

Enhanced Gene Expression in the Forebrain of Hatchling and Juvenile Male Zebra Finches

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ABSTRACT: The molecular mechanisms regulating sexual differentiation of the brain are largely unknown, although progress is being made, particularly in some mammalian systems. To uncover more of the key factors, a screen was conducted for genes involved in sexually dimorphic development of the neural song system in zebra finches. cDNA microarrays were initially used to compare gene expression in the telencephalons of hatchling and juvenile males and females. Then, real-time quantitative polymerase chain reaction (PCR) was employed to confirm sex differences, and the brain regions expressing the cDNAs of interest were localized using *in situ* hybridization. Several genes, including those likely to encode two ribosomal proteins (RPL17

and RPL37), SCAMP1, ZNF216, and a COBW-domain containing protein, showed enhanced expression in the telencephalon of males compared to females. In several cases, expression in the song control nuclei specifically was detected only in males. Interestingly, the sequences of some of these cDNAs shared substantial homology with regions of the chicken Z chromosome (male birds are ZZ, females ZW). Thus, we have identified genes likely to be involved in masculinization of the structure and/or function of the song circuit, some of which could be initial triggers for the sexual differentiation process. © 2005 Wiley Periodicals, Inc. *J Neurobiol* 64: 224–238, 2005

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INTRODUCTION

Sex differences in brain and behavior exist in a wide range of vertebrate species. Masculinization is commonly thought to be stimulated by testosterone secreted by the testes or by metabolites of the hormone synthesized in the brain. This view stems in

part from elegant work on a number of limbic regions of the rodent brain (reviewed in De Vries and Simerly, 2002). Results from several other systems suggest the potential for additional direct roles of genes in sexual differentiation of the brain, yet relatively little is currently known about the mechanisms critical to this process (see Arnold, 2002).

The song system of zebra finches is particularly well suited to the investigation of the molecular basis of sexual differentiation of neural structure and function. Only males of this species sing, and the morphological differences between the sexes are spectacular. Telencephalic song control nuclei are far larger in males, and one (Area X) cannot be identified in females with standard Nissl stains. This morphology and the behavioral function are permanently organized in the first few weeks after hatching (all reviewed in Wade, 2001). Despite these advantages, until very

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recently the genes important for song system development were not studied. In part, that is because information about the genome and molecular tools readily accessible for some model systems (such as mice) were not available for songbirds. However, it is also the case that for many years it appeared that the rodent model for sexual differentiation involving gonadal hormones applied to zebra finches, so research focused on steroids. Testosterone, and especially its metabolite estradiol, masculinize (enlarge) morphology of song control regions and the capacity to sing in adulthood (reviewed in Wade and Arnold, 2004).

In contrast, other studies do not support a role for steroid hormones in differentiation of song system structure and function. Sex differences in plasma steroid levels or brain aromatase have not been consistently detected in zebra finches after hatching (Hutchison et al., 1984; Adkins-Regan et al., 1990; Schlinger and Arnold, 1992; Wade et al., 1995; Saldanha et al., 2000; Wade, 2001). Perhaps more importantly, castration or treatment with inhibitors of androgen or estrogen action or availability have typically not prevented normal masculinization of the song system (Arnold, 1975; Mathews et al., 1988; Adkins-Regan and Ascenzi, 1990; Mathews and Arnold, 1990, 1991; Schlinger and Arnold, 1991; Springer and Wade, 1997; Wade et al., 1999).

In a few cases, certain aspects of masculinization were inhibited with diminished estrogen synthesis. These include the increased expression of androgen receptors in song control nuclei (Kim et al., 2004) and, *in vitro*, the growth of axons between HVC and the robust nucleus of the archopallium, RA (see Reiner et al., 2004, for revised avian nomenclature; Holloway and Clayton, 2001). However, treatments of posthatching birds with aromatase inhibitors produced little or no effect on the morphological and behavioral features most commonly evaluated in studies of sexual differentiation (brain region volume, neuron number and soma size, and song production; Wade and Arnold, 1994; Balthazart et al., 1995; see above). Additionally, the song systems of genetic females that mature with large quantities of functional testicular tissue, even in the absence of ovarian tissue, are neither functionally nor anatomically masculinized (Wade and Arnold, 1996; Wade et al., 1996; Springer and Wade, 1997; Wade et al., 1999).

In the face of this accumulating evidence suggesting a relatively minor role for *gonadal* steroids as the triggers for neural sexual differentiation, researchers have begun to investigate molecular mechanisms. To date, a few studies have identified genes expressed in a sexually dimorphic pattern in the zebra finch brain. These genes include a few located on the avian sex

chromosomes (males are homogametic with two Z chromosomes; females are heterogametic and have one Z and one W chromosome) encoding CHD1Z, CHD1W, and ASW, as well as neurocalcin for which the chromosome location is unknown (Agate et al., 2003, 2004; Veney et al., 2003). However, it is not clear that any of these genes have direct roles in song system differentiation.

Therefore, we developed a cDNA microarray to begin to more efficiently uncover sexually dimorphic gene expression in the developing zebra finch brain (Wade et al., 2004). In the present study, gene expression was directly compared between the sexes using RNA from the telencephalon of males and females on the day of hatching and at day 25, a juvenile stage when song memorization is occurring and morphological differentiation of the song circuit is enhanced (reviewed in Doupe et al., 2004, and Wade and Arnold, 2004). Sex differences reliably detected in this initial screen were validated by real-time quantitative polymerase chain reaction (qPCR). Then, *in situ* hybridization was used to determine whether expression occurred in any of the telencephalic song control nuclei in either sex.

METHODS

Tissue Collection and RNA Preparation

Zebra finches raised in our colony were rapidly decapitated within 24 h of hatching or on posthatching day 25. The two telencephalic hemispheres were lifted off the top of the brain and immediately frozen using dry ice, and the animals were sexed by inspection of the gonads under a dissecting microscope. All samples were stored at -80°C until use.

RNA for use on microarrays was extracted from the whole telencephalon of eight 25-day-old birds of each sex and seven hatchlings of each sex using Trizol (Invitrogen, Carlsbad, CA) per manufacturer's instructions. Each sample from 25-day-old birds was then treated with 40 units of RQ1 RNase-free DNase (Promega, Madison, WI) for 30 min at 37°C . These samples were extracted with phenol and chloroform, ethanol precipitated, and cleaned using the RNeasy Mini Kit (Qiagen, Valencia, CA). Each sample from 1-day-old birds was treated with RNase-free DNase (Qiagen, Valencia, CA) during processing with the RNeasy Mini Kit per manufacturer's instructions, followed by ethanol precipitation. The concentration of all samples was determined by spectrophotometry, and the quality of the RNA was confirmed on 1% agarose gels.

