Multiple and Independent Cessation of Recombination Between Avian Sex Chromosomes

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ABSTRACT

Sex chromosome evolution is assumed to take place by a gradual arrest of recombination between autosomal homologs, followed by genetic decay of one of the chromosomes (Bull 1983; Charlesworth 1996). According to a widely accepted model, this process is triggered initially by the favorable linkage disequilibrium between a sex-determining locus and sexually antagonistic alleles (or between several sex-determining loci), which selects for reduced recombination between these loci. Subsequently, loss of functional genes of one of the chromosomes occurs by the gradual accumulation of mutations or rearrangements. This may be driven by (i) “Muller’s ratchet,” the stochastic elimination of chromosomes with the fewest mutations in a clonal system (e.g., a nonrecombining chromosome; Felsenstein 1974, Charlesworth 1978, 1991; Charlesworth and Charlesworth 1997); (ii) genetic “hitchhiking,” where an advantageous mutation becomes fixed in a population and carries with it a linked neutral or slightly deleterious mutation (Rice 1987); or (iii) background selection against linked deleterious alleles (Charlesworth 1993, 1996; Charlesworth et al. 1993; Orr and Kim 1998). The relative importance of these factors may relate to population size. Other paths of sex chromosome evolution include the direct addition of genes into the nonrecombining region of one sex chromosome through transposition or retrotransposition from autosomal origins (Saxena et al. 1996; Burgoyne 1998; Lahn and Page 1999a).

Signatures of an ancestral state of sex chromosome homology are today evident from a few gene pairs shared between the nonrecombining regions of the two sex chromosomes, e.g., ZFX/ZFY on the X and Y in mammals (Graves 1995a,b; Lahn and Page 1997; Roldan and Gomendio 1999) and MROS3X/MROS3Y and SIX1/SIX1 on the X and Y in the plant Silene latifolia (Delichere et al. 1999; Guttmann and Charlesworth 1999). The avian Z and W sex chromosomes evolved from a different pair of ancestral autosomes than the mammalian X and Y (Fridolfsson et al. 1998). However, despite being female-specific, the avian W is organized in a similar way as the mammalian Y; it is gene poor, generally very small, and rich in heterochromatin and repetitive arrays (Bloom et al. 1993; Ellegren 2000). So far, only two expressed genes have been mapped to the avian W chromosome. One encodes a chromo-helicase DNA-binding protein (CHD1W; Ellegren 1996; Griffiths et al. 1996) and the other the α-subunit of ATP synthase (ATP5AIW; Dvorak et al. 1992; Fridolfsson et al. 1998). Both genes are located within the nonrecombining part of the W chromosome and both also have similar and expressed homologs on the Z chromosome (CHD1Z and ATP5AIZ, respectively; Griffiths and Korn 1997; Fridolfsson et al. 1998). This suggests that CHD1 and ATP5AI were present on the ancestral pair of autosomes that subsequently evolved into avian sex chromosomes and that functional homologs have been retained on W and Z when these
ceeded recombining. Sequence analysis of avian CHD1W and CHD1Z has revealed that they evolve independently; i.e., CHD1W genes from several different bird orders are more similar to each other than to any CHD1Z gene (Ellegren and Fridolfsson 1997; Fridolfsson et al. 1998; Fridolfsson and Ellegren 2000; Garcia-Moreno and Mindell 2000). Differentiation of the ancestral CHD1 gene into CHD1W and CHD1Z must therefore have occurred prior to the split of extant bird lineages (>60–100 million years ago (mya)).

In general, if cessation of recombination between sex chromosomes occurred early in the lineages leading to contemporary vertebrate classes, we should expect homologous gene pairs that are shared between the nonrecombining regions of the two sex chromosomes (e.g., Z and W in birds) to evolve independently; i.e., the pattern found for CHD1W and CHD1Z should be seen. To test this assumption we made a detailed study of the evolution of the avian ATP5A1W and ATP5A1Z genes. Contrary to the expectation, however, these genes were found to cluster within the three bird orders examined. This suggests multiple and parallel events of cessation of recombination between sex chromosomes to have occurred after the split of major avian lineages in Cretaceous.

