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Gene expression profiling during cellular differentiation in the embryonic pituitary gland using cDNA microarrays

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Ellestad, Laura E., Wilfrid Carre, Michael Muchow, Sultan A. Jenkins, Xiaofei Wang, Larry A. Cogburn, and Tom E. Porter. Gene expression profiling during cellular differentiation in the embryonic pituitary gland using cDNA microarrays. *Physiol Genomics* 25: 414–425, 2006. First published February 21, 2006; doi:10.1152/physiolgenomics.00248.2005.—The anterior pituitary is comprised of five major hormone-secreting cell types that differentiate during embryonic development in a temporally distinct manner. Microarrays containing 5,128 unique cDNAs expressed in the chicken neuroendocrine system were produced and used to identify genes with potential involvement in the onset of thyroid-stimulating hormone β-subunit (TSHβ), growth hormone (GH), and prolactin (PRL) mRNA during embryonic development. We identified 352 cDNAs that were differentially expressed ($P \leq 0.05$) on embryonic *day 10* (e10), e12, e14, or e17, the period of thyrotroph, somatotroph, and lactotroph differentiation. Self-organizing maps were used to identify genes that may function to initiate hormone gene transcription. Consistent with cellular ontogeny, TSHβ mRNA increased steadily between e10 and e17, GH mRNA increased between e12 and e17, and PRL mRNA did not increase until e17. Expression of 141 genes increased in a manner similar to TSHβ mRNA, and 64 genes decreased between e10 and e17. Although genes with these expression profiles are likely involved in development of the pituitary gland as a whole, some of these could be specifically associated with thyrotroph differentiation. Similarly, the expression profiles of 69 and 61 genes indicate a potential involvement in the induction of GH and PRL mRNA, respectively. Quantitative real-time RT-PCR was used to confirm microarray results for 31 genes. This is the first study to evaluate changes in anterior pituitary gene expression during embryonic development of any species using microarrays, and numerous transcription factors and signaling molecules not previously implicated in pituitary development were identified.

embryo; thyrotropin; somatotropin; prolactin

THE ADENOHYPOPHYSIS, or anterior pituitary gland, consists of at least five major cell types that are phenotypically characterized by the trophic hormones they synthesize and secrete. These cell types do not arise simultaneously but rather differentiate during embryonic development in a temporally distinct manner that is similar across vertebrate species (3, 26, 27, 45). The first pituitary marker to appear in rodents (22, 48) and chickens (24) is the α-glycoprotein subunit (α-GSU) that is common to luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). However, corticotrophs, which produce adrenocorticotropic hormone (ACTH), are the initial hormone-secreting cell type to differentiate in chickens, rats, and mice (22, 44, 48). The next cell types to appear are gonadotrophs, which secrete LH and FSH, and thyrotrophs, which produce TSH (22, 31, 35, 48). The final two cell types to differentiate in the developing pituitary gland are somatotrophs, which secrete growth hormone (GH), and lactotrophs, which produce prolactin (PRL) (21, 22, 37, 40, 44, 61).

Development and differentiation of the anterior pituitary gland is divided into three major stages (55): gland commitment from the oral ectoderm, formation of Rathke's pouch, and emergence of the terminally differentiated cell types. Considerable progress has been made in identifying genes that are critical for pituitary organogenesis (reviewed in Refs. 27, 33, 45, 46, 62). Signaling molecules and transcription factors such as sonic hedgehog, bone morphogenetic protein-4, fibroblast growth factor-8, Wnt5a, Wnt4, Hesx1, Lhx3, Lhx4, Pitx1, Pitx2, and Pax6 play crucial roles in the first two stages of adenohypophysial development. Other transcription factors like Prop-1, T-pit, Gata-2, and Pit-1 are necessary for cell lineage commitment and cell type-specific gene expression. However, it is likely that a large number of signaling molecules and transcription factors necessary for normal pituitary development remain unidentified.

Many of the genes implicated in pituitary development have been discovered through analysis of genetic mutations and investigated using traditional molecular biology techniques, but these approaches are limited by the number of genes that can be studied at one time. With the advent of high-throughput technologies such as DNA microarrays, it should be possible to discover additional genes necessary for pituitary gland development. Only two studies have been published using cDNA microarrays to detect transcripts differentially expressed in the normal pituitary gland. The first study (60) was designed to detect transcripts enriched in the mouse pituitary gland during the time of progenitor cell expansion, just before terminal differentiation; the second study (30) focused on genes that are differentially expressed in fetal and adult human pituitary glands that may regulate gene expression of the pituitary hormones. To our knowledge, no study using DNA microarrays has been published that investigates gene expression profiles during terminal differentiation of pituitary cell types in the embryo. Therefore, the objective of the current study was to use a neuroendocrine system-specific cDNA microarray developed in our laboratory to identify genes that are involved in proliferation and differentiation of cell types in the Pit-1 lineage (thyrotrophs, somatotrophs, and lactotrophs) during chick embryonic development. In particular, we were interested in identifying candidate genes that could play a role in the increase in hormone mRNA expression that occurs during this process.

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Although much of the work investigating adenohypophysis organogenesis has been conducted in rodents (reviewed in Refs. 6, 15, 27, 33, 45, 46, 62), the chicken is an excellent model for studying the development of the pituitary gland because the accessibility to the embryo readily allows in ovo manipulation. Furthermore, the chicken embryo develops in the absence of influence from the maternal endocrine system (34). The recent acquisition of a large expressed sequence tag (EST) collection (20) and completion of the genome sequence (20a) has elevated the chicken's status as a widely used model organism for developmental biology and allowed it to be used in large-scale screens to assess gene function during embryonic development (5, 7, 50).

In the chicken embryonic pituitary gland, cell types in the Pit-1 lineage arise during the latter one-half of the 21-day incubation period. Pituitary RNA extracted from embryonic *day 10* (e10), e12, e14, and e17 chickens was amplified and analyzed with microarrays containing 5,128 unique cDNAs expressed in the neuroendocrine system, which is ~25% of the genes identified in chickens (20, 20a). Differentially expressed genes were analyzed with self-organizing maps (SOMS) to identify expression patterns that indicate potential involvement in thyrotroph, somatotroph, and lactotroph differentiation. Quantitative real-time RT-PCR (qRT-PCR) was used to verify expression profiles determined by SOMS analysis of microarray data. Candidate genes that include transcription factors and signaling molecules were identified.

MATERIALS AND METHODS

Animals and tissue collection. The Avian × Avian broiler strain chicken embryos used in these studies were purchased from Allen's Hatchery (Seaford, DE). The day eggs were placed in a 37.5°C humidified incubator was designated as e0. The typical incubation period for chicken embryos is 21 days. Embryos were removed from the incubator on e10, e12, e14, and e17, and their anterior pituitary glands were isolated using a dissecting microscope. To yield sufficient RNA from each developmental age for amplification and analysis, pituitaries from 16 e10, 8 e12, 4 e14, and 2 e17 embryos were pooled for each replicate sample, and 4 replicate samples were collected at each age ($n = 4$). For the preliminary experiment, three replicate samples ($n = 3$) of one pituitary gland each were collected from e17 and posthatch *day 3* (d3) chickens. All procedures involving the use of animals were approved by the Institutional Animal Care and Use Committee at the University of Maryland. Pituitary glands were immediately frozen in liquid nitrogen after collection and stored at −80°C until RNA extraction.

RNA extraction and amplification. Total cellular RNA (T-RNA) was isolated from pituitary glands using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and quantified by measuring absorbance at 260 nm; the quality was verified using a bioanalyzer (Agilent Technologies, Palo Alto, CA). Chicken embryonic anterior pituitary glands do not yield sufficient T-RNA for microarray analysis; therefore, a previously detailed (41) modification of the Eberwine procedure (38) for amplifying mRNA was employed. Briefly, 0.5 μg of total RNA was reverse transcribed with SuperScript II (Invitrogen, Carlsbad, CA) and an oligo(dT) primer containing a T7 promoter site (5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGT$_{24}$-3′; Affymetrix, Santa Clara, CA). After second-strand synthesis, the double-stranded cDNA was phenol-chloroform extracted, purified using a Microcon-30 column (Millipore, Billerica, MA), and used as a template for in vitro transcription with the T7 MEGAscript Kit (Ambion, Austin, TX) according to the manufacturer's protocol. The resulting aRNA was phenol-chloroform extracted, purified with a Spin Column-30 (Sigma, St. Louis, MO), and quantified using the RiboGreen RNA Quantitation Kit (Invitrogen).

