The Origin and Differentiation of the Heteromorphic Sex Chromosomes Z, W, X, and Y in the Frog *Rana rugosa*, Inferred from the Sequences of a Sex-Linked Gene, ADP/ATP Translocase

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Sex chromosomes of the Japanese frog *Rana rugosa* are heteromorphic in the male (XX/XY) or in the female (ZZ/ZW) in two geographic forms, whereas they are still homomorphic in both sexes in two other forms (Hiroshima and Isehara types). To make clear the origin and differentiation mechanisms of the heteromorphic sex chromosomes, we isolated a sex-linked gene, ADP/ATP translocase, and constructed a phylogenetic tree of the genes derived from the sex chromosomes. The tree shows that the Hiroshima gene diverges first, and the rest form two clusters: one includes the Y and Z genes and the other includes the X, W, and Isehara genes. The Hiroshima gene shares more sequence similarity with the Y and Z genes than with the X, W, and Isehara genes. This suggests that the Y and Z sex chromosomes originate from the Hiroshima type, whereas the X and W chromosomes originate from the Isehara-type sex chromosome. Thus, we infer that hybridization between two ancestral forms, with the Hiroshima-type sex chromosome in one and the Isehara-type sex chromosome in the other, was the primary event causing differentiation of the heteromorphic sex chromosomes.

Introduction

The frog *Rana rugosa* is distributed widely in Japan and is unique in its sex chromosome differentiation (see fig. 1). The species comprises four geographic forms differing in the morphology of sex-determining chromosome 7 (Nishioka, Miura, and Saitoh 1993; Nishioka et al. 1994). Three of these forms have an XX/XY sex-determining system; one, inhabiting the Tokai-Chubu Region (central Japan), has heteromorphic X and Y chromosomes, and the remaining two, found in western Japan and the Kanto Region (central Japan, east of the Tokai-Chubu), have homomorphic X and Y chromosomes in both sexes (here called the Hiroshima and Isehara types, respectively). The last form, distributed in the Tohoku-Hokuriku Region (northeastern Japan), is characterized by a ZZ/ZW system, with the W chromosome differentiating morphologically from the Z. These six kinds of sex chromosome can be classified into three morphs: subtelocentric (Y, Z, and Hiroshima), more subtelocentric (Isehara), and metacentric (X and W). Chromosome banding and lampbrush chromosome pairing configuration studies (Nishioka et al. 1994; Miura et al. 1997*a*, 1997*b*) have proven that the more subtelocentric morph could be created from the subtelocentric morph by a pericentric inversion, and the metacentric morph could be created from the more subtelocentric morph by another pericentric inversion.

According to the well-known route of sex chromosome differentiation, the two forms of XX/XY and ZZ/ZW with morphologically differentiated sex chromosomes may have evolved from either of the two forms with undifferentiated sex chromosomes soon after the evolution of the species *Rana rugosa*. The sex-chromosomal origin is quite recent. Therefore, it should be possible to understand through this species a primary mechanism of sex chromosome differentiation. We have hitherto studied isozyme variation among populations, chromosomal morphology, and sex differentiation of interform hybrids in order to elucidate which type of sex chromosome (or which form) is ancestral and the evolution of heteromorphic systems. However, the following questions remain unresolved. First, does similarity of chromosomal morphology indicate the same origin? The Z, Y, and Hiroshima-type sex chromosomes are similar to each other, and the X and W chromosomes are similar to each other. Second, how did the Isehara-type sex chromosome take part in the differentiation events? This type of sex chromosome is not shared among any of the other three forms. An approach to resolving these questions and understanding the differentiation of sex chromosomes is to construct a sex-linked gene tree, which would tell us the phylogenetic relationships of the sex chromosomes.

In the present study, we isolated a sex-linked gene, ADP/ATP translocase, using differential display of RNAs from ZZ and WW tadpoles and determined the nucleotide sequences of the genes derived from the Z, W, X, Y, Hiroshima-type, and Isehara-type sex chromosomes. On the basis of the reconstructed phylogenetic gene tree, we infer the phylogeny of the sex chromosomes and discuss the primary mechanism of sex chromosome differentiation in this species.

Abbreviations: AAT, ADP/ATP translocase; *AAT*, ADP/ATP translocase gene.

Keywords: sex chromosome, ADP/ATP translocase, frog, *Rana rugosa*.

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Materials and Methods

Production of ZZ and WW Tadpoles

ZZ and WW tadpoles were produced gynogenetically. Eggs laid by a ZW female were inseminated with UV-irradiated sperm, kept at room temperature for 15

