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Review

The monotreme genome: a patchwork of reptile, mammal and unique features?☆

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Abstract

The first specimen of platypus (*Ornithorhynchus anatinus*) that reached Britain in the late 18th century was regarded a scientific hoax. Over decades the anatomical characteristics of these unique mammals, such as egg laying and the existence of mammary glands, were hotly debated before they were accepted. Within the last 40 years, more and more details of monotreme physiology, histology, reproduction and genetics have been revealed. Some show similarities with birds or reptiles, some with therian mammals, but many are very specific to monotremes. The genome is no exception to monotreme uniqueness. An early opinion was that the karyotype, composed of a few large chromosomes and many small ones, resembled bird and reptile macro- and micro-chromosomes. However, the platypus genome also features characteristics that are not present in other mammals, such as a complex translocation system. The sex chromosome system is still not resolved. Nothing is known about dosage compensation and, unlike in therian mammals, there seems to be no genomic imprinting. In this article we will recount the mysteries of the monotreme genome and describe how we are using recently developed technology to identify chromosomes in mitosis, meiosis and sperm, to map genes to chromosomes, to unravel the sex chromosome system and the translocation chain and investigate X inactivation and genomic imprinting in monotremes.

Keywords: Monotremes; Chromosome and sex chromosome evolution; Translocation chain; Comparative mapping; Genomic imprinting; X inactivation

1. Evolution and systematics of monotremes

Since the discovery of the platypus and echidnas in Australia by European explorers more than 200 years ago, monotremes have presented biologists with a contradictory mixture of reptilian, eutherian and specialized characters. Generally classified in a single order Monotremata of the mammalian Subclass Prototheria, only three species are known: the duck-billed platypus (*Ornithorhynchus anatinus*) and two echidna species, the Australian short-

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beaked echidna (*Tachyglossus aculeatus*) and the long beaked Niugini echidna (*Zaglossus bruijni*).

The fossil record of monotremes comes mostly from Australia and New Guinea. The oldest fossil dates from the Mesozoic 100 MYA (million years ago) (Archer et al., 1985, 1992). Miocene and Pleistocene fossils of a giant echidna were also found in Australia. However, recent discovery of a fossil tooth of an early Pleistocene platypus in southern Argentina shows that the distribution of monotremes was wider and included eastern Gondwana during the late Cretaceous to early Paleocene (Pascual et al., 1992).

Although monotremes were immediately identified as mammals, their evolutionary relationship with other mammal groups is still controversial. Traditionally, they are considered the only extant order of the mammalian subclass Prototheria that diverged from the subclass Theria (marsupials and placental mammals) 150–215 million years ago (MYA). This orthodox view is supported by a vast body of morphological, physiological and anatomical evidence (Carroll, 1988; Rougier et al., 1996; Vaughn, 1986; McKenna and Bell, 1997). However, it is challenged by sequence comparisons of mitochondrial DNA and nuclear 18S rRNA, which suggest that monotremes and marsupials form one group (Marsupionta) that diverged together from eutherians, and split later into the monotreme and marsupial lineages (Janke et al., 1997, 2002). However, more recent and more exhaustive analysis of nuclear genes strongly supports the traditional idea of the Theria clade that includes marsupials and eutherian mammals (Killian et al., 2001b; Belov et al., 2002; Woodburne et al., 2003). These studies date the branching of the monotremes from the mammalian line at 200–210 MYA.

The divergence of platypus and echidnas was dated at between 20 and 45 MYR (Cao et al., 1998) but has been narrowed down by DNA–DNA hybridisation as well as mtDNA and Immunoglobulin sequences to be 21–25 MYA (Westerman and Edwards, 1992; Kirsch and Mayer, 1998, Belov, personal communication).

2. The monotreme karyotype and sex chromosomes

The size of the haploid genome of monotremes is 3.06 pg in platypus and 2.98 pg in the short beaked echidna (Bick and Jackson, 1967b). This computes to a haploid genome size of 3060 Mb (millions of base pairs, or Megabases) for platypus and 2980 Mb for echidna. This compares to a 3.5 pg (3500 Mb) genome in humans and falls well within the range of mammal genome sizes (between 1.8 pg in bats up to 7 pg in some rodents), but outside the range in birds of approximately 1.2 pg (http://www.genomesize.com).

However, the organisation of the genomes of platypus and echidna into chromosomes that are visible in mitosis and meiosis shows some remarkable differences. At the first encounter with monotreme chromosomes, early cytologists considered that the karyotypes of platypus and echidna were similar to those of reptiles and birds, which contain tiny dot like microchromosomes as well as normal sized macroelements (Matthey, 1949; White, 1973). However, VanBrink (1959) considered that the small chromosomes of monotremes were much larger than sauropsid microchromosomes, and there was a more continuous distribution of chromosome size, in contrast to the bimodal distribution in birds and reptiles. The question of whether monotremes have microchromosomes remains and will be answered by comparisons of sequences.

In the early study of VanBrink (1959), without the benefit of G-banding to identify homologous chromosomes, only one larger chromosome was identified without a homologue in the male mitotic metaphase spreads. Males were, therefore, identified as the heterogametic sex, as is standard for other mammals. In platypus, 52 chromosomes are counted in diploid cells of males and females suggesting a XX/XY chromosome system. These were early days for mammalian chromosome work: only 3 years earlier, in 1956, the human chromosome number was corrected from $2n=48$ to $2n=46$ (Tjio and Levan, 1956).