RNA for qPCR was extracted from a set of birds different from those used for the microarrays, seven of each sex on the day of hatching and six of each sex at posthatching day 25, in order to provide a truly independent validation of the microarray results. The telencephalons of individual

birds were homogenized, and Trizol was used per manufacturer's instructions. All samples were then treated with RNase-free DNase on the column during use of the RNeasy Mini Kit as was done for the day 1 samples used on arrays. The concentration and quality of all samples was determined as above.

Microarrays

The microarrays contained 2400 cDNAs randomly selected from a normalized telencephalic pSport1 library we developed from males and females at posthatching days 10–60 (Wade et al., 2004). These cDNAs, along with various controls, were located in 16 patches (subarrays) printed in duplicate; GAPDH was spotted once within each patch. The arrays were printed at Michigan State University on glass slides (Telechem, Sunnyvale, CA). They were hybridized as in Wade et al. (2004). Eight slides were used for 25-day-old birds; a male and female were co-hybridized on each. On four slides, the male sample was labeled with Cy3 and the female sample with Cy5; on the other four slides the dyes were reversed. Seven slides were used for birds collected on the day of hatching; on four slides, the female sample was labeled with Cy3, whereas female samples were labeled with Cy5 on the other three slides.

Our intention was only to screen for differences between the sexes. Thus, labeled cDNA from one individual male and female of the same age were tested on each array, but hatchling and 25-day-old individuals were not paired to allow a focused direct comparison of potential differences in gene expression between the ages. The arrays using hatchling and juvenile samples were run at different times and employed slight differences in processing (washes) and software for analysis of the slides (see below). We had initially planned to analyze and report sex differences from 1- and 25-day-old telencephalons separately, but have merged the array data sets because the results were very similar (see below). As the effects of age and sex \times age interactions would be potentially confounded with technical effects due to the separate processing of the hatchling and 25-day-old birds, they are not reported.

RNA (10 μ g) from each bird was converted to cDNA and labeled with Cy3 or Cy5 (Amersham, Piscataway, NJ) using the Atlas PowerScript Fluorescent Labeling Kit (BD Sciences Clontech, San Jose, CA) according to manufacturer's directions with the few exceptions reported in Wade et al. (2004). Labeled samples were purified with the Qiaquick PCR Purification Kit (Qiagen, Valencia, CA). The Cy3- and Cy5-labeled samples to be paired were then combined, concentrated with a Microcon tube (Millipore, Billerica MA) and mixed with SlideHyb #1 buffer (Ambion, Austin, TX) to a total volume of 48 μ L. Slides were hybridized at 54°C overnight. They were then washed in 2 \times standard saline citrate (SSC) with 0.5% sodium dodecyl sulfate (SDS) for 2 times 5 min at 54°C, followed by 0.1 \times SSC with 0.5% SDS at room temperature for 2 times 5 min, and 0.1 \times SSC for 2 times 5 min at room temperature. The slides were dipped briefly in water (day 25 samples) or 0.01 \times SSC (day 1 samples), centrifuged to

dry them, and then scanned using an Affimetrix (Santa Clara CA) 428 slide scanner. Data were generated with GenePix 3.1 (Axon Instruments, now Molecular Devices Corp., Union City, CA) for 25-day-old birds, and GenePix 5.0 for the hatchlings.

Log₂-transformed values (relative fluorescence intensities corrected for background) were normalized for potential dye intensity bias using the loess smoothing procedure advocated by (Yang et al., 2002). Further statistical analysis was based on the two-stage mixed model approach described by Wolfinger et al. (2001) and more recently by Gibson and Wolfinger (2004) using the software SAS[®] PROC MIXED (www.sas.com) on a Pentium IV 3.2 GHz PC. The first-stage statistical model included the main effects and all possible interactions involving the fixed effects of sex, dye, and age as well as the random effects of array and patch within array for GAPDH log intensities in order to globally normalize the data for those systematic effects using the GAPDH spots. More specifically, an anisotropic exponential spatial covariance structure (Cressie, 1993) was modeled on patches within arrays using the REPEATED option of SAS[®] PROC MIXED (Littell et al., 1996, p. 309) to further minimize biases due to spatial effects that typically plague microarray data (Balázs et al., 2003; Qian et al., 2003). An anisotropic model facilitates the specification of different spatial correlations in fluorescence intensities along the patch rows relative to the patch columns, and was found to fit the data much better than the default specification of independently distributed patch effects.

Previous Northern analyses have indicated that GAPDH is equivalently expressed in the telencephalon of male and female zebra finches at numerous ages from posthatching day 5 through adulthood, and this result was confirmed by qPCR at days 1 and 25 in the present study (see below). The fluorescence intensity of each clone of interest was expressed as a deviation from the corresponding (same patch) prediction for each GAPDH spot, thereby creating a GAPDH-residual or -normalized expression. The normalized data (residuals based on GAPDH corrections) were then analyzed by a series of cDNA-specific mixed effects models that included the main effects and all possible two-way interactions of sex, dye, and age as well as the random effects of array within age, sex by array within age, and spot within array, thereby allowing for estimated variance components that are cDNA specific (Wolfinger et al., 2001) and suitably partitioning and accounting for experimental (due to biological replicates) sources as separate from technical sources (due to duplicate spots per clone) of variability.

Analysis of variance (ANOVA)-based two-tailed *t* test statistics were then used to determine unadjusted *P* values for gene expression fold changes between the two sexes, separately for each age and then averaged across them. The false discovery rate (FDR) procedures of Storey and Tibshirani (2003) were used to convert these *P* values to *Q*

Table 1 Primers Used for qPCR (all Listed 5' to 3')

