The human sex-reversing ATRX gene has a homologue on the marsupial Y chromosome, ATRY: Implications for the evolution of mammalian sex determination

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Mutations in the *ATRX* gene on the human X chromosome cause X-linked α-thalassemia and mental retardation. XY patients with deletions or mutations in this gene display varying degrees of sex reversal, implicating *ATRX* in the development of the human testis. To explore further the role of *ATRX* in mammalian sex differentiation, the homologous gene was cloned and characterized in a marsupial. Surprisingly, active homologues of *ATRX* were detected on the marsupial Y as well as the X chromosome. The Y-borne copy (*ATRY*) displays testis-specific expression. This, as well as the sex reversal of ATRX patients, suggests that *ATRY* is involved in testis development in marsupials and may represent an ancestral testis-determining mechanism that predated the evolution of *SRY* as the primary mammalian male sex-determining gene. There is no evidence for a Y-borne *ATRX* homologue in mouse or human, implying that this gene has been lost in eutherians and its role supplanted by the evolution of *SRY* from *SOX3* as the dominant determiner of male differentiation.

The primary mammalian sex-determining event is the development of the testis, which results in the expression of hormones that lead to the masculinization of the developing embryo (1). In eutherian mammals, the *SRY* gene on the Y chromosome (2) has been demonstrated by mutation analysis and transgenesis to be the key switch gene in the sex-determining cascade (3), at least in humans and mice. There is an *SRY* gene on the Y chromosome of marsupial mammals (4), although there is no direct evidence that it is sex determining. So far, no *SRY* homologue has been detected in the more distantly related monotreme mammals (A.P. and P. Kirby, unpublished data), implying that another sex-determining gene must operate in this mammal group.

The existence of other genes in the male-determining pathway is also attested by eutherian species that have lost *SRY*, suggesting that another gene or genes can control male sex differentiation (5, 6). In addition, most sex-reversed XY human females have a normal *SRY* gene and therefore must harbor mutations elsewhere in the sex-determining pathway (7). Normal male development in the absence of *SRY* in human XX males, in addition to two rodent species that lack *Sry*, suggests that the *SRY* gene acts only as a male switch and contributes little or nothing to male development itself. Several sex-reversing genes have been isolated from the human X (8, 9) and autosomes (10–13) and are proposed to lie in the sex-determining pathway (14, 15). Comparisons of such genes between distantly related mammals and other vertebrates have proved valuable to our understanding of the function, as well as the evolution, of genes in a conserved vertebrate sex-determining pathway (16).

Many genes on the human and mouse Y chromosome have homologues on the X. Even *SRY* shares homology with the X-borne *SOX3* gene (17). The presence of these X–Y shared genes, as well as the several genes on the two pseudoautosomal regions (PAR1 and PAR2) shared by the X and Y, supports the hypothesis that mammalian sex chromosomes evolved from a homologous autosome pair (18). The Y chromosome originally contained copies of all of the genes on the X (including *ATRX*) but became degraded as mutations and deletions accumulated in a region in which recombination was suppressed between a sex-determining allele and other male-specific genes (19). Different sets of active genes survive on the Y chromosome in different species; for instance, a homologue of *RPS4X* is present on the Y in all eutherians except rodents, and an active copy of *STS* is present on the Y in rodents but not in primates. The *UBE1X* gene provides examples of every stage of the decline and fall of a Y-borne gene, from a pseudoautosomal gene in monotremes to degraded and deleted fragments in primates (20). Ancient homology explains the origin of the PAR and at least 10 genes and pseudogenes on the human Y chromosome, including at least two (the sex-determining gene *SRY* and the candidate spermatogenesis gene *RBMY*) with male-specific functions (21), although some other male-specific genes may have been recruited from autosomes (22).

Five genes on the human and/or mouse X and Y chromosomes (*SRY/SOX3*, *RBMX/Y*, *SMCX/Y*, *RPS4X/Y*, and *UBE1X/Y*) are also present on the X and Y chromosomes in marsupials. Thus, the Y is monophyletic in all therian mammals. However, several human X–Y shared genes (such as *ZFY/X*, *AMELY/X*, *STS/STSP*, and the pseudoautosomal genes) are autosomal in marsupials (23) and colocalize with other genes from the short arm of the human X distal to Xp11.23 (24). These genes are also autosomal in monotremes. As marsupials and monotremes diverged independently of eutherian mammals 130 million and 180 million years ago, respectively, this implies that these regions were added recently to the eutherian X and Y chromosomes, presumably by translocation to an ancient PAR (25).

