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A platypus' eye view of the mammalian genome

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The genome of monotremes, like the animals themselves, is unique and strange. The importance of monotremes to genomics depends on their position as the earliest offshoot of the mammalian lineage. Although there has been controversy in the literature over the phylogenetic position of monotremes, this traditional interpretation is now confirmed by recent sequence comparisons. Characterizing the monotreme genome will therefore be important for studying the evolution and organization of the mammalian genome, and the proposal to sequence the platypus genome has been received enthusiastically by the genomics community. Recent investigations of X-chromosome inactivation, genomic imprinting and sex chromosome evolution provide good examples of the power of the monotreme genome to inform us about mammalian genome organization and evolution.

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Abbreviations
IGF2   insulin-like growth factor 2
IGF2R   IGF2 receptor
M6P   mannose 6-phosphate
NCBI   National Center for Biotechnology Information

Introduction

Monotremes are an extraordinary group of egg-laying mammals that live in Australasia. There is no question that they are mammals, because they bear fur and suckle their young with milk licked from glands on the abdominal skin. They regulate their body temperature at a lower level (about 31 °C) and with higher flexibility than therian mammals.

Monotremes are usually classified as the mammalian subclass Prototheria, which separated early from the subclass Theriformes, consisting of infraclasses Placentalia (also known as Eutheria) and Marsupialia. There are

only two orders of monotremes: Platypoda (platypus) and Tachyglossa (echidnas) [1]. The duck-billed platypus (*Ornithorhynchus anatinus*) is a toothless semi-aquatic animal about half the size of a cat, which is equipped with a soft and sensitive bill. Males have a poisonous spur. Platypus inhabit rivers and lakes of south and eastern Australia. Echidnas are spiny terrestrial animals that hibernate in winter. The short-beaked echidna (*Tachyglossus aculeatus*) is abundant in all principal habitats in Australia. The much larger and endangered long-beaked echidna (genus *Zaglossus*), which has been recently categorized into three species on the basis of morphological diversity [2], is restricted to New Guinea.

Monotremes have a fascinating mixture of features typical of mammals (fur, mammary glands, regulated body temperature and most skeletal features) and features typical of reptiles and birds (egg laying, fibrillar sperm, absence of teeth, some skeletal features of the skull and shoulder girdle). Their anatomy — especially that of their skeleton und urogenital system — has given monotremes the reputation of being generally plesiomorph (ancestral), and the many highly specialized aspects of their physiology that are adapted to their lifestyle, such as the possession of electro-receptors for localizing prey [3], are often overlooked.

Although there is only one living platypus species, the fossil record of monotremes in Australia, coupled with a single fossil in South America, shows that they existed in various forms and were widespread in eastern Gondwana during the early Cretaceous period [4•]. Echidnas appeared much more recently in the fossil record, in the mid-Tertiary period (about 10–15 million years ago). It is this early divergence that makes monotremes uniquely interesting for studies of genome evolution; thus, we will evaluate the challenges to this traditional grouping.

Here we review recent investigations of X-chromosome inactivation, genomic imprinting and sex chromosome evolution, which provide good examples of the power of the monotreme genome to inform us about mammalian genome organization and evolution.

Challenges to monotreme systematics: the Theria versus Marsupionta debate

The conventional view that monotremes are the oldest branch of the mammalian tree and are placed outside the Theria (marsupials and placentals) is supported by an increasing amount of fossil data, as well as by differences in many morphological and physiological features such as

egg laying, and the conjoining of the uterus and intestine, which are separated in Theria [5]. But some morphological similarities between monotremes and marsupials, such as the possession of epipubic bones, led Gregory [6] and later Kühne [7] to propose a model in which monotremes and marsupials are aligned as sister groups within the mammalian subclass 'Marsupionta'. This model has not gained widespread acceptance because most of the characters used by Gregory and Kühne are considered ancestral or their interpretation is controversial. Fossil data also show that monotremes existed much earlier than Gregory recognized [8].