Clone	Forward Primer	Reverse Primer
23B8	TGAGCTCAAGGGCCTCGAT	CACCTGGATGTGCTCGATCA
12H11	CTCGTTCTTCTCTCACAGTGGCT	GCTGGTCTTAATTGGCAGGA
8E3	GAATTCCCTCACAAATGATACACTCA	CGGATTCTGTTCATGTGCA
7B10	GGCTGCAACAGCTGCTCTCT	GGATTCCGTGAGGGAACGA
8B10	CGCTGCGAGTTCCTGTTGA	TGCCACATCCCTCTGACA
20D11	CCAAGAGTGGTTCAGCTACCTT	ACCATACCTAACATGGCTGCTGTA
22B11	CATGCAACAGAGGGACAGAAGA	TGCCATCTGGTGAATGTCAT
25B8	CAGCAACACAGAACACCTTAACAGA	TCCTTAAATGAGTCACTGTGAGTTCA
7A10	GCTGAACAAAGTCAAACACGAGAT	TCAAACAGCCATTCAGCATCA
6B4	AACATGAGTCTTGTCAACTTTCCCTTTTA	TTCAAAGCTACAGGGCTGGAGT
10A10	CATACAATCAAATAGAAACGAAGGTACAA	GCGTCGGGATGTTTTCTAAGAA
8A10	GTGTGAATCCTGAAGGTTTTGGT	GAAACCCAAGTACAAGGAAGCTCTT
9H3	TCTCTGAACCTGCTGCCGTTACTT	TAGCAGGTGTTGCATGTAAATGGT
5B4	AGGTGCCAGATGCTGTGGA	GCCTTCGTGGAGACAGAGGA
14B10	TACATATAACATTGGGAGGCCATCT	AAATCACTCACCTGGAAACGT
22G5	ACATGCTCTTTCTTGGCTACCTTT	TTCCATTGACCAACAGAATCTGA
7F11	GGCGAGTGTATGGCCTCAA	CCACTGCTTTCTTGTAGCTTTT
8F7	CTCCCACTGCCAATGAAGCT	AAGATCTGATTCTGACAACCCACAT
GAPDH	AAACCAGCCAAGTACGATGACAT	CCATCAGCAGCAGCCTTCA

values in order to adjust for multiple testing. Considering clones with a Q value ≤ 0.05 is equivalent to controlling the FDR $\leq 5\%$. As none of the Q values for the sex by age interactions even approached statistical significance, reported results are based on the average of the two ages. Clones for further analysis were prioritized based on relatively low Q values for the main effect of sex and relatively high mean fold-differences between the sexes.

Gene Identification

The cDNAs spotted on the arrays were initially subjected to high throughput sequencing (5' to 3') at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois (see Wade et al., 2004). Those data are maintained at <http://titan.biotec.uiuc.edu/songbird/> (please note updated URL compared to the previous publication), and have been submitted to Genbank (without full annotation, accession numbers CK234114–CK235953). However, to obtain potentially more accurate data for designing qPCR primers (see below; also Table 1), all plasmids containing the cDNAs of interest were resequenced 3' to 5' at Michigan State University using M13 forward and Big Dye version 3.1 (Applied Biosystems, Foster City, CA). For the cDNAs in which qPCR confirmed the sexually dimorphic expression detected on the arrays, further sequence analysis was conducted. In those cases, the entire insert was sequenced at least twice using vector primers (M13 forward and M13 reverse). When it was necessary to obtain sequence data from the middle of the longer inserts, additional internal primers were designed. Compiled sequence data was compared to existing sequences in Genbank (<http://www.ncbi.nlm.nih.gov/BLAST/>) and to The Institute for Genomic Research (TIGR) chicken gene index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=

g_gallus) using BLASTn. Potential homology to the chicken Z chromosome was also assessed for genes that showed male-biased expression (see below) using the BLAT function at the Chicken Genome Browser Gateway at the University of California Santa Cruz (UCSC) Genome Bioinformatics web site (<http://genome.ucsc.edu/cgi-bin/hgBlat>). The percent identities reported below using this algorithm, which compares cDNA to genomic DNA sequences, are calculated after deleting gaps (those it determines would likely be spliced out).

In many cases, we could assign at least a tentative identification to the genes of interest. However, classifications could not always be made for reasons that include the following: (1) the cDNAs printed on our arrays were synthesized from the 3' end and do not all contain large amounts of protein coding sequence; and (2) the chicken genome, which presumably provides the best available guide for comparison for zebra finch sequences, has not been fully assembled or annotated (see recent reports on the draft genome sequence from the International Chicken Genome Sequencing Consortium, Hillier et al., 2004, and Wallis et al., 2004). The cDNAs are therefore described below by the alphanumeric identifiers used internally by our lab. When possible, gene identifications are also provided. The completed sequences for our cDNA inserts were submitted to Genbank, and the accession numbers are included in Table 2 (see Results).

Real-Time qPCR

Based on the sequence information, primers for qPCR were designed using Primer Express 2.0 (Applied Biosystems, Foster City CA; Table 1). cDNA was simultaneously made from the individual telencephalic samples from all males and females of the same age using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City CA) per man-

Table 2 Sexually Dimorphic Gene Expression Detected Using cDNA Microarrays, Real-Time qPCR, and *In Situ* Hybridization

cDNA*	Tentative Gene Identification	Array Male/Female Ratio (d1 & d25)	Array Q Value (d1 & d25)	d25 qPCR Male/Female Ratio	d25 qPCR P Value	d1 qPCR Male/Female Ratio	d1 qPCR P Value	Enhanced Telencephalic Expression
8E3 (AY833079)	Unknown	1.54	0.015	2.7	0.0008	1.51	0.081	Both sexes: lateral ventricles, RA
23B8 (AY833083)	RPL17	1.67	0.015	1.87	<0.0001	1.81	<0.0001	Both sexes: lateral ventricles, RA Males only: area X, IMAN
7B10 [†] (AY833078)	RPL37	1.64	0.039	1.90	<0.0001	1.97	<0.0001	Both sexes: lateral ventricles Males only: area X, RA
22B11 (AY833080)	SCAMP1	1.39	0.039	1.31	0.0002	1.42	<0.0001	Both sexes: HD, M, hippocampus Males only: E, IMAN, RA, HVC, area X
20D11 [†] (AY833082)	ZNF216	1.36	0.045	2.06	<0.0001	2.25	<0.0001	Both sexes: dorsal to MSt Males only: RA
9H3 [†] (AY833084)	GPP34/GMx33	1.13	0.049	1.74	0.0001	2.05	<0.0001	Both sexes: E
5B4 (AY833086)	Transmembrane protein (M83)	1.37	0.053	1.56	<0.0001	1.63	0.0008	Both sexes: heavy labeling throughout much of telencephalon, but absent from E Males only: light labeling in area X
22G5 [†] (AY833085)	Unknown	1.19	0.053	1.34	0.004	1.6	0.005	Both sexes: Hippocampus
12H11 [†] (AY833081)	COBW domain containing protein	1.47	0.100	1.83	<0.0001	2.11	<0.0001	Both sexes: M, HD, E Males only: IMAN

* Genbank accession numbers in parentheses.