We now show that the sex-reversing gene, *ATRX*, on the human and mouse X chromosome, has an active homologue on the Y chromosome in marsupials, which is expressed specifically in testis. *ATRX* (α-thalassemia and mental retardation associated with the X chromosome; ref. 9), is a member of a helicase

Abbreviations: ATRX, α-thalassemia and mental retardation associated with the X chromosome; ATRY, α-thalassemia and mental retardation-like gene on the Y chromosome; SRY, sex-determining region on the Y chromosome; SOX3, SRY-like HMG box containing gene 3; PAR, pseudoautosomal region; RT-PCR, reverse transcription–PCR.

Data deposition: The sequences reported in this paper has been deposited in the GenBank database (accession no. AF303445).

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superfamily subgroup that contains genes involved in DNA recombination, repair, and regulation of transcription (26). A highly homologous *ATRX* gene has been identified on the mouse X chromosome (27).

Mutations in human *ATRX* cause severe psychomotor retardation, characteristic facial features, α-thalassemia, genital abnormalities, and several other congenital irregularities (9). The level of gonadal dysgenesis in affected individuals is variable, in some cases leading to complete male to female sex reversal and female gender assignment (28). Histological analysis of sex-reversed patients reveals streak gonads, but the absence of Mullerian ducts indicates that AMH (anti-Mullerian hormone) is expressed, confirming the development of Sertoli cells in the gonad (29). The development of the gonad has therefore been interrupted at a stage before testis organization but after the testis-determining signal from *SRY* and the differentiation of Sertoli and Leydig cells. *ATRX* in eutherian mammals therefore functions downstream of *SRY SOX9* and *AMH* in the male development pathway. However, in birds and reptiles, *SOX9* appears to act later in testis differentiation, and the position of *ATRX* in the pathway is unknown (30–32).

The location of the human *ATRX* gene at Xq 13.3 (33) places it within the conserved region of the X chromosome present on the X in all mammalian species. This suggests that it may have had a role (perhaps dosage-regulated) in gonad development in an ancestral mammal. To confirm that *ATRX* was on the X chromosome in an ancestral mammal, the gene was cloned and mapped in the model marsupial, the tammar wallaby. Surprisingly, active homologues of *ATRX* were found to be present, not only on the X but also on the Y chromosome in marsupials.

Materials and Methods

Materials. Tissue and blood samples were collected from the marsupial species *Macropus eugenii* (the tammar wallaby) under Animal Experimentation Ethics Committee permit number LSB96/4.

Southern Hybridization. The 600-bp partial human *ATRX* cDNA clone containing the functional zinc finger domain was kindly supplied by Douglas Higgs (MRC Molecular Hematology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK). Genomic DNA extractions and Southern blotting were carried out according to standard procedures (34). Probes were randomly primed with [^{32}P]dCTP and hybridized to membranes at 55°C in 6× SSPE/0.5% SDS/5× Denhardts/10 mg/ml salmon sperm DNA. Membranes were washed under low stringency conditions of 2× SSC at 55°C.

Isolation of Genomic and cDNA Clones. A λ EMBL 3 genomic library was constructed from DNA extracted from male *M. eugenii* liver. The genomic DNA was partially digested with *Sau 3A* and size selected through a 10–40% glycerol gradient. Fractions were taken, and partially digested DNA in the 15- to 20-kb size range was ligated to λ EMBL 3 arms. The ligation reaction was packaged with Giga-pack gold (Stratagene). The library was titred and plated on four plates at a density of 120,000 plaque-forming units per 22 × 22-cm Nunc plate.

Sequence was obtained by using the Fmol DNA sequencing kit (Promega) and the Bluescript plasmid primers T3 and T7 (35).

