Annu. Rev. Genet. 1996. 30:233–60

MAMMALS THAT BREAK THE RULES: Genetics of Marsupials and Monotremes

Jennifer A. Marshall Graves
School of Genetics and Human Variation, La Trobe University, Melbourne, Victoria 3083, Australia; e-mail: GENJMG@genome.latrobe.edu.au

KEY WORDS: comparative genetics, mammals, marsupials and monotremes, genome evolution, sex chromosomes

ABSTRACT

Marsupials and monotremes, the mammals most distantly related to placental mammals, share essentially the same genome but show major variations in chromosome organization and function. Rules established for the mammalian genome by studies of human and mouse do not always apply to these distantly related mammals, and we must make new and more general laws. Some examples are contradictions to our assumption of frequent genome reshuffling in vertebrate evolution, Ohno's Law of X chromosome conservation, the Lyon Hypothesis of X chromosome inactivation, sex chromosome pairing, several explanations of Haldane's Rule, and the theory that the mammalian Y chromosome contains a male-specific gene with a direct dominant action on sex determination. Significantly, it is not always the marsupials and monotremes (usually considered the weird mammals) that are exceptional. In many features, it appears that humans and, particularly, mice are the weird mammals that break more general mammalian, or even vertebrate rules.

INTRODUCTION

Genetic analysis depends on variation. Much has been learned about mammalian genes and genomes from analysis of mutations and polymorphisms within species, particularly humans and mice. Gene mapping and cloning, greatly accelerated by the Human Genome Project, have enormously increased our understanding of human genome organization, normal gene function, and alterations that cause genetic disease. Breeding and genetic manipulation of

0066-4197/96/1215-0233$08.00

the laboratory mouse offer an unparalleled experimental system to study mammalian genome organization, gene function, and control. Such studies have established the rules of mammalian gene transmission, arrangement, and genome evolution.

Analyses of differences between mammal species provide another rich source of variation that has been inadequately exploited. Comparisons between mammalian genomes provide depth to the Human Genome Project and test the generality of models extrapolated from the mouse. Mammals are a large and varied group. Comparisons between close relatives, such as man and the great apes, or between different mouse species, have enabled us to reconstruct recent evolutionary events. At the other end of the spectrum, comparisons between the most distantly related mammals—eutherians, marsupials, and monotremes—allow us to examine major changes in genome organization and function that occurred long ago, when the mammalian genome was first shaped.

It is these wider comparisons that I examine, for weird mammals may yield weird results, and weird results offer new insights and interpretations. Marsupial and monotreme mammals break long-established rules of mammalian genetics, yet their insubordination allows us to propound yet more general rules that govern mammalian genome organization, function, and evolution.

Mammal Relationships

Mammals evolved about 200 million years ago from synapsid ("mammal-like") reptiles, and are their only living descendants. There are three major extant mammal groups, the Eutheria ("placentals"), Metatheria (marsupials), and Prototheria (monotremes). Marsupials diverged from eutherians 120–150 million years before present (MYrBP), and monotremes even earlier in the 200 million-year history of Class Mammalia (43). The mammalian Infraclass Eutheria is by far the most abundant and diverse group, whose 18 extant orders diverged from each other very rapidly between 50 and 80 MYrBP.

Marsupials are famous for their distinct mode of reproduction, in which immature young are born and complete development attached to a teat, often (but not always) protected in a pouch. Two of the three orders and most of the 250 species are confined to Australasia, but one order is found in South America, with a single species in North America. The most extensively studied marsupials have been small carnivorous dasyurids ("marsupial mice") and larger herbivorous macropodids (kangaroos and wallabies), which represent two Australian orders that diverged 36–50 MYrBP. Two American didelphid marsupials (opossums), which diverged from the Australian marsupials about 80 MYrBP, have also provided useful information. Marsupials are not easy or cheap to study, but three species have been bred in captivity for some years in attempts to develop a "laboratory marsupial."

Monotremes, too, are distinguished by a unique mode of reproduction, laying eggs and feeding their young on milk secreted through glands and sucked from the fur. There are only three extant species, the platypus and two echidnas, all confined to Australasia. The platypus (*Ornithorhynchus anatinus*) and echidna (or spiny anteater, *Tachyglossus aculeatus*), though relatively common, are difficult to study because they are secretive animals in the wild and will not breed in captivity.

