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Evolution

The X chromosome of monotremes shares a highly conserved region with the eutherian and marsupial X chromosomes despite the absence of X chromosome inactivation

(sex chromosomes/chromosome evolution/platypus/comparative mapping)

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ABSTRACT **Eight genes, located on the long arm of the human X chromosome and present on the marsupial X chromosome, were mapped by *in situ* hybridization to the chromosomes of the platypus *Ornithorhynchus anatinus*, one of the three species of monotreme mammals. All were located on the X chromosome. We conclude that the long arm of the human X chromosome represents a highly conserved region that formed part of the X chromosome in a mammalian ancestor at least 150 million years ago. Since three of these genes are located on the long arm of the platypus X chromosome, which is G-band homologous to the Y chromosome and apparently exempt from X chromosome inactivation, the conservation of this region has evidently not depended on isolation by X–Y chromosome differentiation and X chromosome inactivation.**

The X chromosome of eutherian mammals was early observed to constitute about 5% of the haploid genome, regardless of the sizes of the autosomes, and was proposed to bear the same suite of genes in all species (1). It was suggested that this conservation was the result of the isolation of the X chromosome by its involvement in a chromosome-wide inactivation mechanism that might make X chromosome–autosome translocation heterozygotes infertile.

Research has borne out the prediction that the mammalian X chromosome is conserved *in toto*; to our knowledge, there are no exceptions among eutherian mammals to "Ohno's Law" that genes sex-linked in one species will be found to be sex-linked in all (2). We have also found that the genes located on the long arm of the human X chromosome are located on the X chromosome also in a number of marsupial species (3, 4). However, comparative gene mapping among eutherian orders has established that several groups of autosomal genes have also been highly conserved; for instance several genes located on human chromosome 11p are found together in carnivores (5) and even marsupials (6). It is no longer certain, therefore, that the conservation of the mammalian X chromosome demands a special explanation.

Monotremes are egg-laying mammals, belonging to the separate mammalian subclass Prototheria. There are only three species extant; the platypus (*Ornithorhynchus anatinus*) and two echidna (so-called "spiny anteater") species. Monotremes diverged from the marsupial and eutherian lineage (subclass Theria) 150–170 million years before present, early in the 200-million-year history of mammals (7). Comparisons between the X chromosomes of these distantly related mammal groups may, therefore, provide variants of mammalian X chromosome gene content and function that can be used to further test Ohno's hypothesis that the X chromosome is conserved *in toto* and that this conservation depended on X chromosome inactivation.

The cytology of the monotreme sex chromosomes and the details of X chromosome inactivation certainly distinguish this group from therians. The X chromosome of the platypus is somewhat larger (about 6%) than the X chromosome of eutherians; the Y chromosome is nearly as large, and its long arm is G-band homologous to the long arm of the X chromosome, with which it pairs at meiosis (8, 9). The two echidna species, although they have an X_1X_2Y sex chromosome system, have X_1 and Y chromosomes that are almost G-band identical to the X and Y chromosomes of the platypus. The observations that the short arms of the X chromosomes replicate asynchronously in female-derived cells but the long arms replicate synchronously suggest that X chromosome inactivation is confined to the differential region of the short arm of the X chromosome (9).

Gene mapping in monotremes has been virtually impossible, because neither the platypus nor the echidna can be bred in captivity. However, the observation of putative heterozygotes for *PGK* among male echidnas suggests that this gene is autosomal (10), although a pseudoautosomal mode of inheritance would be consistent with a location on the paired long arm of the X (and Y) chromosome. Somatic cell genetic analysis of rodent–platypus cell hybrids has established synteny between *PGK* and *HPRT* but, because of the fragmentation of chromosomes in these hybrids, has not provided a chromosome assignment (11). One gene (*OTC*), which is located on the human Xp chromosome, is on an autosome in the platypus (12), suggesting that the conservation of the X chromosome may not extend to monotremes.

We, therefore, set out to map to platypus chromosomes several genes that are located on the long arm of the human X chromosome (called here "human Xq genes") to determine whether the conservation of the X chromosome extends to monotremes, to determine whether it involves regions exempt from inactivation, and to establish the limits of the conserved segment of the X chromosome. We report here that eight human Xq genes are also located on the X chromosome in monotremes. Of these, three map to the long arm of the monotreme X chromosome, which appears not to be subject to inactivation.