[†] Shows homology to chicken Z-chromosome (see Results for details).

Clones are presented in order beginning with the lowest Q value from the microarray analysis.

Abbreviations: E = entopallium; HD = hyperpallium densocellulare; HVC (none; used as proper name); IMAN = lateral magnocellular nucleus of the anterior nidopallium; M = mesopallium; MSt = medial striatum; RA = robust nucleus of the archopallium.

ufacturer's instructions. Before the individual samples were analyzed, appropriate cDNA and primer concentrations were confirmed as follows: Within each age, primer pairs representing each of the cDNAs of interest, as well as GAPDH, were added at 50–200 nM to 25- μ L reactions run in duplicate. A range of template cDNA (pooled from several individuals) was tested for each of the primer concentrations. These quantities corresponded to 5–25 ng of total RNA determined on aliquots of each sample prior to cDNA synthesis. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was included according to manufacturer's instructions, and the reactions were run with the default program on the ABI Prism 7000 (Applied Biosystems, Foster City CA; 50°C for 2 min, 95°C for 10 min, then 40 repetitions of 95°C for 15 min and 60°C for 1 min). Tubes without template were included at each primer concentration, to be sure that amplifications did not occur, and the dissociation curve was carefully inspected to confirm the absence of primer dimers and other unwanted products. For each set of primers, 100 nM produced clean, detectable amplification, so this concentration was used in a standard curve under the conditions above, with duplicate samples containing the cDNA produced from 0.012 to 96 ng total RNA (along with no template controls). Each of the primer sets of interest was run in parallel with GAPDH, and the efficiency of amplification was in all cases close to 100% and equivalent for the target primer pair and the GAPDH (Applied Biosystems; Livak and Schmittgen, 2001).

Negative reverse-transcriptase (RT⁻) controls were performed as follows: For each bird, total RNA (25 ng) replaced the cDNA in duplicate qPCR reactions for GAPDH under conditions identical to those used for the analysis of sex differences (see below). Amplification was detected in some samples, but in all cases the calculated DNA concentration was more than 4 orders of magnitude less than in samples run simultaneously in which cDNA had been synthesized (RT⁺; difference of more than 15 cycles). Thus, such contamination would be extremely unlikely to affect our results, and would not even been detected for the majority of the expressed sequence tags (ESTs; cDNAs) tested, which produced threshold cycles (C_T s) substantially higher than GAPDH. Importantly, neither at day 1 ($t = 1.89$, $p = 0.083$) nor at day 25 ($t = 0.80$, $p = 0.440$) were sex differences detected in the C_T s of the RT⁻ controls (values for undetectable samples were conservatively set at the last cycle run = 40). In samples from 1-day-old birds, in which the mean difference between males and females was 1.37 cycles, it was in the direction opposite that of all sex differences detected. That is, females had the lower average C_T in the RT⁻ controls, whereas in all reactions in which a sex difference was detected using the cDNA, males showed a greater rate of amplification and thus increased cDNA concentration.

To determine whether expression differed between males and females, samples from six individuals of each sex at posthatching day 25 and seven individuals of each sex at day 1 (in all cases different animals from those used on the arrays) were run in triplicate with each of the primer sets of interest and GAPDH in parallel. With PCR conditions identi-

cal to those above, all primers were used at 100 nM, and the template cDNA corresponded to 25 ng total RNA. The ratio of male to female expression was calculated using $\Delta\Delta C_T$ (Livak and Schmittgen, 2001; Dewing et al., 2003). To test for sex differences, C_T s were averaged across the replicates for each animal, and the values were then analyzed by unpaired t tests (two-tailed values reported, although in each case the direction of the result was predicted by the microarray data). Data on GAPDH were analyzed in the same way. As 18 genes of interest were compared (see below), each at two ages, using a Bonferroni correction, values were considered significantly different if $p < 0.0014$.

In Situ Hybridization

These experiments were run only in 25-day-old animals, as the song control regions are not yet visible on the day of hatching. Telencephalons were analyzed ($n = 2$ per sex) for nine of the ESTs (see below). Whole brains were removed from each animal following rapid decapitation, frozen in cold methyl-butane, and stored at -80°C . Brains were sectioned (20 μm) on a cryostat and thaw-mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). Six sets of slides containing coronal brain sections throughout the telencephalon of each animal were stored with desiccant at -80°C until further processing.

Two adjacent sets of tissue sections (one for antisense and one for sense probes) from each animal were warmed to room temperature for 15 min, briefly rinsed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 15 min, and washed in 0.1% diethylpyrocarbonate-treated water for 5 min. Following a brief rinse in PBS, slides were incubated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, followed by rinsing in PBS, dehydration in a series of ethanols, and then air drying. Slides were pre-hybridized in a solution containing 1 \times hybridization buffer (4 \times SET, 1 \times Denhardt's, 0.2% SDS, 250 $\mu\text{g}/\text{mL}$ tRNA, and 25 $\mu\text{g}/\text{mL}$ 5'-polyadenylic acid) and 50% formamide at 55°C for 1 h, then hybridized overnight at 55°C with 200 μL of a solution containing 1 \times hybridization buffer, 10% dextran-sulfate, 50% formamide, and 5 $\times 10^6$ cpm ^{33}P -UTP-labeled RNA probe (antisense or sense). These probes were prepared using the MAXIscript In Vitro Transcription Kit with SP6/T7 RNA polymerases (Ambion, Austin, TX). For all genes examined except 23B8, SP6 transcribed the antisense and T7 the sense strands. Sequencing confirmed that this cDNA was, due to some sort of artifact during library construction, cloned into the vector in the opposite orientation.

After hybridization, slides were washed sequentially in 4 \times SSC at 55°C for 10 min, 2 \times SSC at room temperature for 30 min, followed by a 30-min incubation in 2 \times SSC with RNase A (20 $\mu\text{g}/\text{mL}$) at 37°C. After RNase A digestion, slides were washed in 2 \times SSC at 37°C for 15 min and in 0.1 \times SSC at 60°C for 10 min, dehydrated in 35, 70, and 95% ethanol containing 0.3 M ammonium acetate and air dried. Slides were exposed to Hyperfilm MP (Amersham Biosciences, Piscataway, NJ) with an intensifying screen (BioMax Transcreen LE; Eastman Kodak, Rochester, NY)

for four days to one week, depending on the level of radioactivity detected from the sections. The slides were exposed to NTB-2 or NTB emulsion (Eastman Kodak, Rochester, NY; NTB was used after the company discontinued NTB-2) for two to six weeks, and then developed and lightly counterstained with cresyl violet. Labeling was confirmed using dark- and bright-field microscopy on the slides. Areas of the telencephalon reported as showing gene expression had specific labeling (antisense far greater than background detected in sense sections) that was consistently seen both in the two individuals of the same sex and in multiple tissue sections containing the brain region.