Production of neuroendocrine system 5K cDNA microarrays. The cDNA library used to make the microarrays was generated from RNA isolated from the hypothalamus, anterior pituitary gland, and pineal gland of chickens ranging in age from e12 to d63 and has been described previously (8, 9, 41). The library was normalized to eliminate redundant clones after 5,568 clones were randomly selected and sequenced, and an additional 3,301 randomly selected clones from the normalized library were sequenced. Overlapping sequences (contigs) that likely represent the same cDNA were identified by comparing all sequences against one another, and clones without sequence identity to other cDNAs (singlets) were also identified. Table 1 summarizes the number of clones sequenced from the nonnormalized and normalized libraries and the GenBank accession numbers for these clones. From the 8,869 clones sequenced, 5,128 unique cDNAs (unigenes) were identified within the neuroendocrine system library. The unigenes were annotated with the name of the highest basic local alignment search tool (BLAST) score from an analysis of GenBank entries by the BLASTX and BLASTN procedures.

The unigene clones were grown overnight in Terrific Broth (Sigma) in 96-well PCR plates and lysed, and the inserts were amplified by PCR from the pCMV·Sport6.1 vector (Invitrogen) using SP6 (5′-GGCCTATTTAGGTGACACTATAG-3′) and T7 (5′-GCTTATAATACGACTCACTATAGGG-3′) vector-specific primers (Sigma Genosys, The Woodlands, TX). PCR products were visualized in 1% agarose gels using ethidium bromide staining. Clones that did not amplify were manually rearrayed into 96-well PCR plates. These cDNA inserts were amplified using ThermalAce DNA polymerase (Invitrogen) with alternate forward (5′-TGGTACCGGTCCGGAATTCC-3′) and reverse (5′-AGCTGGGTACGCGTAAGCTTGG-3′) primers (Sigma Genosys), resulting in amplification of ~50% of the cDNA inserts that did not amplify using traditional PCR. All products were then purified using isopropanol precipitation. PCR products were rearrayed into 384-well plates in 50% DMSO and printed onto glass microscope slides (Full Moon Biosystems, Sunnyvale, CA) as high-density cDNA microarrays at the Delaware Biotechnology Institute (**http://www.dbi.udel.edu/**) using the OmniGrid 100 arrayer (Genomic Solutions, Ann Arbor, MI). In addition, eight spots of salmon testes DNA were spotted to determine a general background for each slide, which was designated as 90% of the lowest median pixel intensity of salmon testes DNA of all slides in a given experiment. An annotated list of clones and their location on the chicken neuroendocrine system 5K microarrays are available at the Gene Expression Omnibus (GEO; **http://www.ncbi.nlm.nih.gov/geo/**) data repository [platform accession no. GPL1744; National Center for Biotechnology Information (NCBI), Rockville, MD]. The GEO file contains links to the cDNA sequence, contig sequence, BLASTN and BLASTX alignments, and chromosomal location.

Table 1. *Summary of library sequencing and composition of clones used for production of neuroendocrine system 5K microarrays*

Library	No. of ESTs	GenBank Accession Nos.
Nonnormalized	5,490	BI389587–BI395077
Nonnormalized	78	CD215425–CD215503
Normalized	2,096	BM489955–BM492051
Normalized	1,205	CD215504–CD216709
Total ESTs	8,869	
Contigs	4,270	
Singlets	858	
Unigenes	5,128	

EST, expressed sequence tag.

Microarray hybridization and data analysis. Samples were hybridized to the microarrays using a reference design (49). For each experiment, an internal reference sample was generated by labeling an aliquot of an RNA pool made from all the RNA samples from that experiment with Cy5. The experimental samples were labeled with Cy3 and hybridized to an array with an aliquot of the Cy5-labeled pool from each study. This design resulted in the use of 6 microarrays for the preliminary experiment (2 ages, $n = 3$) and 16 microarrays (4 ages, $n = 4$) for the e10 through e17 embryonic development study.

Labeling of cDNA with Cy3 and Cy5, microarray hybridization, and image scanning were performed at the University of Maryland Biotechnology Institute's Microarray Core Facility (**http://www.umbi.umd.edu/~cbr/macore/macorestart.htm**; Rockville, MD). Cy3- or Cy5-labeled target cDNA was generated in a two-step process from 1 μg of aRNA using random primers with the Amino Allyl cDNA Labeling Kit (Ambion), followed by coupling of monoreactive Cy3 or Cy5 dyes (Amersham Biosciences, Piscataway, NJ) to the cDNA. Labeled cDNA targets were purified from unincorporated fluorescent dye using the CyScribe GFX Purification Kit (Amersham). Microarrays were hybridized overnight at 42°C with Cy3-labeled experimental samples and an aliquot of the Cy5-labeled reference pool using microarray hybridization buffer (Amersham). The slides were then washed with increasing stringency using salt sodium citrate and scanned with a 418 confocal laser scanner (Affymetrix) at 550 nm for Cy3 and 649 nm for Cy5, generating two TIFF images for each slide. The images for each slide can be found at the GEO data repository (accession nos. GSM72701–GSM72722).

Data from the microarray analysis were processed and normalized using freely available software that is part of the TM4 suite of microarray data analysis applications (43) offered by The Institute for Genomic Research (TIGR, Rockville, MD). The two images for each slide were processed using Spotfinder (version 2.2.4), and the numeric data were exported for data normalization using Microarray Data Analysis System (MIDAS; version 2.18). Data from the Cy3 channel (representing each experimental sample) were Lowess normalized by block without background correction, followed by standard deviation regularization first by block and then by slide, with Cy5 (the pooled RNA sample) as the reference. The data files for the preliminary e17 to d3 experiment (series accession no. GSE3226, sample accession nos. GSM72717–GSM72722) and the e10 to e17 experiment (series accession no. GSE3227, sample accession nos. GSM72701–GSM72716) can be found at the GEO data repository. Data were then statistically analyzed (see *Statistical analysis*, below) as $\log_2$(normalized Cy3/raw Cy5), or $\log_2$ ratio, for each spot, and it was confirmed that the normalized Cy3 value for each statistically significant spot was above the designated background for at least one age in an experiment. Spots whose pixel intensity for all ages was <90% of the lowest median pixel intensity for the salmon DNA control spots (background; 8 spots/slide) among all slides in a given experiment were eliminated from further consideration. The data were then divided by the mean of the highest level of gene expression across the ages in a given experiment for statistical analysis and comparison with the qRT-PCR results (see below).

For the e10 through e17 experiment, the means from each age for differentially expressed spots ($P \leq 0.05$, and highest mean ≥ 160% of the lowest mean) were organized into 16 clusters [*cluster 0* (c0) to c15] by SOMS analysis using GeneCluster version 2.0 (42), a software program developed by the Broad Institute (Cambridge, MA) and freely available from their web site (**http://www.broad.mit.edu/cancer/software/genecluster2/gc2**). A SOMS 4 × 4 grid was chosen to minimize both the variance within individual clusters and the redundancy of similar clusters while maintaining profiles that reflect ontogeny of thyrotrophs, somatotrophs, and lactotrophs. However, there was still some redundancy among the individual clusters, which were further classified into seven groups (*profiles A–G*) containing clusters with similar gene expression profiles across the four ages.

qRT-PCR. Two-step qRT-PCR was used to confirm expression patterns of 14 cDNA spots for the preliminary experiment and 36 spots for the e10 through e17 developmental study. RT reactions were carried out using SuperScript III (Invitrogen) with random primers (Invitrogen) for aRNA or an oligo(dT) primer (5′-CGGAATTCTTTTTTTTTTTTTTTTTTTTV-3′; Sigma Genosys) for T-RNA. RT reactions (20 μl) were done using 300 ng of aRNA for the preliminary study and 1 μg of T-RNA (26 genes) or 500 ng of aRNA (7 genes) for the e10 through e17 study. As a negative control for genomic DNA contamination, a pool of all the RNA from a given experiment was made, and the reaction was conducted as the others except RT was not added. All reactions were diluted to 100 μl (5-fold) before PCR analysis.