Reverse Transcription–PCR (RT-PCR). RNA was isolated from 0.1 g of tissue by using the TRI-Pure RNA isolation kit (Boehringer Mannheim) according to the manufacturer's instructions. Extracted RNA was then treated with 4 units of DNase1 (Boehringer Mannheim) at 37°C for 1 h to remove any contaminating DNA. Before RT-PCR, an aliquot was removed and RNA was reverse-transcribed by using reverse transcriptase (Boehringer Mannheim) at 42°C for 90 min. RT-PCR conditions were as follows: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. Samples were analyzed on 1.5% agarose gel (GIBCO/BRL). Primers are shown 5′-3′ as follows. ATRY primers, ATRY(f)ATTTGTTGTGACTCTTGCCAT and ATRY(r)GCCTTTACCTTCTGTTGCTTT; *ATRX* primers, ATRX(f)CAATAATGGATGAAAACAGCC and ATRX(r)TGCCTGCTTCAAAAATCTTAC. All primers were synthesized by Bresatec (Adelaide, Australia).

Northern Hybridization. RNA from adult female brain and adult testis was extracted and DNase1-treated as described above. Aliquots of 40 μg of RNA were freeze-dried and stored at −80°C. Samples were hydrated in 4.5 μl of di-ethyl-pyrocarbonate-treated water and 16 μl of NorthernMAX loading buffer (Ambion, Sydney). RNA samples were electrophoresed through a 1% agarose/6% formaldehyde gel in 1× 4-morpholinepropanesulfonic acid at 90 V for 5 h. The sizes of hybridizing fragments were determined by running 10 μg of RNA ladder according to manufacturer's instructions (New England Biolabs, Sydney) and poststaining gel with ethidium bromide. Blotting of the gel was carried out according to standard procedures (34). Northern blots were hybridized at 60°C for 24 h in NorthernMAX prehybridized/hybridized solution (Ambion) and washed twice in 0.5× SSC at 60°C.

^{32}P-labeled antisense riboprobes for Northern hybridization were synthesized from tammar wallaby-derived *ATRX* and *ATRY* RT-PCR fragments, according to the manufacturer's instructions (MAXIscript T7/SP6 kit, Ambion).

Cytological Methods. Air-dried chromosome preparations were made from peripheral blood according to standard methods with minor modifications (36).

Chromosome *in situ* suppression hybridization of the genomic λ probes was performed, with minor modifications (37). Digoxygenin-labeled probes were detected with anti-DIG-mouse antibodies (Serva), followed by tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse antibodies (Serva). After chromosome *in situ* suppression hybridization, the chromosome preparations were stained with 4,6-diamidino-2-phenylindole to visualize the chromosomes. Images were collected on a Leitz microscope with a liquid-cooled Photometrics charge-coupled device camera.

Results

Southern Blot Analysis. The human *ATRX* cDNA was used to probe a Southern blot containing *Eco*RI-digested total genomic DNA from male and female *M. eugenii* (tammar wallaby) and *Trichosurus vulpecula* (brushtailed possum). The probe detected weak bands shared between the sexes, suggesting the location of an *ATRX*-like sequence on the marsupial X chromosome or autosomes. Unexpectedly, strongly hybridizing male-specific bands were detected in both marsupial species, indicating that sequences homologous to *ATRX* were located on the marsupial Y chromosome (Fig. 1).

Cloning and Characterizing Marsupial ATRX Homologues. The human cDNA probe was used to screen the wallaby adult liver genomic λ phage library. Two clones were isolated with overlapping restriction fragments. Restriction analysis revealed hybridizing *Eco*RI fragments of sizes identical to the male-specific bands detected on Southern blots. Each fragment was subcloned and sequenced (Fig. 2*a*). The sequence displayed very high homology (80% identity at the nucleotide level and 86–90% similarity at the amino acid level) to human *ATRX* over exon 7 and part of exon 9. This male-specific *ATRX* homologue was named *ATRY*.

