and direction (positive or negative) of gene interactions are determined by perturbing the rate of transcription, one gene at a time. Although gene-by-gene perturbations are easily accomplished in simple organisms (e.g., yeast), genetic interactions in higher organisms (e.g., birds and mammals) are more difficult to demonstrate. The perturbation approach

for identifying gene networks involves the integration of computational models with experimental perturbations and global (or genome-wide) measurements of the responses of the biological system (Tegner and Bjorkegren, 2007). The architecture and dynamics of gene networks are best revealed when prior knowledge of the biological system is incorporated into the inference algorithm. Similarly, pathway analysis focuses on identifying a defined set of biochemical reactions (i.e., metabolism, apoptosis, or growth factor signaling; Klamt and Stelling, 2003; Papin et al., 2003). Metabolic pathway analyses reveal complex biochemical-reaction networks, which ultimately define biological systems. A recent review has described several popular bioinformatic methods and Web-based resources used for incorporating genome-wide transcriptional data into metabolic, cellular, and regulatory networks or pathways (Cavalieri and De Filippo, 2005).

Mapping of gene and regulatory networks requires high-throughput analysis of transcriptional scans, clustering of coregulated genes, and computational searches for functional motifs [i.e., *cis*-regulatory elements and transcription factor (**TF**) binding sites (**TFBS**); Banerjee and Zhang, 2002]. Multiple transcriptional snapshots taken in a time series provide a dynamic dimension of gene expression (Hoffman et al., 2003). Bayesian modeling seems best suited for reconstruction of gene networks from global expression data (van Someren et al., 2002). More recent gene network models integrate meta-analyses of gene expression profiles from different studies and multiple microarray platforms in humans and mice (Kyoon Choi et al., 2004; Hackl et al., 2005; Stahlberg et al., 2005; Stathopoulos and Levine, 2005; Estrada et al., 2006; Mulligan et al., 2006). Rigorous interrogation of the *cis*-regulatory regions and GO annotation of genes provides a more comprehensive view of genetic control over metabolic and developmental processes. Large-scale transcriptional profiling and refined bioinformatics analyses have revealed exquisite detail of the biological processes and molecular networks involved in transcriptional regulation of fat cell development (Hackl et al., 2005). A systems biology approach that integrates gene expression, QTL analysis, and modular gene network modeling in a segregating population affords the greatest power in detecting major genes controlling complex traits (Ghazalpour et al., 2006). This novel approach, called modular QTL analysis, combines expression QTL (**eQTL**) analysis and gene coexpression networks to identify key regulatory loci that control expression of phenotypic traits.

A powerful new computational approach for detecting network motifs in coexpressed gene networks uses graph theory to extract groups of highly interconnected transcripts (cliques) from genetic correlation matrices derived from high-throughput transcriptional scans (Baldwin et al., 2005; Chesler et al., 2005). Genes with similar expression patterns are clustered together, presumably under a common transcriptional control mechanism. Correlations are the most prevalent measure of coexpression and allow construction of a "graph" with genes forming vertexes connected with strength equal to the correlation coefficient. Cliques are completely intercorrelated groups of genes, an ideal definition for a cluster of potentially functionally related and commonly regulated genes (Voy et al., 2006). Tightly connected regions of the correlation graph represent subsets of genes with strong correlations among members, and thus are likely to represent biologically significant interactions. Another component of graph structure exploits the likelihood that coregulated genes share some common TFBS (Allocco et al., 2004). Genes linked by physical interactions in a network, such as TF-gene interactions, have strongly correlated expression levels (Ideker et al., 2001).

Only a few attempts have been made to apply gene network modeling in the chicken, although the importance of gene-gene interactions in determining phenotype has been clearly demonstrated (Carlborg et al., 2003, 2006). Two strong metabolic perturbations—the embryo-to-hatching transition (Glass et al., 2002) and the fasting and refeeding response (Duclos et al., 2004)—have been used to take time-series transcriptional snapshots of the chicken liver. A dynamic Bayesian model for analysis of microarray data and a spanning tree clustering method (Rejto and Tusnady, 2006) have been developed for mapping "functional" clusters of genes that respond to these metabolic perturbations. Some of the metabolic enzymes and transcription factors identified by gene cluster analysis in the liver of the perihatch chick (Cogburn et al., 2003c, 2004) or fasting and refed chickens (Duclos et al., 2004) have been integrated into a working model of transcriptional control of the tricarboxylic acid cycle and fat biosynthesis pathway (Cogburn et al., 2004). Thus, time-series perturbation studies and gene cluster analysis provide powerful methods for revealing the major topography of gene networks that control metabolic pathways in the chicken. Identification of conserved motifs (i.e., TFBS) in the promoter region of coexpressed genes should enhance the identification of genetic regulatory loci that control important production traits in poultry.

Expression QTL or "Genetical Genomics"

Several techniques have been developed to help assess gene function on a genome-wide scale. The most common method is to monitor gene expression levels with cDNA microarrays, whereby genes found to be differentially expressed may contribute to the trait being examined. Proteomics with mass spectrometry provides essentially the same type of information except at the protein level. With a growing number of genomic techniques, it is not surprising to find that 2 or more high-throughput methods have been integrated to harness more power and information. This section reviews attempts to merge transcriptional profiling with traditional QTL analysis to reveal functional genes controlling expression of important phenotypic traits.

An early example was the identification of positional candidate genes for Marek's disease (**MD**) resistance QTL (Liu et al., 2001a). Fourteen QTL for MD resistance were

identified by using an F_2 cross between inbred experimental lines that were relatively resistant or susceptible to MD, a virus-induced lymphoma of chickens (Vallejo et al., 1998; Yonash et al., 1999). Because it is extremely difficult to find the causative gene(s) for each QTL, it was hypothesized that resistance to MD could be accounted for by differences in gene expression. In other words, a positional candidate gene for MD resistance QTL would be one that is within a QTL and is differentially expressed between the resistant and susceptible parental lines following challenge with the MDV. Because this work was done prior to release of the chicken genome sequence, each differentially expressed gene had to be mapped to determine its genomic location. Despite monitoring only 1,200 cDNA spotted on nylon membrane arrays and mapping 15 candidates on the unassembled genome sequence, Liu et al. (2001b) identified a single MD resistance gene, *GH*, and several other promising candidate genes with this approach. With the current availability of an assembled genome sequence and genome-wide DNA microarrays, this approach could be more powerful, simpler to implement, and used for any quantitative trait (Wayne and McIntyre, 2002).

Thus, genetical genomics or eQTL is simply the marriage of traditional linkage analysis and global transcript profiling with cDNA microarrays (de Koning et al., 2005, 2007; Haley and de Koning, 2006). Initially conceived by Jansen and Nap (2001), gene expression, as measured by transcript abundance, is considered as another quantitative trait or phenotype and, in combination with genetic markers spaced throughout the genome, QTL are revealed that account for variation in gene expression. The result is that QTL can be in either *cis* or *trans* with respect to the gene of interest. The simplest interpretation for *cis*-acting QTL is that sequences flanking the gene (e.g., the promoter region) regulate gene expression or transcript stability. On the other hand, *trans*-acting QTL are thought to involve transcription factors or other modulators. Genomic regions with a high proportion of eQTL could represent areas with genes that have common transcriptional regulators, which control important biological pathways. The ability to identify *trans*-acting loci is particularly attractive because it is difficult to identify expression regulators even with a complete genome sequence.

The first study to implement this approach was performed by using yeast (Brem et al., 2002), with additional reports with more information appearing soon afterward (Yvert et al., 2003; Brem and Kruglyak, 2005). Similar eQTL analyses have been done in rodents (Schadt et al., 2003; Chesler et al., 2005; Hubner et al., 2005; Lan et al., 2006), human cells or tissues (Cheung et al., 2003; Schadt et al., 2003; Monks et al., 2004; Morley et al., 2004; Bystrykh et al., 2005), and plants (Schadt et al., 2003; Kirst et al., 2004). For the most part, the results are similar to those found in yeast. Because expression variation is heritable, it is possible to identify eQTL, although many transcripts do not have QTL, in which about one-third of the eQTL act in *cis*, and eQTL typically account for 25 to 50% of the variation, which suggests that transcription is complex.