Primers (Sigma Genosys) used for PCR were designed using Primer Express Software (Applied Biosystems, Foster City, CA) from the contig or singlet sequence. Sequences of primer pairs are listed in Table 2, along with the cDNA spot number and the gene annotation as abbreviated in the Entrez Gene database (NCBI). Spot number corresponds to the identification (ID) column in the GEO data table, and gene name is the annotation of the cDNA using the highest BLASTX (where available) or BLASTN score of the contig or singlet. Levels of β-ACTIN, COL1A1, DDX41, DPYSL3, EFEMP1, EIF4GI, ENDOG, GAPDH, HSP90, NCAM1, NPDC1, OB-RGRP, STMN1, and TUBB4 were determined for the preliminary study, and ARFGAP1, β-ACTIN, CAMKK2, CHGA (2 different cDNAs with the same annotation), CPE (2 different cDNAs with the same annotation), CTL2, DEXRAS1, DUSP1, EDNRA, EFEMP1, EGR1, EIF4GI, ENDOG, GDAP2, GH, GHRHR (2 different cDNAs with the same annotation), GILZ, IGFBP7, JUN, MCR, NPDC1, POMC, PRL, RAB3B, REDD1, SGK, SOX10, STMN1, SYP2A, and TSHβ mRNA levels were measured for the e10 through e17 developmental study. Each PCR reaction (20 μl) contained 2 μl of diluted cDNA, 400 nM each primer, and 10 μl of 2× PCR buffer (described below). All reactions conducted for the preliminary study and those where aRNA was used for the e10 through e17 study (CHGA spot 3296, ENDOG, EFEMP1, DUSP1, CTL2, SYP2A, and EGR1) were performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and 2× PCR buffer containing 100 mM KCl, 20 mM Tris·HCl (pH 9.0), 0.2% Triton X-100, 3.8 mM $MgCl_2$, 0.12 U/μl Taq polymerase, 400 nM dNTPs, 40 nM fluorescein (Invitrogen), and SYBR Green I Nucleic Acid Gel Stain (Invitrogen) diluted 1:10,000. The remainder of the mRNA levels were assessed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and the 2× Quantitect SYBR Green PCR Master Mix (Qiagen). In all cases, a two-step PCR cycle was used: initial denaturation at 95°C for 5 min (iCycler) or 15 min to activate the HotStarTaq in the Quantitect Master Mix (ABI 7900HT), followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s. Dissociation curve analysis and gel electrophoresis were conducted to ensure that a single PCR product of appropriate size was amplified in each reaction. The data were transformed using the equation 2^{-Ct}, where Ct is the threshold cycle, the fractional cycle number when the amount of amplified product reaches a fixed threshold for fluorescence because of binding of SYBR green to the double-stranded PCR product. The data were divided by the mean of the highest level of gene expression across the ages in a given experiment for statistical analysis and comparison with the microarray results.

Statistical analysis. Statistical analyses were performed using Statistical Analysis System (SAS) version 8.02 (SAS Institute, Cary, NC). Normalized microarray data were subjected to one-way ANOVA to identify spots that were differentially expressed on at least one of the developmental ages. Differences were considered significant at $P \leq 0.05$. To further filter out false positives, spots were considered differentially expressed only if the highest mean among the experimental groups was ≥160% of the lowest mean, corresponding to a 0.68-fold change on a $\log_2$ scale. Files for each experiment containing the mean $\log_2$ ratio, the P value, and the fold change for

Table 2. *Primers used for qRT-PCR*

Spot*	Gene Name†	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)
906	ARFGAP1	CTTTTTAGGGATCAGGTTGCTACAGT	CGAGTTTCCTGGCAGGAGAAGT
302	β-ACTIN	CCCAAAGCCAACAGAGAGAAG	ACCATCACCAGAGTCCATCAC
2,954	CAMKK2	CCTGGCTCAGTAATTCCTCCTTAC	GGGATAAGCATAGCTTCAGGTGAT
175	CHGA	GTGAGGGATTCTAGGCTGCAA	GCTACTTCTCCCTGTGCATCCT
3,296	CHGA	AAGCAGAGAGCAACGAGATCAAG	TCTCAGTAGACGTGGCTGCTCTT
5,029	COLA1A	AGTGATTGAGTACAAGACGACGAAGA	CGATGTCAATGCCAAATTCCT
967	CPE	CTTTAGTCCTGCTGTTAGTGGTTGA	CTGACATCATTTTCCACCATTCC
4,945	CPE	GTGCTCTCTGCCAATCTTCATG	TCATCAGGGCAGGAGCTGTAT
1,377	CTL2	CTGTTCTATTTCGACATCACTGCAT	GGGCATTCGGAGACACAGA
5,698	DDX41	TGCAGGAAGTGGAGTATGTGAAAG	CGCATCAACATCTGCCTTCTT
2,379	DEXRAS1	AAACACGTGCAGAAGACACTTCTAAG	CCCAGATCTTGCAAATCAGACA
585	DPYSL3	GAGTGACCGTCTCCTGATTAAAGG	CTCCAATCTGCTTTATGAGACCATCT
5,008	DUSP1	TCGTGTCGCTGACTCCTGAA	ACTACTGAACATGCAGCAGGCTTA
2,985	EDNRA	GCGTGTACTGCCATCTTCTATACACT	CTCGACGCTGCTTAAGGTGTT
5,774	EFEMP1	TGCCCAGGTTCATGATGTTG	GCATTGCGTGTACGTTATGGTT
4,106	EGR1	TCAGCACTTTCAGACATGACATCA	AGTACCAGTGGAAGAGGTGAATGC
2,907	EIF4GI	CTGGAACACTGTGCCCATCA	AGAGCTGGTTGTTGGAGTCGAT
6,743	ENDOG	TATGGAAGGCATCAGAGAACTTGAC	CACGCACAAAGGGAGCAATTA
1,636	GAPDH	AAGGAGTGAGCCAAGCACACA	TCACTGCAGGATGCAGAACTG
6,512	GDAP2	AGGACAAGATCCACTATGTGGAAAG	CCCTGGCATCATACTCAAGAACA
993, 5,800	GH	AGGAAGCTGCTGCCACCTT	CGTTGGCAAACAGGTTGGA
1,542	GHRHR	ACAGCTGGTTGTATTGACCAGATC	GACCATGGCAGAGAAGTCAATTAA
3,488	GHRHR	CATCCGGAATGTGATTTTATAGCA	TCTGAGTTCTCCAGGCATTCAG
1,134	GILZ	GGAGCAGATTAAGGAACTGTTGGA	GGAGCCGTGACTGGAACTTCT
2,218	HSP90	GCAGAGGCTGATAAGAATGACAAA	TAAATGCGGTTGGCATGTGT
508	IGFBP7	ATGTGACAGGAGCACAGATCTACCT	TCTGGATACCATACTGTCCTCGAAT
4,970	JUN	GTGCTGCAAAGCGCATGT	CCTCATCGGAGCAGGAACAG
6,717	MCR	TTGCTCCAGACCTGATTTTTGAT	TGGCACAGTTCAAACATTGCA
574	NCAM1	CCCAGAACCCTTAGATTAATTTTGG	CTAAGCTACCTTGGGAATGGGATAT
3,159, 4,954	NPDC1	CGCCCAGATGTACCACTACCA	CTCCGTCCTCGTTCTCCTCAT
3,273	OB-RGRP	TCTCTCATGAAGTCTCACAGAGGAA	CAATTCAGGCACTTCACACACA
1,185, 2,526	POMC	AGGGACCTCAGGGATCATCAA	TGTTCAAGGGCAGGTTGGA
1,297	PRL	ACCTGTGGGCTGCATTACTCA	AAGTTACTGATGATCCTGGTGCTGTA
6,322	RAB3B	GCCACGTGCAGAGCTTGTATT	CAAACAGCGGTGCAATCATG
4,153	REDDI	CCGTGTGCTTCATTGATTACAGTT	AAACGACACAATTGCATAGGAAGTC
2,869	SGK	CTGATGAGATCTTGTTAGCTCCAAAG	GTAGTACGCAGACCACACGCATA
1,464	SOX10	TCGTGGATATAAGCAAGGTAGATATGC	TGAAACCCAGAGGTGAAAATGAGT
239	STMN1	GAACCGTGAGGCACAAATGG	CACCAGGGTCTTTGCCTTCTT
6,712	SYP2A	GTGACCTTACGAAGCGTAAGCAT	GGCAACAGAACAGAGGGAAATC
2,139	TSHβ	CTACCCCGTGGCCATAAGCT	TCTGTGGCTTGGTGCAGTAGTT
2,746	TUBB4	CGGTGGTGGAGCCCTACA	CATCCGTGTTCTCCACCAGTT