The human cDNA probe was also used to screen a combined male/female pouch young cDNA λ phage library. One clone was isolated and fully sequenced. This cDNA clone spanned human

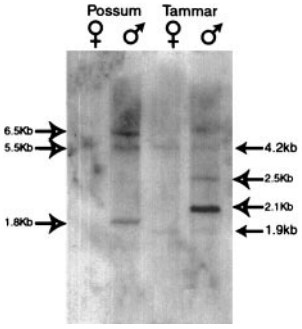

Fig. 1. Southern blot analysis. Detection of *ATRX* homologues in marsupials. Total genomic DNA was extracted from male and female *M. eugenii* (tammar wallaby) and *T. vulpecula* (brushtailed possum) liver tissue and digested with *Eco*RI. The blot was hybridized with a partial human *ATRX* cDNA probe. Unfilled arrows indicate the male-specific bands present in both marsupial species. Filled arrows indicate shared bands between male and female lanes.

ATRX exons 6–9, displaying 80% sequence identity and 82% amino acid similarity to human *ATRX* (Fig. 2*a*). Alignment of the wallaby cDNA clone with the genomic *ATRY* sequence revealed several nucleotide differences, implying that this clone represented an additional *ATRX* homologue in the wallaby genome. This wallaby cDNA clone was used to rescreen the wallaby genomic λ libraries, yielding one new clone. A hybridizing region of the clone was partially sequenced and showed 100% sequence identity to the wallaby cDNA clone over exon 7, indicating that the two clones represented the same gene (Fig. 2*b*). Restriction mapping of this clone showed no overlap with the genomic *ATRY* clone (Fig. 2*c*), and sequencing showed a number of nucleotide differences from *ATRY*. This gene was named *ATRX*.

Mapping Marsupial *ATRX* and ATRY. Fluorescence *in situ* hybridization localized the wallaby *ATRY* genomic clone unambiguously to the Y chromosome in this species, confirming the result of the Southern blot analysis (Fig. 3*a*). Fluorescence *in situ* hybridization mapping of the *ATRX* genomic clone localized it to the wallaby X chromosome (Fig. 3*b*), consistent with its location within the conserved region of the X chromosome in humans.

Expression Analysis. Primers were designed specifically to distinguish the marsupial *ATRY* and *ATRX* sequences. RT-PCR was performed, using these primers, to determine the expression pattern of the two genes in tissues from a developing male embryo, adult male and adult female wallaby. The integrity of each RNA extraction was determined by using primers for *PGK* (phosphoglycerate kinase), which is ubiquitously expressed in the wallaby (38). *ATRX* showed a wide range of expression, being detected in every tissue sampled with the exception of the developing male gonad. However, *ATRY* expression was confined to the developing male gonad and adult testis, with weak expression in the prostate and epididymis (Fig. 4*a*).

Northern Blot Analysis. *ATRX* and *ATRY* antisense riboprobes were hybridized to a Northern blot containing total RNA from adult female brain and adult testis. A single hybridization band (approximately 7.5 kb in size) was detected in both tissues (Fig. 4*b*).

Discussion

We demonstrate here that the sex-reversing human *ATRX* gene has a marsupial homologue, not only on the X but also on the Y chromosome. The demonstration of an *ATRX* homologue on the marsupial X chromosome implies the presence of this gene on the X in the common therian ancestor more than 130 million