MATERIALS AND METHODS

Platypus diploid fibroblast cell lines were established (13) by a plasma clot method using toe-web tissue kindly supplied by David Goldney under permit A579 from New South Wales National Parks and Wildlife. Platypus cell lines were grown

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in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 10% (vol/vol) fetal calf serum (Flow Laboratories and GIBCO), streptomycin (50 μg/ml), penicillin (60 μg/ml), and glutamine (100 μg/ml), at 32°C (the body temperature of the animal) in an atmosphere of 10% CO_2/90% air.

Chromosome preparations were made by arresting cells at metaphase for 3–7 hr with 0.005% colchicine (Commonwealth Serum Laboratories, Melbourne, Australia), harvesting, swelling 7–20 min in 0.05 M KCl, and fixing in several changes of methanol/acetic acid, 3:1 (vol/vol). The suspensions were then dropped onto cleaned microscope slides and air dried.

DNA probes and their sources are listed in Table 1. The identity of each was verified by confirming that the insert size and restriction sites matched those published or provided by the supplier and, for most probes, also whether they detected bands at the equivalent position in human genomic DNA. For *in situ* hybridization, probes were labeled by nick-translation to specific activities of 2–6 × 10^7 cpm/μg, using mixtures of [^{3}H]dATP, [^{3}H]dCTP, and [^{3}H]dGTP (Amersham). The *in situ* hybridization procedure was as described (12), using carefully screened batches of Ilford K2 or Kodak NTB2 nuclear track emulsion. Slides were exposed for 3–6 weeks at 4°C with dessicant, were developed in Kodak D19, and stained with 10% Giemsa. At least 100 well-spread metaphases were scored from slides hybridized with an optimal probe concentration. Only grains overlaying a chromatid were scored as signal. Grain distributions were analyzed using a GLIM program for unique genes (14).

RESULTS

Each of the human probes used for *in situ* hybridization was tested first for a sufficiently strong signal by hybridization to a Southern blot containing DNA from a range of eutherian and marsupial species as well as from a monotreme (the Australian echidna). The membranes were washed and autoradiographed at progressively higher stringencies to establish conditions that gave maximal signal and acceptable background levels. Fig. 1 shows typical "zoo" blots obtained using gene probes that were found to be highly conserved (*RCP* or *PLP*) or moderately to poorly conserved (*F9* or *G6PD*).

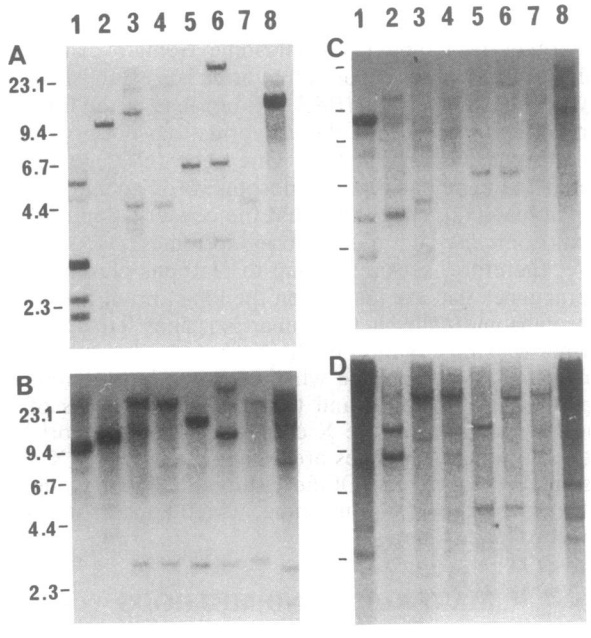

FIG. 1. Southern blot analysis of eutherian (lanes 1 and 2), marsupial (lanes 3–7), and monotreme (lane 8) DNA. Lanes: 1, human; 2, rat; 3, *Macropus rufus*; 4, *Macropus eugenii*; 5, *Dasykaluta rosamondae*; 6, *Pseudantechinus apicalis*; 7, *Trichosurus vulpecula*; 8, *Tachyglossus aculeatus* DNA. (*A*) Digested with *Pst* I and probed with *RCP*. (*B*) Digested with *Bam*HI and probed with *PLP*. (*C*) Digested with *Pst* I and probed with *F9*. (*D*) Digested with *Bam*HI and probed with *G6PD*. Molecular sizes in kilobases are to the left.