*Spot corresponds to the identification (ID) column in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) data table, which can be found at **http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GPL1744**. †Gene names are annotated with the highest basic local alignment search tool (BLAST)X score (where possible) or the highest BLASTN score (if no BlastX hit) of the contig or singlet and abbreviated as in the Entrez Gene database. qRT-PCR, quantitative real-time RT-PCR.

each cDNA spot can be found at the GEO data repository (accession nos. GSE3226 and GSE3227).

For the preliminary experiment, qRT-PCR data were analyzed using a one-tailed *t*-test, and differences were considered significant at $P \leq 0.05$ only if the gene expression changed between e17 and d3 in the same direction as the microarray results. For the e10 through e17 developmental study, qRT-PCR data and the corresponding microarray data expressed on a relative level as described above were analyzed using one-way ANOVA followed by Duncan's new multiple-range test. Differences were considered significant at $P \leq 0.05$.

RESULTS

Validation of RNA amplification procedure. Chicken embryonic pituitary glands do not contain sufficient T-RNA for microarray analysis. To overcome this problem, we employed an RNA amplification protocol that is a modification of the Eberwine procedure (38). In establishing this procedure, we confirmed that microarray results from amplified RNA (aRNA) agree with results from T-RNA. Two samples from a pool of T-RNA and two aRNA samples independently amplified from the same pool were each analyzed with separate cDNA microarrays, and the mean $\log_2$-transformed raw pixel intensities from each spot were correlated. As seen in Fig. 1, results from the aRNA and T-RNA correlated well ($r^2 = 0.96$). However, it should be noted that this correlation might be influenced by the amount of DNA printed in each spot. In addition to correlating microarray results from unamplified T-RNA and aRNA, we also confirmed microarray results obtained from aRNA with qRT-PCR analysis of the original, unamplified T-RNA (see below). Together, our results support the fidelity of the amplification procedure.

Changes in pituitary gene expression between e17 and d3. To further verify that aRNA could be used in our cDNA microarray analysis, we conducted an experiment to determine differential gene expression between e17, when all the major cell types in the chick anterior pituitary gland have differentiated, and d3. The results of this preliminary experiment are

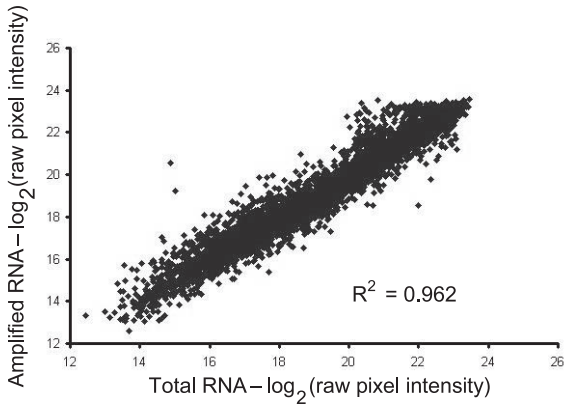

Fig. 1. Correlation of cDNA microarray results between total and amplified pituitary RNA. Pituitary RNA was amplified in 2 independent reactions, and then 1 μg of amplified RNA from each reaction or 25 μg of total RNA were analyzed using 2 arrays each. Pixel intensity was quantified using Spotfinder (TIGR, Rockville, MD) and $\log_2$ transformed, and the mean values for total RNA and amplified RNA were correlated.

summarized in Table 3. It is important to note that greater than 99% of the spots on the array that contained chicken cDNA detected RNA on at least one of the ages, emphasizing the utility of tissue-specific cDNA microarrays for studying global gene expression patterns in the tissues from which the printed cDNA was derived.

Of the 662 cDNAs that were expressed at significantly different levels between the two ages ($P \leq 0.05$, $n = 3$ samples/age), 159 exhibited considerable differences, where the higher mean was at least 160% of the lower mean. These 159 cDNAs represented 156 unique cDNAs because of duplication on the microarray. Expression of the majority of genes increased between e17 and d3. To validate that these changes were detectable by alternative methods, we used qRT-PCR to assess differences in expression for 14 of these genes (see MATERIALS AND METHODS and Table 2). The qRT-PCR results from all of these genes positively correlated with the results of the microarray: if a gene was determined to increase between e17 and d3 by the microarray, it also increased as determined by PCR, and if a gene was determined to decrease between e17 and d3 by the microarray, it decreased by PCR. However, it should be noted that aRNA was used for this qRT-PCR analysis, and any bias introduced into the microarray results from amplification of the RNA is also reflected in the qRT-PCR analysis. A comparison of results from the microarray analysis and qRT-PCR for two genes each that did not change, increased, or decreased between the two ages is shown in Fig. 2. The ability to detect changes in gene expression between e17 and d3 and confirm these changes with qRT-PCR, in conjunction with the highly correlated microarray results from T-RNA and aRNA (Fig. 1), indicate that aRNA can be used with our cDNA microarrays to assess global gene expression in the developing pituitary gland.

A list of all the cDNAs that were differentially expressed between e17 and d3 is shown in Supplemental Table S1 (available at the *Physiological Genomics* web site).[1] The cDNAs are annotated with the gene name of the highest BLASTX

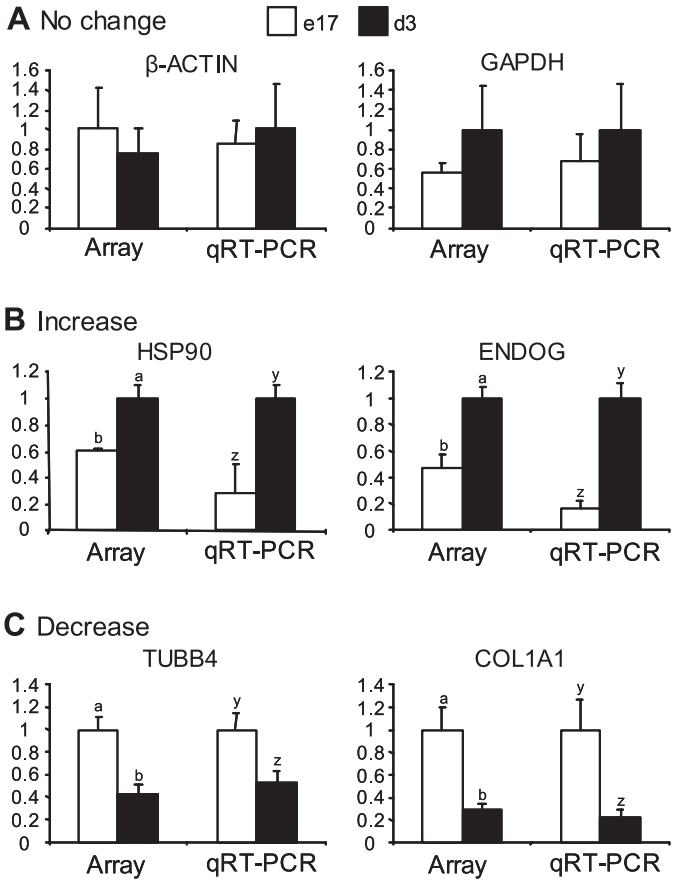

Fig. 2. Microarray and quantitative real-time RT-PCR (qRT-PCR) analysis of gene expression in the anterior pituitary on embryonic *day 17* (e17) and posthatch *day 3* (d3). Shown are results for 6 genes: 2 that did not change between the ages, 2 that increased between e17 and d3, and 2 that decreased during development. *A*: β-actin and glyceraldehyde-3-phosphodehydrogenase (GAPDH) did not change on the microarray. *B*: heat shock protein 90 (HSP90) and endonuclease G (ENDOG) increased between e17 and d3. *C*: tubulin, beta 4 (TUBB4), and collagen, type I, alpha I (COL1A1), decreased between e17 and d3. Values within a given technique with different letters are significantly different from one another ($P \leq 0.05$).

