Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes

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All snake species exhibit genetic sex determination with the ZZ/ZW type of sex chromosomes. To investigate the origin and evolution of snake sex chromosomes, we constructed, by FISH, a cytogenetic map of the Japanese four-striped rat snake (Elaphe quadrivirgata) with 109 cDNA clones. Eleven of the 109 clones were localized to the Z chromosome. All human and chicken homologues of the snake Z-linked genes were located on autosomes, suggesting that the sex chromosomes of snakes, mammals, and birds were all derived from different autosomal pairs of the common ancestor. We mapped the 11 Z-linked genes of E. quadrivirgata to chromosomes of two other species, the Burmese python (Python molurus bivittatus) and the habu (Trimeresurus flavoviridis), to investigate the process of W chromosome differentiation. All and 3 of the 11 clones were localized to both the Z and W chromosomes in P. molurus and E. quadrivirgata, respectively, whereas no cDNA clones were mapped to the W chromosome in T. flavoviridis. Comparative mapping revealed that the sex chromosomes are only slightly differentiated in P. molurus, whereas they are fully differentiated in T. flavoviridis, and E. quadrivirgata is at a transitional stage of sex-chromosome differentiation. The differentiation of sex chromosomes was probably initiated from the distal region on the short arm of the proteosex chromosome of the common ancestor, and then deletion and heterochromatization progressed on the sex-specific chromosome from the phylogenetically primitive boids to the more advanced vipers.
W chromosomes varies among species (3, 18, 19). The Z and W chromosomes are homomorphic in the boid species. In contrast, the W chromosomes are highly degenerated and heterochromatic in the poisonous snakes belonging to the Elapidae and the Viperidae. The colubrid species, which have moderately differentiated sex chromosomes, are at an intermediate stage of sex-chromosome differentiation between the Boidae and the poisonous snakes. Thus, snakes are a good animal model for studying the evolutionary process of sex-chromosome differentiation in vertebrates.

Here, we report a high-resolution cytogenetic map of the Japanese four-striped rat snake constructed with 105 EST clones. We demonstrate the conservation of the linkage homologies of snake chromosomes with human and chicken chromosomes and discuss the genome evolution and the origins of sex chromosomes in amniotes. Furthermore, we compare the structures of sex chromosomes among three snake species, the Japanese four-striped rat snake (Colubridae), the Burmese python (Python molurus bivittatus, Pythonidae) and the habu (Trimeresurus flavoviridis, Viperidae) to track the process of sex-chromosome differentiation during the evolution of snakes. First, the morphologies and G- and C-banded patterns of sex chromosomes were compared. Second, the cDNA clones localized to the sex chromosomes of the Japanese four-striped rat snake were comparatively mapped to the chromosomes of two other species. In addition, we cloned a sex chromosome-specific repetitive DNA sequence from the Japanese four-striped rat snake, which is also conserved in both the python and the habu, and used it as a cytogenetic marker for comparative mapping of sex chromosomes. We also cloned two sexual-differentiation genes, DMRT1 and SOX9, from the habu and determined their chromosomal locations in the three snake species to search for candidate genes of sex determination in snakes. Finally, we discuss the origin and the process of differentiation of snake sex chromosomes.