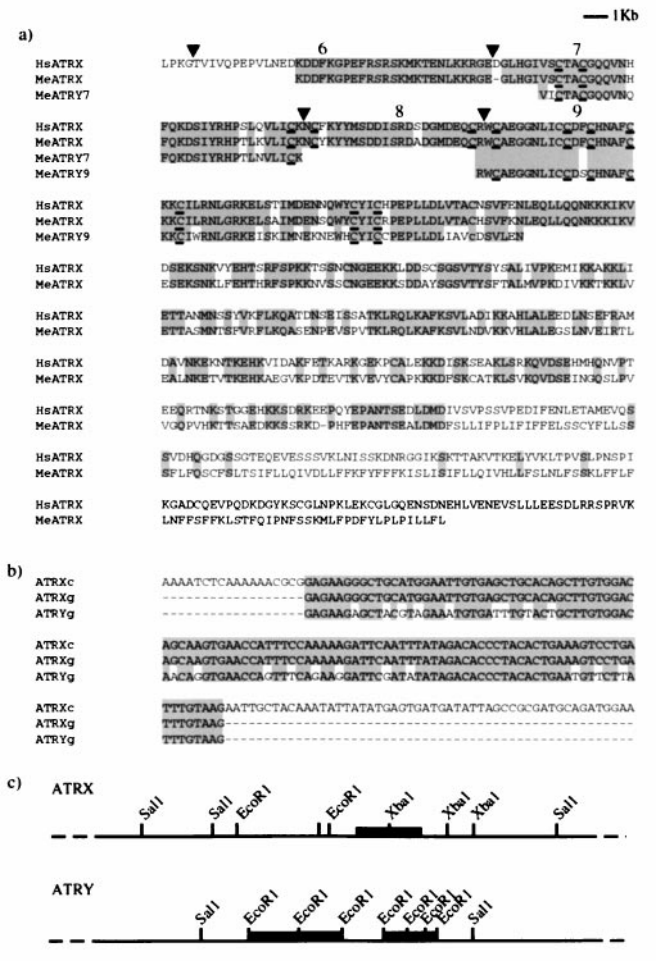

Fig. 2. ATRX/Y sequence analysis. (*a*) Amino acid alignment of human *ATRX* cDNA sequence (39) with tammar wallaby ATRX cDNA clone (MeATRX) and *ATRY* genomic sequence of exons 7 (MeATRY7) and 9 (MeATRY9). Shaded regions denote identity. Solid arrows indicate the position of introns in the human gene, and numbers indicate exon number. Underlined amino acids indicate cysteines located in potential zinc fingers (39). (*b*) Sequence alignment of *ATRX* cDNA clone (ATRXc) with *ATRX* genomic sequence (ATRXg) and *ATRY* genomic sequence (ATRYg). Shaded regions denote identity. (*c*) Restriction enzyme maps of the *ATRX* and *ATRY* genomic clones. Black boxes indicate regions of homology to the human *ATRX* probe.

years ago. As for the mouse *Atrx*, high sequence homology was observed between human and partial wallaby *ATRX* throughout exons 7, 8, and the first part of exon 9, corresponding to the zinc finger domain of the ATRX protein (27, 39).

The surprising finding was that *ATRX* also has a Y-borne homologue *ATRY* in marsupials, although there is no Y homologue in the human or mouse genome. Southern blot analysis implied a male-specific copy, and this was confirmed by isolating and characterizing *ATRY* and mapping it to the marsupial Y chromosome. Although the male-specific bands on the Southern blot are completely accounted for by the restriction analysis of the gene, we cannot distinguish between a single or multiple copies of *ATRY* on the Y chromosome. The *ATRX* and *ATRY* genomic clones isolated from the tammar wallaby represent two different loci, producing distinct, although similar proteins. The sequence of *ATRY* had about 80% homology to marsupial and human *ATRX*, containing conserved intron–exon boundaries and an ORF. This suggests that *ATRY* represents an actively transcribed Y-linked copy of the gene. Although complete sequence of the tammar wallaby *ATRX* and *ATRY* genes is yet to