Table 3. *Summary of gene expression changes in the chick anterior pituitary gland between e17 and d3*

Category	No.
Total spots on the array	7,200
No. of good spots*	7,154
Spots with chicken cDNA	5,985
Spots above background†	5,971
Significant spots ($P \leq 0.05$)	662
Fold change ≥1.6	159
Increased between e17 and d3	117
Decreased between e17 and d3	42
Unique cDNAs	156

*Spots that passed microarray data analysis system (MIDAS) (The Institute for Genomic Research; TIGR, Rockville, MD) quality control filters and were subjected to statistical analysis. †Spots whose integrated pixel intensity in channel A (Cy3) was greater than background for at least 2 of the 3 samples on either age. Background was defined as 90% of the lowest median integrated pixel intensity for the salmon testes DNA control spots (8 spots/slide) among the 6 slides used in the experiment. e17, Embryonic *day 17;* d3, posthatch *day 3*.

[1] The Supplemental Material for this article (Supplemental Tables S1 and S2) is available online at **http://physiolgenomics.physiology.org/cgi/content/full/00248.2005/DC1**.

score wherever possible. The BLASTN annotation is given in cases where the BLASTN analysis revealed sequence similarity but the BLASTX analysis did not. The gene name corresponds to the abbreviation in the Entrez Gene database (NCBI) where possible. Seventeen of these genes (11 that increased and 6 that decreased) had no significant sequence homology to known genes, and 40 genes (29 that increased and 11 that decreased) are of unknown function. Genes with roles in broad metabolic processes such as cell signaling, cell proliferation, cytoskeletal organization, energy metabolism, intracellular trafficking, protein metabolism, and transcriptional regulation were differentially expressed between the two ages. Several of these may play a role in neuroendocrine development. Among the genes whose expression increased between e17 and d3 are potential DNA regulatory proteins that may be involved in development and differentiation of specialized cell types. These included enhancer of zeste homolog 2 (EZH2), activating transcription factor 4 (ATF4), and neurogenic differentiation factor 1 (NEUROD1). In addition, mRNA for four proteins with putative transmembrane domains that could be involved in cell type-specific differentiation increased between the two ages. These were adipose differentiation-related protein (ADRP), OB-receptor gene-related protein (OB-RGRP), monocyte-to-macrophage differentiation-associated protein (MMD), and neural proliferation, differentiation and control gene 1 (NPDC1). Another gene with a known signaling function that increased after hatch was heat shock protein 90 (HSP90). Three of the genes that decreased between e17 and d3 are putative signal transduction molecules that may play a role in embryonic development: stathmin 1 (STMN1), dihydropyrimidinase-like 3 (DPYSL3), and ras-related protein RAS-DVA (RAS-DVA). In addition, two molecules involved in cell-to-cell adhesion decreased during this time: neural cell-adhesion molecule 1 (NCAM1) and immunoglobulin superfamily, member 4 (IGSF4), potentially indicating a morphological maturation of the pituitary gland between the two ages.

Changes in anterior pituitary gene expression associated with hormone ontogeny. An experiment was conducted to investigate changes associated with proliferation and terminal differentiation of cell types of the Pit-1 lineage: thyrotrophs, somatotrophs, and lactotrophs. In particular, we were interested in identifying candidate genes that may play a role in increased transcription of TSHβ, GH, and PRL associated with functional maturation of these cell types, which occurs in the chick between e10 and e17 (19, 25, 40, 61). A summary of the results of this experiment is given in Table 4. As in the preliminary study, almost all of the spots that contained chicken cDNA detected RNA targets on at least one of the four ages. These observations further demonstrate the power of using tissue-specific cDNA microarrays to study developmental changes in a tissue when the microarrays are printed using a developmentally complex cDNA library originating from the tissue of interest. The expression level of 1,681 cDNAs was significantly different on at least one of the ages ($P \leq 0.05$, $n = 4$ samples/age), and 352 of these changed substantially, where the highest mean was ≥160% of the lowest mean. Due to duplication of cDNAs or because multiple cDNAs belong to the same contig, these 352 spots represent 332 unique genes. In 17 cases, duplicate cDNAs are represented on the array; in 3 cases, more than 1 cDNA from a given contig was significantly expressed. In all cases, cDNAs that are either identical or represent the same gene (i.e., are part of the same contig) changed in a consistent manner across the four ages. For example, two identical cDNAs (spots 429 and 4157) for vimentin (VIM) decreased steadily between e10 and e17, and two clones that are part of the same contig identified as β-2-microglobulin (B2M) (spots 2743 and 4884) increased steadily between e10 and e17.

Table 4. *Summary of global gene expression changes that occurred in the chick anterior pituitary gland between e10 and e17*

Category	No.
Total spots on the array	7,200
No. of good spots*	7,194
Spots with chicken cDNA	5,985
Spots above background†	5,840
Significant spots ($P \leq 0.05$)	1,681
Fold change ≥1.6	352
Unique cDNAs represented	332
SOMS clusters	16
Distinct profiles	7

*Spots that passed MIDAS (TIGR, Rockville, MD) quality control filters and were subject to statistical analysis. †Spots whose integrated pixel intensity in channel A (Cy3) was greater than background for at least 3 of the 4 slides on at least 1 of the 4 ages. Background was defined as 90% of the lowest median integrated pixel intensity for the salmon testes DNA control spots (8 spots/slide) among the 16 slides used in the experiment. SOMS, self-organizing maps.

The 352 significant cDNAs with substantial changes in gene expression between e10 and e17 were organized into 16 clusters, c0 through c15, using SOMS analysis (Fig. 3). After evaluating all possible SOMS row and column combinations containing between 9 and 30 clusters, we chose 16 clusters in a 4×4 configuration to minimize both the variance within individual clusters and the redundancy of similar clusters, yet still maintain profiles that reflect thyrotroph, somatotroph, and lactotroph ontogeny. Because multiple clusters with similar developmental expression patterns were still present, we further combined those clusters with similar patterns across the four ages into seven gene expression profiles (*profiles A–G*). *Profile A* (149 cDNAs) contains the majority of genes, those whose expression increased steadily between e10 and e17. *Profile B* contains those genes that did not change until e12 and then steadily increased between e12 and e17 (66 cDNAs). Genes that remained steady and then increased on e17 are clustered into *profile C* (31 cDNAs). *Profile D* represents genes that increased in expression transiently, on e12 and e14 (2 cDNAs). Those genes that decreased steadily throughout development are organized into *profile E* (67 cDNAs). *Profile F* contains genes that increased between e10 and e14 and then decreased on e17 (6 cDNAs). Finally, those genes that showed a predominant decline in expression on e17 are found in *profile G* (31 genes). An annotated list of genes found in each cluster and profile is provided in Supplemental Table S2. Of the 352 cDNAs whose expression changed substantially, 42 of these had no significant sequence homology to known genes and are potentially novel regulators of embryonic pituitary development.