Despite success in other species, some caution should be exercised before implementing eQTL analysis in poultry. First, like most gene expression experiments, only one or a few tissues and time points are monitored in an individual. Therefore, this gives a very limited snapshot of the complete transcriptome; consequently, conclusions must be interpreted with this in mind. Second, significant challenges exist with regard to how to properly analyze the data (Gibson and Weir, 2005). Current methods are limited in their ability to handle nonadditive, epistatic, or other complex gene effects. This is likely to be one of the main reasons why only a subset of genes will have an eQTL. Third, compounding the analysis issue are experimental designs that do not have sufficient statistical power. However, Rosa et al. (2006) recently provided an excellent review of experimental design strategies for using microarrays in genetical genomics studies. It is widely recognized that gene expression measurements are subject to "noise," but without biological and technical replicates, one cannot determine the source of the problem. Without sufficient statistical power, only genes with large expression variation can be mapped. Related to this limited statistical power is the ability to determine whether *cis*-acting QTL are truly *cis*, where gene expression is regulated by the gene sequence itself, or the inability to separate out *trans*-acting eQTL, which are closely linked. Finally, a large eQTL project with a large number of genotyping and microarray assays represent an expensive venture that is beyond the reach of most laboratories. Although new and improved technologies with lower costs per data point are available, the cost of genotyping and genome-wide transcriptional scans needs to be lower for implementation in domestic animals. de Koning and colleagues (2007) have outlined a more focused approach for implementation of genetical genomics in chickens. The integration of fine mapping of putative (or marked) QTL and transcriptional analysis of known candidate genes should enhance detection of *cis*-acting eQTL in the targeted region.

Expression Proteomics

Proteomics is a new discipline in functional genomics that involves global study of proteins expressed in cells, tissues, or body fluids. An introduction to proteomics and its application to functional genomics of the chicken immune system was published earlier (Burgess, 2004). As an essential tool for understanding avian systems biology, proteomics is rapidly increasing our knowledge of proteins and their dynamic interactions across all aspects of avian biology, including host responses to infectious diseases (Liu and Hicks, 2007). "Expression proteomics" is similar to transcriptional analysis because it is high-throughput profiling, which is dependent on a well-annotated genome sequence and multiple supporting technologies (i.e., computational analysis, bioinformatics, and pathway modeling). The expressed proteome is defined by all proteins existing in an organism throughout its life cycle or, on a smaller scale, all proteins found in a

particular cell type under a particular condition, or all proteins in a subcellular organelle (e.g., the mitochondria), or even, in some cases, all proteins in a particular complex (e.g., the ribosome). Because of alternative splicing and regulatory RNA, transcriptomics is more complicated than structural genomics, and expression proteomics is more complicated than transcriptomics. The expressed proteome is constantly changing through its biochemical interactions and posttranslational modifications (acylation, acetylation, glycosylation, phosphorylation on Tyr or Ser-Thr residues or both), which greatly increase the diversity of the proteome and modulate subcellular localizations and protein-protein interactions. The expressed proteome interacts with the genome, the genome of other organisms, and the environment. Proteins also have a complex pattern of localization, which changes in response to stimuli. Such changes in localization are critical to defining protein function and the expressed proteome. Organisms have radically different proteomes in different life stages and in different environmental conditions. One of the most valuable uses for expression proteomics is in biomarker discovery, especially from blood products. Importantly, the proteome in a production animal (e.g., the chicken) is a major component of food consumed by humans (Hayter et al., 2005).

Expression proteomics became possible in the chicken with the release of the chicken genome sequence (International Chicken Genome Sequencing Consortium, 2004). A sequenced and well-annotated genome is fundamental for protein identification in high-throughput screens. As in other functional genomics disciplines, one of the biggest challenges in proteomics is the meaningful analysis of large data sets. With the possible exception of biomarker discovery, large "laundry lists" of proteins have limited value for solving biological problems. Fortunately, the GO was created to organize, model, and understand the biological function of gene products identified by global transcriptional and proteomic profiling (Ashburner et al., 2000).

Beynon and colleagues are considered pioneers in the development of proteomic methods and original investigations of protein dynamics in the chicken muscle (Flannery and Beynon, 1991; Cookson et al., 1992; Flannery et al., 1992). While investigating protein turnover (i.e., synthesis and degradation rates), Beynon's group developed novel methods for absolute quantification in proteomics (Beynon, 2005; Beynon et al., 2005; Doherty et al., 2005) and even less expensive approaches (Hayter et al., 2003). Just as protein turnover is critical to cell physiology, so is protein location; movement from one place to another is critical for cell function and often defines a critical event (i.e., cell death or immune responses). Another group has focused on proteome-scale methods to localize known proteins and then predict the localization of unknown proteins (McCarthy et al., 2005, 2006b; van den Berg et al., 2006). This new work on chicken proteomics is providing a rapid means of functionally annotating proteins in the GO cellular component category (McCarthy et al., 2006a, 2007).

Proteomics is beginning to increase our understanding of how pathogens function in the chicken and eventually will contribute to the design of novel interventions for their control. For example, de Venevelles and colleagues (2004) have begun to build a reference map of *Eimeria tenella* sporozoite proteins. This group identified 16 known and 12 novel proteins in the *E. tenella* sporozoite. Furthermore, *E. tenella* sporozoite proteins were examined as potential targets for their immunogenicity, and approximately 50 proteins were defined as potential antigens. Notably, abundance and immunogenicity are not related in the sporozoite stage. Nanduri and colleagues (2005, 2006) have examined the controversial area of feed additive antibiotics in animals and how these could affect *Pasteurella multocida*, the causative agent of fowl cholera and a human zoonosis. Using an in vitro model of subminimum inhibitory concentrations of antibiotics (amoxicillin, chlortetracycline, and enrofloxacin) and isotope-coded affinity tags, Tatusov et al. (1997) applied proteomics to identify parallel effects on the inhibition of growth kinetics and the suppression of protein expression by "clusters of orthologous group" categories. Potential compensatory mechanisms enabling antibiotic adaptation were identified, which could provide novel therapeutic targets. Perhaps most significantly, this work provides fundamental molecular evidence of how subminimum inhibitory concentrations of antibiotics used as feed additives could inhibit a pathogen.

The chick embryo has been used as a comparative model for the proteomic study of cerebrospinal fluid (**CSF**) and brain disease (Parada et al., 2006a,b). During the early stages of embryogenesis, the CSF plays an essential role in neuroectoderm survival, proliferation, and neurogenesis. In a comparative study of the rat and chicken embryonic CSF proteome, Parada et al. (2006b) found that rat CSF had greater apolipoprotein and a more complex enzyme pattern than chicken CSF. This difference could be related to the greater neural complexity and synaptic plasticity in mammals. Furthermore, 14 of these proteins exist in the adult human CSF proteome and are altered in neurodegenerative diseases or neurological disorders. Proteomics has been used to study the molecular mechanisms of normal and abnormal embryogenesis and to construct a whole-embryo proteome map for the chicken (Agudo et al., 2005). A proteome map of gonadal primordial germ cells from chicken embryos was established as a basis for understanding the mechanism of embryonic germ cell development (Han et al., 2005). This chicken gonadal primordial germ cell proteome map will serve as a reference for germ cell biology and transgenic research. Proteomic profiling of facial development in the chick embryo has been used as a model for studying craniofacial birth defects in humans (Mangum et al., 2005). A microsample proteomics strategy was adopted to analyze the first brachial arch, which is an embryonic structure crucial for facial development. This work showed that ~8% of the craniofacial proteome changed between e3 and e5, when 21 proteins were associated with the rapid growth phase. An analysis of the chicken

serum proteome has revealed plasma protein dynamics in single-comb White Leghorn hens at 8, 19 and 23 wk after hatching (Huang et al., 2006). Expression of 10 proteins increased, whereas 3 proteins decreased as the hens aged. Interestingly, some of these proteins are known to be critical for egg production, leading to speculation that the others may also be important in this process. The chicken is also an excellent model for studying developmental regulation of the crystallin gene in the lens of the eye (Wilmarth et al., 2004). This description of the adult chicken lens proteome included novel forms of β-A2 and β-B2 crystalline. The novel form of β-A2 was the most abundant and resulted from translation of a second Kozak consensus sequence, whereas the novel form of β-B2 resulted from alternate splicing of the crystalline mRNA.