Results and Discussion

Cytogenetic Map of the Japanese Four-Striped Rat Snake. Fifty-three EST clones and one cDNA clone of the SOX9 gene (see Chromosome mapping of DMRT1 and SOX9, below) were newly mapped to the E. quadrivirgata (EQU) chromosomes by the direct R-banding FISH method (Fig. 1). A preliminary cytogenetic map of this species was constructed with 52 EST clones and 3 cDNA clones isolated by RT-PCR in our study (4), and the cytogenetic map constructed in this study consequently defines the locations of a total of 109 cDNA clones (Fig. 2 and Table 1). The 105 EST clones mapped on the snake chromosomes and their accession numbers and chromosomal locations in the snake, human (Homo sapiens: HSA), and chicken (Gallus gallus: GGA) are listed in Table 2, which is published as supporting information on the PNAS web site. The chromosome homologies were investigated among the three species, and the numbers of homologous chromosome segments were found to be 25 and 49 for chromosomes 1–7 and the Z chromosomes between the snake and chicken and between the snake and human, respectively. We had constructed a cytogenetic map of the Chinese soft-shelled turtle (Pelodiscus sinensis) with 92 cDNA clones (4, 20), which revealed that the chromosomes have been highly conserved between the chicken and the turtle, with the six largest chromosomes being almost equivalent to each other. All of the data collectively suggest that the number of chromosome rearrangements that occurred between the snake and chicken was much more than that between the turtle and chicken. The primitive reptiles diverged into two major lineages, Lepidosauria (lizards and snakes) and Archosauromorpha (turtles, crocodilians, and birds), ~260 million years ago (21, 22). The large differences of chromosome numbers between the rat snake (2n = 36) and chicken (2n = 78) probably resulted from two independent events of chromosome rearrangements: the accumulation of fusions between macro- and microchromosomes in the lineage of snakes leading to the increase in chromosome size and the decrease of microchromosomes; and the fission of macrochromosomes that occurred in the lineage of birds, which caused the increase of macro- and microchromosomes. Several types of cytogenetic evidence of the fission and/or fusion events that occurred in the two lineages were found in this study. For instance, the large chromosome segments of the long arm of EQU2 corresponded to three chicken microchromosomes, GGA12, GGA13, and GGA18, and the long arm of EQU3 corresponded to GGA8, GGA20, and GGA26 (Fig. 2). In like manner, the chromosomal segments homologous to GGA19, GGA12, and GGA27 were localized to EQU1p, EQU6p, and EQUZ4, respectively. More comparative mapping data for the snake, chicken, and other amniote species will be needed to decide between the alternatives.

Eleven of 105 EST clones were localized to the Z chromosome of the Japanese four-striped rat snake (Figs. 1a and 2). Three of the 11 genes were also mapped to the W chromosome, and 8 other genes were localized only to the Z chromosome, indicating that certain homologous regions remain between the Z and W chromosomes. No human and chicken homologues of the 11 genes were mapped on the short arm of chromosome 2 (e) and on the long arm of chromosome 2 (f), respectively, in T. flavoviridis. (Scale bar, 10 μm.)
In chicken, GAD2, WAC, KLF6, AMPH, CTNNB1, and RAB5A were located on GGA2p, and TUBG1 and GH1 were located on a pair of microchromosomes, GGA27. On the other hand, the snake homologues of human X-linked genes, EIF2S3, SYAP1, and ATRX, were localized to EQU4, EQU4, and a microchromosome, respectively, and the snake homologues of six chicken Z-linked genes, ZFR, PHAX, C9orf72, UBQLN1, KIAA0368, and TOPORS, were all mapped to EQU2p. These results confirm our finding that the sex chromosomes of snakes, mammals, and birds were derived from different autosomal pairs of the common ancestor and differentiated independently in each lineage.

Comparison of Karyotypes Among Three Snake Species by Chromosome Banding. The G- and C-banded karyotypes of P. molurus, E. quadrivirgata, and T. flavoviridis are shown in Fig. 3. The snake karyotypes are highly conserved, and the most common diploid number is 2n = 36, consisting of eight pairs of macrochromosomes and 10 pairs of microchromosomes (3, 23, 24). The Z chromosomes were the fourth or fifth largest metacentric chromosomes for all three species, whereas the G-banded patterns were different among the species. The sex chromosomes of P. molurus were morphologically homomorphic, and the G-banded patterns of the Z and W chromosomes were the same (Fig. 3a).

In E. quadrivirgata, the W chromosome was submetacentric, and its size was ∼4/5 that of the metacentric Z chromosome (Fig. 3c). The submetacentric W chromosome of T. flavoviridis was ∼2/3 the size of the metacentric Z chromosome (Fig. 3e). In P. molurus, C-positive heterochromatin was localized to the telomeric and centromeric regions on both the Z and W chromosomes (Fig. 3b), and it was found that heterochromatization of the sex-specific W chromosome has not occurred. In contrast, the deletion of euchromatic regions and chromosomal heterochromatization is far advanced on the W chromosomes of E. quadrivirgata and T. flavoviridis. The short arms of the W chromosomes were found to be degenerated in the two species. A large amount of C-positive heterochromatin was distributed on the interstitial region of the long arm of the E. quadrivirgata W chromosome (Fig. 3d). In T. flavoviridis, a large amount of heterochromatin was distributed over the entire long arm and the centromeric region of the W chromosome (Fig. 3f).

Molecular Cloning and Characterization of Sex Chromosome-Specific Repetitive Sequences. A sex chromosome-specific repetitive DNA sequence was isolated from E. quadrivirgata. The chromosomal distribution was examined for 16 clones isolated from the 1.3-kb DNA band of the BamHI digest of E. quadrivirgata genomic DNA, and one clone containing sex chromosome-specific repetitive DNA sequence was identified. The BamHI B4 fragment
(accession no. AB254800) was localized to the distal regions on the long arm of the Z chromosome and the short arm of the W chromosome (Fig. 4a). The size of the fragment was 1,261 bp, and its G+C content was 40.0%, indicating that it was AT-rich.

