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Evolution

Sex chromosome evolution: Platypus gene mapping suggests that part of the human X chromosome was originally autosomal

(sex chromosomes/mammals/genome evolution/X chromosome inactivation)

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ABSTRACT **To investigate the evolution of the mammalian sex chromosomes, we have compared the gene content of the X chromosomes in the mammalian groups most distantly related to man (marsupials and monotremes). Previous work established that genes on the long arm of the human X chromosome are conserved on the X chromosomes in all mammals, revealing that this region was part of an ancient mammalian X chromosome. However, we now report that several genes located on the short arm of the human X chromosome are absent from the platypus X chromosome, as well as from the marsupial X chromosome. Because monotremes and marsupials diverged independently from eutherian mammals, this finding implies that the whole human X short arm region is a relatively recent addition to the X chromosome in eutherian mammals.**

The mammalian X chromosome seems to be extraordinarily conserved in size and gene content, as originally predicted by Ohno (1). Many genes borne on the human X chromosome have also been localized to the X chromosome in a wide variety of other eutherian mammals from several different orders (2). Not a single exception has been reported among eutherian (inaccurately called "placental") mammals.

Marsupials (mammalian infraclass Metatheria) diverged from eutherians 120–150 million years ago (3), and monotremes (subclass Prototheria) diverged from the therian (eutherian and metatherian) lineage 150–170 million years ago, so a comparison of the gene content of the eutherian X chromosome with marsupial and monotreme X chromosomes may provide information about the extent of this conserved region and the time over which this region has remained intact.

A number of enzyme loci sex-linked in man have been found by family studies also to be sex-linked in marsupials (4, 5) and to be assigned to the marsupial X chromosome by somatic-cell genetic analysis (6–9). Southern blot analysis of rodent–marsupial cell hybrids, with probes derived from other genes located on the long arm of the human X chromosome (here referred to as human Xq genes), also assigned all these genes to the X chromosome in several marsupial species, and *in situ* hybridization confirmed this assignment and provided regional localizations (10).

However, a number of markers, including the genes for steroid sulfatase (*STS*), ornithine transcarbamylase (*OTC*), Duchenne muscular dystrophy (*DMD*), synapsin 1 (*SYN1*), DNA polymerase α (*POLA*), cytochrome *b* β chain (*CYBB*), monoamine oxidase A (*MAOA*), and ornithine aminotransferase pseudogene (*OATL1*), located on the short arm of the human X chromosome (referred to here as human Xp genes), were shown to be absent from the X chromosome in marsupials. Rodent–marsupial cell hybrids retaining an intact marsupial X chromosome were found not to express the marsupial form of *STS* (8, 11), and Southern blot analysis of these cell hybrids, as well as *in situ* hybridization, demonstrated that several human Xp probes detected no site on the X chromosome but were localized to an autosome in several marsupial species (12–14).

The finding that these human Xp genes are autosomal in marsupials is consistent with either of two alternative hypotheses. Either (*i*) this human Xp region was part of the X chromosome in the common therian ancestor of the eutherians and marsupials and was translocated to an autosome in the marsupial lineage or (*ii*) this human Xp region was originally autosomal and was translocated to the X chromosome in the eutherian lineage.

We have distinguished between these two hypotheses by examining the location of these genes in even more distantly related mammals, the monotremes, represented by the platypus *Ornithorhynchus anatinus*. Gene mapping in monotremes by family studies has been impossible because the animals do not breed in captivity, and mapping is all but impossible using somatic-cell genetic analysis because rodent–monotreme cell hybrids grow poorly and retain only fragments of monotreme chromosomes (15). However, by carefully monitoring stringency and background, we were able to use heterologous DNA probes (human or rodent) for *in situ* hybridization to platypus chromosomes, demonstrating that nine Xq genes of humans are also located on the X chromosome in the platypus (16).