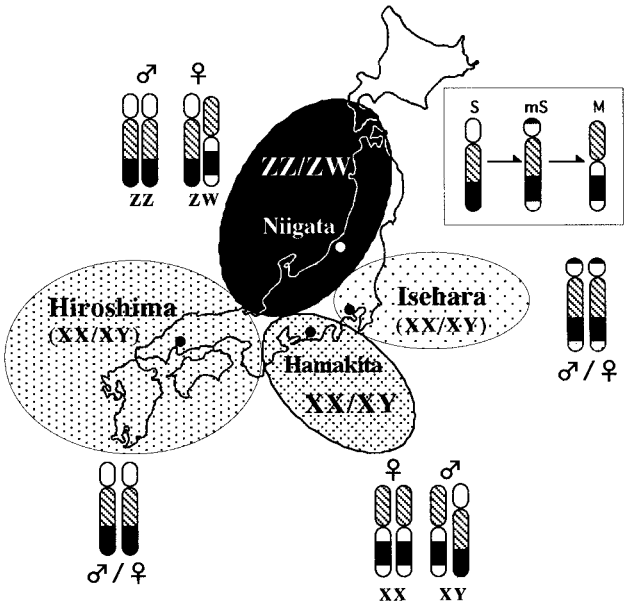

FIG. 1.—Four geographic forms of *Rana rugosa*, differing in the morphology of sex-determining chromosome 7. Three of these, indicated by the dotted areas, have an XX/XY sex-determining system. The fourth form, indicated by the black area, is characterized by a ZZ/ZW system. Three kinds of sex chromosome 7 can be distinguished morphologically in these four geographic forms. The Z, Y, and Hiroshima-type chromosomes are subtelocentric, the W and X chromosomes are metacentric, and the Isehara-type chromosome is more subtelocentric. The structural relationships of the three kinds of sex chromosome are shown boxed at the upper right: S = subtelocentric; mS = more subtelocentric; M = metacentric. A subtelocentric chromosome is divided into three segments, displayed as white, hatched, and black areas, and each of them is arranged by inversions through differentiation (Nishioka, Miura, and Saitoh 1993; Nishioka et al. 1994; Miura et al. 1997*a*, 1997*b*). One pericentric inversion is indicated by an arrow line. The localities from which the four strains are obtained are shown on the map.

min, and then cooled to 4°C for 1.5 h. The WW tadpoles became edematous after hatching and died within 1 week, while the ZZ tadpoles were viable and grew into mature males.

Differential Display of RNA

Total RNA was isolated from whole tadpoles at stage 25 (Shamway 1940) by a guanidinium thiocyanate–CsCl method (Glisin, Crkvenjakov, and Byus 1974; Ullrich et al. 1977).

Differential display was performed essentially as described by Adati et al. (1995). The first-strand cDNA was synthesized after annealing at 25°C by oligo-dT priming using 2.5 μg of total RNA as a template at 42°C for 50 min. The 20 μl of reaction buffer contained 0.5 mM dNTP's, 20 mM Tris-Cl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 2.5 μM anchored primer degenerate at the second base from the 3′ end (5′-GT_{15}VT-3′), and 200 U Superscript II reverse transcriptase (BRL). The mixture was heated at 90°C for 5 min and diluted with 80 μl of TE (10 mM Tris; 0.1 mM EDTA, pH 8.0). Polymerase chain reactions (PCRs) were performed for 20-μl volumes containing 2.0 μl of cDNA solution, 0.5 μM of arbitrary primer, 0.25 μM of anchored primer, 2.5 μM of each dTTP, dCTP, and dGTP, 0.5 μl of α-[^{35}S]-dATP (1,000 Ci/mmol), 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 1 U of *Taq* DNA polymerase (Genetaq, Nippon gene). Thermal cycling was carried out in a PROGRAM TEMP CONTROL SYSTEM PC-700 (ASTEC). Reactions were cycled at 94°C for 3 min, 25°C for 5 min, and 72°C for 5 min for one cycle, at 94°C for 30 s, 30°C for 2 min, and 72°C for 1 min for 30 cycles, and at 72°C for 5 min for an additional final extension. The amplified cDNAs (5 μl) were separated on a 6% polyacrylamide–8 M urea gel in TBE buffer (0.89 M Tris, 0.89 M boric acid, 22 mM EDTA) for 3 h. The gel was dried on a gel drier and exposed with an X-ray film (Fuji) for 12 h.

Southern and Northern Blot Analyses

DNA was prepared from the CsCl fraction obtained by the guanidinium thiocianate–CsCl method of RNA extraction. The CsCl solution containing DNA was dialyzed against 1 l of TE with four to six changes for 12 h. The DNA solution, added with 1/10 volume of 10% SDS, 1/5 volume of 0.5 M EDTA (pH 8.0), RNase A (20 μg/ml), and proteinase K (100 μg/ml), was incubated at 55°C for 4 h, extracted once each with phenol, phenol-chloroform, and chloroform, and then precipitated with 1/5 volume of 10 M ammonium acetate and 2 volumes of 99% ethanol. The pooled DNA was rinsed with 70% ethanol and diluted in TE. The DNA (15 μg) cut with *Pst*I was electrophoresed in 0.8% agarose gel, while RNA (15 μg) was electrophoresed in 1.1% denatured formaldehyde gel. The separated DNA and RNA were transferred to nylon membranes and hybridized with digoxigenin-labeled probe in 0.05 M sodium phosphate buffer containing 5 × SSC, 10% SDS, 2% blocking solution, 0.1% *N*-lauroylsarcosine, and denatured salmon sperm DNA (60 μg/ml) at 68°C for 16 h. The membranes were washed three times in 2 × SSC, 0.1% SDS at room temperature for 5 min and once in 0.1 × SSC, 0.1% SDS at 68°C for 30 min, and they were then exposed to X-ray film after immersion in anti-digoxigenin AP conjugate (1:10,000), washed, and incubated with 1/100 CSPD (Boehringer Mannheim).

DNA Sequencing

The *AAT* insert of lambda gt10 was subcloned into the phage vector M13mp18, and the single-strand DNA was prepared and sequenced with an ABI 373A automated DNA sequencer according to the manufacturer's guide (Perkin Elmer). Both strands of the clone DNA were sequenced.

cDNA Library and Screening

Poly(A)$^+$mRNA was purified using Oligotex-dT30 (TAKARA), and 4 μg was used for the first-strand cDNA synthesis. Libraries were produced with the Amersham kit by oligo-dT priming in Lambda-gt10.

