Journal of Heredity 2005:96(3):212–216
doi:10.1093/jhered/esi025
Advance Access publication December 23, 2004

FoxP2 in Song-Learning Birds and Vocal-Learning Mammals

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Abstract

FoxP2 is the first identified gene that is specifically involved in speech and language development in humans. Population genetic studies of *FoxP2* revealed a selective sweep in recent human history associated with two amino acid substitutions in exon 7. Avian song learning and human language acquisition share many behavioral and neurological similarities. To determine whether *FoxP2* plays a similar role in song-learning birds, we sequenced exon 7 of *FoxP2* in multiple song-learning and nonlearning birds. We show extreme conservation of *FoxP2* sequences in birds, including unusually low rates of synonymous substitutions. However, no amino acid substitutions are shared between the song-learning birds and humans. Furthermore, sequences from vocal-learning whales, dolphins, and bats do not share the human-unique substitutions. While *FoxP2* appears to be under strong functional constraints in mammals and birds, we find no evidence for its role during the evolution of vocal learning in nonhuman animals as in humans.

FoxP2 is a member of the winged helix/forkhead class of transcription factors (Lai et al. 2001; Shu et al. 2001). It is expressed in multiple fetal and adult tissues, with a high expression in certain regions of the fetal brain (Lai et al. 2001; Shu et al. 2001). Mutations in the gene cause severe deficits in mental grammar skills and the orofacial coordination necessary for sound production in affected humans, despite their adequate intelligence and opportunity for language acquisition, suggesting that *FoxP2* is specifically involved in speech development (Lai et al. 2001). *FoxP2* is a highly conserved protein. Between mouse and human there are only 3 amino acid differences (and one insertion/deletion) among 715 amino acids. Surprisingly, two of the three changes occurred after humans split from chimpanzees (Enard et al. 2002; Zhang et al. 2002). The two amino acid substitutions in humans, a Thr (T)-to-Asn (N) change at position 303 and an Asn (N)-to-Ser (S) change at position 325, are both in exon 7. Human population genetics data revealed signals of recent selective sweeps associated with the two substitutions, suggesting that they resulted from adaptive selection. One of the two substitutions, T303N, appears to be unique to humans, as it was not observed in 28 nonhuman mammals examined (Zhang et al. 2002). But N325S was also found in a diverse array of eight carnivores sequenced (Zhang et al. 2002).

Parallels between human and songbird phonological development have led to the use of songbirds as a model for speech development in humans (Goldstein et al. 2003). In both groups, there is a critical period in which juveniles need exposure to species-typical sounds to acquire them, and both have an innate predisposition for receiving species-typical signals. Both groups also have sensory learning phases during which sound patterns are stored in long-term memory and subsequently used to guide motor production (Kuhl 2003). Most birds and mammals do not need prior exposure to their species-specific vocalizations to produce them. *FoxP2* may have an evolutionarily conserved role in brain development. For example, its expression pattern in neural tissue is similar in birds and mammals (Haesler et al. 2004; Lai et al. 2003; Teramitsu et al. 2004). These parallels prompted us to raise the hypothesis that *FoxP2* plays a similar role in song-learning birds. Song-learning has independently evolved three times in birds: in parrots, in oscine passerines, and within hummingbirds (Gahr 2000) (see Figure 1). Here we sequence a portion of the *FoxP2* gene for representative species of each of these groups, as well as representatives of their non-song-learning sister groups, to examine whether there are parallel amino acid substitutions in the *FoxP2* of humans and song-learning birds and, in particular, whether humans and avian song learners have similar substitutions in exon 7.

Materials and Methods

Genomic DNA was isolated with the DNeasy tissue kit following the manufacturer's protocol (Qiagen, Valencia,