We have, therefore, used *in situ* hybridization to localize five human Xp genes to sites on platypus chromosomes. As for marsupials, the human Xp genes were localized to two clusters on platypus autosomes. The autosomal location in monotremes, as well as in marsupials, suggests that the human Xp region was originally autosomal and was translocated to the sex chromosomes in the eutherian lineage.

MATERIALS AND METHODS

Platypus fibroblast lines were established by a plasma clot method, as has been described (17), using toe-web tissue supplied by D. Goldney (Charles Sturt University, Bathurst, NSW, Australia) under permit A579 from the New South Wales National Parks and Wildlife Division. The four fibroblast lines remained diploid throughout these experiments. Cells were grown in Dulbecco's modified Eagle's medium (Flow Laboratories)/10% fetal calf serum (Flow Laboratories and GIBCO)/streptomycin at 50 μg/ml/penicillin at 60

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Abbreviations: STS, steroid sulfatase; OTC, ornithine transcarbamylase; DMD, Duchenne muscular dystrophy; SYN1, synapsin 1; POLA, DNA polymerase α; CYBB, cytochrome *b*, β polypeptide; MAOA, monoamine oxidase A; ZFX and ZFY, X-linked and Y-linked zinc finger proteins, respectively.

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Table 1. Gene loci and DNA probes used

Locus	Position on human X chromosome	Name	Homology to monotreme sequence*	Probe	cDNA origin	Source and ref.
POLA	Xp22.1–p21.3	DNA polymerase α	1/8	pCD-KB pol α	Human	T. Wang (Stanford, CA) (18)
DMD	Xp21.3–p21.1	Duchenne muscular dystrophy	1/1.5	pCA1-A	Human	K. E. Davies (Oxford) (19)
SYN1	Xp11.2	Synapsin 1	1/8	pSYN5 (fragment 5E2)†	Rat	L. De Gennaro (Boston) (20)
CYBB	Xp21.1	Cytochrome *b*-245, β polypeptide (chronic granulomatous disease)	1/2	Clone 100.1†	Human	S. Orkin (Boston) (21)
MAOA	Xp11.3–p11.23	Monoamine oxidase A	1/2.5	HM11	Human	J. Powell (Harrow, U.K.) (22)

*Strength of monotreme signal was compared to signal from species of origin.
†Probes are described in detail in the relevant reference but are not specifically named.

μg/ml/glutamine at 100 μg/ml at 32°C (body temperature of animal) in an atmosphere of 10% CO_2.

Cells were arrested at metaphase for 3–7 hr with 0.005% colchicine (Commonwealth Serum Laboratories, Melbourne, Australia), harvested, treated 7–20 min with 0.05% KCl, and fixed in several changes of methanol/acetic acid, 3:1. Suspensions were dropped onto cleaned microscope slides and air-dried.

The gene names and DNA probes used are listed with their sources in Table 1 (refs. 18–23); identities of the genes were all verified by insert sizing, and some were also verified by restriction analysis and Southern analysis of human or rodent DNA. For Southern blotting, probes were nick-translated with [^{32}P]dATP (3000 Ci/mM, Amersham; 1 Ci = 37 GBq) to an activity of 200–500 μCi/ml. For *in situ* hybridization, probes were nick-translated to specific activities of 2–6 × 10^7 cpm/μg, by using [^{3}H]dATP/[^{3}H]dCTP/[^{3}H]dGTP mixtures.

DNA extraction and Southern blotting procedures were modified from those reported by Reed and Mann (24), by using alkaline transfer, and a hybridization mix consisting of 0.5–6.0× standard saline phosphate/EDTA (SSPE) (depending on the degree of homology between probe and target sequence) (1× SSPE is 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 0.5% Blotto, 0.5% SDS, 0.5% sonicated salmon sperm DNA, and 10% (wt/vol) dextran sulfate. Hybridization was done overnight at 60°C and was followed by a graded series of washes of increased stringency (0.2–4.0× standard saline citrate at temperatures of 50–60°C) depending on probe homology, which was judged for each probe from the signal on zoo blot autoradiographs.

