Detection of Olfactory Receptor Transcripts in Bird Testes

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Abstract

The sense of smell is mediated through olfactory receptors (ORs) expressed in olfactory sensory neurons of the olfactory epithelium. Interestingly, some OR genes also function in another context: they are expressed in nonolfactory testicular tissue and in sperm of mammals and fish where they mediate sperm flagellar motility. The presence of OR transcripts in testicular tissue of both mammals and fish suggests that this is a conserved trait among vertebrates. In birds, sperm competition is widespread and its outcome depends, in part, on sperm motility. Thus, avian testicular OR gene expression might be particularly interesting to study, especially in the context of current ideas on postcopulatory sexual selection. Using reverse transcription–polymerase chain reaction with degenerate primers specific for OR genes and subsequent cloning, we here demonstrate that multiple OR gene transcripts are present in chicken (Gallus gallus domesticus) testes. Moreover, we show that they belong to the class-γ OR gene clade. We discuss the potential significance and evolutionary implications of avian testicular OR gene expression.

Olfactory receptors (ORs) are involved in the detection and discrimination of odors in both invertebrates and vertebrates (Buck and Axel 1991). In mammals, OR genes constitute the largest gene family in the genome (for review, see Niimura and Nei 2006).

OR gene expression is not restricted to the olfactory tissue. Interestingly, transcripts of a subset of OR genes have been detected in testis and sperm (e.g., Parmentier et al. 1992) and other nonolfaction-related tissues of both mammals and fish (for review, see Young and Trask 2002), suggesting that the functions of olfactory receptors are not restricted to the context in which they were first characterized, namely olfaction.

Although the functions of OR genes in many nonolfactory tissues remain unclear, mammalian testicular/sperm OR proteins have been localized to the sperm flagellum midpiece and have been shown to mediate flagellar motility (Spehr et al. 2003). Hence, sperm-expressed OR genes may play an important role in sperm–egg chemotaxis/communication (Spehr et al. 2004).

Although OR genes have been intensively studied in a wide range of species, comparatively little is currently known about avian OR genes. In the red jungle fowl (Gallus gallus) genome, recent estimates of the OR gene repertoire size range between 220 and 550 paralogues (Hillier et al. 2004; Niimura and Nei 2005; Lagerstrom et al. 2006), suggesting that birds may rely more on their sense of smell than is generally thought. Vertebrate OR genes have been classified into 2 distinct groups: class-α (previously termed class I) and class-γ (previously termed class II) (Freitag et al. 1995; Niimura and Nei 2005). A subset of the class-γ OR genes, termed class-γ-c, is greatly expanded in avian genomes (Niimura and Nei 2005; Lagerstrom et al. 2006; Steiger S.S., Fidler A.E., Valcu M., and Kempenaers B. submitted).

Notwithstanding accumulating evidence to the contrary, birds are still widely believed to lack a well-developed sense of smell (for reviews, see Roper 1999; Hagelin 2006). Thus, it is perhaps not surprising that birds have been a neglected group with respect to the study of OR gene expression. In this study, we investigated whether OR gene transcripts could be detected in avian (chicken, Gallus gallus domesticus) testes. We demonstrate that multiple OR transcripts can be detected in chicken testes RNA and show that they fall into the γ-OR clade.

Materials and Methods

Isolation of RNA

Samples of chicken testis, liver, and small intestine tissue were collected after sacrifice and immediately frozen in liquid nitrogen before storage at −80 °C. We homogenized tissue samples using ceramic beads (Precellys Kit CK 14,
Bertin Technologies, Montigny-le-Bretonneux, France) and used a commercial kit to isolate total RNA (RNeasy, Qiagen, Hilden, Germany). Total RNA was digested with RNase-free DNase (DNaseI, Qiagen).

Reverse Transcription–Polymerase Chain Reaction

We used 2 different pairs of degenerate primers, targeting either 1) the non-γ-c OR clade sequences (forward primer ORFor1/reverse primer ORRev1) or 2) the large γ-c OR clade (ORFor2/ORRev2). Primer pairs were designed to anneal to evolutionarily conserved transmembrane (TM) 3 and TM7 coding regions. For more details, see Supplementary Methods. Both primer pairs were predicted to generate products of ~0.5 kb representing approximately half the expected OR full-length coding sequence. To control for cDNA quality, we amplified a chicken β-actin partial coding sequence.

Sequencing and Sequence Analyses

Amplified products were excised from agarose gels and purified before ligation into a T-tailed cloning vector (pGemT-easy, Promega, Madison, WI). Plasmids were purified from individual transformed DH5α colonies by alkaline lysis and sequenced. We deleted vector and primer sequences from the raw sequences and those sequences sharing ≥98.5% identity were considered to be replicated amplifications from the same OR gene transcript and therefore were combined to form a consensus sequence. Sequence identities were calculated using the “sequence identity matrix” function of BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). BLAT searches of the *G. gallus* genome (Build 2.1) with default parameter settings were performed in an attempt to identify homologous regions in the *G. gallus* genome using the UCSC Genome Browser (http://genome.ucsc.edu/).