MATERIALS AND METHODS

DNA work: We collected whole-blood or tissue samples from morphologically sexed birds of six different species: chicken (Gallus domesticus), turkey (Meleagris gallopavo; these two belong to the order Galliformes), eider (Somateria mollissima), black-headed gull (Larus ridibundus), and herring gull (L. argentatus). The three orders sampled from split early in avian radiation and their relationship can probably be represented by a star phylogeny (Sibley and Ahlquist 1990). Within the orders, DNA:DNA hybridization data roughly suggest that chicken and turkey diverged 20–40 mya while eider and black-headed gull <20 mya. Black-headed gull and herring gull belong to the same family and are obviously more closely related than the other two species pairs. Genomic DNA was obtained by proteinase K digestion and phenol-chloroform extraction, according to standard protocols. ATP5A1W and ATP5A1Z genes were amplified using a touchdown-PCR profile, with two different primer combinations. Primers 208F (5'-TCCAAGCAGAAGAAATGTT-3') and 310R (5'-AHTCTG TCATTACAAAACAC-3') amplified the third intron plus most of exon 3 and part of exon 4. Primers 269F (5'-GGAATGTCCCTGAAAYTGGCA-3') and 587R (5'-CAGCTTCT CCTGTCACCRAT-3') amplified the entire fourth intron plus parts of exons 4 and 5 (degenerate nucleotides in primers: H = A/C/T, Y = C/T, and R = G/A). Exons 4 and 5 are 171 and 165 bp, respectively. Exon and intron nomenclature as well as primer designations follows the organization and sequence of the mammalian ATP5A1 gene. The whole gene has as yet not been cloned from birds, but our preliminary data suggest an exon/intron organization identical to that in mammals. PCR reactions (20 µl) were run with ∼100 ng DNA, 0.5 units AmpliTaq Gold (Perkin-Elmer, Norwalk, CT), 0.2 mM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, and 5 pmol of each primer. The PCR conditions used were an initial denaturation of 10 min at 94°C, followed by five touchdown cycles of 94°C for 30 sec, 62°C–55°C for 30 sec (decreasing 1.5°C/cycle), and 72°C for 30 sec, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min 40 sec. A 5-min extension step at 72°C completed the run.

PCR products were purified with QIAquick spin columns (QIAGEN, Chatsworth, CA) and ligated into plasmid vector, using the pGEM-T Easy Vector system (Promega, Madison, WI). Since both primer combinations amplified ATP5A1W as well as ATP5A1Z from female DNA, we discriminated between clones containing the two genes by single-strand conformation polymorphism (SSCP) analysis in native 8% polyacrylamide gels. Amplifications from genomic female and male DNA served as reference in these analyses. Plasmid DNA was purified from clones of interest using the QIAprep Miniprep kit (QIAGEN). DNA sequencing was based on dye terminator cycle sequencing chemistry (Perkin-Elmer) with detection on an ABI 377 instrument (Perkin-Elmer). For each gene, DNA was extracted from two different individuals of each species and sex, amplified, and sequenced as described above.

Sequence analysis: All sequences were aligned using Sequencer 3.0 (Gene Codes Corp.) and Sequence Navigator (Applied Biosystems, Foster City, CA) software. Exon and intron data were treated separately to allow analyzing possible differences in their molecular evolution. For analyses of exon data we combined exon 4 and 5 sequences. Similarly, intron 3 and 4 (whole introns) sequences were combined. MEGA (Kumar et al. 1993) was used for calculating synonymous (silent) substitution rates (applying Jukes-Cantor correction for multiple hits and excluding indels). Before tree construction, Modeltest Version 2.0 (Posada and Crandall 1998) was applied on exon data, using the Tamura and Nei (1993) model with a correction of 0.218 for the gamma distribution. The maximum-likelihood method was employed to construct phylogenetic trees using PAUP* 4.0b2 (Swofford 1998). All trees were tested for bootstrap and quartet puzzling support. Sequences obtained in this study have been deposited in GenBank under the accession nos. AF301554–AF301589.