The monotreme chromosome story became a little more complex when different numbers of chromosomes were counted in cells of echidna males (63) and females (64). This was explained by a XX/XO sex chromosome system, in which a Y chromosome is lacking from the male karyotype (Bick and Jackson, 1967a; Bick et al., 1973).

In 1977, Carolyn Murtagh applied the then new G-banding techniques to chromosome preparations of a number of echidnas and platypus and reported ‘a unique cytogenetic system in monotremes’. She described heteromorphy of some of the large chromosomes. Most extraordinarily, she described six chromosomes with no obvious homologue in the

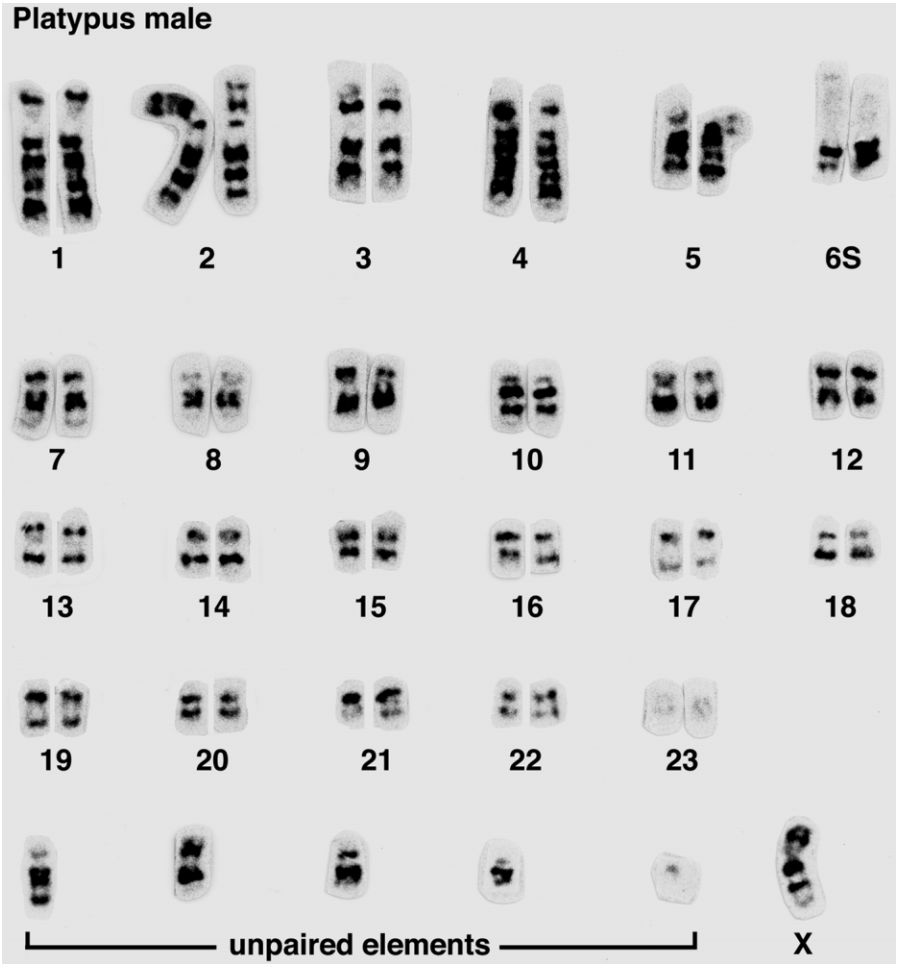


Fig. 1. G-banded karyotype of male platypus. Chromosome 6 bears the nucleolar organizer (6S) and is often heteromorphic.

echidna karyotype and four in platypus. This, with the observation that some chromosomes made a chain at male meiosis in both species, suggested an X_1X_1 X_2X_2 female/X_1X_2Y male sex chromosome system in echidna, in which the Y is fused to an autosome (X_2) (Murtagh, 1977). It was suggested that translocation heterozygosity for a number of chromosomes had been established in monotremes (Bick et al., 1973; Murtagh, 1977). Such a system is known to occur naturally in a few plants and invertebrate species, but was unprecedented in mammals.

Meiosis has been investigated in mice heterozygous for translocations. As in monotremes, the chromosomes of these animals form chains or rings of chromosomes. However, in contrast to monotremes, this results in meiotic arrest and sterility of the heterozygous mice, or the production of high proportions of aneuploid sperm (Eaker et al., 2001) or embryos (Winking et al., 2000; Underkoffler et al., 2002).

The study of monotreme chromosomes was greatly facilitated by the development of methods to culture fibroblasts from platypus toe web and echidna abdominal skin (Wrigley and Graves, 1984). Wrigley and Graves (1988b) prepared monotreme chromosomes and stained them with a barrage of banding methods, including high resolution G-banding, R-banding and late replication banding. The banding patterns revealed a high degree of conservation of the larger chromosomes (including the X) between the echidna and platypus, and revealed no differences between the karyotypes of the two echidna species. For the first time the Nuigini echidna karyotype could be