To examine the genomic organization of the sex chromosome-specific BamHI repeated sequence, the genomic DNA digested with six restriction endonucleases was subjected to Southern blot hybridization with the BamHI B4 fragment as probe (Fig. 4d). A weakly hybridized band corresponding to the monomer unit was observed at 1.3 kb in the BamHI digest. Ladder bands, some of which did not correspond to the sizes of polymeric bands of the BamHI repeated sequence element, were detected 2.5–10 kb, and intense hybridization signals were observed at higher molecular weight than 10 kb. This result indicates that the BamHI sites are conserved in the repetitive DNA sequences but are not frequent in the genome. Many intensely hybridized bands were detected 1.5–23 kb in the MspI digest but not in the HpaII digest. The restriction sites of HpaII and MspI are both "CCGG", and HpaII does not cleave when the second cytosine is methylated, whereas MspI cleaves when the CG sequence is methylated. The difference in hybridization patterns between the MspI and HpaII digests suggests that the BamHI repeated sequence undergoes extensive methylation in the genome.

The BamHI repeated sequence was conserved in the genome of P. molurus and T. flavoviridis and cross-hybridized to the chromosomes of the two species (Fig. 4b and c). The hybridization signals were localized to the distal regions of the short arms of the Z and W chromosomes in the two species. Thus, the nucleotide sequence and chromosomal location of the BamHI repeated sequence is highly conserved in Henophidia and Caenophidia.

**Chromosome Mapping of DMRT1 and SOX9.** DMRT1 and SOX9 are highly conserved in vertebrates as sexual differentiation genes with important roles in testis differentiation (25–27). We molecularly cloned DMRT1 (accession no. AB254801) and SOX9 (accession no. AB254802) from the adult testis of T. flavoviridis by RT-PCR. The primer sets for the DMRT1 and SOX9 genes amplified 1,168-bp and 1,390-bp products, respectively, and their chromosomal locations of the DMRT1 and SOX9 genes were determined for the three species by FISH. In our study (4),

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**Table 1. The list of the genes mapped to microchromosomes of E. quadrivirgata and their chromosomal locations in human and chicken**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Snake</th>
<th>Human*</th>
<th>Chicken†</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEF3</td>
<td>Micro</td>
<td>8p</td>
<td>22</td>
</tr>
<tr>
<td>ASB6</td>
<td>Micro</td>
<td>9q</td>
<td>–</td>
</tr>
<tr>
<td>RPL12</td>
<td>Micro</td>
<td>9q</td>
<td>17</td>
</tr>
<tr>
<td>FLJ25530</td>
<td>Micro</td>
<td>11q</td>
<td>–</td>
</tr>
<tr>
<td>HSPAB</td>
<td>Micro</td>
<td>11q</td>
<td>24</td>
</tr>
<tr>
<td>GLCE</td>
<td>Micro</td>
<td>15q</td>
<td>un</td>
</tr>
<tr>
<td>POLG</td>
<td>Micro</td>
<td>15q</td>
<td>10</td>
</tr>
<tr>
<td>LOC283820</td>
<td>Micro</td>
<td>16p</td>
<td>14</td>
</tr>
<tr>
<td>PARN</td>
<td>Micro</td>
<td>16p</td>
<td>14</td>
</tr>
<tr>
<td>ATRX</td>
<td>Micro</td>
<td>Xq</td>
<td>4</td>
</tr>
</tbody>
</table>

un, the nucleotide sequence of the gene was annotated in the chicken genome sequence, but its chromosomal location has not been yet identified.

*The chromosomal locations were referred from the UniGene database of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov).

†The chromosomal locations of chicken homologues were defined by using the BLASTN program of Ensembl (www.ensembl.org/index.html) and/or the tblastx program of NCBI.
DMRT1 was mapped to the short arm of E. quadrivirgata chromosome 2, which was found here to be homologous to the chicken Z chromosome (Fig. 2). DMRT1 was also localized to the short arm of chromosome 2 in both T. flavoviridis (Fig. 1e) and P. molurus (data not shown) in this study. SOX9 is located on the long arm of chromosome 17 in humans, which contains a segment homologous to the snake Z chromosome (Fig. 2). However, SOX9 was localized to the long arm of chromosome 2 in T. flavoviridis (Fig. 1f) and two other species (data not shown). These results suggest that DMRT1 and SOX9 are not the candidate genes of sex determination situated the furthest upstream in the sex differentiation pathway of snakes.