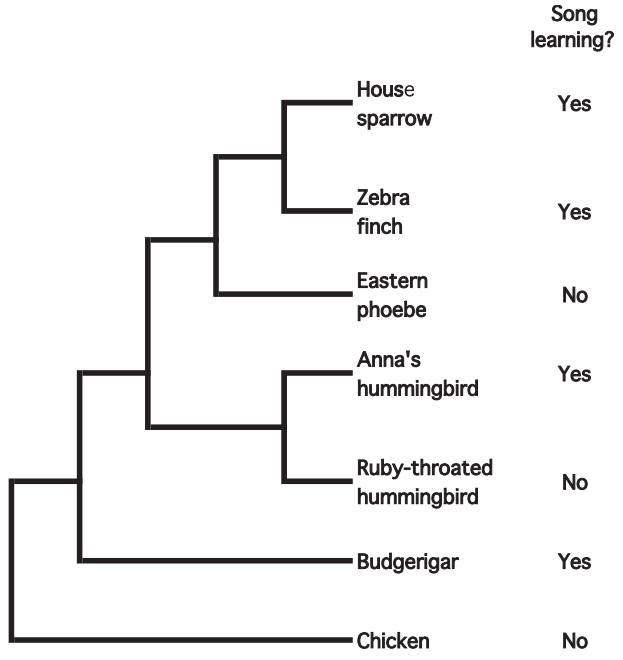

Figure 1. Distribution of song learning among birds used in this study. Song learning is found in all members of the order Psittaciformes (parrots). Budgerigar is a member of Psittaciformes. Song learning is found in all members of the suborder Passeri (oscines). House sparrow and zebra finch are members of Passeri. Within the order Trochiliformes (hummingbirds), song learning has been found in a few species (e.g., Anna's hummingbird) but not in others (e.g., ruby-throated hummingbird). Song learning is absent in all members of the suborder Tyranni (suboscines). Eastern phoebe is a member of Tyranni. Song learning is absent from all members of the order Galliformes. Chicken is a member of Galliformes. Songs, as opposed to calls, are usually complex, multinoted vocalizations produced by males under hormonal regulation during the breeding season for mate attraction and territorial defense. Phylogenetic relationships follow Sibley and Ahlquist (1990).

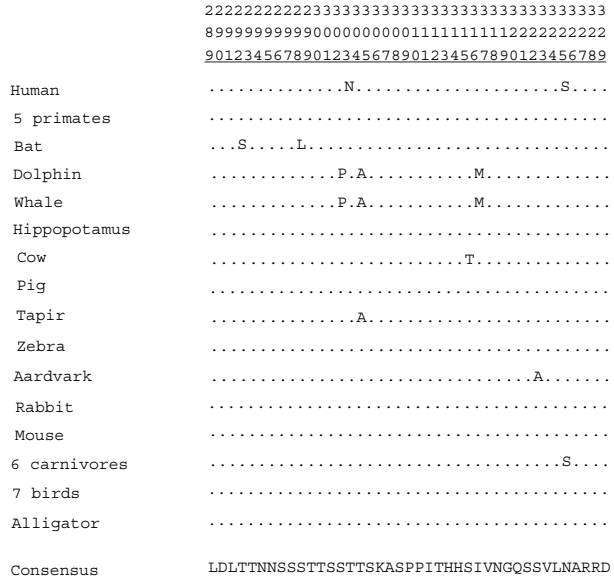

Figure 2. Amino acid sequences of *FoxP2* exon 7. Dots represent amino acids identical to the consensus. Five primates = chimpanzee, bonobo, gorilla, orangutan, and lemur; six carnivores = cat, dog, fox, wolf, bear, and wolverine; seven birds = house sparrow, zebra finch, eastern phoebe, Anna's hummingbird, ruby-throated hummingbird, budgerigar, and chicken. Numbers above the alignment show the amino acid positions in human *FoxP2*.

CA). Tissues from zebra finch (*Taenopygia guttata*), house sparrow (*Passer domesticus*), eastern phoebe (*Sayornis phoebe*), ruby-throated hummingbird (*Archilochus colubris*), Anna's hummingbird (*Calypte anna*), budgerigar (*Melopsittacus undulatus*), and American alligator (*Alligator mississippiensis*) came from the University of Michigan Museum of Zoology. DNA for pygmy hippopotamus (*Hexaprotodon liberiensis*) came from the Zoological Society of San Diego, and that for bottle-nosed dolphin (*Tursiops truncatus*) was a gift from Dr. A. P. Rooney (U.S. Department of Agriculture). Primers for polymerase chain reaction (PCR) amplification of exon 7 of *FoxP2* are 5′-GAAGACAATGGCATTAAACATGGAGG-3′ and 5′-GAATAAAGCTCATGAGATTTACCTGTC-3′. Primers for amplification of cytochrome *b* came from Parson et al. (2000). PCR was conducted with MasterTaq under the manufacturer's recommended conditions (Eppendorf, Hamburg, Germany) and products were sequenced from both directions with the dideoxy chain termination method on an automated sequencer. GenBank accession numbers for the new sequences are AY726626–AY726635 and AY724762–AY724767. After removal of the primer sequences, a total of 124 nucleotides per sequence were compared. Synonymous nucleotide substitution rates were computed by the method of Zhang et al. (1998). Tajima's (1993) test of the molecular clock was computed with the MEGA3 program (Kumar et al. 2004).