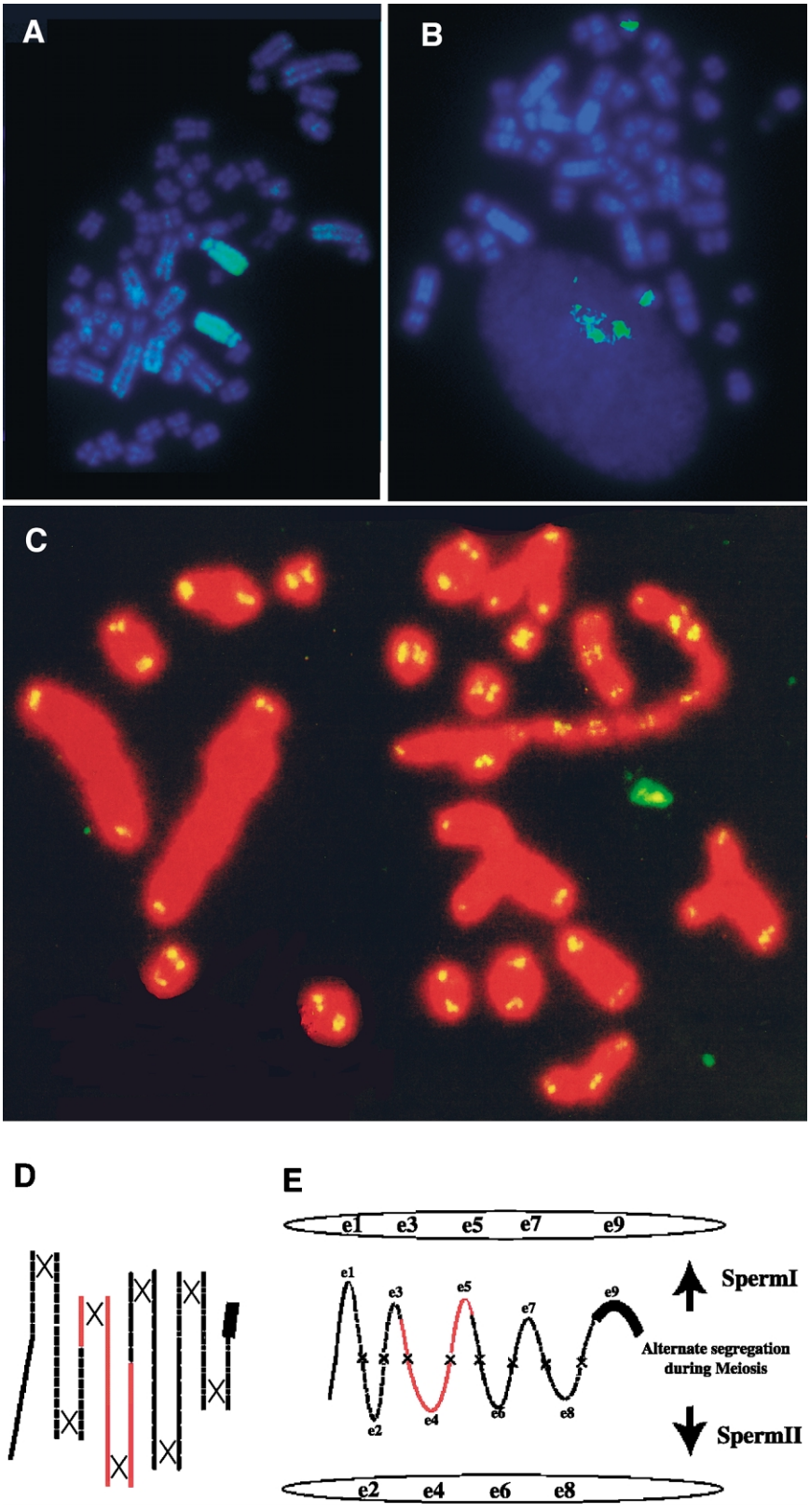

A
B
C
D
E
e1 e3 e5 e7 e9
e1
e3
e5
e7
e9
e2
e4
e6
e8
SpermI
Alternate segregation during Meiosis
SpermII
e2 e4 e6 e8

Fig. 2.

compared, and was found to be virtually identical to its Australian cousin.

Fig. 1 shows the G-banded karyotype of a male platypus. The identity of the Y chromosome (even the presence or absence of a Y chromosome) in males is in doubt, because of the presence of a least four chromosomes in females, and five in males, that show no morphological homologue.

3. The unpaired elements and the meiotic translocation chain

The karyotypes of platypus and echidna are unique among vertebrates in possessing several chromosomes with no obvious homologues (Murtagh, 1977). These seem to be identical between animals, and C-banding confirms that they are not heterochromatic B chromosomes (Wrigley and Graves, 1988b). Together with the X chromosome, these chromosomes form a multivalent chain during the first division of male meiosis (Bick et al., 1973; Bick and Sharman, 1975). The platypus has a chain of eight chromosomes, and echidna a chain of nine (Fig. 2c). In echidna six elements together with an X_1, X_2 and Y could explain the nine elements in the male meiotic chain. In platypus, five chromosomes (including a possible tiny Y chromosome), another two that cannot be distinguished, plus the X must be involved in the chain.

The discovery of a meiotic chain in monotremes provides the only example of such a structure in a vertebrate genome, but translocation chains are known in several plants and invertebrates. Interpretation of the monotreme meiotic chain is, therefore, based on analogy to multivalent meiotic chains described in classic work on the evening primrose *Oenothera* (Cleland, 1962) in which meiotic chains or rings result from balanced heterozygosity for translocations. The chain is formed when homologous regions of each translocated chromosome pair with two different partners, and recombination occurs in the homologous regions at the terminal part of the chromosomes (Fig. 2d). In insect and crustacean groups, translocation heterozygosity involves the sex chromosomes (Luykx and Syren, 1981; Syren and Luykx, 1977). The elements of the monotreme chain are, therefore, expected to be derived from sex chromosomes and homologous autosome pairs.