The chicken bursa of Fabricius, an essential lymphoid tissue for B-cell development (Glick, 1994), was recently used for whole-organ proteomic analysis (McCarthy et al., 2006b). In total, 5,198 proteins were identified in the bursa of Fabricius and classified as B-cell specific (1,753), stroma specific (1,972), and common (1,473) to both classes. These proteins enabled the modeling of differentiation, proliferation, transcriptional activation pathways, and programmed cell death. This work identified 114 TF and, of these, 42 TF had not been identified previously in B cells from any species, which completely changed the transcription factor geography for B cells. Hematopoietic prostaglandin D2 synthase (**PGDS**) was recently identified in a proteomic study of another chicken lymphoid tissue, the Harderian gland (Scott et al., 2005). In humans, PGDS is preferentially expressed in human T helper (**Th**)2, but not Th1, clones (Tanaka et al., 2000). This work suggests that, at the sites of antigen presentation, at least part of the Th2 cell population produces prostaglandin D2, which could be involved in Th2-related immunity in humans. In the chicken, Newcastle disease virus-infectious bronchitis virus vaccination via the ocular route increased hematopoietic PGDS synthesis in the Harderian gland. Notably, immunity to both Newcastle disease virus and infectious bronchitis virus relies on antibody responses, which are driven by Th2 cells.

Signaling in the immune system, as in other systems, relies heavily on reversible phosphorylation, and direct protein analysis is the only way to identify these post-translational modifications. The DT40 B-cell line was engineered to express the "spleen Tyr kinase" (**SYK**) gene (*SYK*) to identify SYK substrates and, in particular, their roles in the nucleus (Zolodz et al., 2004). Spleen Tyr kinase is a protein-Tyr kinase that is widely expressed in hematopoietic cells and is involved in coupling-activated immunoreceptors to downstream signaling events that mediate diverse cellular responses, including proliferation, differentiation, and phagocytosis. In this work, DT40 cells were treated with pervanadate, a potent protein-Tyr phosphatase inhibitor that nonselectively activates SYK. The aim was to detect and identify cytoplasmic and nuclear SYK substrates. Tyrosine-phosphorylated proteins were immunopreciptated by using an antiphosphotyrosine antibody, and the bound proteins were eluted and then analyzed by tandem mass spectrometry. Several known substrates and some candidate substrates for SYK were identified, along with the location of 22 Tyr phosphorylation sites. Proteomics has recently been applied to neoplastic transformation within the immune system to help to classify MD, an important production disease caused by MDV, as a unique in vivo animal model for human Hodgkin's and many non-Hodgkin's lymphomas that overexpress the Hodgkin's disease antigen (**CD30**; Burgess et al., 2004). Plasma proteomics confirms that, just as in the human diseases, a soluble form of CD30 is detectable in chickens with CD30-overexpressing lymphomas. Another group has used a mass spectrometry-based proteomics approach to identify viral proteins expressed in host cells (chick embryo fibroblasts) after infection with MDV (Liu et al., 2006). Proteomic analysis of tryptic digests from MDV-infected chick embryo fibroblast lysates revealed 86 MDV proteins expressed in the infected chicken fibroblasts, which has also helped to annotate the MDV genome.

Muscle proteomics has obvious potential applications for the safe and efficient production of poultry meat and eggs. Expression of soluble proteins was examined in the pectoralis muscle during growth in layer chickens by using stable isotope-labeled Val (Doherty et al., 2004, 2005). Dramatic changes in relative expression levels of many of the proteins were evident over the 27-d experimental period. Ninety protein spots were identified on 2-dimensional gels, 51 spots were matched to known chicken proteins, 12 spots matched proteins from non-avian species, and 11 spots were unknown proteins. The developmental dynamics of breast muscle growth showed a very high degree of complexity: isoenzyme shifts, association with structural elements, and post-translational modifications. The ubiquitin-proteasome system played a critical role in catabolism of skeletal muscle protein in the chicken (Hayter et al., 2005). Apparently, broiler chickens grow faster than layers because of their higher rates of protein accretion and lower rates of intracellular protein catabolism. This work focused on rates of turnover in the 14 individual subunits of the 20S-core particle. Variability in the subunit synthesis rate indicates that some subunits are produced in excess, whereas others could limit the concentration of 20S subunits in the cell.

Hypothalamic proteomes from high- and low-egg-producing chicken strains were compared to gain a better understanding of neuroendocrine regulation in egg production (Kuo et al., 2005). Eight proteins from 430 well-resolved spots on 2-dimensional gels differed quantitatively between lines divergently selected for high or low egg production. These differentially expressed proteins are involved in regulating gene expression, signal transduction, and lipid metabolism. One protein, heterogeneous nuclear ribonucleoprotein, was proposed as a novel molecular marker for high egg production in slow-growth local (Chinese) chickens. Sufficient water loss from the egg is critical for successful hatching rates, and suboptimal water results in accumulation of a subcutaneous gel-

like fluid in the hatchling chicks. This subcutaneous gel contains only a few highly concentrated proteins and is similar in composition to plasma except for the absence of fibrinogen (McLean et al., 2004).

Because feed and its essential nutrients represent the greatest costs in poultry meat production, Corzo and colleagues (2004a,b, 2005) have adopted a proteomics approach to understanding amino acid requirements. The broiler plasma proteome was mapped in 18-d-old commercial broiler chickens in an attempt to identify biomarkers for amino acid deficiencies (Corzo et al., 2004a,b). Imaging mass spectrometry by matrix-assisted laser desorption/ionization time-of-flight was used to identify unique patterns of protein expression in plasma that could indicate dietary Lys deficiency. Blood plasma from broiler chicks fed an adequate or Lys-deficient diet was subjected to direct matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analyses. Plasma from the chicks fed the Lys-adequate diet had a higher number of differentially expressed protein peaks compared with the Lys-deficient chicks. Corzo et al. (2006) have also directly analyzed muscle tissue to identify potential biomarkers of nutritional deficiency. The effect of dietary Met on breast meat accretion and protein expression in the skeletal muscle of broiler chickens was directly analyzed ex vivo (Corzo et al., 2006). Three proteins from pectoral muscle (pyruvate kinase, myosin alkali light chain-1, ribosomal-protein-L-29) were identified as potential biomarkers of dietary Met deficiency. A combined immunologic and proteomic approach was used to investigate protein oxidation in the pectoral and thigh muscle of the chicken (Stagsted et al., 2004). α-Enolase was the predominant carbonyl-reactive species among the water-soluble muscle proteins, although other proteins (e.g., actin, heat shock protein 70, and creatine kinase) contained carbonyls, 3-nitrotyrosine, or both. This was the first evidence of nitrosylation of specific muscle proteins in poultry meat.

Thus, the chicken also serves as an important model organism for developing novel methods in expression proteomics (Burgess, 2004). Expression proteomics has been applied to study avian diseases and other physiological processes (Liu and Hicks, 2007). Potentially useful biomarkers for poultry production can be identified in the tissue and fluids. Finally, expression proteomics enables "proteogenomic" mapping to improve the structural annotation of both the chicken genome and its pathogens.

