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Gene expression profiling in the developing neuroendocrine system of the chick

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Introduction

Levels of messenger RNA (mRNA) in samples are commonly measured one-gene-at-a-time using gene-specific probes and Northern blotting, ribonuclease protection assays, or real time PCR. Although these techniques allow quantification of gene expression, they do not readily afford a comprehensive analysis of the many genes that may be affected among experimental groups. The advent of DNA microarray technology has allowed simultaneous analysis of differences in expression levels of thousands of genes among samples. Research in avian species has been traditionally hampered by a paucity of publicly available probes, often requiring researchers to clone a gene of interest based on sequence homology with known mammalian gene sequences. Experiments in the neuroendocrine system are further hampered by the small size of the neuroendocrine tissues and hence the amount of RNA available for analysis. Recently, a tremendous amount of genetic information has become available for one avian species – the chicken. Public databases such as GenBank now contain partial sequence for approximately 500,000 chicken cDNA clones, and most of the chicken genome has been sequenced. Moreover, several groups have assembled large numbers of chicken cDNA clones and printed these as cDNA microarrays (1, 3-6, 10, 17, 25). These advances have allowed for the first time, large-scale functional genomics analyses of an avian species. In addition, several approaches have been used to increase the amount of RNA available for each sample through amplification of a small amount of starting RNA, thereby allowing microarray analysis of minute samples. We describe here our recent advancements in functional genomics in the chicken neuroendocrine system. We have produced cDNA microarrays containing more than five thousand genes expressed in the neuroendocrine system, validated an RNA amplification protocol

for use with samples from individual embryonic pituitary glands, and used these tools to study changes in gene expression profiles within the hypothalamus and anterior pituitary gland of chickens during late embryonic and early post-hatch development. Our approach and findings will be summarized in this review.

Production of neuroendocrine system cDNA microarrays

High-density cDNA microarrays for the chicken neuroendocrine system were produced in collaboration with Dr. Larry Cogburn at the University of Delaware. The cDNA libraries used to make these arrays have been described elsewhere (5, 6). Briefly, RNA was isolated from the hypothalamus, anterior pituitary gland, and pineal gland of chickens from embryonic day 12 through post-hatch day 63 and pooled. As a result, the RNA pool represented three neuroendocrine tissues and a wide range of developmental stages. This pooled RNA sample was then used to produce a cDNA library, and approximately 5,500 clones from this library were selected at random and sequenced. The library was then normalized to eliminate redundant clones, and an additional 3,300 clones from the normalized library were selected at random and sequenced. All sequences were then compared against one another to identify overlapping sequences (contigs), which likely represent the same cDNA. Clones without sequence identity with other cDNAs (singlets) were also identified. From the 8,869 clones sequenced, 5,128 unique cDNAs (Unigenes) were identified within the neuroendocrine system library. Each Unigene sequence was compared with GenBank by the BLASTX and BLASTN procedures, and the clones annotated with the name of the highest BLAST score. The Unigene clones were then amplified by PCR, and the PCR products were printed onto microscope slides as cDNA microarrays. In addition, multiple copies of selected housekeeping genes, salmon DNA, and water were spotted as quality controls. An annotated list of clones and their location on the chicken neuroendocrine system 5K microarrays is available at:
http://www.ansc.umd.edu/faculty/tpresearch.htm.

Amplification of RNA from small samples

A primary research focus in our laboratory is the development of the anterior pituitary gland, with particular emphasis on the last three cell types to differentiate: the thyrotrophs, somatotrophs, and lactotrophs. However, chicken embryonic pituitary glands yield insufficient total cellular RNA for microarray analysis. Approximately 20-50µg of total RNA (8) or 1-5µg of poly(A) RNA (7) from each sample are required for microarray analysis. To overcome this limitation and allow gene expression profiling with small amounts of tissue or even individual cells, several protocols for amplifying mRNA that are variations on the

Eberwine procedure (18) have been developed (2, 12, 15, 23, 26). The Eberwine protocol uses an oligo(dT) primer containing a T7 RNA polymerase promoter sequence to reverse transcribe mRNA. Synthesis of the complementary strand is then followed by *in vitro* transcription with T7 RNA polymerase, resulting in large amounts of amplified RNA (aRNA).