The availability of DNA technology promised to settle this controversy but has, instead, rekindled the debate because different studies favor different models (Table 1). The first results from platypus mitochondrial DNA supported the Theria hypothesis because the gene order was identical to that of amphibians, fish and placental mammals, but differed from the order of transfer RNA genes in several marsupials [9]. However, the Marsupionta theory enjoyed a renaissance when the sequences of 12 platypus and echidna mitochondrial genes proved to group consistently with marsupials to the exclusion of placentals [10,11]. Support for the Marsupionta hypothesis has also come from DNA–DNA hybridization [8], from phylogenetic analysis of ribosomal RNA sequence [12] and, ironically for the toothless platypus, from analysis of the *Amelogenin* gene, which encodes tooth enamel [13].

The molecular evidence for the Marsupionta hypothesis must be treated with caution. In the DNA–DNA hybridization experiment, the sampling of taxons was limited [8]. Moreover, this technique is considered to be useful only for divergence times of less than 50 million years [14]. The most significant evidence for the Marsupionta hypothesis (the sequence analysis of 12 mitochondrial

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Table 1

Rival theories of monotreme systematics.
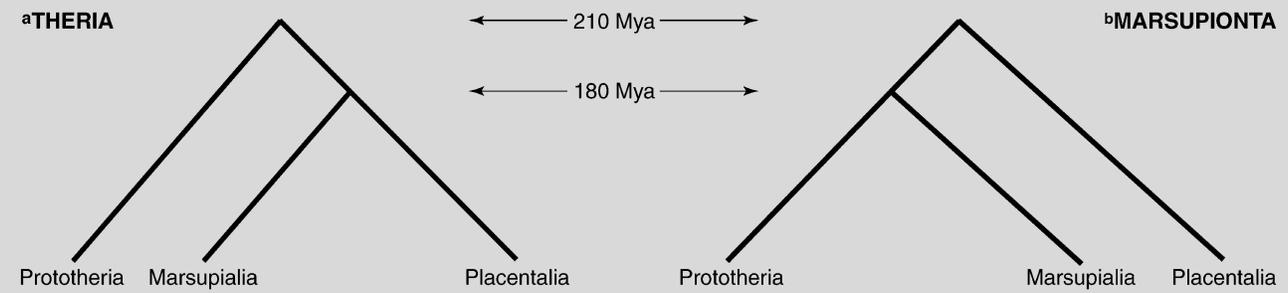
Theria			Marsupionta		
Symbol	Gene	Length (bp)/Species	Symbol	Gene	Length (bp)/Species
Mt	Mitochondrial genes	13856/O	Mt	Mitochondrial genes	9840/O
Mt	Mitochondrial genes	10764/T	Mt	Mitochondrial genes	9840/T
B2M HBB	β2-Microglobulin	939(cDNA)/O 1096(cDNA/)T	AMEL	Amelogenin	362(cDNA)/O,T
APOB	Apolipoprotein B	1300/O 1218/T		DNA–DNA hybridization	
*MHC*1	Major histocompatibility complex class-I	1828(cDNA)/O,T	18S rRNA	Ribosomal RNA	1850(cDNA)/O,T
IgA	Immunoglobulin A	1632(cNDA)/T			
IgG	Immunoglobulin G	1662(cDNA)/O,T			
IgM	Immunoglobulin M	1521(cDNA)/T			
IgE	Immunoglobulin E	470(cDNA)/T			
PRM1	Protamine P1	532/O 555/T			
NT3 BDNF NGF	Neutrophin-3 Brain-derived neutrophic factor Nerve growth factor	130(cDNA)/O,T 256(cDNA)/O,T 127(cDNA)/O,T			
LA	α-Lactalbumin	126aa/O,T			
IGF2	Insulin-like growth factor 2	1106(cDNA)/O,T 819(cDNA)/O,T			
M6P/IGF2R	Mannose 6-phosphate/Insulin-like growth factor 2 receptor	9024(cDNA)/O 1505(cDNA)/T			
	β-Globin	2308/T			
RAG1	Recombination activating gene-1	1182/O,T			

[a]The Theria hypothesis places monotremes outside marsupials and placentals; this conventional view is supported by morphological and growing molecular evidence [9,16••,52-64]. [b]The Marsupionta hypothesis is supported by limited morphological similarities between marsupials and monotremes, as well as by some molecular data [8,10–13]. Abbreviations: Mt, mitochondrial; O, *Ornithoryhnchus anatinus*; T, *Tachyglossus aculeatus*. Mya, million years ago.