Transgenics in Poultry

Definitions and Utility of Transgenic Animals. A transgenic animal is one in which there has been a deliberate modification of its *genome*. Introduction of a foreign gene, termed the "transgene," is transmitted through the germ line; therefore, every cell in the mature animal will carry the transgene. The motives and enthusiasm for developing agriculturally relevant transgenic animals center on the potential to accelerate conventional breeding programs for improvements in desirable production traits. Improved growth characteristics (e.g., muscle deposition, feed efficiency, rate of gain, and body composition) were early goals of transgenic research, and the reality of this approach was well supported by initial reports of the increased growth of mice expressing a *GH* transgene (Palmiter et al., 1982). Other targeted production traits include milk composition, wool production, and disease resistance. However, attempts to apply this technology to production animals have met with limited success: the desired characteristics are frequently improved, but deleterious side effects have limited the commercialization of transgenic animals. Although the technology for generating transgenic animals is well developed for mammals, there is room for improvement, particularly in tissue-specific or developmentally regulated expression. In addition, substantial basic information on the biology of the system to be enhanced or altered is needed to find the ideal target genes for transgenesis.

Technology for the development of transgenic chickens has lagged behind that of mammals (mostly mice), in large part because of the differences in reproductive biology. In mammals, the single-celled oocyte is the starting place for introduction of the transgene, and this ensures that after cell division and embryo formation takes place, all subsequent cells will harbor the introduced gene. Because of the intricate processes involved in egg laying, a single-celled chicken oocyte is not available for manipulation. An egg is certainly readily accessible, but the embryo represents >50,000 cells at this stage. This vast difference has hampered progress until very recently, with the development of viral vectors for the efficient introduction of transgenes and with the establishment of transfectable chicken embryonic stem cell lines. An excellent review of transgenic research in chickens covers developments through 2003 (Mozdziak and Petitte, 2004) and will not be repeated here. Since then, considerable progress has been made by using either retroviruses or transfected embryonic stem cells (**ES**). The improved efficiencies and the ability to generate tissue-specific expression promise to make using transgenic birds a more widely used technique.

Viral Mediated Gene Transfer. Replication-competent and replication-defective retroviral vectors have been used to generate chimeric and transgenic animals for more than 20 yr. Retroviruses are small RNA viruses that, upon infection of dividing cells, are reverse-transcribed into DNA and then integrated into the host genome and passed down from generation to generation. For use in transgenics, nonessential parts of the viral genome are substituted with the gene for transfer, which is subsequently inserted into the host genome along with viral sequences. Retroviruses occur in all classes of vertebrates, and some of the best studied (e.g., Rous sarcoma virus, avian leucosis virus) are well known to chicken biologists.

The use of lentiviral vectors has greatly improved expression of virally introduced transgenes. Lentiviruses belong to a complex retrovirus subfamily that has the ability to incorporate foreign DNA into both dividing and nondividing cells (Naldini et al., 1996) and produce high

titers of viral particles. Lentiviral vectors can accommodate up to 9,000 nucleotides of a foreign gene sequence and produce stable long-term gene expression. Based on high success rates in the production of transgenic mice, lentiviral vectors have been used successfully to create transgenic chickens (McGrew et al., 2004). In this study, an equine infectious anemia lentivirus vector containing the green fluorescent protein (**GFP**) reporter gene was used to infect the embryo, with an efficiency on the order of 100-fold higher than any previously published method. The GFP transgene showed expression in both the G1 and G2 generations (see Figure 1, bottom left). A more recent paper from Sang and colleagues (Lillico et al., 2007) has clearly demonstrated the potential for using transgenic hens to produce large quantities of biologically active pharmaceutical proteins [a humanized monoclonal antibody and interferon (*hIFNβ1a*)] for human therapeutics in egg white. Furthermore, the ovalbumin promoter-driven oviduct-specific expression of these therapeutic proteins does not appear to diminish even after several generations of germ-line transmission. This elegant work has finally paved the way for efficient generation of transgenic chickens and the commercial production of human pharmaceutical proteins in the egg.

The lentivirus system has been refined for tissue-specific expression. Scott and Lois (2005) used human immunodeficiency virus-derived lentiviruses to produce transgenic quail expressing GFP under control of the human synapsin gene I promoter. The transgenic birds express the reporter gene in neurons and have respectable rates of germ-line transmission. The approach should be readily transferable to chickens and is an important advance because it will allow tissue-specific expression of desired transgenes or directed knockout of deleterious genes.

ES. Embryonic stem cells are undifferentiated, pluripotent cells derived from very early, blastocyst-stage embryos. These cells have no predetermined lineage and have the potential to develop into any somatic or germ cell. The key advantages of ES cells are that they can proliferate in cell culture and can integrate foreign DNA at specific, preselected sites, and that cells harboring the introduced DNA can be selected. The ES cells are then injected into early embryos, where they retain totipotency and can develop into all tissues, including germ cells. In that case, the transgene is then transmitted to subsequent generations.

A very significant advance in the application of ES technology to birds focused on the development of chicken ES cell lines (van de Lavoir et al., 2006), which were subsequently applied to develop the chicken as a bioreactor for the production of therapeutic monoclonal antibodies (Zhu et al., 2005). The ES cell lines can be cultured and transfected with plasmid DNA. Introduction of the transfected cells to the subembryonic cavity and culture of the embryo in surrogate shells produced birds with transgene expression in somatic tissues. In a practical application of this approach, the genes encoding a human monoclonal antibody and the regulatory sequences restricting its synthesis to egg white were transfected into ES cells, which were cultured, selected for the transgene, and ultimately introduced into chick embryos. At maturity, chimeric hens lay eggs that contain milligram amounts of antibody, which is purified from the albumin as a human pharmaceutical. This emerging technology opens up new frontiers in poultry science with the potential of using chickens for "pharming" of human proteins.

The recent success in producing transgenic birds now moves the chicken to the forefront for genetic manipulation. We can expect to see research on the introduction of transgenes that will confer disease resistance, reduced phosphate excretion, improved lean body mass, and other desired traits in the near future. In addition, the chicken has great potential for manufacturing human biopharmaceuticals in a very competitive marketplace.

Noncoding RNA and Gene Silencing

MiRNA. Not all expressed genes code for proteins. Animal, plant, and viral genomes encode small, noncoding RNA that regulate gene function by affecting stability or translational efficiency of target mRNA. One class of small RNA, the miRNA, is becoming widely appreciated as a pivotal regulator of gene expression. In animal cells, miRNA appear to repress expression of target genes by translational inhibition or mRNA degradation. The miRNA are derived from primary transcripts that fold into hairpin structures with an imperfect double-stranded (**ds**) characteristic. The primary miRNA transcript (**pri-miRNA**) is processed in the nucleus by the endonuclease Drosha to a precursor miRNA (**pre-miRNA**). The pre-miRNA precursor is then exported to the cytoplasm and further processed by enzymes called Dicers (Lee et al., 2003b), resulting in a ds 22-nucleotide (nt) miRNA. One of the miRNA strands is incorporated into a silencing complex, which binds to mRNA and represses mRNA translation, thereby limiting protein production. This appears to happen in a cooperative manner, with many miRNA binding to a target mRNA (Bartel, 2004). The rapid progress in this field has been the subject of several excellent reviews (Bartel, 2004; Novina and Sharp, 2004; Du and Zamore, 2005).