Phylogenetic Tree Construction

We aligned amino acid sequences predicted from the chicken polymerase chain reaction (PCR) products with the TM3–TM7 predicted protein sequences from 78 previously reported, putatively functional, *G. gallus* OR genes (Niimura and Nei 2005) using ClustalX (Thompson et al. 1997) with default settings. We used the neighbor-joining (NJ) method and Poisson distances to construct phylogenetic trees using the MEGA software package (version 3.0; http://www.megasoftware.net/). The reliability of the phylogenetic tree was evaluated with 1000 bootstrap repeats.

Results

Using a PCR primer pair targeting non-γ-c OR genes (ORFor1/ORRev1), we amplified a discrete band of ~0.5 kb from chicken testis cDNA (Figure 1A). To control for the possibility that the products were amplified from genomic DNA potentially contaminating the RNA preparations, we carried out 2 controls: 1) reactions in which the template was total RNA, without a reverse transcriptase step (data not shown) and 2) reactions in which the template was cDNA derived from chicken liver and small intestine total RNA (Figure 1A). In neither case was a PCR product generated. In contrast, the PCR primer pair targeting the γ-c OR clade (ORFor2/ORRev2) did not generate amplification product from any of the chicken cDNA templates including testis (Figure 1A). It is possible that this result may simply indicate that the reaction conditions were not suitable for the ORFor2/ORRev2 primers. However, this seems highly unlikely because the ORFor2/ORRev2 primers amplified a product of the expected size from a chicken genomic DNA template (Figure 1B). The β-actin primers amplified a product of the expected size (~0.15 kb) from all 3 tissue chicken cDNAs (Figure 1C).

Plasmids carrying the ORFor1/ORRev1 PCR amplification products from testes cDNA were isolated from 20 independent transformed colonies and sequenced. We identified 6 distinct partial OR coding sequences, here denoted GgTestesOR-A–F (GenBank accession numbers EU583984–EU583989). Sequences GgTestesOR-B, -D, and -E are consensus sequences derived from 12, 3, and 2 plasmids, respectively, whereas sequences GgTestesOR-A, -C, and -F were obtained from a single plasmid. We detected no premature stop codons within the predicted open reading frames in any of the OR partial coding sequences, suggesting that all 6 sequences were derived from transcripts encoding functional OR proteins. The 6 OR sequences were between 50–97% and 36–95% identical on the DNA and predicted protein levels, respectively (Supplementary Table 1).

Phylogenetic analysis placed all 6 OR sequences within the evolutionarily older, non-γ-c OR clade (Figure 2). Note
that 3 OR sequences (GgTestesOR-A, -B, and -E) cluster very closely in the phylogenetic tree. Sequence identities among these 3 sequences range from 93% to 97% and 88% to 95% on the DNA and predicted protein levels, respectively (Supplementary Table 1). Furthermore, all 3 sequences align with the same part of the G. gallus genome (Build 2.1) as the best “hit” when used as query sequences in BLAT searches, although the alignments themselves have different percentage identities and differ when inspected by eye (Supplementary Table 2). Surprisingly, every difference in a GgTestesOR-A versus GgTestesOR-B comparison is a C versus T. We examined the electropherogram raw data and see no reason to doubt the quality of the sequence data. In addition, such high rates of C/T differences were not observed in any other pairwise comparisons of sequences derived from the same PCR indicating that they are not an artifact of the experimental procedure.

**Discussion**

Using reverse transcription–polymerase chain reaction (RT-PCR) and subsequent cloning/sequencing, we here show that a minimum of 6 distinct OR genes are transcribed in the chicken (G. gallus domesticus) testis. This number is probably an underestimate because we sequenced only 20 plasmids. Further RT-PCR/sequencing work, or perhaps microarray analyses, could be used to determine whether chicken and other bird species express as many different OR transcripts in their testes as do mammals (e.g., 66 different OR transcripts have been described in mice testis RNA [Zhang et al. 2004]).

Although the testis RNA used in this work was derived from a single bird, we cannot exclude the possibility that one pair of the 3 highly homologous GgTestesOR-A, -B, and -E sequences are allelic. However, this seems unlikely for the following reasons: first, the percentage identity values of the A, B, and E sequences are all below the, albeit arbitrary, “cutoff” values used in previous studies to designate distinct OR genes (e.g., Nef et al. 1996). Second, the frequencies of nucleotide mismatches among those 3 genes (i.e., between 13 and 34 mismatches in approximately half of the coding region) are much higher than those previously reported for mammalian OR gene alleles (i.e., maximally 11 mismatches in full-length coding regions) (Tacher et al. 2005; Moreno-Estrada et al. 2008). Third, the current G. gallus genomic sequence (Build 2.1) is still a draft, albeit an advanced one, so OR sequences may be incorrect and/or absent. Indeed, the GgTestesOR-A, -B, and -E sequences all align, as their best hit, with the same region of G. gallus genome.