Table 2

Sequencing projects in the platypus.

Project	Sequence	Mb	Accession. No	Institution/Reference
ENCODE	Genomic	1,27	NT_086567	NIH, NISC: http://www.nisc.nih.gov./The ENCODE Consortium.
Evolutionary discrimination of mammalian conserved non-genic sequences (CNGs)	58 PCR products		BX936288, CC936500, CC936499, CC936498, CC936497, CC936496, CC936495, CC936494, CC936493, CC936492, CC936491, CC936490, CC936489, CC936488, CC936487, CC936486, CC936485, CC936484, CC936483, CC936482	[21]
BAC sequencing	Genomic	0,76	CR536601, BX936293, BX936280, BX936288, BX295537	Wellcome Trust Sanger Institute

genes) has been challenged on the grounds that differences in pyrimidine frequencies between mitochondrial genomes affect the reconstruction of phylogenetic deep branching. Re-analysis using purines (A+G) and pyrimidines (T+C) pooled into two-state categories (R for A+G and Y for T+C; termed RY-coding), rather than the standard four-state categories (A, C, T and G; termed NT-coding), now clearly favors the Theria hypothesis [15,16••], but suggests that the Prototheria–Theria divergence preceded the Marsupialia–Placentalia divergence by only 10–20 million years. This contrasts with the deeper branching of the monotremes indicated by morphological analyses [16••].

Because nuclear sequences resolve deep-level phylogenetic reconstruction more reliably than do mitochondrial DNA [17], the accumulation of monotreme nuclear gene sequence has proved essential. Indeed, most of the nuclear genes used for phylogenetic analysis clearly support the conventional view that monotremes are grouped outside Theria (Table 1). This view has been confirmed by the recent integration of morphological data and nuclear sequences [18••].

We therefore take exception to the commonly used introductory statement that morphological and molecular data give contradictory views of monotreme phylogeny [11,16••,19]. Although there remain many issues about the evolution of the morphological features of monotremes, most analyses favor the Theria hypothesis. The molecular data also now come down firmly on the side of the Theria hypothesis, especially if the analysis of mitochondrial genes is dismissed as an artifact arising from biased base composition. Analyses of a growing number of nuclear genes confirm the classical view that the monotremes are the earliest branch of the mammalian lineage.

The monotreme genome
The monotreme genome is virtually unexplored territory. The size of the haploid genome of monotremes is about the same size as the human genome (3000 Mb) [20] and well within the range of mammalian genomes

(see the Animal Genome Size Database, http://www.genomesize.com).

Little monotreme sequence is currently available but its accumulation is accelerating. For platypus, 72 entries were submitted to the National Centre for Biotechnology Information (NCBI) in the seven years between 1993 and 2000, and about the same number has been submitted in the six months between January and June 2004, totalling 340 entries. Several recent sequencing projects (Table 2) [21] that include platypus testify to the emerging awareness of the importance of the monotreme genome for understanding the mammalian genome. Available sequences include many immunoglobulin and *MHC* sequences [22•,23], as well as 20 olfactory receptor sequences [24] and the whole mitochondrial genome [10]. For echidna, the total of 150 entries includes the mitochondrial genome [12], and immunoglobulin and *MHC* sequences [22•,23]. Only three entries are listed for *Zaglossus* in the NCBI.