Although identification of the smaller chromosomes is impossible and striking chromosome heteromorphism makes even male/female comparisons difficult by classical cytology, the same set of small unpaired chromosomes apparently occurs also in female mitotic cells (Wrigley and Graves, 1988b). This suggests that the meiotic translocation chain is also formed in female meiosis, although it would not involve the X chromosome.

We now refer to the elements of the chain as e1, e2, etc. to avoid assumptions about the origin of the chromosomes and their role in sex, which is at present unknown (Table 1). For the chromosomes that participate in the multivalent chain the following measurements show the size of the nine echidna and eight platypus elements as a percent of the whole chain (Table 1).

The translocation chain is likely to have evolved from an initial translocation between the partially paired original X and Y chromosome and an autosome. This would normally form a ring of four; however, if the Y chromosome had lost homology with the X over one region, pairing between the differentiated part of the X and the Y would not occur, and a chain would result. A chain formed in this manner would have the Y at the opposite end of the chain from the X (Fig. 2d,e). In platypus, this terminal element e8 is a very small chromosome (Table 1). In echidna, the chain seems to have been enlarged by the insertion of another autosomal pair to make a chain of 10 translocated chromosomes, then the Y may have been lost altogether. Alternatively, fusion of another autosome may have occurred with the terminal Y, which shifted this chromosome towards the start of the chain.

Fig. 2. Monotreme chromosomes. (a) Fluorescence in situ hybridisation (FISH) with the chromosome 4-specific paint. (b) FISH with paint for a smaller metacentric chromosome. Note that the paint in B has a stronger signal in the centromeric region, which is due to enrichment of repetitive sequences in this region. (c) Translocation chain in echidna male meiosis. FISH with telomere-specific sequences marks the ends of chromosomes and shows the chromosome position within the chain. No interstitial telomeric sequences were observed on mitotic chromosomes (not shown). (d) Hypothesized pairing and segregation of elements at echidna male meiosis. The chain contains a set of original chromosomes (e2, e4, e6, e8), and a set of translocated chromosomes (e1, e3, e5, e7, e9). They pair in homologous regions, then form a chain held together by terminal chiasmata. (e) This translocation chain adopts a zig-zag 'alternate' configuration at anaphase I, from which elements segregate to give two types of sperm, containing e-odd or e-even elements.

Table 1
Relative size (%) of meiotic chromosomes in the chain

	e1	e2	e3	e4	e5	e6	e7	e8	e9
Platypus	23.1(X)	12.7	14	10	12.7	8.3	17.9	1.3(Y?)	
Echidna	23.8(X1)	12.2(Y?)	6.8(X2)	8.7	9.4	11.8	7.4	5.3	14.6

(Based on measurements from Bick, 1992 for platypus and Watson et al., 1992b for echidna.)

4. Chromosome segregation and gamete formation

The most puzzling aspect of this translocation chain is to imagine how the animals manage to reproduce successfully. Heterozygosity for just a single translocation in humans and mice causes sub-fertility, since one half of the products of meiosis will be duplicated or deficient for parts of the genome. Random segregation of nine elements (including five translocated chromosomes) would be expected to produce only 1/32 balanced gametes in the echidna, and the formation of a viable zygote by random union of such gametes would, therefore, be extremely improbable (1/1024). Plants and insects with multivalent chains solve this problem by a regular alternate segregation from the chain arrayed in a zig-zag conformation at anaphase I, such that all the original elements go to one pole, and all the translocated elements to the other. Thus, two kinds of gamete, both with balanced complements, are formed (Fig. 2e). Fertilization is restricted by a balanced lethal system to combinations of the two, so that the progeny are all themselves translocation heterozygotes.

A similar system could operate in monotremes. The huge energetic investment in developing a yolk rich egg makes zygotic lethality improbable, and it is more likely that some sort of recognition mechanism prevents the fertilization of incompatible eggs and sperm.

The hypothesis that elements of the chain adopt a regular alternate segregation predicts that two types of sperm will be produced (Fig. 2d). One half should contain the X and other odd-numbered elements, whereas the other half should contain the even-numbered elements (and include any putative Y). The fibrillar monotreme spermhead presents favourable material for investigating chromosome arrangement.

In contrast to the round headed sperm of eutherians and marsupials, spermatozoa of all monotreme species have retained a vermiform shape similar to that of reptiles and birds (Hughes, 1965; Jamieson, 1995). Fine dissection of several stages of spermatid differentiation has recently confirmed homology of most developmental stages between monotremes and reptiles, although monotreme specific features were also discovered (Lin and Jones, 2000).

Previous studies of monotreme sperm answered a venerable question in classical cytology whether chromosomes are arranged randomly in sperm or whether they occupy specific positions. Contradictory observations had been made in several invertebrate species, and initial results from rodent and human sperm revealed no fixed order. Hybridisation of several specific DNA probes to echidna and platypus sperm suggested a non-random tandem distribution (Watson et al., 1996). Fixed chromosome territories were subsequently demonstrated in marsupial sperm using whole chromosome paints (Greaves et al., 2001). Two chromosomes that could be detected by platypus chromosome paints were found consistently at either end of the sperm head (Greaves et al., 2003). There is a consistent evolutionarily conserved arrangement of chromosomes in sperm of closely related marsupial species, and some features, such as the position of the sex chromosomes at the point of first contact with the egg, seem to be conserved in all three mammal groups. In contrast to the conserved chromosome arrangement in mammal sperm (Meyer-Ficca et al., 1998; Luetjens et al., 1999), chromosomes appear to be randomly distributed in the fibrillar sperm of chickens, and there is no special position of the sex chromosomes (Solovei et al., 1998; Greaves et al., 2003).