RESULTS

Microarray and qPCR

Potential sex differences ($P < 0.05$) were detected for 345 cDNAs, but of course many of these would be false positives because of the large number of comparisons. At least three of them represent genes known to be on the sex chromosomes (Hori et al., 2000; O'Neill et al., 2000; Agate et al., 2003; Agate et al., 2004). These and other zebra finch clones were added to the cDNAs on the array that were randomly picked from our telencephalic library as positive controls for various types of investigations. In all cases, sex differences for these added sex-linked cDNAs were revealed in the predicted direction, which indicates the validity of the use of these arrays to screen for differences in the expression of genes in the male and female telencephalon (Wade et al., 2004). Using the FDR procedure to control Type I error rates (Storey and Tibshirani, 2003), 22 clones appeared to show male-biased differential expression ($Q \leq 0.05$), 18 of which were spotted from our telencephalic library (Wade et al., 2004). One of these had a very short insert [~ 40 base pairs (bp)], and was therefore not considered further; the other 17 were evaluated by qPCR. One additional clone (12H11) with significant homology to sequences on the chicken Z chromosome (see below) was also tested.

Of these 18 cDNAs, 8 showed sexual dimorphisms in expression via qPCR in the same direction (male-biased) and of approximately the same magnitude as the arrays (Table 2). In all but one case (8E3), the results were the same in birds at 1 and 25 days of age (Table 2). A significant effect of sex was not detected by qPCR for the remaining 10 cDNAs. However, for one of them, 22G5, the P values were quite low (Table 2). They did not quite reach statistical significance due to the Bonferroni correction for the large number of comparisons that we did. However, because of the relatively low P values that were consistently detected and the fact that the sequence shared strong homology

with the chicken Z chromosome (see below), we decided to use *in situ* hybridization to determine whether it is expressed in the song control nuclei. In all cases, GAPDH expression analyzed in the same samples in simultaneous qPCR reactions showed no sex difference (all $t < 0.89$, all $P > 0.393$).

Clone Identification

Complete sequence data were obtained for all of the cDNAs that remained of interest following qPCR analysis, described here from the longest to the shortest insert. The largest clone that exhibited significant sexual dimorphism was 9H3, 1901 bp. This cDNA showed substantial homology to Golgi phosphoprotein 3 (GPP34) or GMx33 (93% of 526 bp in Genbank; 77% of 931 bp in the TIGR chicken index), two names for the same protein (Dreger, 2003). Ninety percent of 1862 bp in our EST-matched sequence on the chicken Z chromosome. Clone 22G5 contains 1463 bp, but unfortunately did not share homology with a known gene. It was 73% identical (697/946 bp) to an unannotated chicken clone (TIG Rindex), but the most significant match in Genbank was only 90% of 65 bp. This EST was homologous to portions of the chicken Z-chromosome (93% of 833 bp of the cDNA).

The insert for 20D11 is 1025 bp long, and appears to encode zinc finger protein (ZNF) 216. The most significant blast in Genbank indicates a 90% of match of 433 bp, and the TIGR database shows 82% of 974 bp, both to chicken sequences. A large portion of the zebra finch EST is homologous to the chicken Z chromosome (89% over 815bp). Clone 12H11 contains 819 bp, and shares homology with mRNAs encoding proteins containing COBW domains (involved in the synthesis of cobalamin, vitamin B12). Genbank indicates an 88% match for 333 bases to a chicken sequence; the TIGR index indicates an 81% match for 571 bases. A portion of this cDNA (669bp) shows 88% identity with the chicken Z chromosome.

The 748-bp sequence of 5B4 shares homology with proteins containing 5 transmembrane domains. Genbank indicates a 91% match of 336 bp ("similar to transmembrane protein 8; transmembrane protein 6; type I transmembrane protein"); the TIGR index suggests that the sequence is similar to that for human M83, a type I protein (82% of 488 bp). This EST does not show clear homology to a known location of the chicken genome. Clone 23B8, which is 633 bp in length, encodes ribosomal protein (RP) L17 (Genbank: 90% of 561 bp homologous to chicken sequence; TIGR: 89% of 609 bp). The comparison of this EST did not provide a match to any of the chicken chromosomes. The insert for 22B11 is 644 bp long and shares

some homology with human secretory carrier membrane protein 1 (SCAMP1; 82% of 158 bases in Genbank), although the most significant matches in both Genbank (88% of 257 bp) and the TIGR chicken database (87% of 264 bp) are for unannotated chicken clones. Our cDNA shows an 89% match for 630 bp on chicken chromosome 13. Clone 8E3 is 489bp in length. We were unable to determine its identity. The most significant match for an existing sequence in Genbank is 73/79 bp to an unidentified chicken EST, and the TIGR index revealed a similar set of matches to a relatively small proportion of the cDNA (~70 of ~90 bp showed some homology to several different sequences, including unidentified chicken clones, RPL17 and homeobox TLX-3). Additionally, while a relatively large portion of the zebra finch 8E3 EST matched a sequence on a chicken chromosome (77% of 453 bp), the location was unknown. Finally, the 7B10 insert contains 185 bp, and 94% of 161 bases (Genbank) and 93% of 164 bases (TIGR) are shared with the chicken sequence for RPL37. This sequence has 94% (of 161 bp) identity with locations on the chicken Z-chromosome.

***In Situ* Hybridization**

A clear signal was detected in all sections hybridized to the antisense probes, whereas no specific labeling occurred in the alternate sections exposed to the control, sense strands. For all probes, the labeling was consistent in the two individuals of the same sex, and overall the signal was greater in males compared to females (Figs. 1 and 2). In many cases, mRNA expression could be detected in large portions of the forebrain, as well as other structures (e.g., cerebellum, optic tectum). However, the patterns of labeling within the telencephalon were the focus of this investigation. For all of the cDNAs except 9H3 (GPP34/GMx33) and 22G5 (unknown), specific expression was detected in one or more song control nuclei. This expression was most widespread for 22B11 (Fig. 2), and in several cases was detected only in males (Table 2, Figs. 1 and 2; images for 8E3 are not provided due to the similarity of its distribution with 23B8, and 22G5 is not depicted due to the limited nature of its expression).