Some global comparisons are therefore possible. A recent comparative survey of genomic sequences in a range of insects and vertebrates has shown that the platypus genome contains higher frequencies of GC and CG dinucleotides than do placental or marsupial genomes, and has a G+C content even higher than that of the *Fugu* genome. Jabbari and Bernardi [25•] claim that a lower body temperature is correlated with a higher C+G content in platypus, as it is in cold-blooded animals. Clearly, more sequence is needed to test this hypothesis — particularly valuable would be the inclusion of sequence from birds, which have a high body temperature and are evolutionarily closer to reptiles than are monotremes.

Recently the sequencing of the platypus genome has been proposed and has been given a high priority by the National Human Genome Research Institute (http://www.genome.gov/10002154). The complete sequence of a monotreme genome will provide definitive answers to the lingering phylogenetic questions. More importantly, it will help to identify conserved mammalian genes and pathways, and to establish genome changes during

mammalian evolution. It will be equally interesting to compare the sequence of monotremes with those of birds and reptiles, as to compare it with that of other mammals.

Monotreme chromosomes

Not surprisingly the karyotype of monotremes seems to contain a mixture of reptilian, mammalian and specialized features. Early cytologists considered that the karyotypes of platypus and echidna resembled those of reptiles and birds, which contain both tiny dot-like microchromosomes and normal-sized macrochromosomes [26]. Although Van Brink [27] argued that monotremes have a more continuous distribution of chromosome size, there is a striking size difference between the six large chromosomes pairs and the many small chromosomes present in all monotreme karyotypes (Figure 1). The platypus has $2n = 52$ chromosomes in both sexes [28], whereas echidna has $2n = 63$ chromosomes in males and $2n = 64$ chromosomes in females [29]. No differences have been observed in chromosome morphology, or in the number or banding patterns, among different echidna species [30].

The extraordinary discovery [31] of a multivalent chain during male meiosis in monotremes was consistent with the curious observation that several chromosomes in the monotreme karyotype seemed to lack an exact homolog.

Figure 1

Current Opinion in Genetics & Development
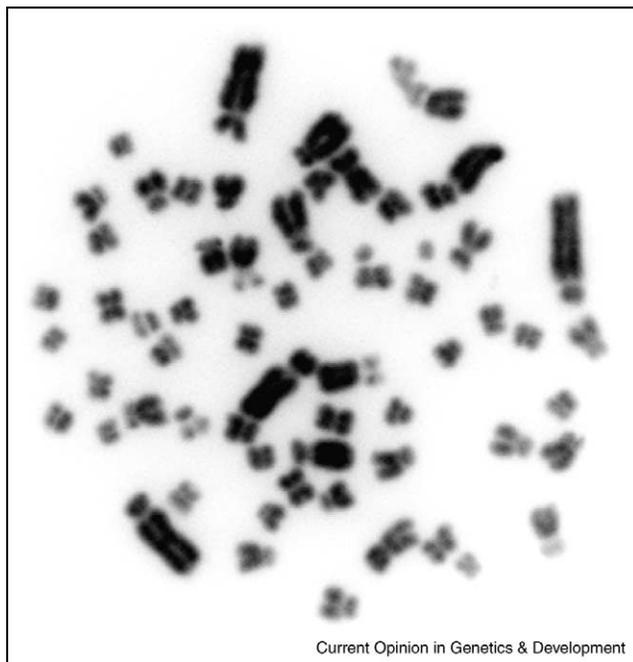
Chromosomes of the male echidna. Shown is a 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)-inverted mitotic metaphase spread. Note the marked differences in size and staining intensity between the six large pairs of chromosomes and the much smaller size of the other 51 chromosomes.

Platypus males were described as having a chain of eight chromosomes at meiosis, and echidna a chain of nine (reviewed in [32•]). An example of the meiotic chain in platypus is shown in Figure 2. Such a large, naturally occurring meiotic chain is unique among mammals. Although there are a few examples of chains involving three sex chromosomes in different mammalian orders, such translocation complexes in mice usually cause severe meiotic defects and result in a mis-segregation of chromosomes and unbalanced sperm. The monotreme chain is more akin to the complex autosomal translocation systems that are known in several plants [33] and the sex-linked complex translocation systems described in invertebrates [34].