By screening about 3.5×10^4 recombinant phages of the ZZ × ZW library with Z1 probe (see *Results*), we obtained a total of 14 positive clones. The sequence of 300 bp from the 3′ end revealed that 12 of the clones had the Z1 sequence, and the remaining two had the W1

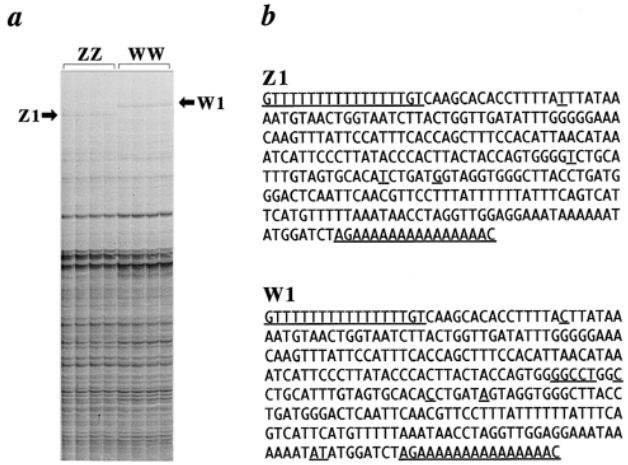

FIG. 2.—Isolation of RNA expressed from the Z and W chromosomes. *a,* Differential display of RNAs from ZZ and WW tadpoles, using one combination primer set of an arbitrary 10mer (5′-TGGATTGGTC-3′) and an 18mer anchored primer degenerate at the second base from the 3′ end (5′-GT_{15}VT-3′). From left to right, the first four lanes are ZZcDNA and the next four are WW. The bands appearing near the top of the gel are termed Z1 and W1 (arrows), respectively. Their mobility is different, as shown in the ZZ and WW lanes. *b,* The nucleotide sequences of the two fragments Z1 and W1, again amplified in the same condition and subcloned into M13mp18. The underlined sequences at both ends are of the anchored primer, indicating that the arbitrary primer is not used for amplification of these fragments. The Z1 and W1 sequences have 96.5% similarity. A database search showed that they were of part of the 3′ noncoding region of ADP/ATP translocase mRNA. The sequence of *Rana sylvatica,* an American brown frog, shows 93.4% similarity to Z1 and 91.4% similarity to W1. Bases different between Z1 and W1 are underlined.

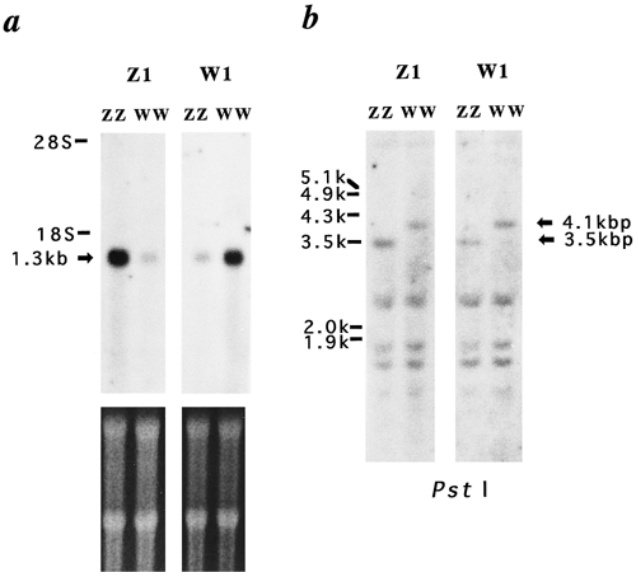

FIG. 3.—Northern and Southern blot analyses showing that the Z1 and W1 cDNAs, respectively, are derived from the Z and W chromosomes. Probes used are shown at the tops of the gels. *a,* ZZ and WW RNAs, hybridized with Z1 and W1, reveal a band of about 1.3 kb. Using the Z1 probe, the band of ZZ is intense, whereas that of the WW is faint. The pattern is reversed when the W1 probe is used. The ethidium bromide–stained RNA gels are shown under the hybridized filter membranes. *b, Pst*l-digested DNAs of ZZ and WW tadpoles reveal six bands when hybridized with the Z1 or W1 fragment. Five of them are identical in size between the ZZ and WW, indicating their autosomal origin, whereas the remaining one differs in size. The band is 3.5 kb in ZZ, whereas it is 4.1 kb in WW. Its intensity is strong when hybridized with the probe from the same origin, but faint with the different one. The DNA membrane hybridized with the Z1 fragment (left) was next reprobed with W1 (right) after stripping off the Z1 probe fragment.

sequence (see *Results*). The 12 Z clones were classified into two kinds, designated Z1 and Z2, with one different nucleotide and a three-base addition. By screening about 1.5×10^4 phages of the XX × XY library, 10 positive clones were obtained. Two showed sequence similar to that of Z1, designated Y, and the remaining eight showed sequences similar to that of W1, designated X. The eight X clones were divided into two kinds, one with three nucleotides added at the 3′ end of the other, and these were designated X2 and X1, respectively. By screening about 2×10^4 and 3×10^4 phages of the Hiroshima and Isehara libraries, we obtained 12 and 13 positive clones, respectively. The sequence of 400 bp of the 3′ noncoding region of the Hiroshima (Hr) was similar to that of Z1, while that of the Isehara (Is) resembled that of W1. No sequence variation was found for clones isolated from the same library.