Marsupials and monotremes represent experiments in mammalian evolution equivalent to that of eutherians. Marsupials have been evolving independently for as long as man and mouse, and they represent just as much diversity of relationships, even if they are less abundant and widespread and are represented by fewer species. Marsupials and monotremes are in no sense "primitive" or to be regarded as "intermediate" in the evolution of eutherians. As for eutherians, some of their features may represent ancient characteristics, whereas others are highly derived.

ORGANIZATION AND EVOLUTION OF THE MAMMALIAN GENOME

Eutherian mammals have an extraordinarily conserved genome of about three billion base pairs, and they probably share most of a set of something like 70,000 genes. This common genome may be divided up into anything from 3 large to 46 small chromosomes. For a long time this karyotypic variation misled us into expecting that gene orders would be scrambled beyond recognition in different lineages.

However, the early application of somatic cell gene mapping in nonhuman eutherians revealed very large regions of homology even between different eutherian orders (72). Over the past decade, linkage mapping, somatic cell genetics, and in situ hybridization have provided quite detailed comparisons of hundreds of loci across more than 30 species (98a), establishing the rules for mammalian genome construction. The primates, carnivores, and artiodactyls, which diverged about 60MYrBP, share a few large genome pieces that have been shuffled between orders. Recent cross-species chromosome painting, using whole chromosome DNA for fluorescence in situ hybridization, has directly demonstrated that only about 30 pieces are rearranged between man and cat and 50 between man and cattle or pig (79, 80, 90, 107). Mouse shares about 90 smaller conserved units with man and other eutherian groups, scrambled by many internal rearrangements. There is a modest sex difference in eutherian recombination frequency, with males having a higher recombination than females in most genome regions.

Marsupial and Monotreme Genomes

Marsupials and monotremes have genome sizes in the range of eutherians, but their karyotypes are very distinctive. Marsupials have a few large chromosomes; indeed, for many years the swamp wallaby held the record for the lowest haploid number ($2n = 5$). Their few, large chromosomes made possible some of the earliest studies of DNA synthesis control, and some of the most thorough studies of karyotype evolution.

Extraordinary karyotypic conservation has enabled an ancestral marsupial karyotype to be deduced, a difficult task for eutherians. A "basic" $2n = 14$ karyotype, with near-identical G-band patterns, is represented in each of the major marsupial groups (82), and other marsupial karyotypes are easily derived from it. As for eutherians, different marsupial groups show different degrees of variation, from the dasyurids with almost no karyotypic variation among many species, to the macropodids with a spread of haploid numbers and chromosome morphologies. At the extreme are the rock wallabies, in which more than 20 different karyotypes are found in a very rapidly diverging species complex (20), offering a unique opportunity to study the role of chromosome change in mammal speciation. However, even among rock wallabies, these differences can be readily accounted for by simple Robertsonian changes.

Monotremes, with their few large chromosomes and small microchromosomes, were at first considered to have rather reptilian karyotypes. However, their small chromosomes are certainly not in the size range of microchromosomes in birds and reptiles. Their real peculiarity is that several small chromosomes are unpaired and form a chain at meiosis, presumably the result of translocation heterozygosity known in plants but unique among mammals (67, 108). Their other unique feature among mammals is a fibrillar sperm head, which has made it possible to study the organization of chromatin in mammalian sperm. In situ hybridization to localize unique and repeated sequences in sperm showed that chromosomes are arranged nonrandomly in tandem, in a sequence that is conserved even between platypus and echidna (100).

The limited karyotypic change in marsupial evolution has usually been regarded as an oddity of a weird group of mammals. However, the monotreme karyotype, too, appears to be stable, for platypus and echidna, which diverged about 70 MYrBP, have almost identical karyotypes (101, 108). It is the variability of eutherian karyotypes that is out of line. Indeed, comparative gene mapping presents a picture of very conserved vertebrate gene arrangements, in which eutherians, particularly rodents, are the oddity.

Gene Maps of Distantly Related Mammals

It was long assumed that, since human and mouse showed so many changes in gene arrangement, marsupial and monotreme genomes would have been

shuffled beyond recognition. Comparative gene mapping has proved that they have not.

Gene mapping in marsupials began with family studies that identified sex-linked genes and provided some autosomal linkages [reviewed in (33)]. Recent identification of DNA markers in intersubspecific crosses will greatly facilitate linkage analysis (61).