DISCUSSION

Summary: A Potential Role for Several Genes in Sexual Differentiation of the Brain

We uncovered at least eight genes, newly identified in the zebra finch, with increased expression in male

compared to female telencephalons. These results were detected via microarray and qPCR, and *in situ* hybridization revealed enhanced mRNA within song nuclei, in six cases only in males. Most of these genes were expressed in the anterior forebrain pathway of 25-day-old males, which is critical for song acquisition in juveniles (Bottjer et al., 1984; Scharff and Nottebohm, 1991). Specifically, mRNAs likely to encode RPL17, RPL37, and SCAMP1 were detected in area X, and putative RPL17, SCAMP1, and a COBW-domain containing gene were expressed in the IMAN of males, but not females. Five ESTs were also detected in the motor pathway. The genes likely to encode RPL37, SCAMP1, and ZNF216 were seen in the RA of males only, and RPL17, as well as the unknown 8E3, were detected in the RA of both sexes (more animals are needed to determine whether this local expression is increased in males). Putative SCAMP1 mRNA was also expressed in the male HVC.

These results are consistent with the hypothesis that the genes are involved in masculinization of the regions critical for song learning and production. It is also possible that this differential gene expression is a consequence, rather than cause, of sexual differentiation; inhibiting transcription can address that issue. However, because increased expression in males was detected in the telencephalon on the day of hatching, as well as in song control regions during a juvenile period of enhanced structural and functional differentiation, it is reasonable to hypothesize that the genes have some influence on these processes. The cellular mechanisms underlying sexual differentiation of the brain regions differ somewhat. For example, enhanced cell death in females plays a major role in IMAN and RA, whereas increased addition of new neurons in males is more important for HVC and area X (reviewed in Wade and Arnold, 2004). In all cases, the present results are consistent with the idea that expression of one or more of the genes we identified facilitates neuronal survival in song control regions. In support of this possibility, the increased gene expression was apparent in the RA and IMAN of males before the regions are sexually dimorphic in neuron number (Kim and DeVoogd, 1989; Nordeen et al., 1992), so the sex differences in gene expression cannot be accounted for by prior loss of specific cell types in females.

The sex differences in mRNA detected by *in situ* hybridization must be quantified and analyzed statistically, which will require additional animals. However, the cases in which expression in song nuclei appeared enhanced in males and no signal over the surrounding telencephalon was detected for females hold particular promise. These cDNAs also showed significantly greater expression in males compared to females using

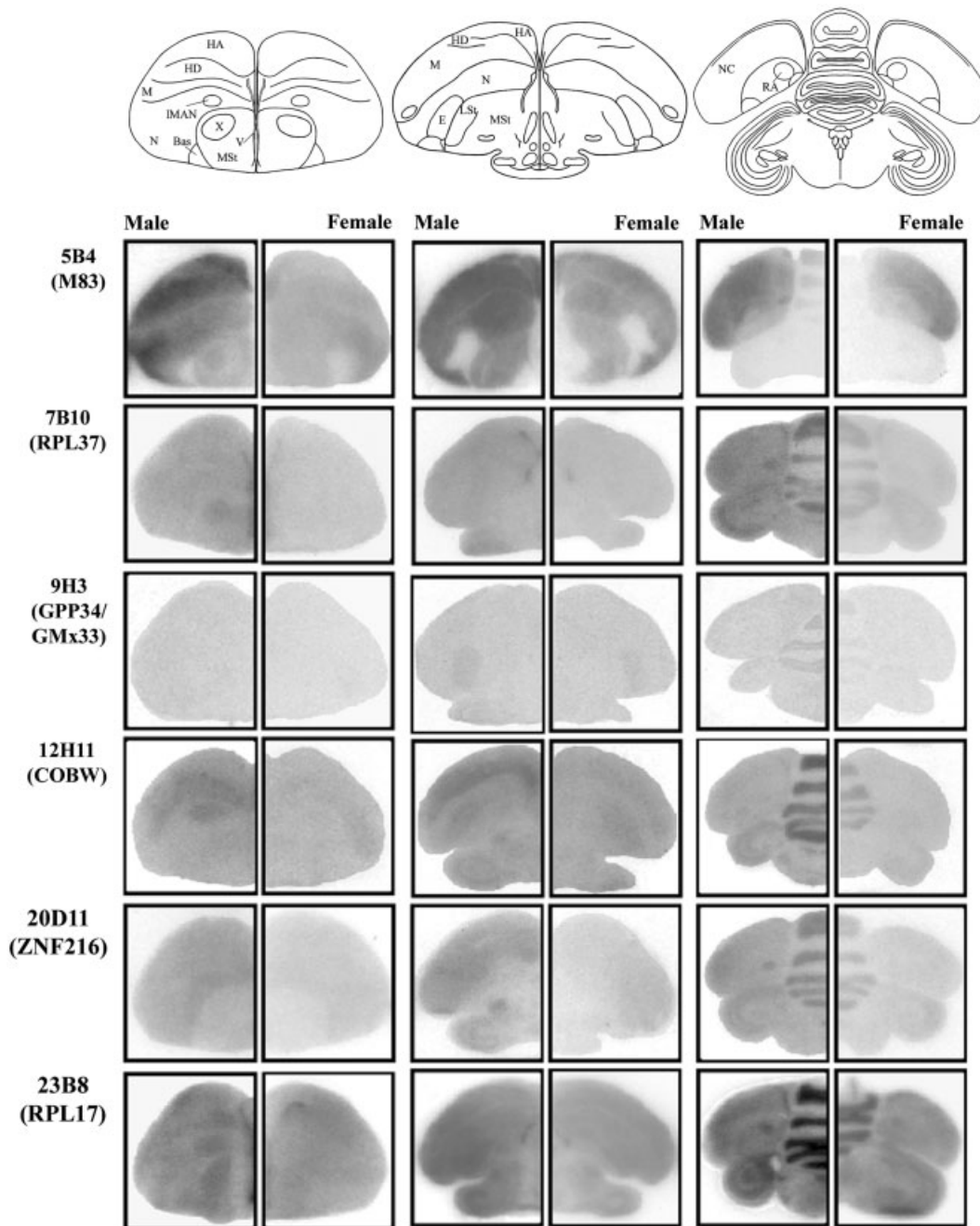


Figure 1 Images from *in situ* hybridization films depicting mRNA expression at three rostro-caudal levels in coronal sections of 25-day-old male and female zebra finch brains hybridized to antisense probes. Each cDNA used as template is identified in the left-hand column (tentative gene identification in parentheses). Abbreviations: HA = hyperpallium apicale; HD = hyperpallium densocellulare; M = mesopallium; IMAN = lateral magnocellular nucleus of the anterior nidopallium; X = area X; N = nidopallium; Bas = nucleus basorostralis pallii; MSSt = medial striatum; E = entopallium; LSt = lateral striatum; NC = nidopallium caudale; RA = robust nucleus of the archopallium; V = ventricle.