Results

A Frog Sex-Linked Gene, ADP/ATP Translocase

The sex chromosome constitutions of the four geographic forms of *R. rugosa* are shown in figure 1. The localities of the strains used in this study are shown on the map: Niigata for ZZ/ZW, Hamakita for XX/XY, Isehara for Isehara form, and Hiroshima for Hiroshima form. To isolate a gene located in the Z and W chromosomes, we carried out differential display of RNAs from ZZ and WW tadpoles. By PCR using the 10-oligomer arbitrary primer on one hand and an 18-oligomer anchored primer on the other, a fragment of cDNA was identified which showed a different mobility in the denaturing gel between the ZZ and WW lanes (fig. 2*a*). The nucleotide sequences determined for the Z1 and W1 fragments, which were isolated from the ZZ and WW RNAs, respectively, revealed a high similarity with the known ADP/ATP translocase (*AAT*) of the American brown frog, *Rana sylvatica*. Between the Z1 and W1 sequences, similarity was 96.5%, with two- and five-base insertions in the W1 sequence (fig. 2*b*).

To confirm the sex-chromosomal location of these two clones, we performed Southern and northern hybridizations with DNA and RNA from ZZ and WW tadpoles (fig. 3). The RNA showed a single band of about 1.3 kb. In the case of ZZ, this band was more intense than that for WW with the Z1 probe, whereas the pattern was reversed with the W1 probe (fig. 3*a*). Of six *Pst*l fragments present in both ZZ and WW DNAs, five were identical in size, thus showing their autosomal origin. In contrast, the size of the remaining fragment was different (3.5 kb in ZZ and 4.1 kb in WW). Particularly, the 3.5 kb of ZZ revealed a stronger intensity than the 4.1 kb of WW with the Z1 probe, whereas the pattern was reversed with the W1 probe (fig. 3*b*). These results show

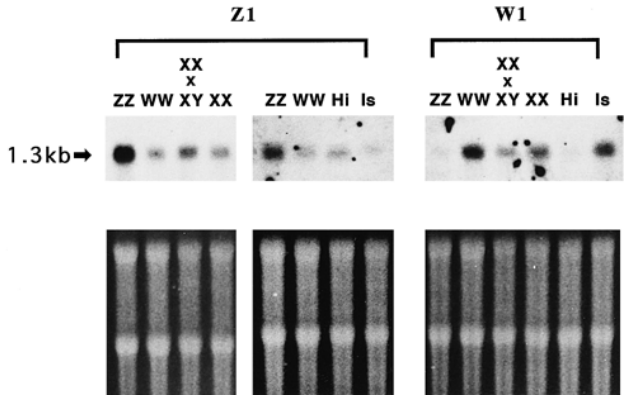

FIG. 4.—Northern blot analysis showing similarities between the ADP/ATP translocase RNAs from X, Y, Hiroshima- and Isehara-type chromosomes and those from the Z and W chromosomes. The probes used are shown at the top. Using the Z1 probe, XY RNA of the offspring from a cross of XX female × XY male shows a stronger signal than the XX RNA, while this pattern is reversed with the W1 probe, indicating that the Y *AAT* has more similarity with the Z, whereas the X *AAT* has more with the W. The signal of Hiroshima RNA is faint when hybridized with both the Z1 and W1 probes, but it is a little stronger with the Z1 probe, showing somewhat less similarity to the Z *AAT*. The Isehara RNA reveals a strong signal with the W1 probe, but a faint signal with the Z1 probe, showing that Isehara *AAT* is more similar to the W *AAT* than to the Z *AAT*. The ethidium bromide–stained gels are shown under the hybridized membranes.

unambiguously that Z1 originates from the Z chromosome and W1 originates from the W chromosome.

Northern Blot Analysis of ADP/ATP Translocase RNAs from Six Kinds of Sex Chromosome

To elucidate the similarities between ADP/ATP translocase (AAT) RNAs from the X, Y, Hr, and Is sex chromosomes and those from the Z and W chromosomes, we performed northern blot analyses (fig. 4 and table 1). RNAs were all prepared from tadpoles at stage 25 (Shamway 1940). The RNA of offspring gynogenetically produced from an XX female was used as XX RNA. As RNA from the Y chromosome alone cannot be isolated, the RNA of offspring produced from the cross XX × XY was used as XY RNA. Each of the Hr and Is RNAs was prepared from tadpoles from one cross. When the Z1 probe was used, the XY RNA signal was stronger than that of the XX RNA, the Hr signal was weaker than both of them, and the Isehara signal the weakest of all. In contrast, when the W1 probe was used, the signal strength pattern was reversed: the XX RNA signal was stronger than that of the XY RNA, the Is signal was as strong as that of XX, and the Hr signal was the weakest. These results show that the *AAT* of Y is more similar to that of Z than to W, while the X *AAT* is more similar to that of W than to that of Z. The Hr *AAT* is more similar to that of Z than to that of W, but the similarity is low. The Is *AAT* is more similar to that of W than to that of Z (table 1).