Even limited data were sufficient to establish that the marsupial genome follows its own rules of recombination. Linkage mapping in a dasyurid species showed that recombination rates are strikingly different in male and female marsupials. Rather than the minor male deficit in recombination standard for eutherians, marsupial females have far less recombination than males (4, 96) as the result of sex-dependent distribution of chiasmata. What this signifies is unknown, but this major variation in chromosome behavior during meiosis may help clarify the molecular basis of initiation of recombination.

Comparative gene mapping in marsupials and monotremes accelerated greatly with the application of somatic cell genetic analysis and in situ hybridization (33), especially in monotremes in which classical family studies are impossible. Valuable data have been wrung from the few cell hybrids that stably retain marsupial or monotreme chromosomes, and assignments have been made using in situ hybridization with human probes, or cloned wallaby or platypus homologues to conserved human genes. Several genes clustered on human chromosomes have been located together on marsupial and monotreme chromosomes. For instance, three human chromosome (HSA) 11p genes were found to map together (89), demonstrating a highly conserved segment. HSA 21 genes mapped into two autosomal clusters in marsupials and monotremes (58), allowing the evolution of human chromosome 21 from two ancestral blocks to be deduced.

This degree of conservation between such distantly related genomes may seem surprising, but in fact, comparative gene mapping of conserved loci that can be identified over vast evolutionary distances has now established homologies that extend far beyond mammals. Groups of genes that are together in human are now found to be clustered in chicken, and even in fish (107). Thus the vertebrate genome appears to have remained very stable for something like 400 million years, and differences between eutherian genomes look more and more like recent and trivial perturbations. It seems that eutherians, especially mouse, are the rule-breakers.

Much more detail has been obtained for gene content and arrangement within marsupial and montreme sex chromosomes, and it is here that distantly related mammals have provided a completely new outlook on the organization, evolution, and even function of part of the mammalian genome.

MAMMALIAN SEX CHROMOSOMES

Mammals share an XX female:XY male system of chromosomal sex determination in which the Y is male determining and the X is highly conserved because of its participation in X inactivation. Rules governing the organization and behavior of X and Y chromosomes were formulated by detailed studies in mouse and human. Marsupial and monotreme sex chromosomes show variation in size, gene content, and pairing relationships suggesting that marsupials, rather than eutherians, retain an original mammalian X and Y. In addition, marsupials offer informative variation on inactivation and even on the role of the X and Y in sex determination.

The X chromosome is unique in the genome because of its extreme conservation and the inactivation of one X in females. Ohno showed three decades ago that among eutherian mammals, the X chromosome was extraordinarily conserved in size, comprising about 5% of the haploid genome regardless of the sizes of autosomes (73). Exceptions include large chunks of heterochromatin, or are the products of recent X-autosome fusion. Ohno's Law has since been upheld by findings that gene content of the differentiated part of the X is virtually invariant in a variety of eutherians (98a). Ohno's suggestion that the X is protected from rearrangement because it lacks a pairing partner and is subject to a chromosome-wide inactivation makes excellent sense, since rearrangement would alter dosage relationships and therefore be selected against.

The Y chromosome is quite the opposite, being small and genetically impoverished. It contains few genes other than the testis-determining factor, believed to act as a master switch in male differentiation, and one or more gene(s) required for spermatogenesis. Almost all the genes on the human and mouse Y have close relatives on the X, supporting Ohno's hypothesis that they were both derived from an original autosomal pair.

Sex chromosomes are exceptional in that they are not present in duplicate in the male genome and therefore must solve problems of pairing at meiosis and dosage differences in somatic cells. Segregation of the X and Y at meiosis is accomplished in most eutherians by pairing at one end within a short genetically homologous pseudoautosomal region (PAR) (8). Deletion or rearrangement of the PAR causes failure of meiosis and spermatogenesis, at least in man and mouse. The problems of male:female dosage differences for X-borne genes are solved by inactivation of one X chromosome (56), and there is evidence that failure of inactivation causes very severe effects (63). The Lyon hypothesis that one or other X is randomly inactivated during early embryogenesis has been confirmed by many studies in human and mouse (26).

Marsupials and monotremes flout Ohno's Law, challenge Lyon's hypothesis, and break all the rules of pairing and segregation. They provide stringent

tests of the credentials of putative sex-determining and differentiating genes. In the following sections, I show how these transgressions have led to deeper understanding of the evolution and function of mammalian sex chromosomes.