5. Comparative gene mapping

The karyotypes of different mammal species were traditionally thought to demonstrate bewildering plasticity. However, comparative gene mapping and chromosome painting between species has revealed extraordinary conservation of the mammalian genome. Relatively few rearrange-

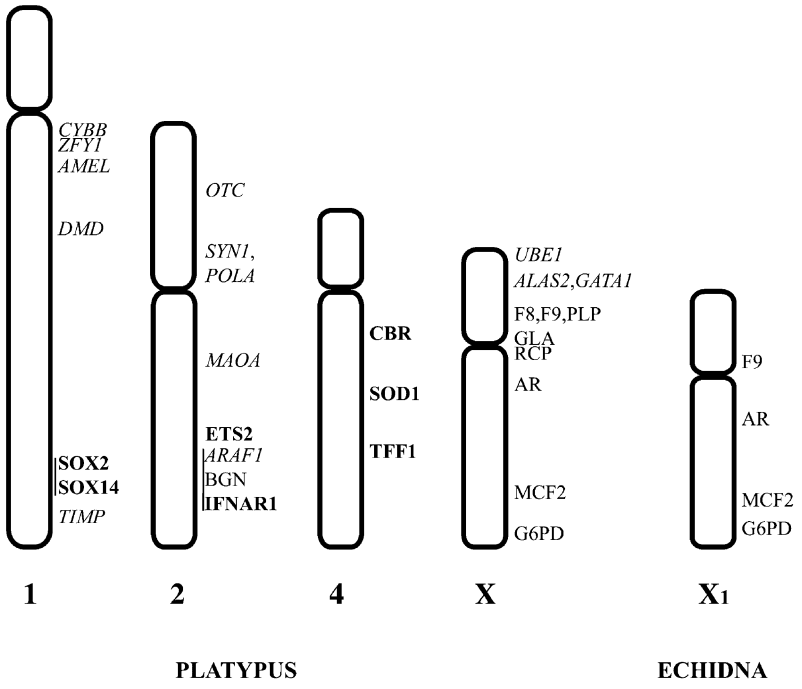


Fig. 3. Comparative gene map showing localisation of human genes in platypus and echidna. Genes mapping to the long arm and pericentric region of the human X chromosome are in Roman print, in italics are genes mapping to the human X short arm, and in bold print are human autosomal genes. The grouped genes indicate identical physical localization.

ments have occurred between even distantly related eutherian species (e.g. eight rearrangements between human and cat, Rettenberger et al., 1995), or between distantly related marsupial species (e.g. only seven rearrangements between American didelphids and Australian species, Rens et al., 2001).

Chromosome number and morphology do not change continuously with time—there is no 'chromosome rearrangement clock' analogous to the 'molecular clock' that describes DNA sequence changes. Some species groups display extraordinary karyotype stability (e.g. the cats, and dasyurid marsupials), while others have changed very rapidly (e.g. dogs, gibbons and some macropodid marsupials). Some chromosomes have been more conserved during mammalian evolution than others (Scherthan et al., 1994; Chowdhary et al., 1998).

There has been little gene mapping in monotremes. The difficulty of captive breeding really rules out family studies. Some information was obtained from somatic cell hybrids formed by fusion of monotreme and rodent cells (Graves and Wrigley, 1986), which segregated monotreme chromosomes. However, most map assignments have been via radioactive, or more recently, fluorescence in situ hybridization (Fig. 3, Table 2, Graves, 1998).

6. The monotreme X and the origin of sex chromosomes

The mammalian X chromosome is the most conserved chromosome in mammalian evolution, perhaps as a result the chromosome-wide dosage compensation system that has been established (Ohno, 1967). There are almost no exceptions to 'Ohno's Law' that genes on the X in one eutherian species are X-borne in others (Wakefield and Graves, 1996). Recently, comparative mapping of a number of X chromosomal genes in pufferfish and zebrafish (which diverged from mammals more than 400 MYA) has demonstrated that large regions of mammalian X are still intact in these species (Grützner et al., 2002; Postlethwait et al., 2000).

Mapping human X-borne genes in marsupials and monotremes, however, provided an important exception to Ohno's Law. Although 11 genes on the long arm of the human X and the pericentric