Whether the monotreme chain represents a sex-linked or autosomal translocation system has been long debated. Reports of unpaired chromosomes in female and male karyotypes would favor a chain built up from autosomal translocations in both sexes [30]. However the first element of the translocation chain has been clearly identified as an X chromosome, defined by its presence in two copies in females and a single copy in males. Comparative mapping showed that this chromosome shares homology with part of the human X chromosome (reviewed in [32•]). It therefore seemed likely that the chain represents a complex sex-linked translocation system. Chromosome painting of platypus flow-sorted chromosomes has been used to resolve this mystery. The platypus meiotic chain consists of 10 sex chromosomes (5X and 5Y chromosomes). Interestingly, this complex sex chromosome system shows similarities with the mammalian XY and the avian ZW sex chromosome systems [35••].

Why monotremes have such a translocation system is unclear. Most of the classic explanations of the genesis of translocation chains speculate on the selective advantages of enforced heterozygosity — for example, to increase genetic relatedness in siblings (i.e. in eusocial termites) — which does not apply to monotremes. It has been also suggested that constant heterozygosity might be beneficial in inbred populations [36], but too little is known about the genetic variability within and between populations of monotremes to evaluate this proposal.

X-chromosome inactivation in monotremes?

As in other mammals, the presence of a large X chromosome in two copies in females and a single copy in males presents a problem of unequal gene dosage. This problem is solved in marsupials and placentals by silencing of one of the X chromosomes to achieve dosage compensation. In placentals, X-chromosome inactivation occurs early in female embryogenesis and is stable and heritable through subsequent mitoses [37]. Silencing of one or other of the X chromosomes involves delayed DNA replication, DNA methylation and deacetylation of histone H4, as well as a

Figure 2
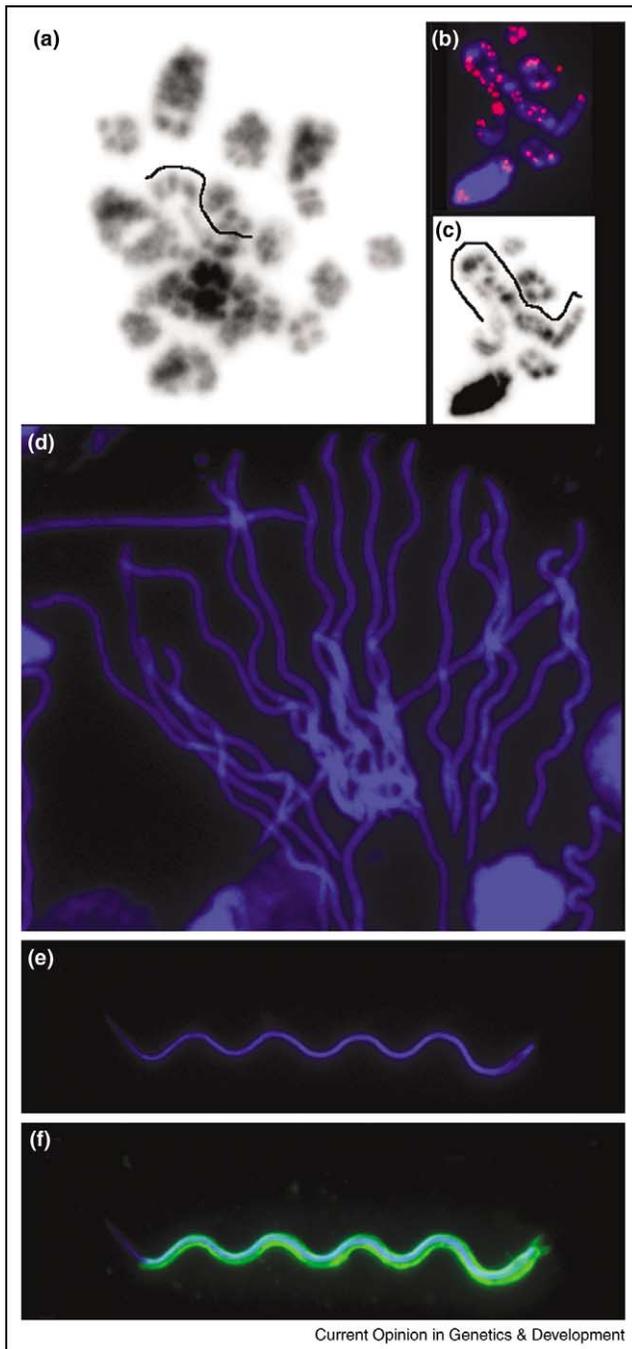
Meiosis and sperm in platypus. **(a)** Meiotic metaphase I cell in which the autosomes are paired. Below the bold line, parts of the meiotic translocation chain are visible. **(b)** Meiotic chain in the platypus. Hybridization of a telomeric repeat (taaccc)$_7$ labeled with a red fluorochrome (Cy3) as a marker to visualize the individual elements of the chain. **(c)** DAPI-inverted picture of the chain in (a). **(d)** Sperm bundle of platypus sperm stained with DAPI. **(e)** Single sperm, with the spermhead pointing left. In contrast to the round-headed sperm of eutherians and marsupials, spermatozoa of all monotreme species have a vermiform shape similar to that of reptiles and birds. Studies of the stages of spermatid differentiation have confirmed the homology of many developmental stages between monotremes and reptiles, although monotreme-specific features have been also discovered [65].