For the *in situ* hybridization, probes had to be nick-translated to a high specific activity and hybridized over a range of concentrations; optimal probe concentration was then determined for each probe by estimating the signal-to-noise ratio over a range of probe concentrations. Stringency was monitored over a careful series of washes, and each batch of emulsion was checked for background immediately prior to dipping. With these precautions, *in situ* hybridization to platypus chromosomes using heterologous probes yielded significant and consistent localizations for 8 of the 10 human Xq probes attempted.

Fig. 2 shows typical platypus metaphase preparations after *in situ* hybridization with probes for *G6PD* and *RCP*.

Fig. 3 shows the grain density over each of the seven large platypus chromosomes (chromosomes 1–6 and X) and over groups A and B of the small chromosomes, which are combined because they are difficult to distinguish even by G-banding (15). Each of the probes produced by far the strongest signal over the X chromosome, mapping *GLA*, *PLP*, *F8*, *F9*, *RCP*, *G6PD*, *GDX*, and *P3* to the X chromosome in this species. Two probes, *RCP* and *PLP*, also produced significant signals on chromosomes 1 and 2. At

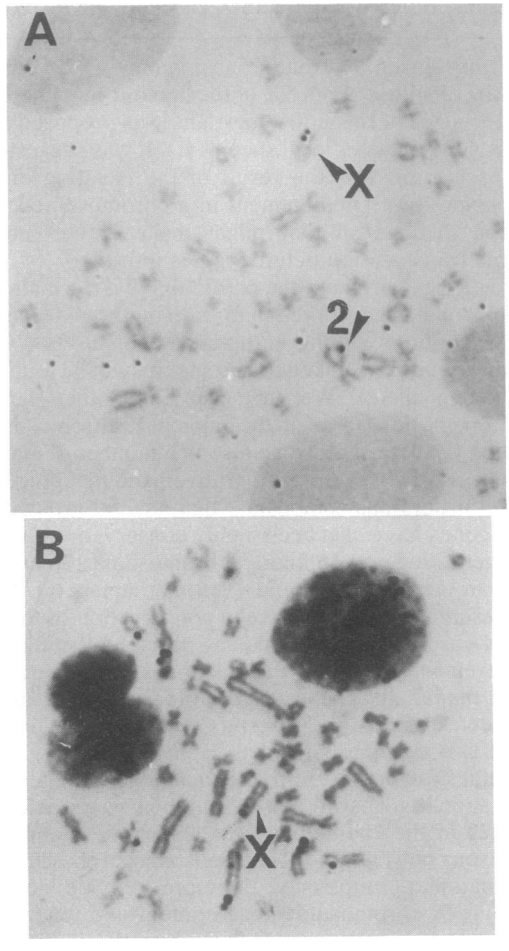

FIG. 2. Metaphase chromosomes of *O. anatinus* after *in situ* hybridization. (*A*) *RCP* probe; arrowheads indicate grains over major site on chromosome Xp and minor site on chromosome 2q. (*B*) *G6PD* probe; arrowhead indicates grains over site on chromosome Xq.