Table 2
Genes from the human X chromosome mapped in platypus or echidna

Gene	Gene name	Reference
AMEL	AMELOGENIN	Watson et al., 1992a
ALAS2	DELTA-AMINOLEVULINATE SYNTHASE 2	Wilcox et al., 1996
AR	ANDROGEN RECEPTOR	Spencer et al., 1991, Watson et al., 1992c
ARAF1	V-RAF MURINE SARCOMA 3611 VIRAL ONCOGENE HOMOLOG 1	Wilcox et al., 1996
BGN	BIGLYCAN	Wilcox et al., 1996
CYBB	CYTOCHROME b-245, BETA POLYPEPTIDE	Watson et al., 1991
CBR1	CARBONYL REDUCTASE 1	Maccarone et al., 1992
DMD	MUSCULAR DYSTROPHY, DUCHENNE TYPE	Watson et al., 1991
ETS2	V-ETS AVIAN ERYTHROBLASTOSIS VIRUS E26 ONCOGENE HOMOLOG 2	Maccarone et al., 1992
F8	COAGULATION FACTOR VIIIC	Watson et al., 1990
F9	COAGULATION FACTOR IX	Watson et al., 1990, Watson et al., 1992c
GATA1	GATA-BINDING PROTEIN 1	Wilcox et al., 1996
GLA	GALACTOSIDASE ALPHA	Watson et al., 1990
G6PD	GLUCOSE-6-PHOSPHATE DEHYDROGENASE	Watson et al., 1990 Watson et al., 1992c
IFNAR1	INTERFERON ALPHA, BETA, AND OMEGA, RECEPTOR 1	Maccarone et al., 1992
MAOA	MONOAMINE OXIDASE A	Watson et al., 1991
MCF2	ONCOGENE MCF2	Watson et al., 1990, Watson et al., 1992c
OTC	ORNITHINE CARBAMOYLTRANSFERASE	Sinclair et al., 1987
PLP	PROTEOLIPID PROTEIN	Watson et al., 1990
POLA	DNA POLYMERASE ALPHA	Watson et al., 1991
RCP	RED AND GREEN PIGMENT GENES	Watson et al., 1990
SOD1	SUPEROXIDE DISMUTASE 1	Maccarone et al., 1992
SOX2	SRY-BOX 2	Kirby et al., 2002
SOX14	SRY-BOX 14	Kirby et al., 2002
SYN1	SYNAPSIN I	Watson et al., 1991
TFF1 (*BCEI*)	TREFOIL FACTOR 1 (BREAST CANCER ESTROGEN INDUCIBLE SEQUENCE)	Maccarone et al., 1992
TIMP	TISSUE INHIBITOR OF METALLOPROTEINASE 1	Watson et al., 1991
UBE1	UBIQUITIN-ACTIVATING ENZYME 1	Mitchell et al., 1998
ZFY	ZINC FINGER PROTEIN	Watson et al., 1993

region mapped to the X in marsupials and monotremes, 13 genes on the short arm of the human X mapped to autosomes (Fig. 3 and Table 2) (Watson et al., 1990, 1991, reviewed Graves, 1995). These mapping results led to a new and refined view of the evolution of the mammalian X chromosome, in which the region of the X conserved in all mammals represents the original X, and the region autosomal in marsupials and monotremes represents a region added recently in the eutherian lineage.

As first proposed by Susumo Ohno, the mammal X and Y chromosomes derived from an ordinary pair of autosomes after one partner acquired a sex-determining gene. This proto-Y was degraded because of genetic isolation engendered by the accumulation of other male-advantage genes (Ohno, 1967; Charlesworth, 1991). On this view, the Y chromosome is merely a degraded X (Graves, 2000). Indeed, comparative mapping of genes on the human Y shows that it, too is composed of ancient and added regions (Waters et al., 2001). Comparisons of genes on the human Y with genes on the Y in distantly related species tell a sorry story of mutation and inactivation; most of the original 1500 genes have disappeared, and the few survivors have acquired male-specific functions in sex determination and spermatogenesis. Even the testis determining gene *SRY* has been shown to originate from a brain-development gene on the X (Foster and Graves, 1994).

The question of the Y chromosome and sex determination is still unresolved in monotremes. Initially it was thought that both echidna and platypus have an XX/XO sex chromosome system because different chromosome numbers were

reported in male and female animals (Bick and Jackson, 1967a). However, although platypus have the same chromosome number in both sexes, no male-specific genes have yet been discovered in monotremes (Graves, 2002). No homologue of the human testis-determining gene *SRY* can be found, and *UBE1*, which has different forms on the X and Y in therians and marsupials and maps to the monotreme X, identifies no male-specific copy (Mitchell et al., 1998). It therefore remains possible that there is no Y chromosome in monotremes, and that an entirely different master gene is used to control monotreme sex determination, perhaps by virtue of its different dosage in males and females.

7. Molecular cytology of monotreme chromosomes

Development of powerful new techniques during the last 15 years has created an era of molecular cytogenetics. The ability to map genes directly by fluorescence in situ hybridisation (FISH), and the generation of DNA probes from whole sorted or microdissected chromosomes (chromosome paints) has allowed confirmation of the vast body of comparative gene mapping and comparative banding studies in different species (e.g. for primates: Dutrillaux, 1979; Ried et al., 1993). Moreover, these resources and techniques have allowed expansion of comparative chromosome maps to more distantly related species, and species, which have undergone massive chromosome changes (Yang et al., 1997).

Especially valuable have been the beautiful chromosome painting techniques. Comparative chromosome painting relies on the cross hybridisation of chromosome-specific DNA from one species onto the chromosomes of another (reviewed O'Brien et al., 1999; Wienberg and Stanyon, 1997). Where species are closely related (for example hybridization of mouse chromosome paints to rat or hamster; Grützner et al., 1999; Stanyon et al., 1999), this approach directly reveals homologies of chromosomes and chromosome regions across species. Cross-hybridization between more distantly related species (for instance dasyurids and macropodid marsupials or even American and Australian marsupials; De Leo et al., 1999; Rens et al., 2001) may require more finesse because the DNA–DNA hybridisation on which the technique is based will be weaker because of the sequence divergence between probe and target.