In situ hybridization was done as described (12, 16), with carefully screened batches of emulsion (Amersham LM-1). Slides were exposed for 2–6 weeks at 4°C with desiccant, then were developed with Ilford Phenisol, and stained with 20% filtered Giemsa stain. For each probe, optimal probe concentration was determined from the signal-to-noise ratio on slides hybridized with a range of concentrations; then 100–200 well-spread metaphases were scored from the slides with satisfactory signal and the lowest background. Only grains overlapping a chromatid were scored as signal. Grain distributions were analyzed using a GLIM program (a software package designed for exploratory fitting of generalized linear models by maximum likelihood, ref. 25).

The six largest pairs of autosomes and the X chromosome are readily identifiable in the platypus karyotype. However, the rest of the chromosomes are very small and not readily distinguishable even with G-banding or late-replication banding (26). Silver grain scores over these chromosomes were, therefore, pooled into two classes, class A (chromosomes 7–16 and the unpaired element a, which together constitute 34.3% of the genome) and class B (chromosomes 17–23 and unpaired elements b, c, and d, which constitute 20.4% of the genome). The necessity of allocating grains to these large classes A or B meant that minor hybridization sites over one of these small chromosomes would be difficult to detect.

RESULTS

Each of the probes used for *in situ* hybridization was tested first for the strength of signal by hybridization to a Southern blot containing DNA from eutherian, marsupial, and monotreme species. Fig. 1 shows these zoo blots probed with DMD, CYBB, and POLA, which show, respectively, high (DMD), moderate (CYBB), and low (POLA) homology to monotreme sequences.

In situ hybridization yielded clear assignments to the platypus chromosomes for all five probes used (Fig. 2). All probes detected major hybridization sites on autosomes, and none detected any site on the X chromosome.

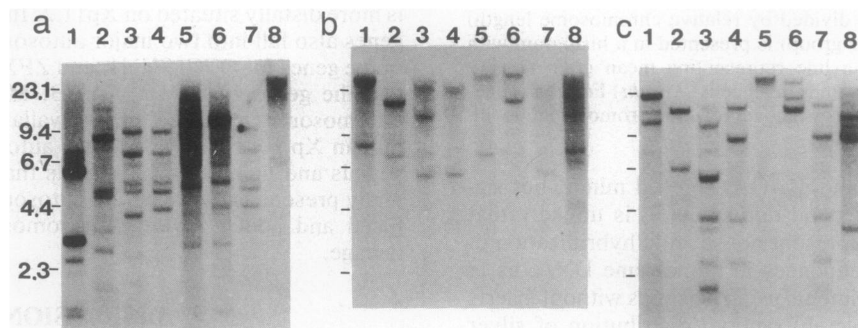

FIG. 1. Southern analysis of eutherian (lanes 1 and 2), marsupial (lanes 3–7), and monotreme (lane 8) DNA. Lanes: 1, human; 2, rat; 3, *Macropus rufus* (red kangaroo); 4, *Macropus eugenii* (Tammar wallaby); 5, *Dasykaluta rosamondae* (Rosamond antechinus); 6, *Antechinus apicalis* (dibbler); 7, *Trichosurus vulpecula* (brush-tailed possum); 8, *Tachyglossus aculeatus* (Australian echidna). DNA was cut with *Hin*dIII and probed with POLA (*a*), cut with *Hin*dIII and probed with DMD clone pCI (*b*), and cut with *Pst* I and probed with CYBB (*c*). Molecular size markers (in kb) are at left.