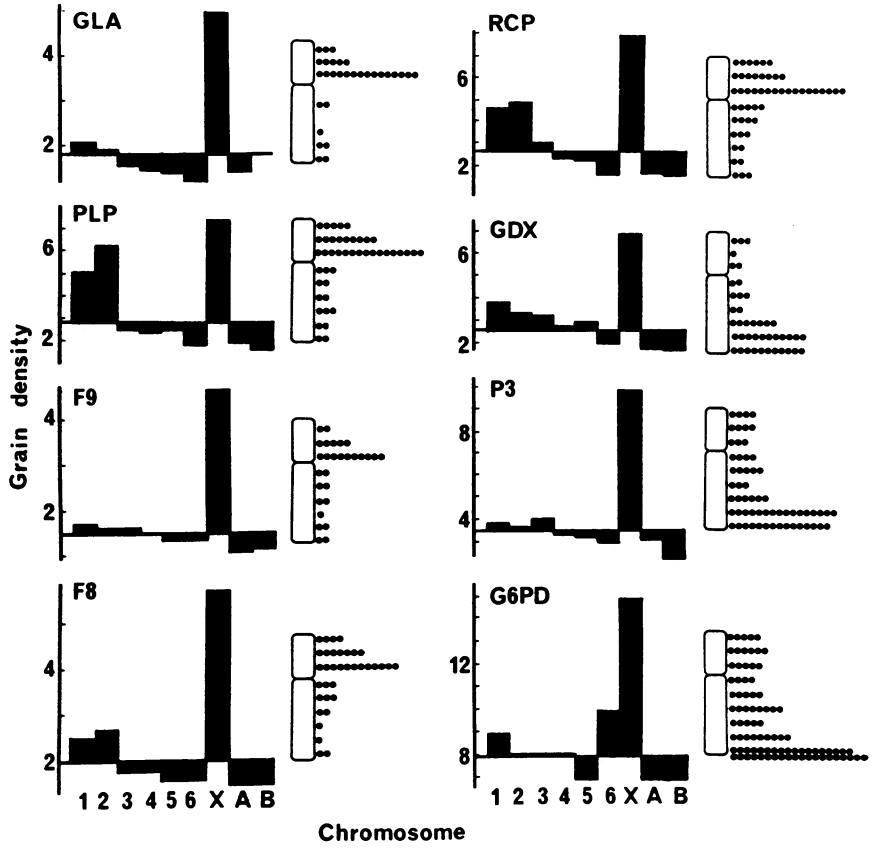

FIG. 3. Density and distribution of grains for eight genes. The grain density [(number of grains corrected for relative chromosome length) $\times 10^2$] over each chromosome is presented as a histogram (on the left) and the grain distribution (totaled over 100 cells) over the X chromosome (on the right) are shown.

present it is not known whether these "secondary sites" represent pseudogenes or closely related functional sequences. However, we have found that a number of the human Xq (and Xp) probes show secondary hybridization sites to both the platypus and the tammar wallaby chromosomes.

The distribution of grains over the X chromosome for each probe (Fig. 3) enabled regional localization (Table 1) and construction of a rough map of the platypus X chromosome (Fig. 4).

DISCUSSION

We have demonstrated by *in situ* hybridization that eight genes, located on the long arm of the human X chromosome, are localized also to the platypus X chromosome. These must necessarily remain tentative assignments, since there is no opportunity to confirm them by an independent method. It would obviously be desirable to repeat these experiments using preparations from male platypus, because the important conclusion that the platypus Xq and Yq are homologous and exempt from X chromosome inactivation depends on cytological evidence. At present this is not technically possible because the Y chromosome is difficult to distinguish from chromosome 7 or from other autosomal pairs included in the class A chromosomal complement, even in late replication banded preparations (9, 15).

The localization of these eight genes to the monotreme X chromosome as well as to the marsupial X chromosome (4) suggests that at least a very large region of the conserved

Table 1. Loci and probes used in this study

Locus	Position on human X chromosome	Locus name	Homology	Source	Ref.	Chromosome location in platypus*
GLA	q21.3–q22	Galactosidase, α	M	E. Ginns (Bethesda, MD)	16	Xp1
PLP	q21.3–q22	Proteolipid protein	H	L. Naismith (Toronto)	17	Xp1
F9	q26.3–q27.1	Coaggulation factor IX	ML	K. H. Choo (Melbourne, Australia)	18	Xp1
F8	q28	Coaggulation factor VIIIC	ML	J. Gitschier (San Francisco)	19	Xp1
RCP	q28	Red cone pigment	H	J. Nathans (Baltimore)	20	Xp1
GDX	q28	Unknown	M	D. Toniolo (Pavia, Italy)	21	Xq5
P3	q28	Unknown	M	D. Toniolo	22	Xq5
G6PD	q28	Glucose-6-phosphate dehydrogenase	H	D. Toniolo	23	Xq5–6

Homology to monotreme sequences: H, high; M, medium; L, low.
*The platypus X chromosome is here divided into nine roughly equivalent regions, p1–3 on the short arm and q1–6 on the long arm (numbering out from the centromere).

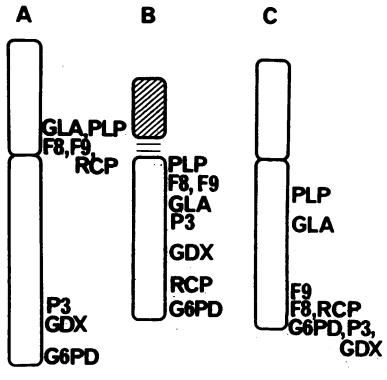

FIG. 4. Positions of the eight human Xq genes on the X chromosome in platypus (*A*), *M. eugenii* (*B*), and human (*C*).

eutherian X chromosome is included on the X chromosome in all three groups of extant mammals. This region is, therefore, likely to have been a part of the X chromosome in the common ancestor of the therian and prototherian mammals more than 150 million years ago.