The same RNA samples that were hybridized to the arrays were analyzed using qRT-PCR to verify gene expression pro-

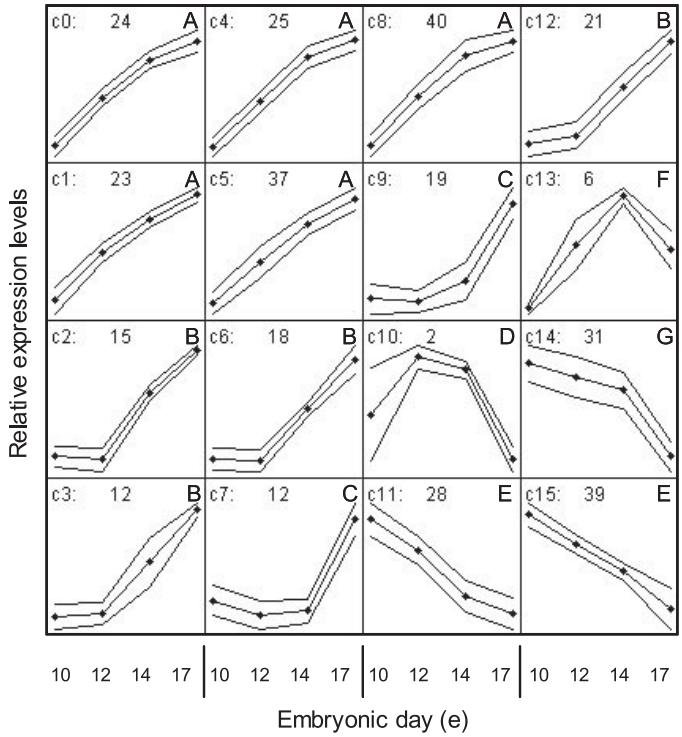

Fig. 3. Self-organizing maps (SOMS) analysis showing means of differentially expressed genes ($P \leq 0.05$, greatest mean $\geq 160\%$ of the lowest mean) between e10 and e17. The expression levels of 352 cDNAs were organized into 16 clusters [*cluster 0* (c0) through c15] using GeneCluster version 2.0. The 16 clusters were further combined into 7 gene expression profiles (*profiles A–G*) that contained clusters that exhibited similar gene expression patterns across the 4 ages. The cluster no. is denoted at *top left* of each cluster, the no. of cDNAs organized into that cluster is at *top middle*, and the profile into which that cluster was grouped is indicated at *top right*. For each cluster, the relative expression level is on the *y*-axis, and the age (e10, e12, e14, and e17) is on the *x*-axis. For example, there are 24 cDNAs in c0 that increased steadily in expression between e10 and e17, and this cluster is part of expression *profile A*.

files revealed by microarray and SOMS analysis. We quantified mRNA levels of 33 genes, representing 36 spots on the array (see MATERIALS AND METHODS and Table 2). At least two genes were evaluated from each expression profile, including three genes that did not change in the microarray analysis. The expression pattern of 31 genes agreed between the two methods. Of the two that did not agree, one was in *profile E* [calcium/calmodulin-dependent protein kinase kinase 2 β-isoform 2 (CAMKK2)], and one was in *profile C* [ADP-ribosylation factor GTPase-activating protein 1 (ARFGAP1)]. These two genes are signaling molecules whose primary mode of action does not involve regulation at the transcriptional level. In both cases, the PCR product that was printed on the array has been sequenced and is correct. Therefore, it is possible that the primers used for qRT-PCR amplified a closely related mRNA that did not change in a similar manner. Microarray and qRT-PCR analyses of selected genes for each profile are shown in Fig. 4. Most of the qRT-PCR analyses were performed on T-RNA, but when the T-RNA was exhausted, additional qRT-PCR reactions were performed on aRNA. Although the use of aRNA to confirm microarray results for a limited number of genes in this experiment (7 of 33 total) may not allow validation of the amplification procedure itself, the expression pattern of the majority of genes was confirmed by qRT-PCR analysis of T-RNA. Of these, 24 of 26 genes exhibited similar developmental profiles between e10 and e17, further validating the use of aRNA in our microarray analysis.

In microarray and qRT-PCR analyses, TSHβ mRNA levels increased steadily between e10 and e17 (Fig. 4 and Supplemental Table S2), a result consistent with previous findings (19, 35). Genes that exhibit a similar expression pattern (those in *profile A*) or an inverse pattern (those in *profile E*) may be involved in the functional expression of this gene (Fig. 3). For example, transcription factors such as SRY-box transcription factor SOX-10 (SOX10), which increased between e10 and e17, or protooncogene c-jun (JUN), which decreased between e10 and e17, may play a role in the transcription of factors important in regulating TSHβ expression. In addition, several genes with putative cell-signaling roles are contained in these two profiles. N-myc downstream-regulated gene 1 (NDRG1), Rho GTPase-activating protein 21 (ARHGAP21), Rho GTPase-activating protein 23 (ARHGAP23), and protein kinase C, delta (PRKCD) are among the genes that increase between e10 and e17 in a manner similar to TSHβ; DPYSL3, a transmembrane protein with EGF-like and two follistatin-like domains 1 (TMEFF1), and KH domain containing, RNA binding, signal transduction-associated 1 (KHDRBS1) decreased steadily between e10 and e17. *Profiles A* and *B* are also likely to contain genes that are involved in general maturation and development of the pituitary gland and do not necessarily play a role in TSHβ gene expression. For example, *profile A* contains genes like NPDC1, chromogranin A (CHG A), chromogranin B (CHG B), and carboxypeptidase E (CPE) which are likely involved in the development of the pituitary as a mature secretory gland. *Profile E* contains many genes that indicate that mitotic activity in the gland is decreasing during this time, including several actin and tubulin proteins, cyclin-dependent kinase 1 (CDK1), and proliferating cell nuclear antigen (PCNA).

GH gene expression as determined by both microarray and qRT-PCR increased between e12 and e17 (Fig. 4 and Supplemental Table S2), consistent with the ontogeny of somatotrophs in the embryonic chick pituitary gland (25, 40). Genes with a similar expression pattern that are potentially involved in initiating GH gene expression in somatotrophs are clustered in *profile B* (Fig. 3). Genes in *profiles D* and *F*, which increased transiently on e12 and e14 just before GH expression and decreased again on e17, may also play a role in the functional differentiation of somatotrophs. Several genes with known or putative transcription factor and cell-signaling functions are contained in these profiles. *Profile B* contains EZH2, glucocorticoid-induced leucine zipper (GILZ), RAS-DVA, dexamethasone-induced ras-related protein 1 (DEXRAS1), MMD, regulated in development and DNA damage responses 1 (REDD1), stress-induced phosphoprotein 1 (STIP1), annexin A2 (ANXA2), and FK506-binding protein 5 (FKBP5). Inducible transcription factor early growth response 1 (EGR1) is in *profile D*, and two potential clones for the growth hormone-releasing hormone receptor (GHRHR) are in *profile F*.