There is strong evolutionary conservation of the sequence of these molecules, although recent studies do predict the presence of species-specific miRNA (Bentwich et al., 2005). Studies on chicken miRNA are very limited. One hundred twenty-two homologs of miRNA were predicted from analysis of the chicken genome sequence (International Chicken Genome Sequencing Consortium, 2004). In an analysis of the chicken transcriptome, 23 miRNA were found in chicken EST databases, and 3 pri-miRNA operons were described that are highly conserved with humans (Hubbard et al., 2005). In situ hybridization studies with chick embryos have confirmed the expression and, in some instances, the tissue specificity, of several miRNA (Hubbard et al., 2005). High-throughput technology has been used to map expression of 135

miRNA by whole-mount in situ hybridization, providing a comprehensive overview of miRNA expression in chick embryos (Darnell et al., 2006; Antin et al., 2007). Typically, the isolation and identification of small RNA involve purification and cDNA production, which is followed by the cloning and sequencing of single molecules or longer "concatamers" of these molecules (Lagos-Quintana et al., 2001; Reinhart et al., 2002; Aravin et al., 2003). This approach was used to clone several previously predicted chicken miRNA (Xu et al., 2006). A more contemporary approach is to use deep-sequencing technology (Margulies et al., 2005). Applications of this technology for the discovery of small RNA were developed by the Green and Meyers laboratories (Lu et al., 2005; Henderson et al., 2006), and this approach has been applied to discover chicken miRNA as well as MDV-encoded miRNA (Burnside et al., 2006). Microribonucleic acids are a missing link in functional genomics, and elucidation of their role in phenotypic expression will no doubt be an exciting chapter in 21st century chicken biology.

RNA Interference. Ribonucleic acid interference generally refers to one or more of several related mechanisms by which the expression of RNA can inhibit gene function in a sequence-specific manner. A key component of RNA interference (**RNai**) is the existence and recognition of some form of ds RNA (Fire et al., 1998; Waterhouse et al., 1998), although this ds RNA can be the stem of a hairpin loop within a single strand of RNA. The first example of a mechanistic role for ds RNA was probably the regulation of colicin E1 plasmid replication in *E. coli* via the interaction of RNA transcribed from opposite strands near the origin of replication (Tomizawa and Itoh, 1982). Later, expression of "antisense" RNA complementary to thymidine kinase mRNA was used to specifically inhibit gene function, presumably through formation of ds RNA (Izant and Weintraub, 1984). However, gene knock-down via antisense RNA expression was not routinely successful, especially in vertebrate systems, in which it was found to generate non-sequence-specific effects attributable to interferon induction. Conversely, others (Matzke et al., 1989; Napoli et al., 1990) found that plant genes could be silenced by expressing an "extra" transgenic copy of the endogenous gene sequence. These observations are now known to result from an evolutionarily conserved pathway used to produce both miRNA (described above) and analogous small interfering RNA (**siRNA**). Both of these small RNA are produced by cleavage of RNA with a ds character (either stems of hairpins or true ds RNA) by RNases of the Dicer family (reviewed in Du and Zamore, 2005). The miRNA are only partially complementary to their target mRNA, and they usually inhibit expression by blocking translation, although they may also reduce target mRNA levels. The siRNA can be fully complementary to their target RNA and thus can silence the gene(s) giving rise to the siRNA itself in a feedback loop. These generally lead to endonucleolytic cleavage of the target RNA by "slicer" activity of an associated *Argonaute* family protein (reviewed in Tolia and Joshua-Tor, 2007) in an RNA-induced silencing complex. In addition, some siRNA act in the nucleus to silence genes by blocking or reducing their transcription. Here, the siRNA appear to act in another ribonucleoprotein complex (RNA-induced transcriptional silencing) that is tethered by siRNA homology to a nascent transcript and that functions to induce the formation of heterochromatin at the gene(s) being silenced (Buhler et al., 2006).

Ribonucleic acid interference is likely to play a major role in the regulation of normal gene expression through a complex interacting network of miRNA and siRNA. Of particular relevance to the topic of functional genomics is the experimental application of RNAi to create "knock-down" mutations, a form of reverse genetics in which one chooses the target gene and then explores the phenotypic consequences of reducing or eliminating its expression. Once a candidate gene is targeted, the question becomes how to deliver RNAi to mediate gene silencing. In chickens, one generally cannot deliver ds RNA because of its interferon-inducing side effects. However, one can transfect synthetic siRNA, most often choosing 21- to 29-bp duplexes with 2-nt overhanging ends similar to the natural products of dicer enzymes (Elbashir et al., 2001). Although the resultant effect is transient, because of the eventual decay of the siRNA, this approach is very useful in cultured cells and has even been applied to live mice (Palliser et al., 2006). Whether it would work in live chickens is not yet certain. Gene knock-downs of longer effect can be generated by using DNA plasmids designed to transcribe short-hairpin RNA (**shRNA**) whose ds stems contain the target sequence, thus relying on the cell's native Dicer activity to remove the loop and other extraneous nucleotides and generate the functional siRNA duplex (Brummelkamp et al., 2002a). Alternatively, viral vectors can deliver the shRNA transgene, with retroviruses, including lentiviruses, being among the most popular (Brummelkamp et al., 2002b). Although most plasmids and vectors originally used RNA polymerase III promoters to express transcripts that contained little more than the shRNA itself, new vectors have been designed to mimic the natural miRNA processing pathway more exactly. These use RNA polymerase II promoters to generate pre-miRNA-like initial transcripts with the knock-down target sequence replacing a natural miRNA duplex region (Silva et al., 2005). Regardless of the delivery method, one must choose specific ~19-nt targets within the gene to be silenced. Target sequences differing by as little as one nucleotide can vary widely in activity, and a variety of experimental and computational methods have been developed for target selection (Pei and Tuschl, 2006). Even with the use of such programs, not all targets will be successful, and those that are will vary in the level of gene silencing for reasons that remain unclear. However, in some cases this can be a blessing, because it can generate a series of "alleles" exhibiting varying protein levels attributable to variable gene silencing.

Applications of RNAi in chickens have focused mainly on questions of embryonic development or inhibition of viral pathogens. The first report of RNAi in chicken cells was that of Hu and colleagues (2002), who used transfec-

tion of cultured cells or embryo electroporation to introduce synthetic siRNA targeted against Rous sarcoma virus to reduce viral growth and pathogenesis. Others (Ge et al., 2003b) also showed that synthetic siRNA could be used to inhibit influenza virus production in both cell lines and chick embryos. At about the same time, several groups used electroporation to introduce ds RNA (Pekarik et al., 2003), shRNA-producing DNA plasmids (Katahira and Nakamura, 2003), or a retroviral vector (Kawakami et al., 2003) into chick embryos to reduce expression of genes acting in developmental pathways. Thus, RNAi has become a key tool for assessing gene function during avian embryogenesis (Dai et al., 2005; Das et al., 2006; Harpavat and Cepko, 2006). Similar to earlier studies in mammalian cells (Silva et al., 2005), Das et al. (2006) and Chen et al. (2007) developed chicken retroviral vectors that expressed the siRNA target as part of a modified miRNA cassette.

The RNAi systems must be able to alter phenotypes by gene silencing in live birds for full realization of their potential in functional genomics, especially with regard to agricultural interests. To date, such studies are yet to be published. However, recent advances in avian transgenic technology (McGrew et al., 2004; van de Lavoir et al., 2006; Lillico et al., 2007) should make such experiments feasible. In particular, lentiviral vectors have been shown to be effective in generating germline transgenic chickens (McGrew et al., 2004) and, at least for mammals, in delivering RNAi constructs (reviewed in Morris and Rossi, 2006). Retroviral vectors make it feasible to generate libraries of recombinant viruses that, among them, target every gene known in the species of interest (reviewed in Chang et al., 2006; Echeverri and Perrimon, 2006; Root et al., 2006). Not only do such libraries spare the prospective user the job of constructing his or her own virus, they allow for high-throughput screens that target multiple genes for silencing or that can select for a phenotype of interest and determine the gene targeted after the fact. When a chicken RNAi library eventually becomes available, it will be a critical asset for progress in avian functional genomics. Despite the fact that it is relatively easy to eliminate selected genes by homologous recombination in certain chicken cell lines (Buerstedde and Takeda, 1991), it has not yet been possible to make transgenic knock-out chickens in the fashion that is now routine for mice. Thus, the ability to make knock-down chickens with RNAi technology is a critical need. In particular, this may be the only way to rigorously verify "candidate" gene alleles that are proposed to encode quantitative traits, once those candidates are identified by positional cloning or other strategies. Therefore, RNAi is a major key to the future of functional genomic analysis in the chicken.