Sequence Alignment of the Full-Length ADP/ATP Translocase cDNAs from the Six Kinds of Sex Chromosome

To determine the complete nucleotide sequences of AAT cDNAs from the six different number 7 sex chromosomes, we screened four cDNA libraries constructed using tadpole RNAs from a ZW × ZZ cross, an XX × XY cross, a Hiroshima cross, and an Isehara cross. Finally, we determined the complete nucleotide sequences of the eight kinds of clone (Hr, Hiroshima; Y, Y chromosome; Z1 and Z2, Z chromosome; Is, Isehara; X1 and X2, X chromosome; W, W chromosome). The sequence alignment is shown in figure 5. The cDNAs have 46–53 bp in the 5′ noncoding region, followed by 894 bp encoding 298 amino acids and 361–374 bp of the 3′ noncoding region. The aligned clones all start at +9, matching with the shortest Is clone starting at +9. Length variation of a T nucleotide cluster is found in the 3′ noncoding region: the number of thymines are 21 in the Y *AAT*, 18 in those of Hr and Is, 16 in those of X1, X2, and W, 14 in that of Z2, and 11 in that of Z1. The similarity of *AAT*s ranges from 97.6% (between Z1 and W) to 99.4% (between the Hr and Y).

Table 1
Quantitative Estimate of Similarity Among ADP/ATP Translocase RNAs Derived from Six Kinds of Sex Chromosome

	PROBE DNA		
RNA	Z1	Z1	W1
ZZ	1.00[a]	1.00	0.16
WW	0.27	0.25	1.00
XX × XY	0.40	—	0.71
XX	0.30	—	0.97
Hr	—[b]	0.27	0.11
Is	—	0.14	0.77

[a] The intensity of each signal appearing on the X-ray films shown in figure 4 is measured using a film scanner (Beckman DU70) and is shown as a ratio to the ZZ *AAT* value on the Z1-probe filter and to the WW *AAT* value on the W1-probe filter.

[b] "—" indicates absence of sample on the filters.

A Phylogenetic Gene Tree of ADP/ATP Translocase

We constructed a gene tree using the neighbor-joining (NJ) method (Saitoh and Nei 1987) on the basis of the pairwise distance of Jukes and Cantor (1969) and also using the maximum-likelihood method (Felsenstein 1981). *Rana sylvatica AAT* is used here as an outgroup. Since the trees constructed by the two methods have the same topology, the NJ tree alone is shown (fig. 6). In *R. rugosa,* the Hr *AAT* diverges first and the rest form two clusters, one containing Y, Z1, and Z2 *AAT*s and the other containing X1, X2, W, and Isehara *AAT*s. For understanding the gene phylogeny and the phylogeny of sex chromosome 7, the most important finding is that the Y *AAT* is more closely related to the Z's and not the X's. This is also the case with the Z and W *AAT*s. This suggests that the Y chromosome had already diverged from the X chromosome, and that the Z chromosome had diverged from the W chromosome, before the formation of the heteromorphic pairs in the present populations. In addition, the X *AAT* is the closest to the W *AAT,* and both are closer to the Is *AAT,* than to the others, suggesting that the metacentric chromosomes X and W share a common origin with the more subtelo-