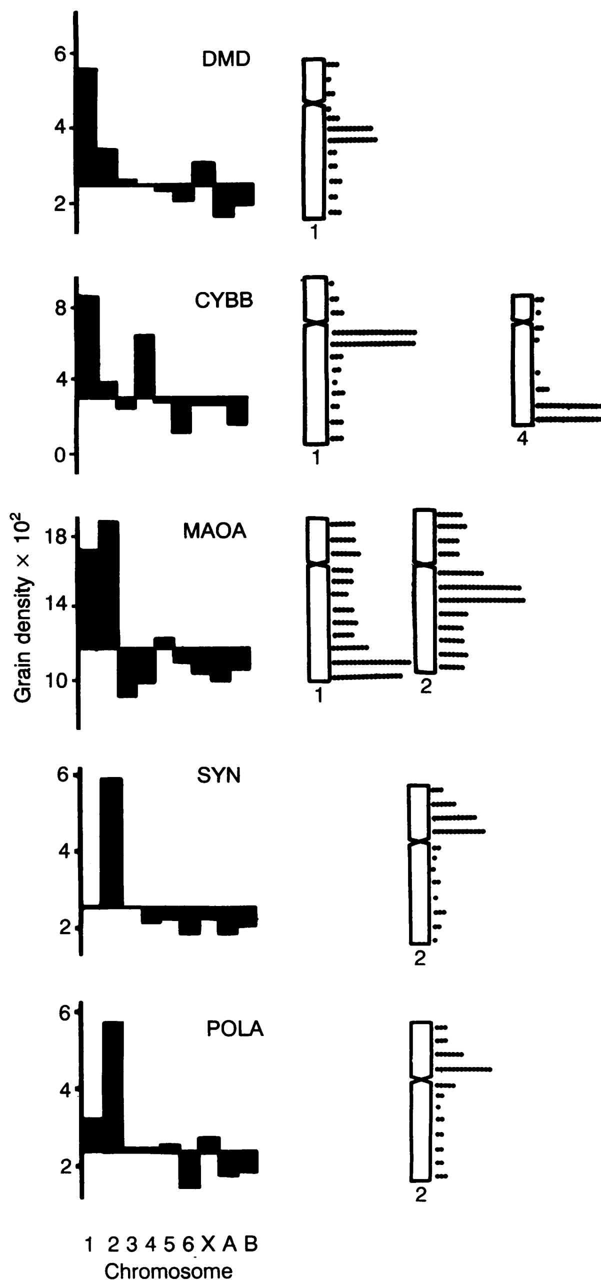

FIG. 2. *In situ* hybridization of DMD, CYBB, MAOA, SYN1, and POLA human cDNA probes to platypus chromosomes 1–6, X chromosome, and groups A and B of small, individually unidentifiable chromosomes. (*Left*) For each of the five genes, the grain density (number of grains divided by relative chromosome length) over each chromosome (or group) is presented in a histogram with values above and below a line representing mean grain density (averaged over all chromosomes in all cells). (*Right*) For each gene, regional distribution of grains is shown for the chromosome(s) with significant positive signal.

Two probes (CYBB and MAOA) showed minor, but significant, signals on a second autosome. It is unlikely that these secondary sites represent nonspecific hybridization of plasmid to repetitive sequences in monotreme DNA, as *in situ* hybridization experiments using plasmids without inserts as probes give a completely random distribution of silver grains over the chromosomes (data not shown). Without cloning each, we cannot determine whether these "secondary sites" [which we have described also for human Xq probes in marsupials and the platypus (10, 16)] represent pseudogenes or closely related functional sequences. It

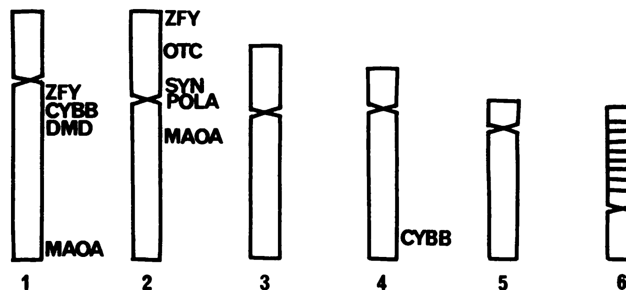

FIG. 3. Location of human Xp genes on chromosomes of the platypus. Horizontal bars on chromosome 6 indicate location of an extended nucleolar organizer region.