The protocol we have adapted in our laboratory is described here to assist other scientists working with small tissue samples, such as comparative endocrinologists, to more easily integrate RNA amplification into their own research. Starting RNA quality is important for microarray analysis, and we routinely use RNeasy® Mini and Midi Kits (Qiagen; Valencia, CA) for our experiments. To initiate first strand synthesis for amplification of RNA, 0.5µg of total RNA is mixed with 1µl of 50µM T7-oligo(dT) primer:
(5'-GCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGT$_{24}$-3'),
(Affymetrix; Santa Clara, CA) in a final volume of 11µl. The mixture is incubated at 70^{o}C for 6min, followed by incubation at 4^{o}C for 2min. Subsequently, 4µl 5X first-strand buffer (Invitrogen; Carlsbad, CA), 2µl 0.1M DTT (Invitrogen), 1µl 10mM dNTPs (Fermentas; Hanover, MD), 1µl RNAse inhibitor (40U/µl, Invitrogen) and 1µl SuperScript II reverse transcriptase (200U/ul, Invitrogen) are added, and the samples are incubated at 42^{o}C for 1h. Next, for second strand synthesis, 30µl 5X second-strand buffer (Invitrogen), 3µl 10mM dNTPs (Fermentas), 4µl DNA polymerase I (10U/µl, Invitrogen), 1µl RNAse H (2U/µl, Invitrogen), 1µl *E. coli* DNA ligase (10U/µl, Invitrogen) and 92µl RNAse-free water are added, and the mixture is incubated at 16^{o}C for 2h. T4 DNA Polymerase is then added (2µl, 5U/µl; Invitrogen), and the samples are incubated at 16^{o}C for 10 min. The double-stranded cDNA is phenol-chloroform extracted using a Phase Lock Gel™ Light tube (Eppendorf; Hamburg, Germany) and washed 2 times with 500µl of RNAse-free water in a Microcon-30 column (Millipore; Billerica, MA). The cDNA is subsequently lyophilized to dryness, and then used as template for an *in vitro* transcription reaction using the T7 MEGAscript kit (Ambion; Austin, TX) according to the manufacturer's protocol. The samples are incubated for 5h at 37^{o}C, and the aRNA is phenol-chloroform extracted using a Phase Lock Gel™ Heavy tube (Eppendorf) and passed through a Spin Column-30 (Sigma; Saint Louis, MO). The resulting aRNA is quantified by optical density at 260nm and with the RiboGreen RNA Quantitation Kit (Invitrogen) and is visualized by ethidium bromide staining in a formaldehyde agarose gel. The resulting aRNA appears in the gel evenly distributed in size between 0.15 and 2.5 kilobases. From 500ng of starting total RNA, the procedure typically

yields 10-15µg of aRNA, which is equivalent to the same amount of mRNA.

In establishing the RNA amplification procedure in our laboratory, we verified that microarray results obtained from aRNA agree with those obtained from total RNA. Pituitary RNA was amplified by the above procedure in two independent reactions. Two samples from a pool of total RNA and two aRNA samples amplified from the same pool were each analyzed with separate microarrays, and the mean $\log_2$-transformed raw pixel intensities from each spot were highly correlated (r^2=0.96). The high degree of correlation between microarray results using either total RNA or using aRNA validated the RNA amplification procedure for use with our microarrays.

Microarray hybridization and data analysis

This section will briefly summarize the general approach used for the studies described in more detail later in this chapter. In performing the microarray analyses, each target RNA sample was first labeled with fluorescently tagged nucleotides for detection with a laser scanner. Cy3- or Cy5-labeled cDNA was made from 1µg aRNA (e.g. pituitary samples) or 25µg total RNA (e.g. hypothalamus samples) in a two-step process using the Amino Allyl cDNA Labeling Kit (Ambion) according to the manufacturer's protocol. Total RNA was primed with an oligo(dT) primer, while aRNA was primed using random primers due to the antisense orientation of the aRNA. After hybridization and washing, the slides were scanned with an Affymetrix 418 confocal laser scanner to generate two TIFF images for each slide, one for Cy3 and one for Cy5.

As the absorption/emission spectra of Cy3 and Cy5 are sufficiently different, two samples of cDNAs labeled with Cy3 and Cy5 can be hybridized simultaneously to the same microarray. In our experiments, we used a reference design (22), so that for each study a pool of all of the samples was generated and labeled with Cy5 as an internal reference sample. Each experimental sample was then labeled with Cy3 and hybridized to an array with an aliquot of the reference sample labeled with Cy5.