Table 3

Features of X-chromosome inactivation in mammals.

Feature	Eutheria	Marsupialia	Monotremata	Birds
Dosage compensation	+	+	+	+
Inactivation of Z/X	+, Random	+, Paternal	?	−
Late replication	+	+	Xp	−
Methylation of CpG	+	−	?	+?[a]
Underacetylation	+	+	?	?

[a] Differential methylated sites have been detected between males and females [43].

growing list of variant histones (recently reviewed in [38,39]).

In marsupials and monotremes, the X chromosome has been identified, and homology to part of the eutherian X chromosome has been demonstrated. Inactivation of the X chromosome does occur in marsupials [40], but it is significantly different in that the X chromosome that is inactivated is not random but is invariably the paternal one (Table 3). The observation that it is always the paternal X that is inactive in extraembryonic membranes of rodents [41] suggests that paternal X-chromosome inactivation was the ancestral mechanism. Marsupial X-chromosome inactivation is also incomplete and tissue-specific, and the molecular mechanism may be less complex, involving histone H4 deacetylation [42] but not DNA methylation (Table 3).

The question of whether X-chromosome inactivation also occurs in monotremes is unresolved. Replication differences have been observed on one arm of the X chromosome in lymphocytes, but these are not consistent with known homology patterns. With semi-quantitative RT-PCR, we and our colleagues [32•] recently demonstrated that dosage compensation between males and females also occurs in monotremes. Three genes, *UBE1*, *AR* and *G6PD*, representing different regions of the platypus X chromosome were cloned and sequenced, and their levels of expression were compared in cells from five males and five females. All genes showed equal levels of expression in the five male and five female cell lines. It remains unclear whether dosage compensation is achieved by upregulation of the single allele in males, by downregulation of both alleles in females or by inactivation of one allele in females as occurs in therian mammals.

It is possible that the dosage compensation system in monotremes is more similar to that in bird sex chromosomes, which seems not to involve the inactivation or asynchronous replication of one Z chromosome (Table 3)

(f) Hybridization of sperm with an α-tubulin antibody labeled with a green fluorochrome (fluorescein isothiocyanate, FITC). In the same way as for chicken, a sheath of microtubules covers the sperm.

Table 4

Imprinting of the *IGF2-M6P/IGF2R* system.

Gene	Eutheria	Marsupialia	Monotremata	Birds
IGF2 Expression	Paternal	Paternal	Biallelic	Biallelic
M6P/IGF2R Expression	Maternal/Biallelic[a]	Maternal	Biallelic	Biallelic
M6P/IGF2R Binding *IGF2*	Strong	Weak	No	No

[a] Maternal (e.g. in Artiodactyls), Biallelic (e.g. Primates).