seems likely that the major signal detected by CYBB and MAOA probes represents the corresponding monotreme functional gene, which will have been most conserved across the very large evolutionary distance between the species used as probe source and target. The SYN1 probe detected a single autosomal site in the platypus; however, in humans an X-linked and an autosomal *SYN1* gene can be detected. Although an additional SYN1 site could exist on a class A or B X-linked chromosome in the platypus, the previous localization of SYN1 to a single autosomal site near to other human Xp markers for ornithine transcarbamylase (OTC) and X-linked zinc finger protein (ZFX) in both macropodid and dasyurid marsupials (12, 13) suggests that this site represents a functional gene homologous to the *SYN1* locus on human Xp.

Fig. 3 shows the positions on platypus chromosomes of these five human Xp genes, as well as of two other human Xp genes *OTC* and *ZFX* or the gene encoding the Y-linked zinc finger protein (ZFY) reported separately (ref. 12; J.M.W., unpublished work). All positions are autosomal. Although none of these genes are located on the X chromosome in the platypus, it appears that the associations of some of these genes are retained. Three human Xp genes, *CYBB, DMD,* and *ZFX*, which are located (in this order) on the human X chromosome in the region Xp21.1–22.1, were all localized to the proximal region of platypus chromosome 1q. Our *in situ* localizations do not have sufficient resolution to determine whether order has been conserved. Because at least three internal rearrangements of the X chromosome have occurred even between human and mouse (27), it would not be surprising were the order different in the platypus. In humans, OTC and POLA probes also map to the same region of the short arm of the X chromosome as do CYBB, DMD, and ZFX probes. However, in the platypus OTC and POLA probes locate these genes together proximally on chromosome 2p close to the localization of SYN1, which, in humans, is more distally situated on Xp11.2. In marsupials, human Xp genes also fall into two major autosomal clusters, consisting of the genes for OTC, SYN1, and ZFX/Y on chromosome 1p and the genes for DMD, CYBB, MAOA, and ZFX/Y on chromosome 5p of the Tammar wallaby (14). The location of human Xp genes in two similar autosomal clusters in marsupials and monotremes suggests that this region was originally present as two separate autosomal blocks that became fused and added to the X chromosome in the eutherian lineage.

DISCUSSION

Seven human Xp genes *OTC, ZFX/ZFY, MAOA, SYN1, DMD, POLA,* and *CYBB* map to the X chromosome in all eutherian species (2) and have been considered a part of a highly conserved mammalian X chromosome (1). However, all these genes are now shown to map to autosomes in the

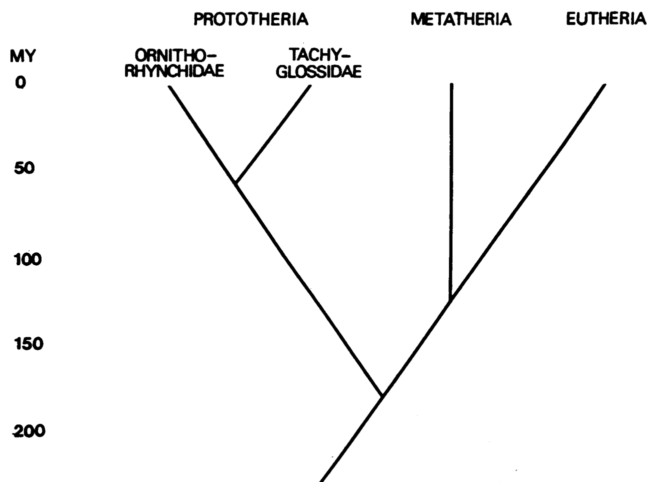

FIG. 4. Relationships between extant mammalian groups: Eutheria ("placental" mammals), Metatheria (marsupials), and the two families of Prototheria (monotremes). MY, million years.