Many software packages exist for analyzing microarray results. The Institute for Genomic Research (TIGR) offers TM4, a suite of freely available microarray data analysis applications, through their website (www.tigr.org/software/tm4) (21). We used two of the programs in this suite, Spotfinder, an image processing program, and MIDAS, a program for data normalization and analysis. For the developmental studies described below, the two TIFF images from the microarray scanner were loaded into Spotfinder version 2.2.4, and then the numeric values for each spot in the microarray were exported as a tab-delimited MEV file for data normalization using MIDAS version 2.18. Data from the Cy3

channel (representing each experimental sample) were Lowess normalized by block, followed by standard deviation regularization first by block then by slide, with Cy5 (the reference sample) as the reference. Finally, the normalized output from each slide was exported as a tab-delimited MEV (text) file. This normalization process reduces systematic variation introduced in microarray printing and processing.

Following normalization of the microarray results, the data were subjected to statistical analysis. Normalized data were analyzed by one-way analysis of variance (Statistical Analysis System, version 8.02; SAS Institute, Cary, NC) to identify spots that were differentially expressed on at least one of the developmental ages. Data were analyzed as the $\log_2$-ratio, or $\log_2$(normalized Cy3/raw Cy5), and differences were considered significant at $P<0.05$. As a further filter for false positives, spots were considered differentially expressed only if the highest mean among the experimental groups was greater than 175% of the lowest mean.

Significant spots were then subjected to self-organizing maps (SOMS) analysis using GeneCluster 2-version 2.0 (20), a software program developed by the Whitehead Institute/MIT Center for Genome Research and available free from their website:
(www.broad.mit.edu/cancer/software/genecluster2/gc2).
SOMS organizes the data so that general patterns of gene expression can be visualized, and it is particularly useful for evaluating gene expression profiles in time-course experiments.

Gene expression profiles in the hypothalamus around hatching

Hatching represents a major physiological change for the developing chick. Upon hatching, the primary source of nourishment shifts from egg yolk to ingested feed; the chick acquires the capacity to regulate body temperature and is exposed to increased light intensity and a daily photoperiod that affects its behavior. Each of these processes – regulation of feed intake, maintenance of body temperature, photoreception and entrainment to light – involves neuronal pathways located in the hypothalamus (13, 14, 16). In our first study with our neuroendocrine system 5K microarrays, we characterized changes in gene expression in the hypothalamus during late embryonic to early post-hatch development. Total RNA was extracted from the hypothalami of chicks on embryonic day (e) 17, e19, post-hatch day (d) 1 and d3. Each sample (25μg) of total RNA was compared against an internal reference sample pooled from all samples, using the neuroendocrine system 5K microarrays as described above. The microarray results were normalized and the data analyzed by analysis of variance. Among the 5,128 cDNAs represented on the microarrays, the expression of 344

spots changed significantly ($P<0.05$; n=3 samples/age) during this period of development. Of these, 105 changed substantially (greatest mean > 175% of the lowest mean). We included this filter to focus on significant changes that could be readily quantified by standard techniques and to decrease the incidence of false positives. Profiles of expression patterns among these 105 genes were identified by SOMS analysis. Expression levels for the 105 genes were placed into 20 clusters. These ranged in patterns of expression from a general increase to a general decrease in expression levels during development. Genes exhibiting an increase in expression levels after hatching included genes involved in myelination, such as myelin basic protein and myelin proteolipid protein. Other genes exhibited transient increases or decreases in levels of expression on e19 or d1. Some of our findings are presented in Fig. 1. Shown are results for myelin basic protein (MBP), dopamine and cAMP-regulated phosphoprotein (DARPP), and bone morphogenic protein 7 (BMP7). MBP and DARPP were in clusters 5 and 16, respectively, each containing 7 genes. Each of these clusters of genes exhibited the greatest increase in expression after hatching. BMP7 was part of cluster 7 that contained 4 genes which showed decreased levels of expression on d1. Levels of MBP, DARPP, and BMP7 mRNA were also measured in the same samples by quantitative real-time RT-PCR (Real-Time PCR). Results from Real-Time PCR agreed with those from the microarray analysis and with the expression profiles for their respective clusters (Fig. 1). Based on these findings, we believe that our microarray and SOMS analyses accurately describe gene expression profiles. Our findings from this study indicate that substantial structural and functional changes occur in the hypothalamus around hatching.