Other Functional Genomics Methods

Whole-Mount In Situ Hybridization. Gene expression mapping with high-throughput whole-mount in situ hybridization is a powerful method of identifying novel functional genes involved in embryogenesis (Bell et al., 2004). A chicken gene discovery project called *Gallus* EST In Situ Hybridization Analysis (**GEISHA**) has established a database of EST sequences, annotated embryonic genes, and high-resolution images of gene expression patterns in e1 to e3 embryos (http://geisha.arizona.edu/geisha/; Antin et al., 2007). Tissue-specific and temporal patterns of more than 100 newly discovered miRNA genes have been mapped in the early chick embryo by whole-mount in situ hybridization screening (Ason et al., 2006; Darnell et al., 2006). Thus, high-throughput whole-mount in situ hybridization screens provide unparalleled views of the spatial and temporal organization of gene expression patterns during organogenesis of the chick embryo. The GEISHA project provides the avian research community with a freely accessible database of chicken whole-mount in situ hybridization images, gene annotation, and associated genomics metadata (Antin et al., 2007).

Metabolomics. Metabolomics is another new discipline initiated in the postgenomic era from the need to integrate metabolites into the control of gene expression and functional activity of most proteins. Metabolomics is the comprehensive and quantitative study of all metabolites within a cell, organ, or organism and represents the final bridge between the other "omics" technologies (genomics, transcriptomics, and proteomics) and phenotype. High-throughput metabolomic data enable calculation of enzyme activity and even prediction of mutations in known or unknown genes encoding the enzymes (Wu et al., 2005). The vast knowledge of biochemistry and metabolic pathways compiled in the last century is integrated with global metabolic profiling to provide a dynamic connection between genome function and phenotype (German et al., 2005; Go et al., 2005). Nutritional genomics has great potential for development of metabolite markers for prevention and treatment of diet-related diseases and "personalized" medicine in the future. Recent publications present some analytical methods used in metabolic footprinting (Hollywood et al., 2006) and the computational prediction of regulatory control over metabolic pathways (Kummel et al., 2006) from high-content metabolomic data. One focus of metabolomics is lipidomics—the system-based study of all lipids, their reactants, and functions within biological systems (Watson, 2006). The human metabolite database provides Web-based access to human metabolites, metabolic pathway information, and metabolism data (Wishart et al., 2007). Global lipid profiling provides a powerful tool from the functional genomics arsenal for understanding genetic and environmental influences in the development of metabolic disorders (i.e., diabetes and obesity) and cardiovascular diseases (Pietilainen et al., 2007). A recent lipidomics study, contrasting the plasma lipid profiles of the 4 divergently selected lines introduced earlier (the FL vs. LL and the HG vs. LG), represents the first application of metabolomics in poultry (Walzem et al., 2007). This study revealed basic differences in lipid metabolism related to the genetic background of the founder lines, whereas distinct shifts in lipid metabolism led to the divergence in adiposity observed among these unique ex-

perimental lines. Integration of metabolomic data with global transcriptional and QTL analyses should greatly improve our understanding of the genotype-phenotype relationship in chickens. The implications of emerging metabolomics for understanding complex biological systems in agriculture are vast and include the functional genomics of animals, plants, and their ultimate benefactor—humans (Dixon et al., 2006).

BIOINFORMATICS AND COMPUTATIONAL BIOLOGY

Gene Ontology Annotation

The genome of an organism fundamentally defines all its characteristics by providing the blueprint for the functional molecules that control life (i.e., genes and proteins). Publicly accessible, curated databases provide structural and functionally annotated genome sequences, which are critical for genomics research. Two value-added databases used for functional genomics research in the chicken are the UniProt Knowledgebase (**UniProtKB**; http://www.ebi.ac.uk/swissprot/; Apweiler et al., 2004) and the GO database (http://www.geneontology.org/; Gene Ontology Consortium, 2001). The UniProtKB database is the central access point for extensive curated protein information, including function, classification, and cross-references. The UniProtKB data are derived from many sources, but one of its most valuable links for functional genomics and systems biology is to the GO database (Gene Ontology Consortium, 2001), which is the central repository for functional gene annotations.

Functional Annotation of the Genome. The GO project was established as the GO Consortium in 1998 to use the vast volume of accessible biological information for functional annotation of gene products (Gene Ontology Consortium, 2001; Lewis, 2005). The primary mission of the GO project is to facilitate the use of the genome information and enable understanding of the functional units of the genome (genes and proteins). The GO Consortium initially included only scientists representing 3 model organisms: the *Saccharomyces* Genome database; FlyBase, the *Drosophila* genome database; and the Mouse Genome database. However, every species has unique genes and gene products that share sequence homology with different species. Because organisms share relatively few core gene orthologs with an identical function, the GO Consortium now includes numerous prokaryotes and eukaryotes. The GO Consortium also includes TIGR (http://www.tigr.org/db.shtml), the European Bioinformatics Institute GOA project (http://www.ebi.ac.uk/GOA/), and AgBase (www.Agbase.msstate.edu), an associate GO Consortium member that represents GO for the chicken (ChickGO; McCarthy et al., 2006a, 2007).

Curators use both peer-reviewed literature and experimental data to describe gene product function in a structured way. Gene products are classified by molecular function, biological process, and cellular compartment, primarily by using peer-reviewed literature and proteomic data or, if these are unavailable, by homology to orthologous products in other species. Genes and gene products for every organism are functionally annotated by using controlled structured vocabularies (ontologies). Gene Ontology has developed 3 ontologies for functional annotation: *molecular function*, *biological process*, and *cellular component*. Genes and gene products can have one or more molecular functions and biological processes, and they can be associated with one or more cellular components. *Molecular function* describes activities at the molecular level and does not specify where, when, or in what context the activity occurs. One or more molecular functions can contribute to a *biological process*. To distinguish between a biological process and a molecular function, a biological process must have more than one distinct step and not be equivalent to a pathway. A *cellular component* is part of some larger object, which can be an anatomical structure or a gene product group (Ashburner and Lewis, 2002; Ashburner et al., 2000).

The existing GO enables cross-product annotations that maximize the utility of an individual ontology while avoiding redundancy. In livestock, very specific processes and functions represent economically important production traits, which could rely on such "cross-products," further emphasizing the importance of representing livestock in the GO Consortium. Gene Ontology annotation has been minimally used in chickens, because using GO annotation in the chicken first required researchers to functionally annotate their own data sets from transcriptional or proteomic profiling studies. Gene Ontology annotations are primarily used for associating functional genomics data sets (i.e., differentially expressed gene lists from microarray experiments) with molecular and cellular functions. The GO Consortium has developed "GO Slim" subsets of the GO. The GO Slim data sets act as "ontology summaries" for modeling, and they provide high-level views of gene function. It is critical to understand that the user must create a GO Slim data set to fit a specific experiment. At AgBase, high-throughput tools are available to use the chicken GO annotations in analyses of functional genomics data. The tools are designed for batch process input and to accept several input formats. Details on the use of GO tools and creating GO Slim data sets are available in recent publications describing the GO database structure and the availability of Web-based resources and tools (McCarthy et al., 2006a, 2007). Two recent studies of genes in embryonic gonads and primordial germ cells of chickens (Kim et al., 2007) and the brain of songbirds (Wada et al., 2006) provide excellent examples of GO annotation used in functional analysis and interpretation of avian gene expression profiles.

Comparative Genomics for Functional Annotation

From an evolutionary viewpoint, all species are interrelated to a certain degree and share many similar or common molecular mechanisms to carry out biological activities. Comparative genomics is applied not only to study

the evolution of species, but also to expand our knowledge of biological processes across multiple species. Because the research resources invested in nonavian species are far greater than those invested in avian species, transferring the discoveries from other species into our knowledge of avian species can significantly advance our understanding. Comparative genomics approaches are proving very effective in identifying novel genes and functional elements in complex genomes. Bioinformatics tools allow investigators to detect and annotate genes by using comparative approaches. Genome sequence, sequence similarity, chromosomal location, phylogenetic trees, and conserved sequences are integrated to effectively detect and annotate novel genes in a new model organism, such as the chicken.