It is possible now to map the extent of this highly conserved region of the mammalian X chromosome in representative species of the three groups of mammals (Fig. 4). The conserved genes are distributed over most or all of the long arm of the human X and the *M. eugenii* X chromosomes, which seem to have an equivalent gene content. However, these genes are located in two rather discrete regions of the platypus X chromosome, one proximal on the short arm (region p1) and the other distal on the long arm (regions q5 and 6). Large apparently euchromatic regions (15) on the short and the long arms of the platypus X chromosome are so far devoid of mapped genes. Whether this blank space will be filled with other genes located on the eutherian X chromosome is as yet unknown; however, since the monotreme X chromosome is larger than the eutherian X chromosome, and at least one gene (*OTC*) located on the short arm of the human X chromosome is autosomal in monotremes (12), it seems likely that genes that are autosomal in eutherians could prove to lie in these regions of the monotreme X chromosome.

It is seen from Fig. 4 that although gene order has obviously not been conserved (hardly surprising, since gene order is not conserved even between primates and rodents) some of the genes map in conserved clusters in two or in all three groups (e.g., *F8–F9*, as well as *GLA–PLP* are close in all three, and *G6PD–GDX–P3* are located together in human and platypus). These conserved associations are, therefore, likely to reflect the close associations of these genes in an ancestral mammal.

Our gene mapping data can be used to answer the question of whether the conservation of this region of the X chromosome is related to its involvement in X chromosome inactivation, as suggested originally by Ohno (1). Our demonstration that the marsupial X chromosome represents a conserved region (4) was at least consistent with this hypothesis, for the entire marsupial X chromosome is differentiated from the (often minute) Y chromosome and appears to be subject to X chromosome inactivation, at least in blood-cell lineages (24, 25). It is of particular interest to examine gene content and location in the monotreme X chromosome, since cytological studies suggest that only part of this large X chromosome is differentiated from the Y chromosome and inactivated. Whereas the short arm has no homologue on the Y chromosome and appears to replicate asynchronously, at least in blood cells, the long arm is G-band homologous to and pairs with the long arm of the Y chromosomes. The synchronous replication of the Xq chromosome in female-derived cells suggests that this region is exempt from inactivation (9), which is consistent with the conclusion that it is paired with the Y chromosome and, therefore, requires no dosage compensation. If this is indeed the case, our finding that at least three genes map to the distal region of the long arm of the platypus X chromosome would contradict the hypothesis that conservation of the X chromosome depended on X chromosome inactivation.

We, therefore, propose that a large region of the ancestral mammalian X chromosome has been conserved in each of the three mammalian groups over a period of 170 million years and that this conservation has not depended on X chromosome inactivation. If this extraordinary conservation of the X chromosome cannot, after all, be ascribed to its participation in the X chromosome inactivation system, how can it be explained? It is, of course, possible that parts of the monotreme Xq chromosome are inactivated by some other mechanism of dosage compensation that lacks the cytological manifestation of late DNA synthesis and that this protects the X chromosome from rearrangement. However, this seems unlikely, given that there is strong cytological evidence for Xq–Yq chromosome homology and pairing; if the Yq chromosome proves to be genetically, as well as cytologically, homologous to the Xq chromosome, there will be two doses of all the genes on this region in both sexes, which means that this region would not need to be dosage-compensated.

Alternatively, the conservation of the X chromosome may not be so extraordinary after all. There is growing evidence that large autosomal regions may be conserved even between distantly related eutherian mammals; for instance, human chromosomes 11 and 12 are entirely represented by cytologically similar chromosomes with identical genetic constitution in the cat, from which it diverged about 80 million years before present (5). In addition, autosomal syntenic groups are shared among eutherians and marsupials (6), monotremes (26), and even fish (27). The genome rearrangement observed in some eutherian orders (e.g., rodents) may be rather atypical of mammals, for the entire infraclass Metatheria is karyotypically extremely conserved (28), as is the karyotype of subclass Protheria (15). We suggest, therefore, that the X chromosome may simply be an especially well-defined example of a highly conserved mammalian genome.

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