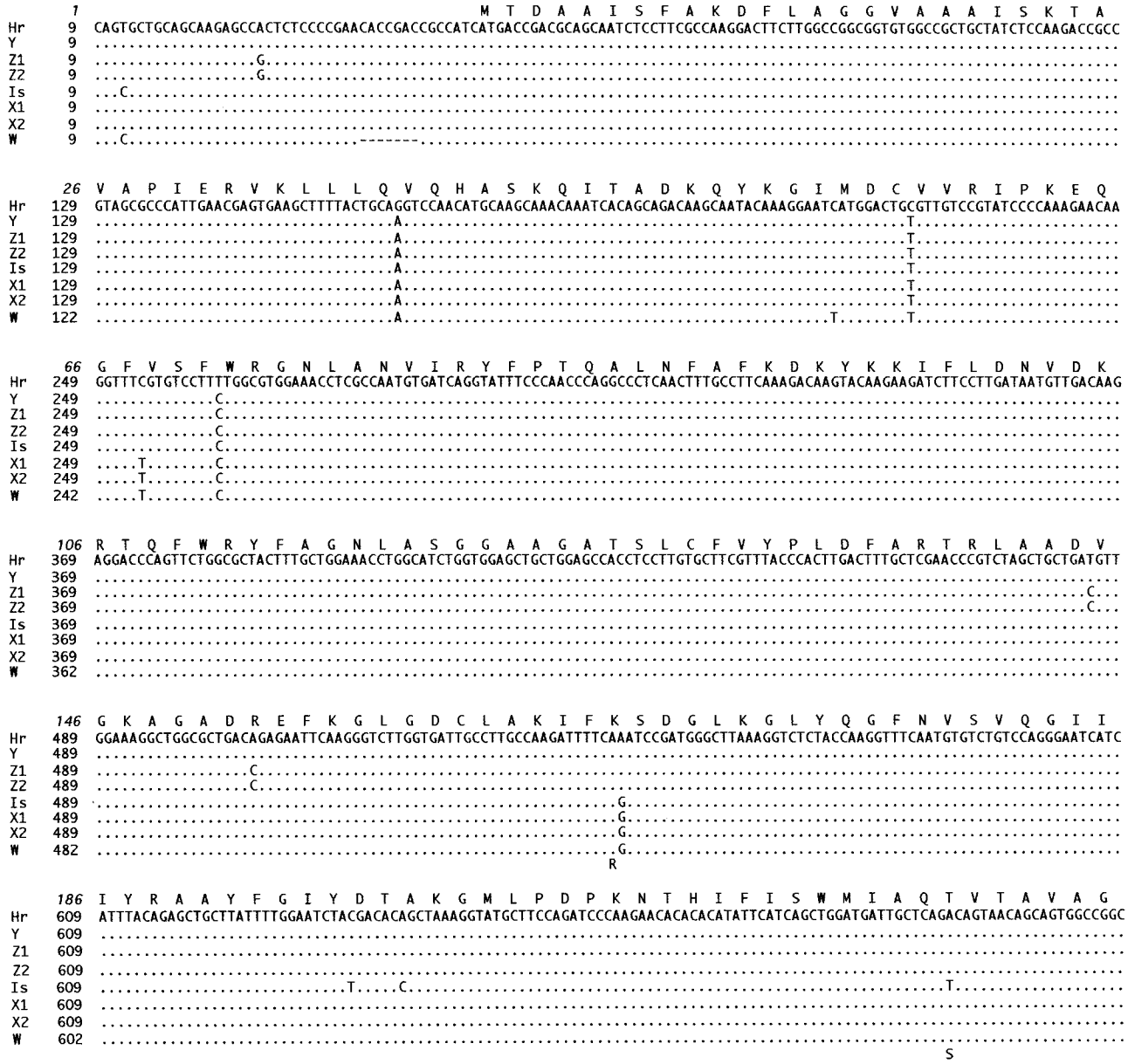

FIG. 5.—Nucleotide sequences of the ADP/ATP translocase cDNAs from Z, W, X, Y, Hiroshima, and Isehara chromosomes. Each of the Z and X cDNAs provides two kinds of clones isolated from the same library. All the sequences start at position 9 and finish at the 3′ end of the transcript, without poly A sequence. Conceptual translation of the Hiroshima sequence is provided. Amino acids changed by nonsynonymous substitutions are shown below the alignment. A dot indicates identity to the Hiroshima sequence; dashes indicate insertions or deletions required for the alignment. The positions of the first nucleotide of each lane and of its translation (italics) are provided at left. Note the nucleotide at position 550 (543 in W). It is adenine encoding lysine in Hiroshima (Hr), Y, Z1, and Z2, whereas it is guanine encoding arginine in Isehara (Is), X1, X2, and W. In addition, the five-base insertion GGCCT at the 3′ noncoding region is present only in Is, X, and W. Accession numbers for Hr, Is, W, X1, X2, Y, Z1, and Z2, respectively, are AB008456–AB008463.

centric Is type. This gene tree is supported by the following characteristics of the *AAT* sequences. First, amino acid residue 166 is lysine in the AATs from the Hr, Y, and Z chromosomes, whereas it is arginine in the Is, X, and W AATs (fig. 5). The lysine of AAT is highly conserved throughout eukaryotic evolution (Brandolin et al. 1993). Therefore, the AATs of Hr, Y, and Z that have lysine at this conserved position are probably an ancestral type in this species. Second, five-base insertion in the 3′ noncoding region is found in the X, W, and Is *AAT*s, but not in those of Hr, Y, or Z, and not in *R. sylvatica.* This also suggests that the *AAT* without the insertion is ancestral and, judging from the sequence downstream of the insert, the Hiroshima *AAT* is probably the most basal. The insert GGCCT is followed by the same five nucleotides in the W, X, and Is *AAT*s (fig. 5). The insertion probably resulted from duplication of the downstream five bases. Although the Hr *AAT* still has a template sequence of GGCCT, it has already been replaced with GGTCT in the Y and Z *AAT*s.