Expression profiles in the anterior pituitary associated with hormone ontogeny

The results from the hypothalamic study described above, demonstrate the utility of our neuroendocrine system microarrays. To determine whether differences in gene expression can be detected using amplified RNA (aRNA) and our neuroendocrine system microarrays, a preliminary study exploring differences in gene expression between e17 and d3 was conducted. Pituitary RNA from e17 and d3 chickens (500ng) was amplified, and 1µg of aRNA analyzed with the neuroendocrine system 5K microarrays. Expression of 116 cDNAs changed significantly ($P<0.05$, n=3, highest mean >175% of lowest mean), and these represented 113 genes due to duplication of cDNAs or genes on the array. Of these, expression levels of 90 genes increased and levels for 23 genes decreased between e17 and d3. Results for three genes were confirmed by Real-Time PCR. The microarray results and Real-Time PCR data from both aRNA and non-amplified total RNA from three

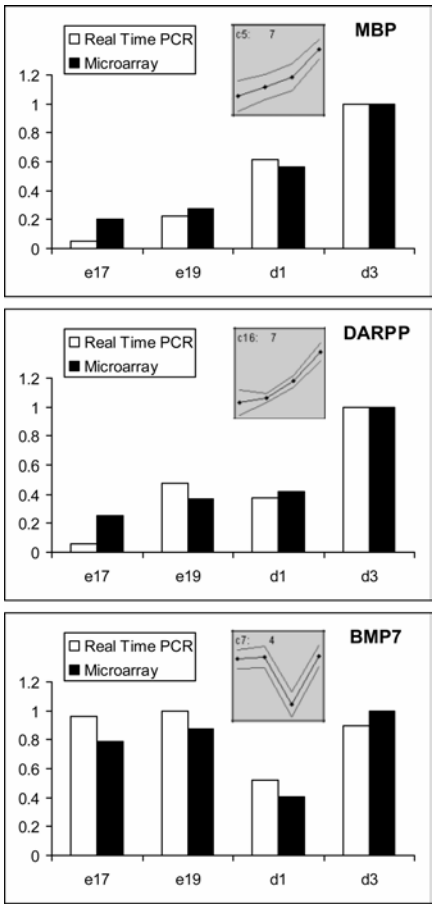

Fig. 1 Comparison of microarray analysis for hypothalamic expression levels for three genes with results from Real-Time PCR from embryonic day 17 (e17), and 19 (e19) through post-hatch day1 (d1) and 3 (d3). Results are presented for Myelin Basic Protein (MBP), Dopamine and cAMP Regulated Phosphoprotein (DARPP), and Bone Morphogenic Protein 7 (BMP7). The expression profile cluster from self organized maps (SOMS) analysis are presented in the insert for each gene.

genes are shown in Fig. 2. Growth hormone (GH) expression increased, and alpha-1-collagen mRNA levels decreased during development, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) did not change between e17 and d3. Results from the microarrays and from Real-Time PCR performed on aRNA and non-amplified RNA were in congruence. These findings indicated that all three analyses yielded similar findings

and further validated the RNA amplification procedure and the neuroendocrine system microarrays.

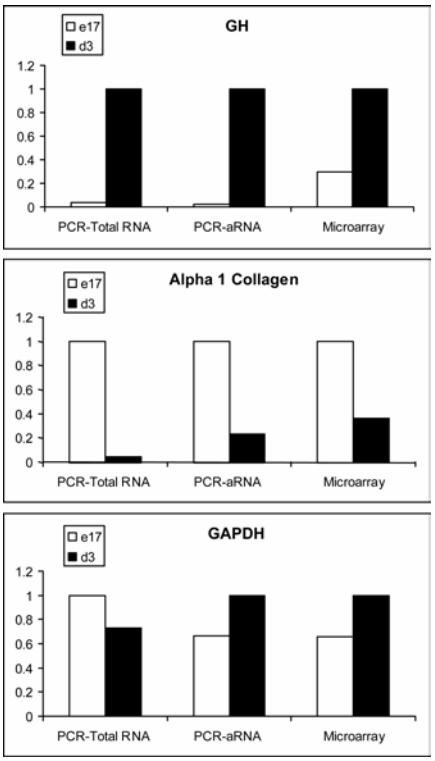

Fig. 2 A comparison of results from microarray analyses with amplified RNA (aRNA) with Real-Time PCR measurements of aRNA and non-amplified total RNA from e17 and d3 pituitary samples. Results are shown for growth hormone (GH), alpha 1 collagen, and glyceraldehyde 3 phosphodehydrogenase (GAPDH).