both microarray and qPCR analyses. For these two techniques, different sets of individuals at two ages were used; the increased expression in males compared to females for this subset of genes therefore seems quite reliable. However, because microarrays and

qPCR used RNA extracted from homogenates of the whole telencephalon, it is entirely possible that increased gene expression in males is not limited to the song circuit. Indeed, the *in situ* hybridization data suggest that it is probably more widespread (see Figures 1

22B11 (SCAMP1)

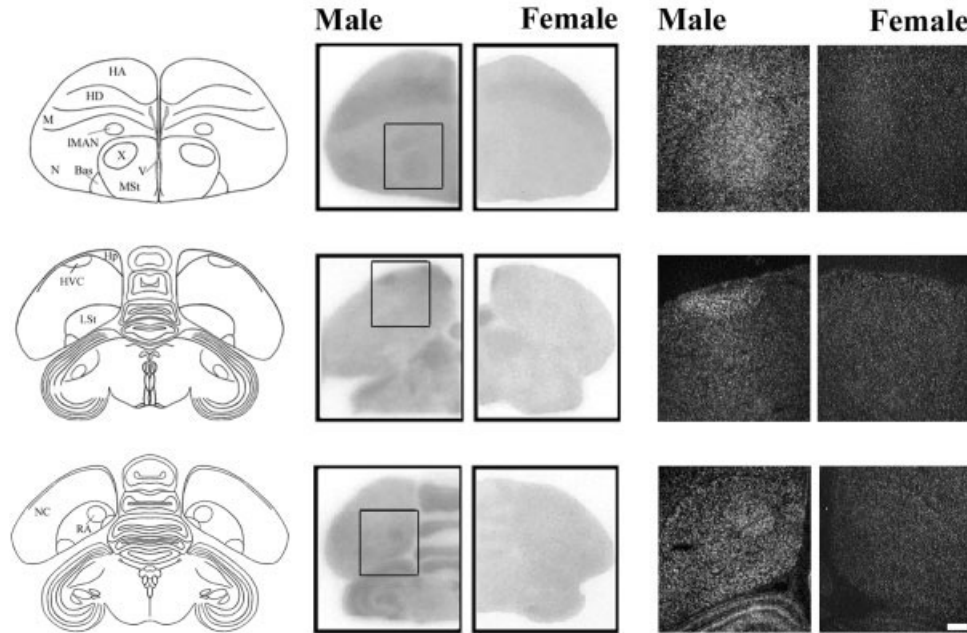


Figure 2 Images from *in situ* hybridization depicting sexually dimorphic mRNA expression for 22B11 (tentatively identified as SCAMP1) in the song system of 25-day-old zebra finches. The left column indicates the three rostro-caudal levels of the brain depicted. Abbreviations are listed in the caption for Figure 1, plus Hp = hippocampus. The panels in the middle are from film. The boxes highlight male-specific labeling in song control nuclei; matching dark-field photomicrographs from emulsion-coated slides from these regions are shown on the right. The images from females are from comparable locations. The scale bar (in bottom right photo) for all darkfield images = 300 μm .

and 2). This type of result might be expected for genes on the Z chromosome (see below), but the data are compatible with the idea that any of the genes exhibiting sexually dimorphic expression in the telencephalon as a whole could influence masculinization of the song control nuclei. Certainly, however, the hypothesis is most compelling for those in which expression was increased compared to the surrounding tissue in one or more of these regions exclusively in males.

Available Information About the Function of the Genes Newly Identified in the Zebra Finch Brain

A critical issue in determining whether the genes exhibiting sexually dimorphic expression in the present study are involved in masculinization is understanding their specific functions (or those of their protein products), including which other factors they interact with and whether those molecules are localized to the song control regions. At present, these points are unfortunately not easy to address. In

two cases (8E3 and 22G5), comparisons to existing sequences did not provide potential identifications, and for some of the shorter inserts, for which we have less protein coding sequence, it is possible that our tentative identifications are not accurate. Obtaining more 5' sequence will be useful. However, the similarity to sequences currently available is in most cases quite strong. Greater difficulty is imposed by the limited information available in the literature on the genes we have tentatively identified. Still, some tantalizing results suggest potential roles, and as these directions are pursued, we will learn not only more about mechanisms regulating sexual differentiation, but also about the genes themselves.

The functions of ribosomal proteins are in many cases unknown, but levels of mRNA expression are probably determined largely by the rates of growth and proliferation of cells (Bévort and Leffers, 2000; Nomura et al., 1984). Thus, the increased expression of RPL17 and RPL37 in the male area X, and of RPL37 and perhaps RPL17 in RA, is consistent with the maturation of surviving cells. Unlike most ribosomal proteins, RPL17 is initially upregulated during

retinoic acid induced neuronal differentiation of human NTERA2 cells (Bévort and Leffers, 2000). Thus, it is conceivable that RPL17 influences the differentiation of neurons. Although the authors suggest that the change in RPL17 expression *in vitro* may not be specifically due to retinoic acid, it is intriguing that neurons in RA, and those in HVC that project to Area X, express the retinaldehyde-specific ALDH. The activity of this enzyme in the production of retinoic acid in HVC is required for juvenile song to become stereotyped (Denisenko-Nehrbass et al., 2000).

The two genes that likely encode ribosomal proteins, and an unknown (8E3), also showed heavy labeling neighboring the lateral ventricles, where enhanced cell proliferation occurs in juvenile birds. In particular locations, this mitotic activity is increased in males compared to females, and may contribute to the neurons that join the song control regions (DeWulf and Bottjer, 2002). Interestingly, during development this region also expresses 17 α -hydroxylase/17,20 lyase (CYP17), the enzyme required for androgen synthesis (London et al., 2003). It is therefore possible that some interaction between these genes and androgen facilitates neurogenesis. To our knowledge, RPL37 expression has not previously been reported in brain, but it is increased in prostate cancer (Vaarala et al., 1998). This idea is intriguing because of the dependence of the survival of prostate cancer on androgen (Sirotnak et al., 2004). In zebra finches, androgen receptors are expressed during development in both Area X and RA, in which RPL37 is apparently expressed only in males. The receptors are also expressed in HVC, which projects to both of these regions, and in IMAN (Gahr and Metzdorf, 1999; Kim et al., 2004). Thus, it is possible that a relationship exists between androgen receptors and this ribosomal protein.