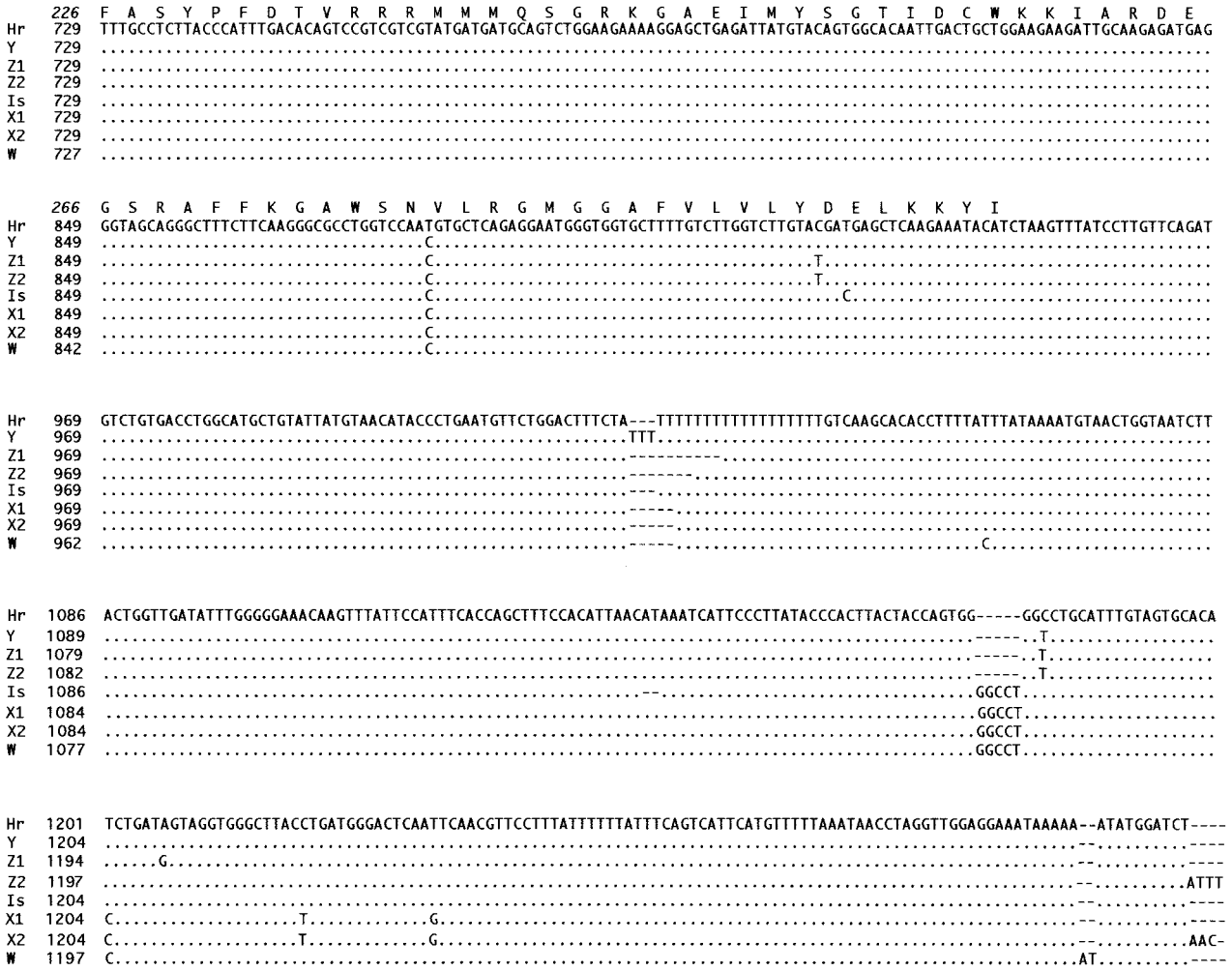

FIG. 5 *(Continued)*

Discussion

The gene encoding ADP/ATP translocase was found to be located in the sex chromosomes of the frog *R. rugosa*. In amphibians, no sex-linked genes have hitherto been isolated. Here, for the first time for amphibians, the structure of the sex-linked gene ADP/ATP translocase and its sequence differences between Z and W, and between X and Y sex chromosomes have been demonstrated. ADP/ATP translocase is located in the mitochondrial inner membrane of eukaryotic cells and catalyzes transmembrane exchange between cytosolic ADP and mitochondrial ATP. In humans, three transcriptionally active genes, *ANT1*, *ANT2*, and *ANT3*, and at least nine pseudogenes are present (Cozens, Runswick, and Walker 1989; Chen et al. 1990). The three active genes share 88%–92% identities with each other at the amino acid level. *ANT1* is located in chromosome 4 (Fan, Yang, and Lin 1992), while *ANT2* is in the X chromosome and *ANT3* is in the X and Y chromosomes (Chen et al. 1990; Schiebel et al. 1993; Slim et al. 1993). The frog AAT shares 87.2%, 88.6%, and 91.9% amino acid identities with human ANT1, ANT2, and ANT3, respectively. *ANT3* is considered to have an autosomal origin, because it is localized in the short arm of the X chromosome and is not subject to X chromosome inactivation (Schiebel et al. 1993; Slim et al. 1993). Thus, the sex-linkage of *AAT* (*ANT3*) found both in humans and frogs seems to be due to convergence in the evolution of their sex chromosomes.

On the basis of the phylogenetic *AAT* tree, we infer the following phylogeny of the number 7 sex chromosomes in *R. rugosa* (fig. 7). The sex chromosome in the ancestral form was subtelocentric. Perhaps part of the population became geographically isolated from the rest and developed the more subtelocentric sex chromosome by an inversion. At this time, the sex chromosomes of the two forms were still homomorphic in both sexes. Sometime later, the two forms again came into contact and hybridized with each other. The hybrid population was then isolated from the two parent populations, and it achieved independent differentiation. Within the hybrid population, the more subtelocentric sex chromosome changed into the metacentric chromosome by a second inversion. Finally, the hybrid population was separated into two geographically isolated and genetically differentiated forms: one developed a metacentric W and a subtelocentric Z, establishing a ZZ/ZW system, and the other established an XX/XY system composed