Next, a study was conducted to investigate changes in gene expression in the anterior pituitary gland that occur around the age of differentiation and proliferation of the last three major pituitary cell types, the thyrotrophs, somatotrophs, and lactotrophs. Thyrotrophs are first detectable on embryonic day (e) 6.5 and substantially increase on e11.5 (24), and levels of thyroid-stimulating hormone β (TSHβ) mRNA increase between e11 and e19 (9). Somatotrophs are first apparent on e14 and become a significant population by e16 (19), and GH mRNA levels increase between e14 and e16 (11). Lactotrophs differentiate by e17 (27),

and prolactin (PRL) gene expression increases between e16 and e18 (11). Pituitary RNA was extracted and amplified from embryonic chicks of four ages, e10, e12, e14, and e17, and aRNA was labeled and hybridized to microarrays. Expression of 245 cDNAs, representing 233 genes, changed significantly during this period of embryonic development ($P<0.05$, $n=4$, highest mean >175% of lowest mean). The data were organized by SOMS into 18 clusters. Real-Time PCR analysis verified expression patterns for genes in 3 of these clusters. Fig. 3 shows the microarray and Real-Time PCR results from total RNA for TSHβ, GH, and PRL. In agreement with previous reports, an increase in TSHβ mRNA levels occurred between e10 and e12, GH expression increased between e12 and e14, and PRL mRNA levels did not increase until e17. These results confirmed that the neuroendocrine system cDNA microarrays can be used to detect changes in pituitary gene expression and are therefore potentially useful in identifying global gene expression patterns that have not yet been elucidated. Particularly, examination of the clusters whose expression profiles indicate potential involvement in thyrotroph, somatotroph, and lactotroph differentiation should lead to the identification of candidate genes that may play a role in these processes. For example, TSHβ, GH, and PRL were in clusters containing 27, 12, and 10 genes, respectively. Among the other genes in these clusters are cDNAs for numerous transcription factors, signaling molecules, and morphogenic factors.

Summary

We have begun applying functional genomics to studies of gene expression in the developing chick neuroendocrine system. Microarrays containing cDNAs for more than five thousand genes expressed in the chicken hypothalamus, pineal and pituitary have been produced, and a protocol for amplifying small amounts of RNA from avian neuroendocrine tissues has been established. We have used our chicken neuroendocrine system 5K microarrays to characterize profiles of gene expression in the hypothalamus from e17 to d3 and in the anterior pituitary gland from e10 through d3. Clusters of gene expression were identified indicating that structural and functional maturation continues in the hypothalamus after hatching. In the anterior pituitary, clusters of genes were identified whose expression suggests potential involvement in the initiation of TSHβ, GH and PRL gene expression. Our future studies are aimed at characterizing gene expression profiles in embryonic pituitary cells treated with corticosterone to induce GH cell differentiation, in the hypothalamus and pituitary of chickens divergently selected for high and low body weight or for high and low body fat, and in the hypothalamus of chickens subjected to fasting and refeeding.

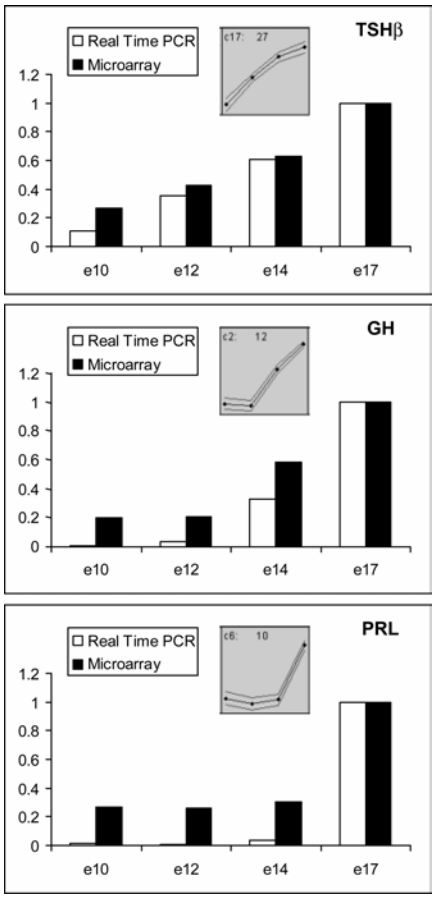

Fig. 3 A comparison of microarray and real-time PCR analyses of gene expression in the anterior pituitary from embryonic day 10 (e10) through e17. The results for thyroid-stimulating hormone β-subunit (TSHβ), growth hormone (GH), and prolactin (PRL) mRNAs are shown. Microarray analysis was performed on amplified RNA, while real-time PCR was performed on total RNA. The clusters containing each gene from SOMS analyses of gene expression profiles are shown in the inserts.

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