RESULTS

We sequenced exons 4 and 5 of ATP5A1W and ATP5A1Z from six different bird species, two from each of three major avian lineages—Galliformes (chicken and turkey), Anseriformes (eider and goldeneye), and Ciconiiformes (black-headed gull and herring gull; see Figure 1). A phylogenetic analysis of these sequences revealed an unexpected pattern. In contrast to the situation for avian CHD1 genes, the overall topology of the tree mainly reflected the genetic relationships between species rather than the chromosomal origin of individual genes (Figure 2). For instance, waterfowl ATP5A1W were more similar to waterfowl ATP5A1Z than to ATP5A1W from other lineages. Within each lineage, though, individual ATP5A1W and ATP5A1Z sequences tended to cluster separately. The similarity of ATP5A1W and ATP5A1Z within orders, relative to that between orders, would indicate that ATP5A1W and ATP5A1Z did not evolve independently during early avian evolution, but that they are doing so now within the three lineages studied.

One possible interpretation of this observation is that gene conversion between ATP5A1W and ATP5A1Z has
Figure 1.—Alignment of exon 4 and 5 sequences of avian ATP5A1W and ATP5A1Z genes. The species are chicken (Gd, Gallus domesticus), turkey (Mg, Meleagris gallopavo), eider (Sm, Somateria mollissima), goldeneye (Bc, Bucephala clangula), black-headed gull (Lr, Larus ridibundus), and herring gull (La, Larus argentatus). Z and W, respectively, denote chromosomal origin of genes. Identical positions are denoted with dashes. The amino acid sequence is given above the DNA master sequence. An arrow denotes the boundary between exons 4 and 5.

had a homogenizing effect on their evolution in each of the three lineages. It has been argued that gene conversion may occur mainly in coding parts of genes (Liskay et al. 1987; this is also supported by some empirical data, Pamilo and Bianchi 1993; see further discussion below), and we therefore analyzed the phylogenetic relationships of two introns (~915 and 100 bp, respectively) immediately flanking exon 4 of ATP5A1W and ATP5A1Z. Again, however, ATP5A1W and ATP5A1Z did not cluster on separate branches (Figure 3). With strong bootstrap support, the ATP5A1W genes of each lineage were more related to the ATP5A1Z genes of the same lineage than to ATP5A1W from other lineages. Also in this case, though, each individual ATP5A1W sequence was most related to the other ATP5A1W sequenced from that lineage. Thus, from analysis of a total of ~1360 bp continuous coding as well as noncoding sequence of avian ATP5A1W and ATP5A1Z, we conclude that the two genes have evolved nonindependently in a manner that at some point must have involved frequent genetic change between the Z and W chromosomes, but that this exchange has now ceased.

When did the avian ATP5A1W and ATP5A1Z genes cease to recombine (i.e., start to diverge)? One possible
way of answering this question would be to apply a molecular clock to our divergence data. The frequencies of synonymous substitution between ATP5A1W and ATP5A1Z for the three orders analyzed in this study were 0.047 ± 0.022 (Ciconiiformes), 0.139 ± 0.009 (Galliformes), and 0.229 ± 0.021 (Anseriformes) substitutions per site. The contrasting frequencies seen among orders suggest that not only has recombination independently ceased in the different orders, but that this has also happened at different times in the different orders. It is somewhat difficult to estimate divergence times from these data due to the absence of good fossil records needed for calibration of the avian molecular clock. However, applying the number of 3.5 synonymous substitutions per site per 10⁸ years, which is frequently used as a mammalian average (Li 1997), ATP5A1W/ATP5A1Z divergence times of ~13 (Ciconiiformes), 40 (Galliformes), and 65 mya (Anseriformes) would be suggested. Importantly, the estimated ATP5A1W/ATP5A1Z divergence times are less than the estimated time of divergence of major avian orders in Cretaceous (Cooper and Penny 1997). This lends further support to the idea that the ancestral ATP5A1 gene independently ceased to recombine in different avian orders after they had split from a common early avian ancestor.