Results and Discussion

We found the exon 7 amino acid sequences for eight crocodilians (seven birds and one alligator) to be identical to one another and to the mouse (Figure 2). In contrast, humans have one unique amino acid substitution (T303N) and share one with carnivores (N325S). The nucleotide sequences of the eight crocodilians were also remarkably similar (Figure 3). Six birds had identical sequences, while budgerigar had two third-position synonymous differences. Alligator had two other synonymous changes. The average number of synonymous differences between the alligator and the seven birds was 2.3 for the 124 bp region compared (Figure 3). In contrast, the average number of synonymous differences among eight mammalian species of eight

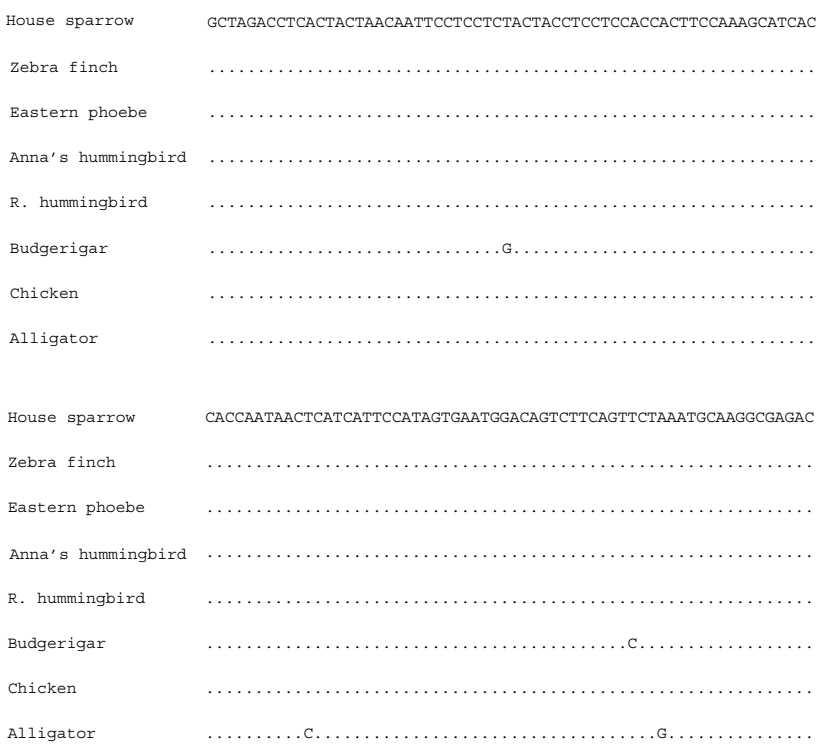

Figure 3. Exon 7 nucleotides of *FoxP2* for alligator and seven bird species. Dots represent nucleotides identical to house sparrow. R. hummingbird = ruby-throated hummingbird.

different orders (chimpanzee, dog, cow, bat, mole, tapir, rabbit, and aardvark) was 3.7. Birds and alligators are estimated to have diverged 240 million years ago (MYA) (Benton 1993), while placental mammalian orders originated approximately 90 MYA (Benton and Ayala 2003). Thus the synonymous substitution rate in this region is approximately 4.3 times lower in crocodilians than in mammals. To exclude the possibility that the low sequence divergence of birds may be due to cross contamination, we reisolated the genomic DNAs and synthesized new primers for amplification and sequencing. We found no differences between our first and second set of sequences. Furthermore, we verified the identity of our avian genomic DNA by sequencing a portion of the mitochondrial cytochrome *b* (*Cytb*) gene and compared our sequences with those available in GenBank. In all cases our *Cytb* sequences matched their closest phylogenetic relatives among the GenBank sequences (Figure 4).

While preparing this manuscript we found the newly released complete zebra finch and budgerigar *FoxP2* coding sequences in GenBank (AY549148 and AY66101). A low level of synonymous change was also seen in these complete avian *FoxP2* sequences. The number of synonymous substitutions per synonymous site (d_s) is 0.069 $\pm$ 0.010 between zebra finch and budgerigar. Between human and mouse, d_s is 0.255 $\pm$ 0.026. Assuming an identical divergence date for both species pairs (i.e., approximately 90 MYA) (Benton and Ayala 2003; van Tuinen and Hedges 2001), synonymous substitutions in *FoxP2* are 3.7 times slower in birds than in mammals ($P < .001$).

For the complete *FoxP2* protein sequences of human, mouse, chicken, zebra finch, and budgerigar, there were no uniquely shared substitutions between the vocal-learning animals or between the two vocal-learning birds (Figure 5). Relative rate tests among chicken, zebra finch, and budgerigar (with mouse as the outgroup) showed no significant differences in avian amino acid substitution rates (Tajima's test; $P > .05$) and a lower d_n than d_s was observed for pairwise comparisons among the three birds.