The level of PRL mRNA remained low until e17 in both microarray and qRT-PCR analyses (Fig. 4 and Supplemental Table S2), which is consistent with data reported previously (25, 61). In SOMS analysis, genes with this same expression pattern are in *profile C* (Fig. 3). It is also likely that genes with

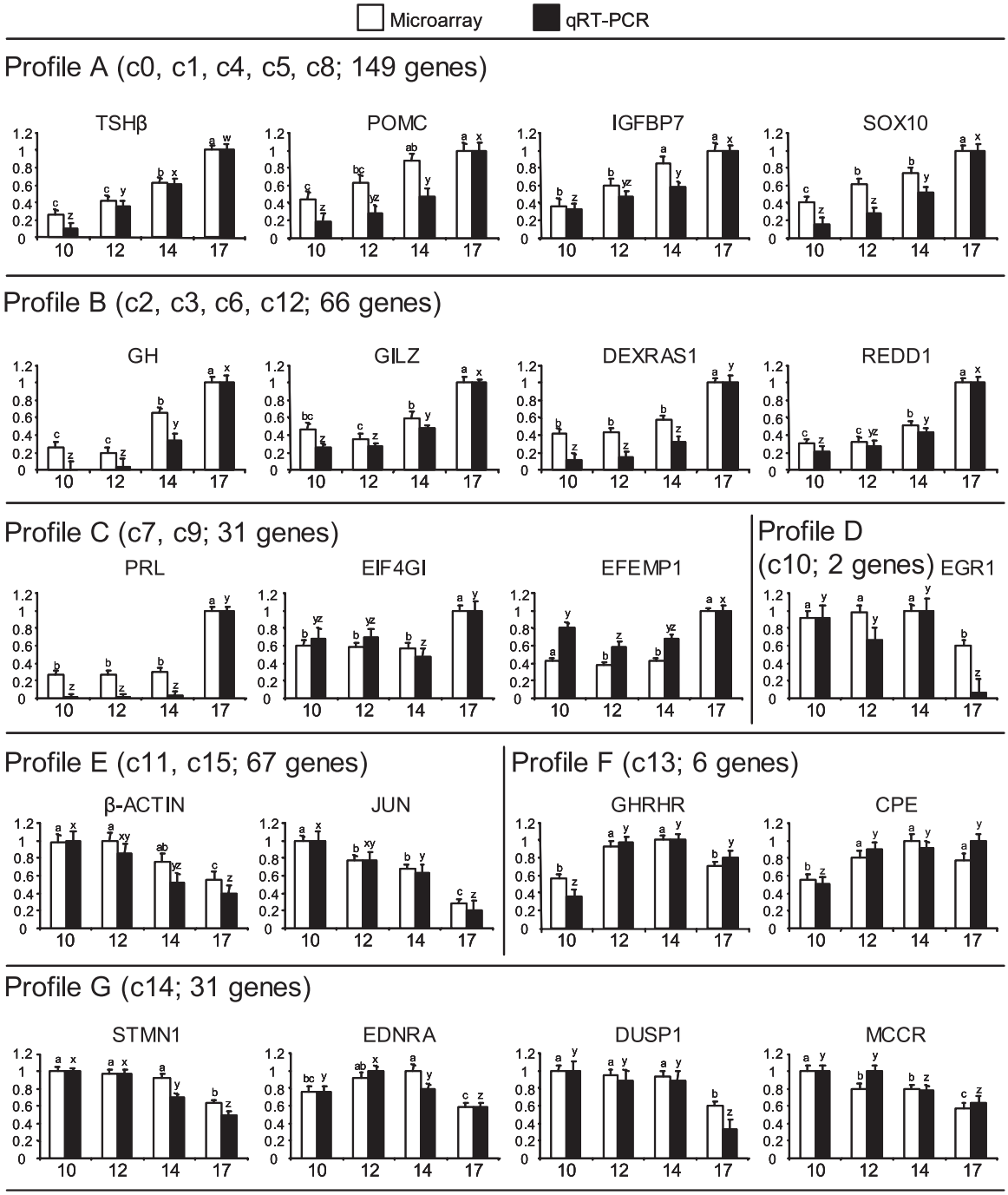

Fig. 4. Comparison of microarray analysis of anterior pituitary expression levels for 20 genes with results from qRT-PCR analysis on e10, e12, e14, and e17. Data were normalized to the highest expression level for each technique. Results are presented for thyroid-stimulating hormone, beta chain (TSHβ), proopiomelanocortin (POMC), insulin-like growth factor-binding protein 7 (IGFBP7), SRY-box transcription factor SOX-10 (SOX10), growth hormone (GH), glucocorticoid-induced leucine zipper (GILZ), dexamethasone-induced ras-related protein 1 (DEXRAS1), regulated in development and DNA damage responses 1 (REDD1), prolactin (PRL), eukaryotic translation initiation factor 4G I (EIF4GI), EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), inducible transcription factor early growth response 1 (EGR1), β-actin, protooncogene c-jun (JUN), growth hormone-releasing hormone receptor (GHRHR spot 3488), carboxypeptidase E (CPE spot 4945), stathmin (STMN1), endothelin type A receptor (EDNRA), dual-specificity phosphatase 1 (DUSP1), and mineralocorticoid receptor (MCR). The qRT-PCR analysis for all genes except EFEMP1, EGR1, and DUSP1 was conducted on total RNA, and for these genes amplified RNA was used. Values within a given technique with different letters are significantly different from one another ($P \leq 0.05$).

an inverse pattern, or those in *profile G* that exhibited a decrease in expression on e17, may be involved in the increase in PRL mRNA associated with lactotroph differentiation. Some of the genes contained in *profile C* are putative signaling molecules and transcription factors such as ADRP, choline transporter-like 2 protein (CTL2), RAB8A, OB-RGRP, and REST corepressor 1 (RCOR1). In *profile G*, potential signaling molecules and transcription factor candidates for initiating PRL gene expression are STMN1, endothelin type A receptor (EDNRA), tyrosine 3-monooxygenase/tryptophan 5-monoox-

ygenase activation protein, gamma polypeptide (YWHAG), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), dual-specificity phosphatase 1 (DUSP1), and mineralocorticoid receptor (MCR).

DISCUSSION

We have used a custom neuroendocrine cDNA microarray to assess global gene expression profiles in the anterior pituitary during chick embryonic development. Our goal was to identify genes associated with the increase in TSHβ, GH, and PRL mRNA expression corresponding with terminal differentiation of thyrotrophs, somatotrophs, and lactotrophs. The microarrays were produced from 5,128 cDNAs expressed in the chicken neuroendocrine system. The primary and normalized cDNA libraries used to produce the microarrays were made from a pooled RNA sample containing pituitary RNA as early as e12 (8). Thus the arrays were designed to study development of the neuroendocrine system, and, indeed, greater than 99% of the cDNA probes detected measurable amounts of pituitary RNA on at least one embryonic age. Because embryonic pituitary glands yield insufficient RNA for microarray analysis, we first amplified the mRNA. We verified that microarray data from pituitary T-RNA and aRNA were correlated and conducted a preliminary study investigating changes occurring between e17 and d3, in which microarray results were confirmed by qRT-PCR. In the subsequent e10 to e17 experiment, microarray results for 31 of 33 genes were confirmed by qRT-PCR analysis. Furthermore, we were able to detect changes in pituitary hormone mRNA levels between e10 and e17 that are consistent with previous reports. These observations indicate that changes in gene expression detected by microarray analysis, and their organization into the patterns revealed by SOMS, accurately reflect anterior pituitary gene expression during the latter one-half of embryonic development in the chick.

Although thyrotrophs first appear in the chick pituitary gland as early as e6.5, their numbers increase considerably on e11.5 (53) and continue to increase through e17 (32), which is consistent with an increase in the level of TSHβ mRNA that occurs between e9 and e19 (19, 35). In both microarray and qRT-PCR analyses, TSHβ mRNA levels rose steadily between e10 and e17. It is likely that genes with a similar expression pattern (*profile A*) or an inverse expression pattern (*profile E*) may influence TSHβ gene expression associated with functional differentiation of thyrotrophs. Proopiomelanocortin (POMC) mRNA levels also increased steadily between e10 and e17, so genes contained in *profiles A* and *E* may also be associated with changes in POMC gene expression. There were 205 unique genes in these two expression profiles, and mRNA levels of 141 genes increased between e10 and e17 similar to TSHβ and POMC. In these two profiles, 30 genes had no significant homology to known genes, and 48 of the identified genes had unknown functions. The remaining mRNAs were associated with general cellular metabolism (65 genes), cell cycle regulation (8 genes), secretion (5 genes), signal transduction (38 genes), and transcription (11 genes). It is difficult to distinguish those genes potentially involved in TSHβ and POMC expression from those that are involved in development of the gland as a whole. Some genes within *profile A* are thought to be associated with formation of secretory vesicles [CHGA and CHGB (16)] and prohormone processing [CPE (12)] and might reflect development of the pituitary into a functional secretory gland. Many of the genes downregulated in *profile E* are those with roles in cell proliferation (i.e., cytoskeletal proteins, cyclin-dependent kinases, and PCNA), likely reflecting the decrease in mitotic activity associated with terminal differentiation of hormone-secreting cell types during this time (47). It is tempting to speculate that some of these genes could be involved in TSHβ or POMC mRNA expression. A transcription factor shown to increase by both microarray and qRT-PCR analysis in a manner similar to TSHβ and POMC is SOX10. As SOX proteins are known to form functional associations with POU-domain transcription factors (1) like Pit-1, this protein may play a role in inducing TSHβ gene expression.