DISCUSSION

Our data are compatible with the three avian lineages diverging before ATP5A1 ceased to recombine (i.e., started to differentiate into ATP5A1W and ATP5A1Z). This would for the first time provide gene-based phylogenetic evidence, from one of the major vertebrate classes, that at least the final stages of differentiation into heteromorphic sex chromosomes have occurred on several independent occasions after the split of extant lineages. Although no such data are yet available from other classes, this might be a common feature of vertebrate sex chromosomes. It has recently been shown that the human X chromosome is characterized by four “evolutionary strata,” each stratum representing a distinct chromosomal region in which suppression of recombination was established during a specific time (Lahn and Page 1999b). The most recently evolving stratum is estimated to have differentiated at about the time when the simian and prosimian lineages diverged, <50 mya. There is only limited map information on the chromosomal location of genes from this stratum in more distant lineages (Roldan and Gomendio 1999), so it remains to be tested whether a similar process has taken place in other lineages.

The hypothesis of avian sex chromosome differentiation being incomplete prior to the split of extant lineages receives strong support from several recent observations. First, ratites (Palaeognathae), i.e., the ostrich and its allies, traditionally considered to be the most primitive avian lineage (Cracraft 1981), have Z and W sex chromosomes that are hard to distinguish on the basis of size and banding pattern (Ansari et al. 1988).
Moreover, chromosome painting reveals the ratite Z to be more or less identical to the ratite W (Shetty et al. 1999), and physical mapping of a handful of genes Z-linked in other birds shows them to be on both Z and W in ratites (Ogawa et al. 1998). Also, cytological studies reveal recombination nodules over most of the ratite W and Z chromosomes (Pigozzi and Solari 1997). Accepting the ratite clade as basal in the avian phylogeny is consistent with a model in which the Z and W sex chromosomes had not differentiated in full when ratites split off from other birds (Neognathae; Figure 4a). Subsequently, sex chromosome differentiation may have taken place in the Neognathae lineage, being partly accomplished prior to the divergence of extant neognath lineages. Full sex chromosome differentiation may then have been independently achieved in different lineages, differentiating ATP5A1 into ATP5A1W and ATP5A1Z. In line with this idea, we have not been able to detect a female-specific copy of ATP5A1 in ostriches despite applying numerous primer pairs from various parts of the gene. This strongly suggests that ATP5A1W and ATP5A1Z are indeed not differentiated in ratites, i.e., that ATP5A1 still recombines.

The situation is, however, complicated by the fact that the precise sequence in which early avian lineages diverged is a matter of discussion. Recent data from whole mitochondrial genome sequences suggest that ratites are not basal in the avian phylogeny (Hårild et al. 1997). While now supported by several studies (Mindell et al. 1997; Hårild et al. 1998; Hårild and Arnason 1999), the idea of the ratite lineage clustering within other extant bird lineages poses an inherent problem in that ratites do not have differentiated sex chromosomes, unlike birds of other lineages. It may seem unlikely that avian sex chromosomes were once differentiated but, by some mechanism, reverted to an “autosomic” stage in ratites. This possibility cannot be completely excluded, though, as examples of the initial chromosomal system for sex determination being replaced by a single-gene system on another chromosome are known from insects (Traut and Willhoeft 1990; Schmidt et al. 1999). However, the data presented here provide an attractive alternative explanation. If avian sex chromosome differentiation, as manifested by the cessation of recombination at the ATP5A1 locus, has occurred on several independent occasions in different lineages, full differentiation might not have taken place before ratites diverged from other lineages (Figure 4b). Differentiated sex chromosomes in birds may in this sense not completely reflect identity-by-descent but rather identity-by-state.

Additional supports for the hypothesis of multiple and independent cessation of recombination at the ATP5A1 locus come from the physical location of genes on the avian Z chromosome. In chicken, CHD1Z is located at Zq16-21 (Fridolfsson et al. 1998) and is thus quite distant from the pseudoautosomal region (PAR)
at the terminal part of the Zp arm (Solari et al. 1998). It is reasonable to assume that CHD1W and CHD1Z stopped recombining early in the process of avian sex chromosome differentiation, assuming that differentiation proceeded directionally, with genes nearest the present PAR becoming differentiated last, as postulated for the human X chromosome (Lahn and Page 1999b). 