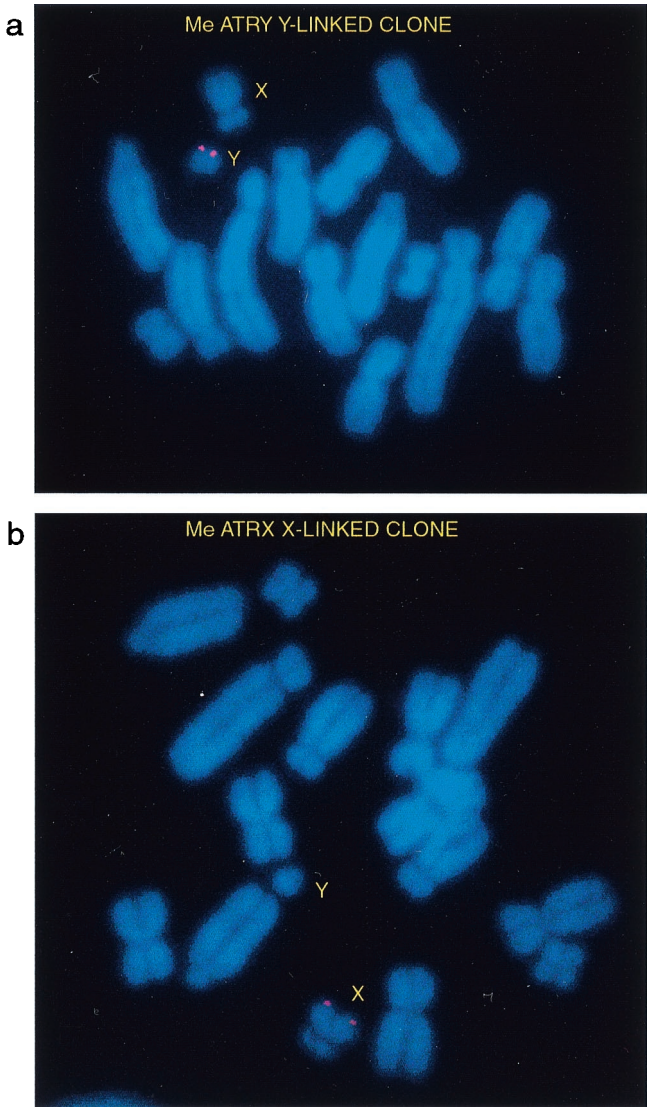

Fig. 3. Fluorescence *in situ* hybridization of the *ATRX/Y* genomic clones in cells from a male tammar wallaby. (*a*) The male-specific clone (*ATRY*) produced strong signals on the Y and no signals on the X. (*b*) The clone showing shared female and male bands on the Southern analysis (*ATRX*) hybridized to the X chromosome and showed no cross-hybridization with the Y.

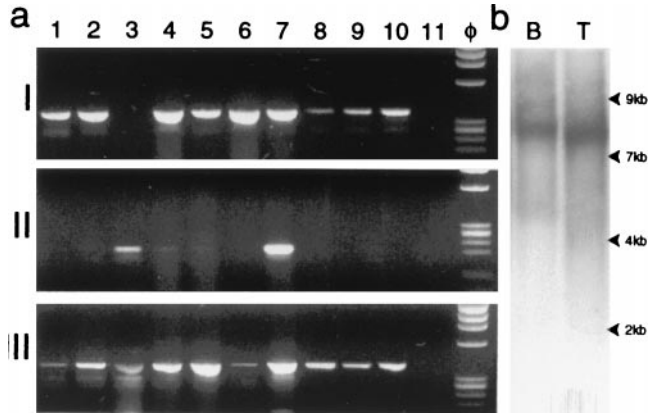

Fig. 4. Expression analysis of *ATRX* and *ATRY*. (*a*) RT-PCR using primers to *ATRX* (I), *ATRY* (II), and a control *PGK* (III) to confirm the integrity of the RNA. Lanes 1–3, male pouch young tissue at day 15 after birth (1, adrenal gland; 2, mesonephros; 3, developing testis). Lanes 4–7, adult male tissues (4, epididymus; 5, prostate; 6, spleen; 7, testis). Lanes 8–10, adult female tissues (8, brain; 9, kidney; 10, lung). Lane 11 represents a control PCR in which template DNA was omitted. The DNA marker ϕX-174-*Hin*fI (Boehringer Mannheim) is represented in the lanes marked ϕ. *ATRX* transcripts were detected in every tissue sampled with the exception of the developing testis (I). *ATRY* expression was detected only in the developing and adult testis and was absent in all other tissues (II). (*b*) Northern blot of adult female brain RNA (B) and adult testis RNA (T) probed with the ATRY riboprobe. In both brain and testis, a single hybridization band of approximately 7.5 kb was detected. The positions of RNA marker size standards (New England Biolabs) are indicated by arrows.

be determined, both appeared to have a transcript length similar to human and mouse *ATRX* genes (7,479 bp; refs. 27 and 39). This was demonstrated by the single hybridization band seen in Northern blots of adult testis RNA, where both *ATRX* and *ATRY* have been shown by RT-PCR to be expressed.

Despite the similarities in their transcripts, marsupial *ATRX* and *ATRY* were shown to have completely different and complementary expression patterns in the wallaby. *ATRX* is transcribed everywhere except the developing male gonad, whereas *ATRY* is expressed predominantly in the testis. The *ATRX* expression pattern observed in humans appears to be a combination of the patterns of marsupial *ATRX* and *ATRY*, being detected in a wide range of adult and embryonic tissues including the testis (9, 39).