Among mammals, only humans, bats, whales, and dolphins are vocal-learning animals (Haesler et al. 2004). Our previous study showed that whale, bat, and human do not share any amino acid changes in exon 7 of *FoxP2* (Zhang et al. 2002). Additional sequences from vocal-learning (dolphin) and non-vocal-learning (hippopotamus) cetartiodactyls show that whales and dolphins share three amino acid substitutions while their closest relative, the hippopotamus, is identical to mouse. Notably, the human-unique substitution (T303N) was flanked by two changes in both whale and dolphin (S302P and T304A).

Though strong purifying selection can explain the absence of nonsynonymous changes in crocodilian *FoxP2* sequences, synonymous changes should be nearly neutral and accrue at the rate of mutation. Several recent studies in mammals, however, found evidence for purifying selection at synonymous sites (Chamary and Hurst 2004; Hellmann et al. 2003). For example, Duan et al. (2003) found that mutations at synonymous sites in *dopamine receptor D2* (*DRD2*) affected messenger RNA (mRNA) secondary structure and gene expression. Purifying selection

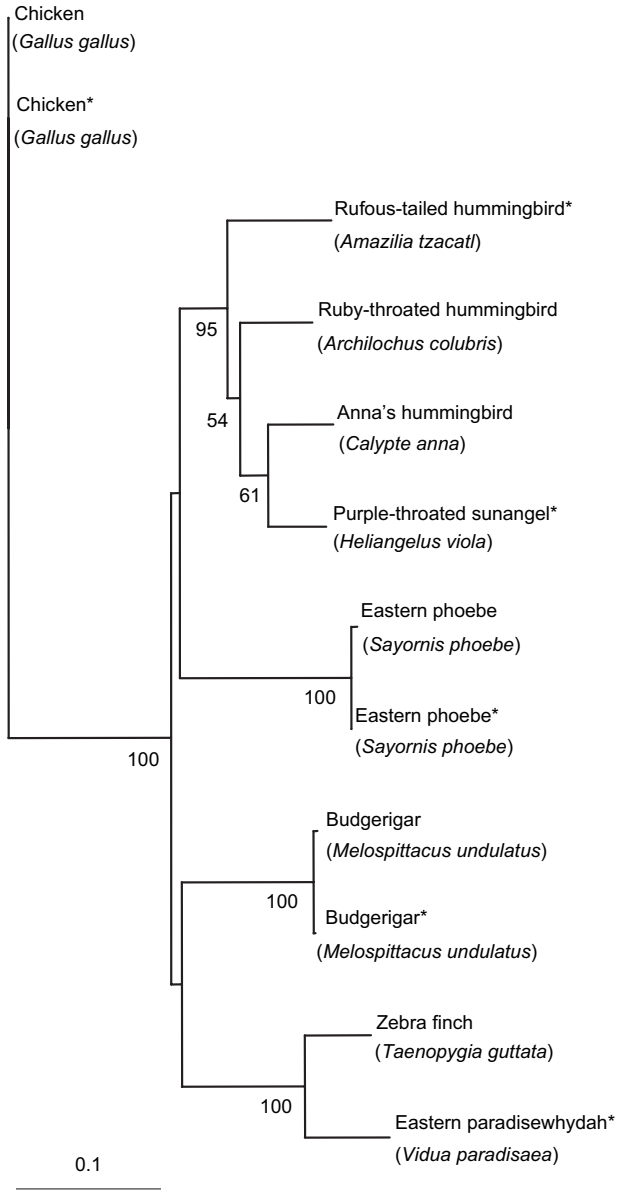

Figure 4. Neighbor-joining tree based on general reversible distances from pairwise comparisons of 307 nucleotides of the *Cyth* gene. Bootstrap percentages higher than 50 (from 2000 replications) are shown below nodes. *Indicates the following GenBank blastn best matches: *Gallus gallus*, AY029583; *Amazilla tzacatl*, ATU89180; *Heliangelus viola*, AF022675; *Sayornis phoebe*, AF447614; *Melopsittacus undulatus*, S46672; *Vidua paradisea*, VPU18865. All other sequences were determined in this study.

can also act on silent substitutions when codons with abundant transfer RNAs (tRNAs) are preferentially used in highly expressed genes (Ikemura 1982). However, *FoxP2* is not a highly expressed gene, and codon usage bias probably does not occur in birds (Ouenzar et al. 1988). Furthermore, the effective number of codons (Wright 1990), which ranges from 20 when one codon is used per amino acid in the coding sequence to 61 when all codons are used, is relatively large in both birds and mammals. The effective number of codons is 47.4, 48.7, 54.9 and 53.6 in zebra finch, budgerigar, human, and mouse, respectively. Other explanations for selection-driven codon usage could be regulation of gene expression levels via CpG islands, alternative exon splicing, and antisense transcripts (Hurst and Pal 2001).