Without intrachromosomal rearrangements on the Z, any gene that ceased to recombine after CHD1 should then be closer to the PAR. ATP5A1Z has been mapped to the terminal part of chicken Zp (Fridolfsson et al. 1998), very close to the PAR, which is thus compatible with a model of a gradually contracting PAR and a recent pseudoautosomal origin of ATP5A1. This resembles the situation for the human sex chromosomes, where the frequency of synonymous substitution between XY gene pairs gradually increases from the evolutionary stratum closest to the PAR to the stratum that is most distant (Lahn and Page 1999b). Similarly, the degree of divergence between CHD1W and CHD1Z clearly exceeds that between ATP5A1W and ATP5A1Z (Carmichael et al. 2000; Fridolfsson and Ellegren 2000). Using data from several different avian orders, Garcia-Moreno and Mindell (2000) recently estimated that CHD1W and CHD1Z ceased to recombine 125 mya, i.e., likely prior to early avian radiation and prior to our estimated times of divergence of ATP5A1W and ATP5A1Z (13–65 mya).

An alternative interpretation to our observations is that the avian sex chromosomes diverged in full, including ATP5A1 diverging into ATP5A1W/ATP5A1Z, prior to the split of extant neognath lineages. Subsequently, several independent gene conversion events, in different lineages, could have homogenized the molecular evolution of the ATP5A1W/ATP5A1Z genes. Gene conversion between homologous genes on the sex chromosomes has been documented for one gene pair on the mammalian X and Y chromosomes, the ZFY/ZFX genes (Hayashida et al. 1992; Pamilo and Bianchi 1993; Pecon Slattery et al. 2000). We cannot formally reject gene conversion as an explanation for the observed pattern in birds but we think that the following arguments can be raised against the idea. First, if ATP5A1 had ceased to recombine prior to the split of the three avian lineages studied herein, three independent gene conversion events in the same genomic region and in the very same part of the ATP5A1 genes would have been required to obtain the phylogenetic relationships between ATP5A1W and ATP5A1Z genes seen in Figures 2 and 3. This may be considered an unlikely scenario. Second, the fact that the interdependence of avian ATP5A1W/ATP5A1Z genes involves both exon and intron sequences might not be expected from gene conversion. It seems reasonable to assume that the possibility of successful conversion is affected by the degree of sequence conservation (Liskay et al. 1987). Thus, the rapid divergence of noncoding sequences, including point mutations as well as insertion/deletion mutations, should impose obstacles to intronic gene conversion between genes that have been separated for a long time. Under the hypothesis that the ATP5A1W/ATP5A1Z genes diverged prior to the split of contemporary neognath lineages, it may therefore seem unlikely that their intron sequences were conserved to an extent that allowed several independent events of gene conversion in the lineages we studied. One problem with this argument, however, is that gene conversion could be initiated in conserved coding regions and then spread to involve adjacent introns, through branch migration. While this is plausible in theory, empirical data from, for instance, major histocompatibility complex (Mhc; reviewed by Martinsohn et al. 1999) and ZFY/ZFX genes (Hayashida et al. 1992; Pamilo and Bianchi 1993; Pecon Slattery et al. 2000) indicate that gene conversion generally involves only short genomic regions (less than a few hundred base pairs; we analyzed 1.3 kb of continuous sequence) and is commonly restricted to exonic sequences. We therefore favor the idea that independent sex chromosome divergence is a more parsimonious interpretation of our data than frequent and independent gene conversion events.

In conclusion, we postulate that avian sex chromosomes have diverged at several independent occasions, after the split of major extant lineages. This might suggest that once sex chromosome differentiation has been initiated, it will commonly be a process that proceeds until the pseudoautosomal region has contracted to a minimum size required for proper pairing of sex chromosomes at meiosis. In fact, in some organisms one of the sex chromosomes has been entirely lost.

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