Comparative Cytogenetic Maps of Sex Chromosome-Linked Genes. The E. quadrivirgata cDNA clones of 11 Z-linked genes were successfully localized to the chromosomes of P. molurus and T. flavoviridis (Fig. 1c and d). Fig. 5 shows the comparative cytogenetic maps of sex chromosomes in the three species. The order of the Z-linked genes was identical among the three species except that the location of AMPH was different between E. quadrivirgata and the two other species. In P. molurus and T. flavoviridis, MYST2, GHI, and TUBG1 were all localized to the short arm of the Z chromosome, and AMPH was localized to the long arm, whereas all four genes were located on the long arm of the E. quadrivirgata Z chromosome. These results suggest that the order of the four genes on the Z chromosome of the common ancestor has been conserved in P. molurus and T. flavoviridis and that a small pericentric inversion occurred in the region containing AMPH on the E. quadrivirgata Z chromosome.

All 11 cDNA clones were mapped to both the Z and W chromosomes in P. molurus, and the order of the genes was identical between the Z and W chromosome. In E. quadrivirgata, the hybridization signals on the W chromosome were observed for only three clones, CTNNB1, RAB5A, and WAC, and the genes were localized to the proximal C-negative euchromatic region on the long arm (Fig. 3d). No cDNA clones were mapped to the W chromosome of T. flavoviridis. The chromosome segments that contained the W homologues of the Z-linked genes were probably deleted during the process of W chromosome differentiation in E. quadrivirgata and T. flavoviridis and were subsequently heterochromatized with the amplification of the repetitive sequences. The other possibility is a decrease of the hybridization efficiency due to the divergence in nucleotide sequence between the Z- and W-linked genes by the cessation of meiotic recombination.

Evolution of Sex Chromosomes in Snakes. Morphologically undifferentiated sex chromosomes have been described in several organisms, such as the medaka fish (28–30) and papaya (31). The Y chromosome of the medaka is completely homologous to its counterpart except for a 250-kb male-specific chromosomal region containing the male-determining DMY/DMRT1Yb gene (28–30). The male-specific region of the papaya Y chromosome accounts for ~10% of the chromosome (31). These instances lead us to suppose that the differentiated region between the Z and W chromosome of P. molurus, which possibly contains sex-determining gene(s), is too small to be detected by banding techniques and comparative FISH mapping. In E. quadrivirgata and T. flavoviridis, the short arm of the W chromosome is extensively degenerated, and almost no homology between the Z and W chromosomes remains except for the telomeric regions, where the BamHI repeat element is localized. Homology to the Z chromosome is partially preserved in the region near the centromere on the long arm of the heterochromatic W chromosome in E. quadrivirgata, whereas no homology on the long arm was detected between the Z and W chromosomes in T. flavoviridis. These results suggest that the differentiation of sex chromosomes was initiated from a distal region on the short arm of the protosex chromosome in the common ancestor through the occurrence of a sex differentiator on only one of an autosomal pair. The cessation of meiotic recombination because of chromosome rearrangements occurring in the sex-specific region is considered to favor the accumulation of gene mutations. This accumulation should lead to the partial deletion of euchromatic regions and heterochromatization with the accumulation of repetitive DNA sequences on the sex-specific chromosome, such as extended from the short arm to the long arm of the W chromosome in the E. quadrivirgata and T. flavoviridis lineages. After the divergence of the two lineages, the degeneration might have become more advanced independently in the T. flavoviridis lineage.

Materials and Methods

Animals. One adult female of the Japanese four-striped rat snake (E. quadrivirgata, Colubridae) was captured in the field in Japan and used for chromosome banding, FISH mapping, and Southern blot hybridization. The same individual was also used in our previous study (4). One adult female each of the Burmese python (P. molurus bivittatus, Pythonidae) and the habu (T. flavoviridis, Viperidae), which were bred at the Japan Snake Institute, was used for chromosome banding and FISH. The original collection locality of the individual of P. molurus bivittatus is unknown. The individual of T. flavoviridis was originally captured in Tokunoshima Island in Japan. A testis of one male T. flavoviridis originally captured on Okinawa Island, Japan, was used for molecular cloning of the DMRT1 and SOX9 genes.