The most parsimonious explanation for the presence of a Y-borne copy in marsupials is that *ATRX/Y* was present on the ancestral proto-X/Y. The X- and Y-borne copies differentiated as the Y was progressively degraded, and the Y-borne copy in marsupials (and perhaps ancestral eutherians) retained a selectable function. However, *ATRY* was degraded and lost from the Y early in eutherian evolution. This demonstrates loss of a gene from the Y early in eutherian evolution, although there are examples of more recent loss from the Y in the primate lineage (e.g., *UBE1Y*) and the rodent lineage (e.g., *RPS4Y*).

The evolutionary history of *ATRX/ATRY* does not conform to a model for the evolution of male-specific genes on the Y chromosome recently put forward by Lahn and Page (22), in which genes on the human Y chromosome were proposed to represent two classes with different evolutionary histories. Class I comprises genes shared between the X and the Y, which were derived from the ancient homologous proto-X/Y and show widespread expression. Class II comprises Y-specific multicopy genes that were derived by transposition from other chromosomes. These are expressed exclusively in the testis and have functions in male-specific development, such as testis determination and spermatogenesis. Clearly, *ATRX/ATRY* violates this distinction, being present on both the X and Y chromosomes in marsupials, yet showing testis-specific expression. In this, it is not the only exception. The candidate spermatogenesis gene *RBMY* is present in multiple copies and testis specific, although it has now been shown to have a homologue *RBMX* on the X (21), and even the eutherian testis determining factor *SRY* also has a homologue, *SOX3*, on the X (17), which is expressed in the central nervous system. Thus, it appears that most or all of the genes on the mammalian Y chromosome, including those with male-specific functions, are relics of ancient X–Y homology. Differential degradation of the Y in different lineages means that spermatogenesis, and even testis determination, may be controlled by different genes in different species.

The retention of *ATRY* on the Y in marsupials is particularly interesting in view of the involvement of human *ATRX* in male sex differentiation. *ATRX* apparently functions in the eutherian sexual development pathway downstream of *SRY*, *SOX9*, and *AMH*, possibly acting at the level of testis organization. Marsupial *ATRY* is expressed in the developing testis, suggesting a role in testis determination and/or differentiation, although weak

expression in prostate and epididymis could suggest a wider role in development of the whole male urogenital system. It would be interesting to discover whether *ATRX* is expressed before *SOX9* in nonmammalian vertebrates, consistent with a more direct role in testis determination, which may have been retained by ATRY in marsupials.

The complementary expression patterns of marsupial *ATRX* and *ATRY* are unique for X- and Y-borne copies of genes. The most parsimonious explanation is that the ancestral *ATR* gene had a general role in the development of several organs (including testis) in the ancestor to both groups. On differentiation from its X-borne homologue in marsupials, *ATRY* acquired a male-specific, dominant role in testis differentiation and/or determination, and *ATRX* lost this function. This led to conservation of *ATRY* on the Y chromosome in the marsupial lineage, in which it still fulfils a critical function in testis development. In fact, *ATRY*, being testis-specific, may be a better candidate sex-determining gene in this mammal group than *SRY*, which is poorly conserved and ubiquitously expressed in marsupials (40). Perhaps *ATRY* has functions in sex determination as well as sex differentiation in marsupials in the same way that many other genes in the eutherian sex-determining pathway work at multiple levels (14, 15). Localization of *ATR* homologues in monotremes will be of great interest, as these mammals appear to have no Y-borne *SRY* gene and may still determine sex through the action of the *ATRX/Y* locus.

ATR homologues appear to have a conserved role in the development of the mammalian testis that is independent of their location in the genome. There is no evidence that *ATRY* has been retained in any eutherian mammal. When and why was it lost? One possibility is that continued expression of *ATRX* in the testis may have led to a redundancy of *ATRY* function, which permitted the loss of *ATRY* from the eutherian Y chromosome (41). Another possibility is that *ATRY* originally had a testis-determining function in eutherians as well as marsupials, but this function was supplanted by the differentiation of *SRY*, and *ATRY* was lost in a manner analogous to the loss of the *SRY* gene from mole voles and spiny rats (5, 6).

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