Because of the isolated position of monotremes, it has not proved possible to use comparative chromosome painting to relate eutherian or marsupial paints to monotreme chromosomes. Chromosome paints prepared from isolated monotreme chromosomes, however, are the ideal tools to unravel the relationships between platypus and echidna chromosomes. They can also be used to resolve the complex translocation system. They will allow identification of sex chromosomes and the unpaired elements, not only on metaphase chromosomes, but also at different stages of meiosis and spermatogenesis. It will be possible to map the elements within the meiotic chain and determine if they are homologous in platypus and echidna. It will be very interesting to learn how this complex system has evolved, if translocated chromosomes are homologues, how the different numbers of elements of the meiotic translocation chain evolved and if their position in the chain is conserved between species.

Chromosome paints for platypus were recently made as part of our collaboration with the Molecular Cytogenetic Group of the Department of Clinical Veterinary Medicine, University of Cambridge. These paints were prepared by flow-sorting platypus chromosome using a fluorescence activated flow sorter, or microdissecting them. DNA from isolated chromosomes was amplified by degenerate oligonucleotide primed PCR and hybridized by fluorescence in situ hybridisation (for methods see Rens et al., 1999; Grützner et al., 2001). An example is given in Fig. 2a,b: A single paint—e.g. from one of the larger chromosomes, hybridises only to chromosome 4 in platypus metaphase spreads (Fig. 2a), while another paint from a smaller metacentric chromosome identifies specifically the homologous chromosome in the platypus metaphase (Fig. 2b).

8. Genomic imprinting in monotremes?

Stable alterations of gene expression that arise during development and are subsequently retained during mitosis are said to be epigenetic. These alterations do not change the DNA sequence, but modify DNA or chromosomal protein. Important changes include DNA methylation and histone modifications.

There are two major types of epigenetic modifications that are crucial for mammalian development: genomic imprinting and X chromosome inactivation. The hallmark of both is monoallelic gene expression.

Genomic imprinting results in parent of origin-dependent expression of a gene. In humans and mouse, over 70 genes with diverse functions affecting growth, differentiation and behaviour are expressed only if they come from the mother, or from the father (reviewed Murphy and Jirtle, 2003). For instance, the *IGF2* gene (coding for an insulin-like growth factor) is expressed only from the paternal allele; the silent maternal allele is said to be 'imprinted'. Failure of imprinting of the maternal allele leads to embryonic overgrowth (Vrana et al., 1998). Another gene *M6P/IGF2R* (Mannose 6-phosphate/insulin-like growth factor 2 receptor) that suppresses fetal growth is expressed predominately from the maternal allele. This antagonistic regulation suggests that genomic imprinting evolved as a 'parental tug-of-war', in which the paternal genome promotes embryonic growth and the maternal genome limits it in order to protect the mother's long-term reproductive output.

The origin and evolution of genomic imprinting as a regulator of embryonic development is proposed to relate closely to placental functions (Haig and Graham, 1991). Therefore, it becomes more and more interesting to investigate whether genes that are imprinted in eutherians are also imprinted in marsupials, which have a less elaborate and invasive placenta, and particularly in monotremes, the only egg-laying mammals (John and Surani, 2000).

Genomic imprinting of *IGF2* and *M6P/IGF2R* has been demonstrated in marsupials, but in bird embryos *IGF2* is biallelically expressed (Killian et al., 2000; O'Neill et al., 2000). No imprinting was found in monotremes for *IGF2* and *M6P/IGF2R*, but it is not clear whether the latter gene has the same function as in therians (Killian et al., 2001a). The observation that monotreme (like amphibian and chicken) *M6P/IGF2R* product does not bind and regulate *IGF2* would obviate the need for its imprinting, so the question of whether imprinting occurs in monotremes is not yet settled. The interaction of these gene products to regulate embryonic growth and the selection for their imprinting must have occurred later in mammalian evolution after the divergence of prototherian from the therian lineage (Killian et al., 2001a).

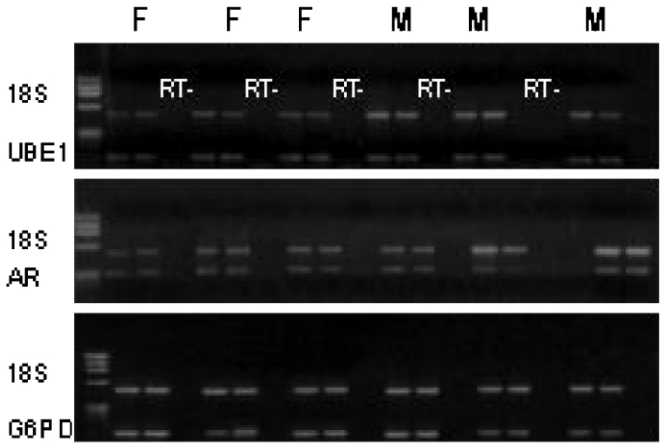
9. X chromosome inactivation in monotremes?

Dosage compensation to achieve the same level of activity for X-borne genes in males and females may be achieved in different ways. In Drosophila, X chromosomal genes are up-regulated in males, whereas in mammals one copy of the X chromosome is silenced in females.