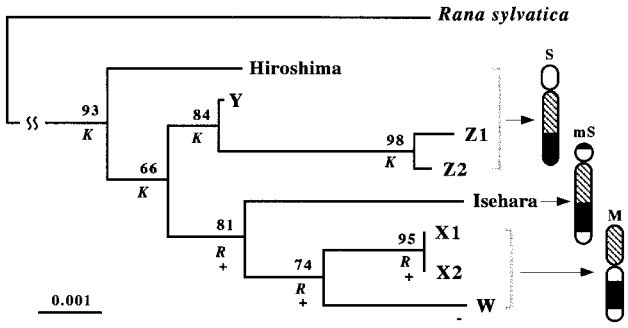

FIG. 6.—A phylogenetic tree of the ADP/ATP translocase gene from the six kinds of sex-determining chromosome 7. This tree was constructed using programs in PHYLIP, version 3.57 (Felsenstein 1989), by the neighbor-joining method (Saitou and Nei 1987) based on the pairwise distance of Jukes and Cantor (1969), and rooted using *Rana sylvatica AAT* (accession number U44832) as an outgroup. The percentage of 1,000 bootstrap replicates is shown at the node. The 166th amino acid is indicated by *K*(lysine) or *R*(arginine); "+" indicates the five-base GGCCT insertion in the 3′ noncoding region, and "−" indicates the seven-base CACCGAC deletion in the 5′ noncoding region. The three kinds of chromosome 7 are shown on the right: S = subtelocentric; mS = more subtelocentric; M = metacentric. Based on the tree, the Hiroshima gene is basal and the other genes form two clusters, one including the Y, Z1, and Z2 genes and the other including the Isehara, X1, X2, and W genes. These clusters coincided well with the morphology of the number 7 sex chromosomes. It is evident that the Z and Y chromosomes, respectively, were already isolated from their partners, W and X, before the formation of the heteromorphic pairs.

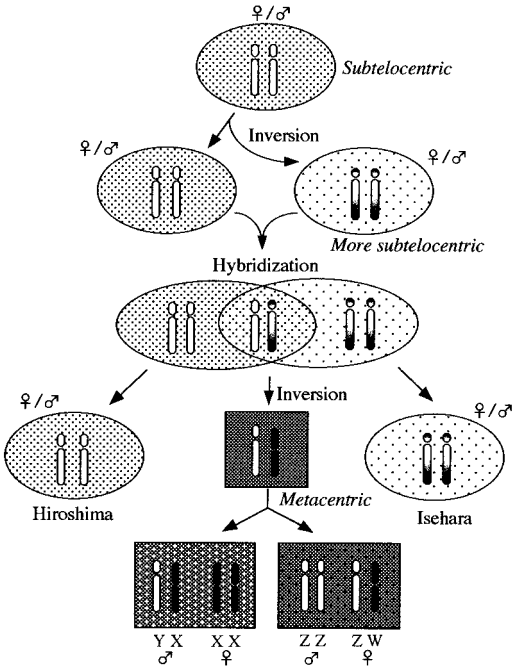

FIG. 7.—A phylogenetic pathway of the sex-determining chromosome 7 inferred from the ADP/ATP translocase gene tree. The subtelocentric chromosome is shown by a white area, the more subtelocentric by shaded area, and the metacentric by black area. For a detailed explanation, see the text.

of a metacentric X and a subtelocentric Y. Thus, we conclude that hybridization between the two ancestral forms with undifferentiated sex chromosomes would have been the primary event causing differentiation of the heteromorphic sex chromosomes. The two derived forms, ZZ/ZW and XX/XY, are found to follow a male basis in gonadal differentiation during early development: The ZW and XX female gonads show a testis-like structure at the early stage of their ovarian differentiation. Single W (dominant) and double X (recessive) chromosomes may inhibit the testis pathway, occurring by default, and then lead the gonad toward an ovary formation. The W and X chromosomes, not the Z or Y, probably defined the types of sex-determination in the two forms.

Here, we can assume two pathways for X and W chromosome differentiation from the single metacentric chromosome. First, the original metacentric chromosome gained a dominant ovary-determining function by the second inversion, and established the ZZ/ZW sex chromosome system using the subtelocentric chromosome as the Z chromosome. Then, in the form diverged from the ancestral hybrid form, the W chromosome changed into the present X chromosome with a recessive ovary-determining function by backcrossing with the western Japan invaders, and established the XX/XY system using the subtelocentric chromosome as the Y chromosome. This scenario is based on the following experimental data. The Y *AAT* has more sequence similarity to Hr than to Z. Also, isozyme study of the four Japanese forms shows that the heteromorphic XX/XY form shares many enzyme genotypes with the western Japan (Hiroshima) form (Nishioka et al. 1993). These results suggest that gene flow from the west to the XX/XY form occurred in the past and still continues. In fact, there are no definite geographical barriers between the two forms now. Moreover, in the artificial crosses between the Hiroshima and Hirosaki (ZZ/ZW) populations, the latter of which is situated in the north end of Honshu, males bearing the W chromosome appeared: 1.6% of the hybrids (Nishioka, Miura, and Saitoh 1993) and 6.25% of the backcrosses with the W chromosome (unpublished data) were males. This shows that the backcrossing of hybrids with the western Japan form weakens the ovary-determining function of the W chromosome. Second, the original metacentric chromosome gained a recessive ovary-determining function by the inversion and established the XX/XY sex chromosome system using the subtelocentric chromosome as the Y chromosome. Then, in the diverged group, the X chromosome changed into the present W chromosome with a dominant ovary-determining function, which was gained by mutation or duplication of the ovary-determining gene, and at last established the ZZ/ZW system. This is analogous to the human dose-sensitive sex-reversal (DSS) locus located on the X chromosome (Bardoni et al. 1994; Zanaria et al. 1994). DSS can override the effect of the Y chromosome and produce an XY female when present in two active copies (Berstein, Koo, and Wachtel 1980). Consequently, it remains to be shown by which pathway the X and W chromosomes have evolved.

As a second step to understanding the mechanisms whereby sex chromosome differentiation and sex deter-

mination have evolved in this species, we now intend to isolate the key gene that is involved in ovary determination and localized on the W chromosome, and to examine the role of Z and Y chromosome in testis determination.

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