Conserved gene sequences and their chromosomal arrangement provide valuable information for comparative genomics. The conservation among relatively closely related species includes similarity in gene sequence and in the chromosomal location of homologous genes. During the course of evolution, chromosomal rearrangement, gene duplication, gene divergence, and gene loss has occurred. Paralogous genes (or paralogs) are genes that have been duplicated within the same species and that often diverge to give different functions, whereas orthologous genes (or orthologs) are homologous genes that have descended from the same gene of a common ancestor and usually share a common function across species (Brinkman and Leipe, 2001). Gene arrangement in chromosomes is usually conserved in vertebrates, and can be classified into conserved synteny (homologous genes of 2 species in the same chromosomal locations, regardless of gene order), conserved segment (uninterrupted by other chromosomal segments), and conserved order (same linear orientation; Andersson et al., 1996). These conserved characteristics allow identification of novel genes and gene families in a species with a completed genome sequence.

Comparative genomics also incorporates similarity-based gene discovery and intrinsic DNA sequence analysis to increase the specificity of gene detection (Windsor and Mitchell-Olds, 2006). Intrinsic gene prediction, also called ab initio prediction, is based only on the DNA sequence; for example, Genscan software (Burge and Karlin, 1997) uses this approach to predict gene structure. Gene prediction is highly sensitive but with low specificity and is generally incapable of identifying small open reading frames and noncoding sequences. On the other hand, extrinsic or comparative gene prediction programs include transcriptional evidence (Ensembl; Hubbard et al., 2002) and sequence similarity among species [Twin-Scan (Korf et al., 2001) and syntenic gene prediction 2 programs (Parra et al., 2003)] to improve specificity.

Another application of comparative genomics in chickens is the systematic annotation of genes, in which a large number of genes have an unknown function. Many chicken genes have very low sequence homology to mammalian genes and some are avian specific, which creates a great challenge for automated electronic gene annotation. In the absence of systematic biochemical analysis, the prediction of functional elements in the genome of a new model organism depends heavily on computational and comparative analyses (Jones, 2006). Comparative approaches also identify regulatory sequences (TFBS and miRNA) that control gene expression. However, predicting regulatory sequences in the genome is much more difficult than gene prediction. Regulatory sequences are *cis*-acting modules (i.e., promoters and enhancers) that regulate the timing and abundance of gene expression. Genome sequences harboring these functional elements seem to be conserved among related species. Genome sequences of several related species are aligned to identify evolutionary conserved sequences that contain transcription regulatory elements. This process is called "phylogenetic footprinting" (Tagle et al., 1988). Comparative analyses of the human, mouse, rat, and dog genome sequences have identified many common regulatory motifs in promoters and 3′-untranslated regions (Xie et al., 2005). Pairwise sequence comparison between evolutionarily distant species represents a powerful method for identification of functional noncoding sequences that regulate gene expression (Ahituv et al., 2005). Mulan is another Web-based multiple-sequence local alignment and visualization tool developed for detection of evolutionarily conserved TFBS in multiple-species alignments (Ovcharenko et al., 2005). The short sequence and inherent variability of real regulatory motifs makes their detection difficult by computational searches unless accompanied by robust statistical confirmation and experimental verification (Friberg et al., 2005; Elnitski et al., 2006; GuhaThakurta, 2006). Recently, more than a dozen of the most widely used computational tools were rigorously evaluated for efficiency in predicting TFBS motifs in genomic DNA (Tompa et al., 2005). However, the most reliable method of detecting protein (TF) binding to DNA (promoter regions) is chromatin immunoprecipitation (ChIP) coupled with microarray or "ChIP-on-chip" analysis (Ren et al., 2000; Elnitski et al., 2006; Jones, 2006). The use of numerous bioinformatics tools and high-performance computational methods for analyzing and mining the chicken genome sequence will continue to advance our understanding of avian functional genomics.

A major justification for sequencing the genome of multiple organisms is to gain a clear understanding of the evolution of genome size and genomic structures and to have access to the sequence (primarily coding and regulatory regions) of all genes. For example, the chicken genome is about one-third the size of the human genome, yet is thought to contain about the same number of genes (International Chicken Genome Sequencing Consortium, 2004). This initial assembly of the chicken genome sequence has provided compelling answers to some obvious questions regarding the avian phenotype and the loss or gain of gene families that have occurred during the parallel evolution of birds and mammals. For example, what genes are truly avian specific and responsible for specific features such as feathers, absence of mastication (teeth) and lactation, egg formation and oviposition, or

even flight? The speculation that the condensed genome size of birds reflects an evolutionary response to the physiological demands of flight is quite interesting (Ellegren, 2005). A recent phylogenomic analysis of amniote genome structure based on avian and nonavian dinosaurs suggests that the original reduction in avian genome size and other avian characteristics evolved prior to flight, although the high energetic requirements of flight in birds led to the additional shrinkage in avian genome size (Organ et al., 2007). Furthermore, comparative analysis of avian (chicken) and mammalian genomes should provide new insight into the ecology and evolution of bird populations found in the natural environment. Comprehensive cross-species analysis of gene catalogs and genome sequences enables the discovery of novel genes and conserved gene families and improves functional annotation in the absence of experimental evidence (Windsor and Mitchell-Olds, 2006). Comparative genomic analysis provides a powerful tool for understanding the evolutionary conservation of genes and gene families and for discovery of the functional noncoding genome sequence (Nobrega and Pennacchio, 2004). For example, an initial analysis of noncoding RNA in the chicken transcriptome revealed 14 pri-miRNA that encode 23 distinct miRNA in the chicken (Hubbard et al., 2005).

Numerous gene families in the adaptive and innate immune system of the chicken have been identified by a comparative genomics approach. An early bioinformatics study of the large chicken EST collection of 330,388 EST (Boardman et al., 2002) revealed that almost half of the known mammalian components of the TLR signaling pathway, including 5 TLR family members, are conserved in the chicken (Lynn et al., 2003). Five chicken TLR family members (*TLR1-1*, *TLR1-2*, *TLR3*, *TLR5*, and *TLR7*) were also identified from a search of human- and mouse-like TLR sequences in chicken EST databases (Yilmaz et al., 2005). With the exception of *TLR1-1* and *TLR5*, the chicken *TLR* isoforms were expressed in all of the 8 tissues examined. The complete molecular phylogenetic analysis of all known vertebrate *TLR* genes, including those from the chicken, zebra fish, puffer fish, flounder, rainbow trout, goldfish, opossum, mouse, rat, and human, enabled construction of a molecular tree illustrating the evolution of this highly conserved gene family (Roach et al., 2005). Six *TLR* gene families have evolved in vertebrates, which recognize distinct molecular patterns representing several classes of pathogens and provoke appropriate adaptive or innate immune responses. Two notable observations are that chicken *TLR15* is molecularly distinct from all other vertebrate TLR (although it could have evolved from the ancestral *TLR1* gene) and that a homolog of *TLR9* (involved in the recognition of nucleic acids and heme) is absent in the chicken.