SCAMP1 is expressed at high levels in brain and is enriched in synaptic vesicles; like other members of the SCAMP family, it is involved in plasma membrane trafficking (Fernández-Chacón and Südhof, 2000). Interestingly, SCAMP4 is reduced in the ventromedial hypothalamus of female rats during proestrus compared to diestrus. In parallel, progesterone treatment after estradiol priming decreases ventromedial hypothalamic SCAMP4 (Krebs and Pfaff, 2001). This brain region is critical in the hormonal induction of female receptivity. Thus, some precedent exists for interactions between steroid hormones and the SCAMP family in a brain area in which hormones modulate a reproductive function (albeit one quite different from courtship song). Given SCAMP1's widespread distribution in the androgen receptor expressing song control nuclei of males, like the ribosomal proteins it may

interact with testosterone to facilitate morphological or functional differentiation. Androgen may masculinize the expression of these genes, which then have downstream effects, or these genes may influence androgen receptors. These ideas can be tested by characterizing time courses and selectively manipulating gene expression and steroid levels.

The COBW gene encodes an enzyme in the pathway for vitamin B12 (cobalamin) biosynthesis. While the cDNA we isolated shares some homology with the gene for this enzyme, its function in the zebra finch brain is unclear, as *de novo* synthesis of cobalamin occurs only in prokaryotes (Scott et al., 2003). Nonetheless, vitamin B12 is critical to vertebrate brain development. In humans, B12-deficient children can show a variety of disorders including those associated with movement and cognition and inhibited brain growth, but the source of this vitamin is dietary (Stabler and Allen, 2004). Limited information is available for birds, but excess B12 impairs long-term memory formation in chicks (Crowe and Ross, 1997).

It is even more difficult to speculate on the role of the other ESTs that showed enhanced expression in males compared to females. For example, ZNF216 has unknown biological functions, but is expressed in the fetal human cochlea and at high levels in several tissues in mouse, including brain (Scott et al., 1998). Under certain conditions *in vitro*, ZNF216 may inhibit the anti-apoptotic transcription factor NF κ B (Huang et al., 2004). On the surface this might seem inconsistent with increased neuronal survival in the RA of males, the song control region that expresses this gene. However, no information is available about the role it might play in the intact brain. Similarly, the function of GPP34/GMx33 is unknown. The protein was recently identified, and is well conserved across species. It is located in the cytoplasm of cells, associated with the membrane of the Golgi apparatus, and has been found in multiple tissues in the rat, including brain (Wu et al., 2000; Bell et al., 2001). Finally, the M83 gene encodes a type I protein with 5 membrane-spanning domains. It was isolated from hematopoietic cells as a glycosylated cell surface protein, and appears to play a role in the regulation of leukocytes, although no ligands have been identified (Motohashi et al., 2000).

The Potential for Some of the Newly Identified Genes to be Located on a Sex Chromosome

While more work must be done before we can understand the specific roles newly identified genes

may play in sexual differentiation of the brain, we are particularly intrigued by those sharing homology with the chicken Z chromosome. Until the zebra finch genome is fully sequenced and its chromosomes mapped, we will not know for sure whether the genes are sex-linked in this species. However, if 7B10 (RPL37), 20D11 (ZNF216), 9H3 (GPP34/GMx33), 12H11 (COBW-domain containing protein), and 22G5 (unidentified) are on the zebra finch Z chromosome, they are in a position to stimulate masculine development, as males have two copies of Z genes and females only have one. Some support exists for sex chromosome genes contributing to song system differentiation, primarily from a gynandromorphic zebra finch. This bird was split at the midline, with masculine features, including a testis, on the right, and feminine features, including an ovary, on the left. In the brain, W-chromosome genes were expressed almost exclusively on the left side, and Z-linked genes were increased on the right. The song system was also lateralized; HVC was far larger on the right than the left. This finding supports the idea that dimorphic gene expression within HVC masculinizes, or perhaps defeminizes its structure, although the fact that other song control regions on the left side were masculinized compared to normal females suggests that some diffusible factor(s) may also play a role (Agate et al., 2003).

Genes and Steroid Hormones Likely Both Contribute to Masculinization

The dogma, derived primarily from studies on rodents, is that a particular diffusible factor, testosterone from the testes, masculinizes the morphology and function of brain regions associated with male reproductive behaviors. The gonadal testosterone is frequently aromatized in the brain—thus the active masculinizing hormone is estradiol (reviewed in De Vries and Simerly, 2002). For a long time, this paradigm was thought to masculinize the zebra finch song system, largely because estradiol administered to females after hatching substantially increases the size of the song control nuclei and allows females to sing in adulthood. Various data, however, suggest that the mammalian dogma may not apply to this avian system (reviewed in Wade and Arnold, 2004; see Introduction). Still, increasing evidence suggests that estradiol, synthesized in the brain rather than the gonads, may be important, and that its masculinizing effects may involve androgen receptors in song control nuclei, which are expressed to a greater extent in

males than females as early as the regions can be identified (Gahr and Metzdorf, 1999; Holloway and Clayton, 2001; Grisham et al., 2002; Kim et al., 2004).

If gonadal hormones are not the source, then genes on the sex chromosomes are strong candidates for directly triggering sexual differentiation. Thus, it is tempting to speculate that one or more of the zebra finch genes identified in our screen that are most likely located on the Z chromosome initiate the masculinization. It is also possible that these genes, as well as those we have identified that are probably located on autosomes and the ~20 genes previously localized to the song control nuclei (reviewed in Clayton, 2004), are in some way responsible for maintaining this process until the song system has matured. While a large amount of work is clearly required to fully detail the roles these genes play in songbird brain development, the discovery of the genes documented here raises a number of possibilities for regulation of song system differentiation. One would predict that genes important to this process (1) are expressed within song control regions or in areas likely to contribute cells to them, (2) are locally expressed at a higher level or exclusively by one sex, and (3) have the potential to influence steroid hormone action in these brain areas, given their sensitivity to androgens and estrogens. We have identified several clones that meet these criteria, and now are in a position to characterize in detail the roles they play, individually and in concert with other factors, in particular steroid hormones.

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