In the chick anterior pituitary gland, somatotrophs are first detected between e12 and e14 and become a significant population by e16 (40), with GH mRNA levels increasing between e14 and e16 (25). Microarray and qRT-PCR data from this study are consistent with these reports, as GH mRNA increased between e12 and e17 and was clustered into *profile B*. In addition to the 61 genes organized into *profile B*, another 8 genes in *profiles D* and *F* exhibited patterns of expression that could indicate an involvement in initiation of GH gene expression during somatotroph differentiation. The genes in *profile D* increased transiently on e12 and e14, the time when GH-producing cells are just beginning to appear in the anterior pituitary. Similarly, those genes in *profile F* increased steadily between e10 and e14 before decreasing on e17. Genes with these expression profiles could have a direct positive effect on GH promoter activity or may act to inhibit expression of a protein that represses GH gene transcription. Of the 46 genes with significant homology to genes with known or putative functions, 22 are involved in general cellular metabolism, 1 plays a role in secretion, 19 are signaling molecules, and 4 are transcriptional regulators. Transcriptional regulators and signaling proteins are the most likely candidates to play a role in initiating GH gene expression during somatotroph differentiation. GHRHR is important in functional differentiation or proliferation of GH-producing cells in mammals, as is evident from the phenotype of the little mouse, which has a mutation in the GHRHR that disrupts its function and exhibits a reduction in the number of pituitary somatotrophs (17, 29).

Glucocorticoids are capable of inducing somatotroph differentiation in embryonic chickens and fetal rats (reviewed in Ref. 39). In chickens (4) and rats (36), inhibition of protein synthesis blocks glucocorticoid induction of GH mRNA, implying induction of an intermediary protein necessary for GH expression. A number of candidate genes identified in the current study that increased between e12 and e14 in a manner similar to GH are induced by glucocorticoids, an observation that is consistent with the rise in serum glucocorticoids that occurs around e14 in the chick (23). Those genes that are known to be directly inducible by glucocorticoids include the putative transcription factor GILZ (13) and the signaling molecules DEXRAS1 (57), REDD1 (59), and FKBP5 (58). We have shown previously that manumycin A, a RAS inhibitor, partially blocks corticosterone induction of GH mRNA in chick embryonic pituitary cells (4). RAS-DVA also exhibits a profile similar to GH and is another protein that could be involved in

GH gene expression during the final stages of somatotroph differentiation. EGR1 was organized into *profile D*, exhibiting a transient increase on e12 and e14, and may be associated with somatotroph differentiation, as EGR1 knockout mice exhibit reduced numbers of somatotrophs (54). However, pituitary glands from EGR1 knockout mice also contain gonadotrophs that are defective in LHβ production (28, 54), and EGR1 has been identified as a positive regulator of LHβ gene transcription (56), indicating that effects of EGR1 are not restricted to somatotrophs.

In the present study, we found that PRL mRNA levels did not increase until e17. This agrees with ontogeny of lactotroph differentiation and PRL gene expression, which occur around e17 (25, 61). Genes with a similar expression pattern are organized into *profile C* and may include factors that positively regulate the PRL promoter. In contrast, *profile G* represents genes with an inverse expression pattern, where mRNA levels decreased on e17, and may include genes with an inhibitory effect on PRL expression. These two clusters contain 61 genes that are potential candidates for regulating the increase in PRL mRNA levels associated with lactotroph differentiation. Of these, 12 genes are of unknown function and 8 have no significant homology to known genes. The remaining mRNAs play roles in cellular metabolism (23 genes), are putative signaling molecules (13 genes), or regulate transcription (5 genes). Ras signaling is involved in PRL gene expression in mammalian lactotrophs, and JUN overexpression antagonizes this response and inhibits PRL promoter activity (10). In the present study, JUN mRNA decreased between e10 and e17 (*profile E*), and this may allow the increase in PRL mRNA that occurs on e17. The level of EDNRA mRNA, a signaling receptor expressed in the anterior pituitary that is involved in repression of PRL release by endothelins in mammals (51), decreased substantially on e17. This decrease in EDNRA expression may be important for allowing PRL mRNA to increase during lactotroph differentiation and proliferation.

All of the anterior pituitary cell types have differentiated by e17, and the period of development after this age likely includes functional maturation of pituitary cell types. A comparison of late embryos (e17) with hatchling chicks (d3) revealed that expression of 114 genes increased and 42 genes decreased during this period. Of those genes that have known or putative functions, 62 are associated with general cellular metabolism, 4 play a role in cell cycle regulation, 27 have been implicated in cell signaling, and 6 are transcriptional regulators. Seven of the genes associated with cellular metabolism that are upregulated between e17 and d3 reflect the development of the pituitary into a mature secretory gland. Genes with positive roles in protein synthesis (eukaryotic translation initiation factor 4G I), translocation of proteins into the endoplasmic reticulum (signal sequence receptor, gamma), glycoprotein protein folding (glucose-regulated thiol oxidoreductase protein) and glycosylation (sialyltransferase 1), and trafficking through the secretory pathway (ADP-ribosylation factor GTPase-activating protein 1, RAB8A, and dynactin 1) increased between e17 and d3. HSP90 plays a role in several intracellular signaling pathways, including steroid hormone and protein kinase signaling, and the increase in pituitary HSP90 that occurs after hatch may reflect an increasing responsiveness to circulating steroids that occurs as the gland matures.

Of the many transcription factors implicated in cellular differentiation of the anterior pituitary gland (e.g., T-pit, Lhx3, Pitx1 and -2, Prop-1, Gata-2, and Pit-1), only Pitx2, Gata-2, and Pit-1 are represented on our neuroendocrine cDNA microarray. Of these, levels of Pit-1 ($P = 0.89$) and Pitx2 ($P = 0.45$) mRNA did not change between e10 and e17, and Gata-2 ($P = 0.57$) and Pitx2 ($P = 0.18$) were not statistically different between e17 and d3. Gata-2 expression increased by 50% between e10 and e14 ($P = 0.02$) and remained relatively constant between e14 and e17. This expression pattern is consistent with the established role in mammals for Gata-2, in combination with Pit-1, as a regulator of TSHβ (14, 18), as TSHβ mRNA levels were shown to increase between e10 and e17. Although Pit-1 gene expression significantly increased between e17 and d3 ($P = 0.03$), it was only 40% greater on d3 than on e17. The increase in Pit-1 mRNA after hatch is consistent with previous data demonstrating an increase in Pit-1 mRNA between hatching and 4 wk of age in chickens (52). The lack of an increase in Pit-1 mRNA between e10 and e17 is also consistent with observations that Pit-1 is expressed during embryonic development on e5 and maintained at high levels until hatch (58a). However, our results do not indicate that an increase in Pit-1 initiates GH or PRL gene expression on e14 or e17, respectively.

In conclusion, we used a neuroendocrine system cDNA microarray to define gene expression profiles during the time of thyrotroph, somatotroph, and lactotroph differentiation in the chick embryonic anterior pituitary gland. We identified a large number of candidate genes that could play an important role in initiation of hormone transcription in these cell types. Many of these genes have been identified as putative transcription factors and signaling molecules in mammalian species, and it remains to be determined whether these factors play a similar role in birds. This is the first microarray analysis of changes in anterior pituitary gene expression during embryonic development in any species, and we have identified several candidate genes with potential roles in thyrotroph, somatotroph, and lactotroph functional maturation for future study. Future studies can investigate the functional significance of selected candidate genes by defining the effect of their overexpression on mRNA levels of the pituitary hormones.

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