[43]. It has been proposed that alleles on the single Z of females are upregulated by a gene on the W chromosome (the female-specific heteromorphic sex chromosome) [44]. Although Z and X chromosomes are not homologous, the absence of clear-cut asynchronous replication in monotremes leaves open the possibility that dosage compensation in monotremes is achieved in a manner similar to that in birds.

Genomic imprinting in monotremes?

Genomic imprinting describes the parent-specific mono-allelic expression of a gene. In human and mouse, more than 70 genes with diverse functions affecting growth, differentiation and behavior are expressed only if they come from the mother or from the father. As for the unpaired chromosomes, in which 5X chromosomes co-segregate to one pole and the 5Y to the other, genomic imprinting undermines the concept of diploidy and, therefore, protection against recessive mutations [45••].

Several theories have been put forward to explain genomic imprinting, but the generally accepted hypothesis is the 'conflict or kinship' theory. The best-known aspect of this model is that gene imprinting arises from a parental conflict in which it is in the best interest of the paternal genome to promote embryonic growth, but in the best interest of the maternal genome to limit growth to protect the long-term reproductive output of the mother [46].

The classic example of such an antagonistic system is the well studied interaction between the insulin-like growth factor 2 (IGF2) and the mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) (Table 4). The *IGF2* gene is expressed only from the paternal allele in placental mammals and a failure to imprint the maternal allele leads to embryonic overgrowth [47]. M6P/IGF2R, which suppresses fetal growth by binding IGF2 for degradation, is expressed predominantly from the maternal allele [48].

In placentals, the significant maternal contribution to embryonic growth is obvious through a large well-developed placenta [49]. It is therefore interesting to investigate the IGF2–M6P/IGF2 pathway in marsupials, which are born at an earlier stage, and in monotremes, which lay eggs, because both groups have significantly less intra-uterine embryo development than placental mammals.

Genomic imprinting of *IGF2* and binding between M6P/IGF2R and IGF2 have been demonstrated in marsupials, but no imprinting has been detected in monotremes. The IGF2-binding site in M6P/IGF2R of monotremes has more sequence similarity to that of birds, in which no binding occurs [50–52]. The lack of binding between M6P/IGF2R and IGF2 prevents their antagonistic inter-action and makes the imprinting of *M6P/IGF2R* needless. This suggests that the first step in the evolution of *IGF2* imprinting in viviparous mammals was suppression of the maternal allele. M6P/IGF2R later developed binding affinities for IGF2 and was subsequently imprinted. Loss of *M6P/IGF2R* imprinting in some eutherian lineages is likely to have happened independently [45••].

Thus, comparisons between monotremes and therian mammals can unscramble the steps by which a complex regulatory system such as genomic imprinting was assembled. Correlations with developmental events, although difficult to interpret, may offer clues to the selective advantage of imprinting.

Conclusions

Monotremes offer a unique perspective on the mammalian genome. The special value of these egg-laying mammals is derived from their position at the base of the mammalian lineage — a position that has been challenged once again by selected molecular data, as well as by some morphological similarities to marsupials. However, an assessment of the accumulating sequence of the nuclear genes, coupled with doubts about the interpretation of mitochondrial DNA comparisons, provides convincing evidence for the traditional Theria hypothesis of mammalian phylogeny.

Comparisons of the chromosomes and gene arrangement in monotremes with those in birds and mammals promises to identify ancestral features of the mammalian genome and to resolve outstanding issues such as the origins of the mammalian sex chromosomes and of chicken micro-chromosomes. Although little sequence is available from monotremes so far, sequencing of the platypus genome is imminent. This will enable alignments between the most distantly related mammals, which will help to identify conserved genes and the sequences that regulate them, as well as the mechanism and evolution of complex regulatory systems such as dosage compensation

and genomic imprinting. Finally, genome comparisons between monotremes and other vertebrates may inform us about the genome changes that underpin the specialized features that distinguish this extraordinary group of mammals.

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