In humans and mouse, the molecular mechanism of X inactivation is relatively well understood. Eutherian X chromosome inactivation occurs early in female embryogenesis and is stable and heritable through subsequent mitosis (Lyon, 1961). In the embryo, the choice of X to be inactivated is random. X inactivation in eutherians represents transcriptional repression of virtually the entire X, although it has been found recently that several genes escape inactivation (Brown et al., 1997; Carrel and Willard, 1999).

Eutherian X chromosome inactivation involves delayed DNA replication, DNA methylation and histone H4 deacetylation, as well as a growing list of variant histones (Costanzi and Pehrson, 1998). An X inactivation centre (XIC), which is the *cis* regulating master switch locus essential for the initiation of X inactivation, contains the *XIST* gene (X-inactive-specific transcript) that is expressed from the inactive X chromosome and codes a RNA that coats the inactive X chromosome (Brockdorff et al., 1991; Brown et al., 1992). *XIST* itself appears to be regulated by an antisense transcript that is controlled by methylation. *XIST* shares many characteristics with imprinted genes, including antisense transcript and control by DNA methylation.

X chromosome inactivation also occurs in marsupials. One X is late replicating (Graves, 1967), and females heterozygous for isozyme variants of sex-linked enzymes express only one allele (Richardson et al., 1973). However, there are several characteristics that set it apart (reviewed Cooper et al., 1993). Most significantly, X inactivation in marsupials is not random, but invariably paternally imprinted (Sharman, 1971); indeed, this was the first evidence of genomic imprinting in a mammal. The later observation that in extraembryonic membranes of rodents, it is always the paternal X that is inactive (Takagi et al., 1978) suggests that paternal X inactivation was the ancestral mecha-



	Female (5)	Male (5)	Female:Male	p value
UBE1	0.58	0.73	0.93	0.64
AR	0.84	0.75	0.85	0.14
G6PD	0.51	0.65	0.78	0.11

Fig. 4. Semi-quantitative RT-PCR of *UBE1*, *AR* and *G6PD*. Reactions for each sample were performed in duplicate. A reaction for each sample minus reverse transcriptase was carried out as a control for genomic DNA contamination. Expression levels were normalized to 18s rRNA.

nism. Marsupial X chromosome inactivation is also incomplete and tissue-specific (VandeBerg et al., 1987), and the molecular mechanism may be less complex, involving histone H4 deacetylation (Wakefield et al., 1997) but not DNA methylation (Piper et al., 1993). *XIST* has not been cloned in marsupials, but mapping of flanking genes suggests that the region that contains it is conserved (Wakefield, unpublished).

The question of X inactivation is unresolved for monotremes. Replication studies using tritium autoradiography in monotremes revealed differences in replication timing of the X, but also of a number of larger autosomal pairs in female echidna (Murtagh, 1977). In a later study (Wrigley and Graves, 1988a), late replication banding, as well as autoradiography of female platypus and echidna fibroblasts revealed no replication asynchrony, but approximately 50% of metaphases from echidna lymphocytes showed asynchronous replication. However, these results are hard to interpret, since asynchrony was limited to the short arm of the X, which is paired with element e2 in both species, and should require no dosage compensation.

In the absence of isozyme variants and captive breeding, it is difficult to study the expression of sex linked genes in monotremes. However, using semi quantitative RT-PCR we demonstrate here that dosage compensation occurs also in monotremes (Fig. 4). Three genes representing different regions of the platypus X chromosome have been isolated and sequenced, and their levels of expression compared in males and females. The expression of *UBE1* (from the paired short arm) and two genes from the unpaired long arm of the platypus X chromosome (*AR* and *G6PD*) in fibroblast cell lines derived from five different males and five different females were compared using semi-quantitative RT PCR (Farazmand et al., 2001). Equal levels of expression in male and female fibroblast cell lines (Fig. 4) imply that dosage compensation occurs in monotremes. It remains an open question if this is done by upregulation of the single allele

in males or by inactivation of the second allele in females.

10. Outlook

The monotreme genome provides unique opportunities for genome analysis. As the earliest offshoot within the mammalian lineage, monotremes provide invaluable outgroups to therians. The structure of the monotreme genome has and will provide us with crucial information about ancestral features of our own genome and those of other therians. The comparison of gene maps and chromosome painting will reveal ancient chromosome rearrangements, and allow us to deduce the karyotype of the ancestral mammal. Comparison of the molecular mechanisms that control genomic imprinting and X chromosome inactivation will allow us to reconstruct the assembly of these complex systems, and to provide information on how they operate in all mammals.

Comparative mapping of genes and DNA sequences between monotreme, marsupial and eutherian species—and also between birds and monotremes—will indicate which parts of the genome have been conserved and which are ancestral, and which are unique to monotremes. The translocation system is likely to be an example of specific development in monotremes. It will be important to determine whether the chromosomes of this translocation system are homologous between platypus and echidna in order to show how the different numbers of elements within this system arose.

The clarification of the sex chromosome system, especially the identification of a Y chromosome will be crucial for our understanding of the evolution of sex chromosomes in mammals. The identification of a new sex-determining gene will add to our knowledge of the sex-determining pathway in all mammals.

Research on the monotreme genome will also provide critical data on the evolution of sex chromosomes, imprinting and X inactivation in mammals. X inactivation and genomic imprinting share characteristics that suggest that they are evolutionarily related. However, the developmental system unique to the only egg-laying mammals provides excellent opportunities to study the evolution of genomic imprinting and X chromosome inactivation, which is believed to have evolved in close relation to placental development and heteromorphic sex chromosomes.

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