Although we did not find parallel amino acid changes between humans and other vocal-learning animals, the study of *FoxP2* in nonhuman vocal learners is only beginning. There is now tantalizing evidence of differential *FoxP2* expression in song-associated brain regions during periods of song remodeling in zebra finches (Haesler et al. 2004; but see Teramitsu et al. 2004), and the extreme sequence conservation in *FoxP2* remains to be explained. The molecular function of the human-unique substitution is yet to be determined and it will be interesting to compare the conserved and altered roles of *FoxP2* in various mammals and birds of vocal learners and nonlearners.

Acknowledgments

We thank the University of Michigan Museum of Zoology and the Zoological Society of San Diego for providing animal tissues and DNA samples. This work was supported by a start-up fund of the University of Michigan and National Institutes of Health grant GM67030 (to J.Z.). This paper is based on a presentation at the symposium entitled "Genomes and Evolution 2004," cosponsored by the American Genetic Association and the International Society of Molecular Biology and Evolution at The Pennsylvania State University, State College, PA, June 17–20, 2004.

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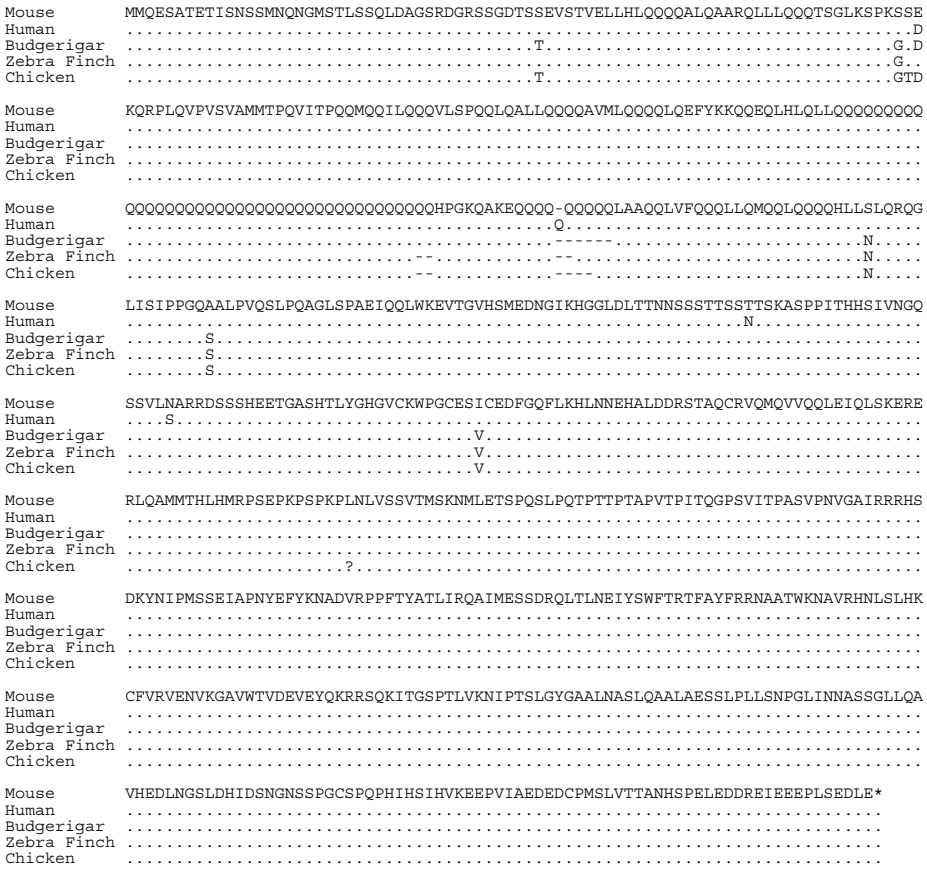

Figure 5. Alignment of complete *FoxP2* protein sequences for mouse (AAH58960), human (AAH18016), budgerigar (AAR28684), zebra finch (AAS55874), and chicken (compiled from blastn hits of the chicken genome sequence). Dots represent identical amino acids to the mouse sequence and dashes represent alignment gaps.

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Corresponding Editor: Shozo Yokoyama