DNA Probes. A large number of EST clones of E. quadrivirgata were obtained from the brain cDNA library in our study (4). We selected 53 additional EST clones of snake homologues of human genes with high E-value (<2e<sup>−35</sup>) and used them for
chromosome mapping. The *T. flavoviridis* homologues of the *DMRT1* and *SOX9* genes were molecularly cloned as described (4). The primer sets for *DMRT1* were synthesized based on the sequence of *E. quadrirugata* (accession no. AB179698). The degenerate primer sets for *SOX9* were newly designed based on the conserved regions among *Eublepharis macularius*, *Calotes versicolor*, *Alligator mississippiensis*, and *G. gallus* (accession nos. AF217252, AF061784, AF106572, and AB012236, respectively). The following primer pair were used in the PCRs: Primers for *DMRT1*: forward, 5'-AGT GAC GAG GTG GGC TTC TA-3'; reverse, 5'-ATC TGG ACT GCT GGG TG-3'. Primers for *SOX9*: forward, 5'-CCG GCC AAC ATG TCG GA-3'; reverse, 5'-GTG AGC TGN GTG TAG ACN GG-3'. The PCR conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 35 s, and, finally, 72°C for 5 min for a final extension. The PCR products were electrophoresed on 3% agarose gels, and the bands of the expected size were isolated and subcloned by using TOP10 competent cells (Invitrogen, Carlsbad, CA). The DNA Extraction and Cloning of Repetitive DNA. Genomic DNA was extracted from liver tissue of the female *E. quadrirugata*. The genomic DNA was digested with restriction endonucleases (BamHI, BglII, BglIII, HaeIII, HpaII, and MspI). The DNAs were fractionated by electrophoresis on 1% and 3% agarose gels. The prominent DNA bands of repetitive sequences were separated and eluted from the gels, and the DNA fragments were eluted and subcloned into pBluescript II SK(-) (Stratagene, La Jolla, CA) and then transferred into Escherichia coli TOP10 competent cells (Invitrogen, Carlsbad, CA). The nucleotide sequences of the clones that produced fluorescence hybridization signals were sequenced.

**Southern Blot Hybridization**. The genomic DNA of *E. quadrirugata* was digested with six restriction endonucleases, BamHI, BglII, DraI, HaeIII, HpaII, and MspI. The DNAs were electrophoresed on 3% agarose gel, and the DNA fragments were transferred onto a nylon membrane (Roche Diagnostics, Basel, Switzerland). The repeated sequence element of *E. quadrirugata* was labeled with digoxigenin-dUTP by using a PCR DIG Labeling mix (Roche Diagnostics) and hybridized to the membrane in DIG Easy Hyb (Roche Diagnostics) overnight at 42°C. After hybridization, the membrane was washed sequentially at 42°C in 2× SSC with 0.1% SDS, 1× SSC with 0.1% SDS, 0.5× SSC with 0.1% SDS, and 0.1× SSC with 0.1% SDS for 15 min each and was reacted with anti-digoxigenin-AP, Fab fragments (Roche Diagnostics). Then the membrane was reacted with CDP-Star (Roche Diagnostics) and exposed to BioMax MS autoradiography film (Kodak, Rochester, NY).

**Chromosome Preparation and FISH**. Chromosome preparation and FISH were performed according to our previous studies (4, 32). Chromosome preparations were made from blood lymphocytes and/or fibroblast cells taken from heart tissue. The cultured cells were treated with BrdU during late S phase for differential replication banding. R-banded chromosomes were obtained by exposure of chromosome slides to UV light after staining with Hoechst 33258. For G- and C-banding analyses, chromosome preparations were made from the cells cultured without BrdU treatment. The G- and C-banded chromosomes were obtained with the GTG (G bands by trypsin using Giemsa) method (33) and the CBG (C bands by barium hydroxide using Giemsa) method (34), respectively.

The probe DNAs were labeled by nick translation with biotin-16-dUTP (Roche Diagnostics). The hybridization was carried out at 37°C for 1 or 2 days. The slides hybridized with genomic DNA clones were stained with fluoresceinated avidin (Roche Diagnostics) and then reacted with goat anti-biotin antibody (Vector Laboratories, Burlingame, CA) and then stained with fluoresceinated anti-goat IgG (Nordic Immunology, Tilburg, The Netherlands). FISH images were observed under a fluorescence microscope (Nikon, Tokyo, Japan) using B-2A and UV-2A filter sets. Kodak Ektachrome ASA 100 films were used for microphotography.

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