**Figure 2** NJ phylogenetic tree generated from an alignment of the predicted proteins derived from 78 putatively functional Gallus gallus OR genes (sequences from Niimura and Nei [2005]) and the 6 testes-expressed OR genes generated in the work (black dots). Scale bar indicates the number of amino acid substitutions per site. Numbers on tree branches show bootstrap values obtained from 1000 replications (only values >90% are shown).
(Build 2.1), but this region of chromosome 5 contains approximately 2500 sequence gaps (i.e., 4.6% of chromosome 5, mean length ca., 1.0 kb), and the largest gap is estimated to be approximately 1.5 Mb (UCSC Genome Browser; http://genome.ucsc.edu/). As homologous mammalian OR genes tend to occur in clusters (Glusman et al. 2001; Godfrey et al. 2004), it is possible that additional OR sequences will be found in the chromosome 5 “gaps” in further drafts of the *G. gallus* genome.

Further, it is becoming apparent that the genome of the domesticated chicken (*G. gallus domesticus*) is probably not a simple, direct derivative from that of the red jungle fowl (*G. gallus*) but, rather, may have been formed by hybridization of *G. gallus* and one or more other members of the genus *Gallus* (Eriksson et al. 2008). If this hybridization scenario is correct, then genuinely orthologous OR sequences from the chicken (*G. gallus domesticus*) and *G. gallus* may not be identical. In addition, concerted evolution may cause OR genes to become very similar presenting a further challenge to the BLAT search program (Sharon et al. 1999; Steiger et al. submitted).

We further demonstrated that the 6 OR transcripts amplified can be placed in the OR group-γ but not in a subclade, group-γ-c, whose expansion is apparently specific to bird genomes (Niimura and Nei 2005, Steiger et al. submitted). In mammals, testes-expressed OR genes do not appear to belong to a single clade (Vanderhaegen et al. 1993). Similarly, we have found that the 6 chicken testes-expressed ORs identified in this study were distributed throughout the group-γ clade. Nonetheless, there is some evidence of a bias in the group-γ clade genes expressed in the chicken testes because 4 sequences (GgTestesOR-A, -B, -D, and -E) are placed in a single subclade. Furthermore, 3 of these sequences (GgTestesOR-B, -D, and -E) were recovered multiple times within the data set, which may indicate higher levels of expression compared with the other 3 OR genes that were found in a single plasmid. This observation should not be over-interpreted as biases inherent in the PCR make extrapolations between ratios in the final PCR products and those in the original template problematic. Because we did not detect any testes-expressed OR genes from the group-γ-c clade, we speculate that this clade may be predominantly, or exclusively, used in olfaction, whereas ORs in other clades may have additional, nonolfactory functions. If correct, one would expect differing evolutionary selective pressures to be acting on these 2 hypothetically functionally different categories. OR genes only used in olfaction are expected to be under more relaxed, or perhaps positive, selection and therefore may evolve more rapidly than OR genes that are constrained by having multiple functions. Indeed, there is evidence from mammals that testes-expressed ORs are evolutionarily more conserved than ORs expressed in olfactory tissue (Branscomb et al. 2000).

Spehr et al. (2003) showed that testicular/sperm OR proteins are localized in the sperm flagellum midpiece and influence flagellar motility. This finding suggests that sperm-expressed OR genes may play an important role in sperm–egg chemotaxis/communication (Spehr et al. 2004). It is reasonable to hypothesize that these findings can be extrapolated to birds. Interestingly, in birds, sperm motility positively correlates with fertilization success and thus, male fitness (Birkhead et al. 1999). Sperm motility is of particular importance when ejaculates of different males compete for fertilization, a phenomenon described as sperm competition (Parker 1970). In many avian species, females mate with more than one male (Petrie and Kempenaers 1998); hence, avian sperm competition is common. However, little is currently known about the underlying mechanisms of postcopulatory selection. Thus, further studies of the evolution and function of OR genes expressed in avian testes may prove a fruitful area of research.

### Supplementary Material

Supplementary methods and tables 1–2 can be found at http://www.jhered.oxfordjournals.org/.

### Funding

The Max Planck Society.

### Acknowledgments

We thank Ursula Holter for expert laboratory assistance. We thank Jakob Müller and Vitam Kodelja for discussion and 2 anonymous reviewers for constructive comments. Oswald Rottmann (Versuchsgut Thalhausen, Technical University, Munich) generously provided chicken tissue.

### References


Received December 12, 2007
Accepted April 22, 2008

Corresponding Editor: Jerry Dodgson