platypus, as well as in several marsupial species (12–14). Their autosomal location in marsupials was initially explained by the loss of this region from the ancestral mammalian X chromosome, leaving a smaller marsupial X chromosome (12, 26, 28). However, their absence also from the X chromosome in monotremes is difficult to explain on this hypothesis. Because monotremes diverged earlier from the therian lineage and marsupials diverged subsequently from eutherians (ref. 3 and Fig. 4), this hypothesis would require that the region was lost independently from the X chromosome in the monotreme and the marsupial lineages. A more parsimonious explanation is that the region was autosomal in the common mammalian ancestor >150 million years ago.

We propose, therefore, that the X chromosome of the common ancestor of the three extant groups of mammals included the highly conserved region represented by the human Xq chromosome and shared by all mammalian X chromosomes, but not the region represented by the human Xp chromosome, which is borne on the X chromosome only in eutherian mammals.

If it is accepted that this region was originally autosomal but is now on the X chromosome in all eutherian mammals tested, the region must have been translocated to the X chromosome early during the eutherian radiation (between the 50–60 million years that separate primates and rodents and the 150 million years that marsupials and eutherians have been evolving independently). The location of human Xp genes in two distinct autosomal clusters (similar, although not identical) in marsupial and monotremes suggests that this region was originally present as two separate blocks that were either fused by a rearrangement between two autosomes and subsequently added to the X chromosome or were translocated to the X chromosome by successive X–autosome rearrangements. It would be worthwhile to explore these alternative hypotheses by looking for exceptions to the conservation of the human Xp region on the X chromosome among eutherian groups (e.g., Artiodactyls and Edentates) thought more distantly related than primates and rodents (29, 30).

Because translocation of a large autosomal region to the small ancestral X chromosome would have created gene-dosage differences between male and female or produced an XY_1Y_2 system, the exchange would probably have added an autosomal region to a region shared by the X and Y chromosomes. Translocation to this pseudoautosomal region of one sex chromosome would be followed by rapid transfer to the other, via recombination. The very tiny pseudoautosomal region in present-day eutherian sex chromosomes may be a relic of this translocated region or part of an original pseudoautosomal region; it will be necessary to map the homologues of human pseudoautosomal genes in marsupials and monotremes to distinguish between these two hypotheses.

Our hypothesis that the human Xp region was autosomal 60–150 million years ago requires that this region must originally have been paired and not subject to X chromosome inactivation. Translocation to the pseudoautosomal region of the X and Y chromosomes in the eutherian lineage must, therefore, have been followed by deletion or inactivation of genes on the Y chromosome and recruitment of their X-linked alleles into the X inactivation system. The observation that the extent of this differentiation and inactivation is different among monotremes, marsupials, and eutherians (28) and even between human and mouse (31) suggests that X-Y differentiation and recruitment into the X inactivation system have occurred in a stepwise fashion. Reports of genes (in different regions of the short arm and in the proximal region of the long arm of the human X chromosome) that share homology with sequences on the Y chromosome and escape inactivation (32–36) suggest that the process is still incomplete in some regions of the human X chromosome, perhaps because these genes lack sequences that respond to a signal spreading from an X inactivation center. The observation that some genes that escape inactivation in humans are inactivated in mouse suggests that the inactivation system may be more completely evolved in mouse (31).

In conclusion, comparisons of gene maps of the sex chromosomes of widely divergent mammals have allowed us to deduce the gene content of the ancestral mammalian X chromosome and have revealed the major rearrangements that occurred to produce the X chromosomes of present-day mammals. The evolutionary history of the mammalian sex chromosomes may help us to understand their function in sex determination and dosage compensation.

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