A comprehensive in silico analysis of 450,000 chicken EST sequences has allowed the identification of 185 immune-related genes in the chicken, including 95 immune gene sequences not previously found in GenBank (Smith et al., 2004). Surprisingly, most of the EST sequences that represent such a broad range of immune-related genes were derived from nonlymphoid tissues in healthy birds. Comparative analysis of the chicken, mouse, and human genomes has revealed 23 chemokine and 14 chemokine receptor sequences in the chicken genome (Wang et al., 2005). The phylogeny, sequence conservation, and high synteny indicate that chicken chemokines and their receptors share common ancestors with the mouse and human genes. Another comparative genomic analysis has revealed a large number of highly conserved genes in the adaptive immune system of the chicken, including 23 interleukins, 8 interferons, 2 transforming growth factors, 24 chemokines, and 10 members of the tumor necrosis factor superfamily (Kaiser et al., 2005). This comprehensive analysis has provided a global view of the repertoire of cytokines and chemokines of the chicken and considerable insight into unique features of its immune system. Two independent genome-wide screens of the chicken genome sequence and clustered chicken EST sequences have identified a single highly conserved cluster of β-defensin genes on *GGA*3 (Lynn et al., 2004; Xiao et al., 2004). The β-defensin genes, formerly called gallinacins in the chicken (Lynn et al., 2007), encode a family of antimicrobial peptides involved in innate immune responses, primarily in the gastrointestinal and reproductive tracts (Hasenstein et al., 2006; Milona et al., 2007). One member of this gene family, *AvBD9*, appears to be involved in adipogenesis, because expression of *AvBD9* is up-regulated in the liver of FL chickens and hypothyroid slightly obese chickens (Cogburn et al., 2003c; Wang et al., 2007). Furthermore, 2 single-nucleotide polymorphisms identified in chicken *AvBD9* are associated with abdominal fatness traits in the F_2 resource population from a FL $\times$ LL intercross and have potential as molecular markers for fatness in the chicken (Cogburn et al., 2003a). Bioinformatic mining of the chicken EST collection and comparative genome analysis has led to the discovery of other novel gene families involved in innate immunity, such as the biotin-binding proteins found in eggs (Niskanen et al., 2005) and the collectins (Hogenkamp et al., 2006). A comparative genomic study of the serpin (Ser protease inhibitor) superfamily in the human, chicken, and zebra fish has provided a new perspective on molecular evolution of the clade B serpins [or ovalbumin-related serpins (*ov-serpins*)] and the elaboration of a younger serpin (*ovalbumin*) in the chicken (Benarafa and Remold-O'Donnell, 2005)]. Chicken ovalbumin is a bird-specific clade B serpin (lacking Ser protease activity) that serves as a storage protein for embryonic development in the avian egg. This novel function could have supported the early adaptation of birds to new habitats and lifestyles. The highly conserved clade B serpin gene cluster is located on *GGA*2 (10 members), whereas the human genome has 2 *ov-serpin* loci: *HSA*6 (3 members) and *HSA*18 (10 members). Duplication of the ancestral *SERPINB12* gene occurred independently in chickens, which gave rise to 3 paralogs (*gene X*, *gene Y*, and *ovalbumin*) and in humans, which have 5 *SERPINB12* paralogs (*SERPINB13*, *SERPINB4*, *SERPINB3*, *SERPINB11*, and *SERPINB7*). An orthologous comparison of chicken, mouse, and human genomes has

enabled the identification of 197 putative candidate genes located in probable QTL on *GGAZ* (Ankra-Badu and Aggrey, 2005).

Thus, comparative studies that integrate multiple vertebrate genomes provide considerable insight into the evolution of highly conserved gene families and evidential support for the functional annotation of genes and proteins. As highlighted by the examples above, comparative genomic analyses also will play an essential role in identifying functional components (genes, proteins, and networks) for systems biology (Roach et al., 2005) in the chicken and other avian species.

Systems Biology

The final frontier of structural and functional genomics is systems biology, "the 21st century science" (Hood, 2003), which integrates transcriptomic, proteomic, and metabolomic interactions into all biological processes and systems that support the life of an organism (Ideker et al., 2001; Auffray et al., 2003; Ge et al., 2003a). The newest additions to the systems biology repertoire are the interactome (Cusick et al., 2005; Rachlin et al., 2006) and the reactome (http://www.reactome.org/; Joshi-Tope et al., 2005; Vastrik et al., 2007). The interactome represents the computational and experimental identification of all protein-protein interactions in integrated biological systems. The reactome is an interactive curated knowledgebase of all biological pathways and processes in an organism. Systems biology offers the virtual reality of complex integrated biological systems and, to some, it represents "the renaissance of physiology and end of naive reductionism" (Strange, 2005). Both physiology and systems biology focus on the integrative and holistic analysis of functional components (genes, proteins, and metabolites) in biological systems that make up the phenome of living organisms. The systems biology approach, which integrates multiple disciplines and multiple high-content data sets, holds great promise for the identification of diseases, discovery of new drugs, evaluation of pharmaceutical intervention, and eventual practice of "personalized medicine" (Butcher et al., 2004; Cho et al., 2006; van der Greef et al., 2007). Efforts are already underway to develop strict standards and quality assurance for the collection, storage, and use of complex data sets generated by the various high-throughput omics technologies (transcriptomics, proteomics, and metabolomics) to maximize the utilization of integrated knowledge gained from systems biology (Ideker et al., 2001; Brazma et al., 2006). Although systems biology has not yet arrived on the doorstep of poultry science, this "big science" of the 21st century will certainly influence most aspects of biological, agricultural, and medical investigation in the near future.

CONCLUSIONS AND OUTLOOK

During the first 7 years of the 21st century, we have amassed the cDNA sequence of most chicken genes, completed the chicken genome sequence, and begun large-scale exploration of the functional genome of the chicken with powerful tools supported by a bioinformatic and computational infrastructure freely available on the World Wide Web. Functional genomics represents the integration of information from genome sequence and structure, gene and protein expression, and metabolite profiles with knowledge databases by using computational and bioinformatics tools (Figure 1). This figure provides a road map of functional genomics in the chicken, and using these tools and knowledge of biological pathways has implications for altering the phenotype of the bird to meet production demands. A major premise of emerging systems biology is that an understanding of the genotype and the biological processes that contribute to expression of the phenotype should allow reconstruction of the phenotype. This systemic approach to understanding complex biological systems, as embraced by emerging systems biology, has been the hallmark of physiology for centuries. Functional genomics offers the genetic blueprint and the knowledge gained from all of the complex interactions among the genomic, proteomic, and metabolic domains of a model organism, such as the chicken, that materialize as the phenotype(s). The intersection of these 3 omic domains constitutes the reactome of the organism. We now have the genomic parts list of the chicken, although we do not yet comprehend what all of these genetic parts are or how they interact. Poultry breeding programs developed during the last century have been very efficient at modifying the chicken genotype to improve phenotypes. For the most part, previous improvements in growth rate and other important production traits were achieved by quantitative genetic selection without knowledge of the underlying genes. Nonetheless, some specific alleles (either useful or deleterious ones) have either been introduced into or been excluded from flocks, depending on regional and global market requirements (i.e., alleles for feather or skin color, sex identification at hatching, fast or slow feathering, dwarfism). Transcriptional profiling with microarrays now provides experimental evidence for the function of thousands of genes simultaneously. Genetical genomics incorporates genome-wide gene expression as a quantitative trait (i.e., eQTL) into QTL analysis in an attempt to identify functional candidate genes that could contribute in either a *cis* or *trans* manner to the expression of important production traits. The integration of transcriptomic, proteomic, and metabolic data into gene networks and biological pathways should provide considerable insight into genetic control of phenotypic traits in the chicken. Emerging technologies (i.e., RNA silencing and transgenics) could eventually be used to change the phenotype of the chicken to meet changing market demands. Recently, transgenic chickens have been designed that produce pharmaceuticals for humans in the egg; therefore, we should be able to modify bird genotypes to meet new target phenotypes. However, the new "designed" chicken genotypes must be rigorously tested to ensure that the intended improvements do not compromise existing productive performance and health.

One can predict that integration of functional genomics and, ultimately, systems biology with classical advances in productive performance will ensure that the chicken remains as a primary food source for humans. Furthermore, these technological revolutions and forthcoming ones will guarantee the continuing role of the domestic chicken as a model organism for our understanding of biological processes and the intervention of human diseases, particularly metabolic disorders (i.e., diabetes and obesity).

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