Ohno's Law and the Evolution of the Mammalian X

Like eutherians, marsupials and monotremes have heteromorphic X and Y chromosomes, but their size, pairing relationships, and gene content differ in revealing ways. Marsupials have a small basic X (about 3% of the haploid complement) and a tiny Y that do not appear to undergo homologous pairing at meiosis. Monotremes are at the other extreme, having large X and Y chromosomes, which pair over the entire short arm of the X and long arm of the Y (67).

The gene contents of marsupial and monotreme X chromosomes provided the first exceptions to Ohno's Law, for only a subset of genes on the conserved eutherian X map to the X in these groups. Family studies, somatic cell genetic analysis, and in situ hybridization show that genes from the long arm and pericentric region of the human X all map to the marsupial and monotreme X (35, 91, 106). This conserved region of the X (XCR) must represent an original mammalian X that has been retained for at least 170 million years. However, it is difficult to concur with Ohno in ascribing conservation to constraints imposed by X inactivation, since in monotremes several XCR genes map to the large PAR, which is paired and needs no inactivation (103).

Ohno's Law is decisively broken by genes on the short arm of the human X, for their homologues are clustered on two or more autosomes in both marsupials and monotremes (104). Since these mammal groups diverged independently from eutherians, the most parsimonious explanation is that a region (XRA) was recently added to the eutherian X, after the divergence of the marsupials, but before the major eutherian radiations. The finding that genes on human Xp map to at least three autosomal clusters in marsupials and monotremes suggests that there might have been at least three additions.

Thus the eutherian X chromosome is composed of an original X conserved between all mammals, and a region recently added to the X in the eutherian lineage. Again, eutherians, rather than their distant relatives, are the lawbreakers.

Origins of the Mammalian Y Chromosome

The eutherian Y chromosome is composed of a differentiated region containing male-determining and fertility genes, and a pseudoautosomal region that pairs with the X and is vital for fertility. What was the evolutionary origin of these regions of the Y?

In marsupials, the Y chromosome is testis determining (84), and also contains at least four genes shared with the human and/or mouse Y (1, 24, 65; ML

Delbridge, personal communication). Marsupial and eutherian Y chromosomes are therefore likely to have a common evolutionary origin.

However, the marsupial Y is very much smaller than even the smallest eutherian Y. In dasyurid marsupials, especially, the Y is a minute euchromatic element, with a calculated size of only 10–12 Mb, compared to the 60 Mb of the human Y. Thus the marsupial Y may provide us with a model mammalian Y chromosome, free of the repetitive elements that make the human and mouse Y difficult to characterize in detail.

Comparative mapping of genes shared between the X and Y in eutherians shows that much of the increased size of the eutherian Y is due to recent additions to both sex chromosomes. Most of the genes with copies on the X and Y chromosomes in humans and mouse lie within the recently added XRA and map to the same clusters on marsupial and monotreme autosomes as do other XRA genes (102). This implies that autosomal regions were added, not only to the eutherian X, but also to the Y. It is unlikely that additions to the X and Y were independent events, so the best explanation is that the region was added initially to an ancient PAR of one partially differentiated sex chromosome, then recombined onto the other (30). The X and Y containing the added region therefore shared an enlarged PAR. The Y too must therefore contain a conserved region (YCR) and a recently added region (YRA), though these have been scrambled in evolution.

If the Y chromosome were originally homologous to the X and to sizeable autosomal regions added to it, why is it now small and genetically depauperate? It is hard to imagine how loss of gene function could confer a selective advantage, and there has been much debate about the forces driving degeneration of the Y. The key concept is that regions of the Y become genetically isolated when they are no longer able to recombine with the X. These regions can then be progressively degraded as the result of genetic drift (Muller's ratchet), or hitchhiking with a favorable mutation (15). The degradation of genes that were originally homologous on the proto-X and Y is well illustrated by *UBE1Y*. This gene maps to the large PAR in monotremes, recognizes differentiated but active copies on the X and Y in marsupials, mouse, and other eutherians, but is completely lost in humans (64). Thus a gene may show every stage in the evolution of male-specific functions, from a pseudoautosomal gene through differentiation of a Y-borne homologue and the acquisition of a male-specific function, to its eventual inactivation and loss.

Eutherian X and Y chromosomes are thus the result of cyclical addition and gradual attrition (30). A rearrangement added an autosomal region to the PAR of one sex chromosome, whence it was recombined onto the other. Initially, the added region was paired and not inactivated, but was gradually subjected

to mutation, deletion, and rearrangement. The X has therefore been enlarged in stages, whereas the Y has gone through cycles of incremental enlargement and progressive attrition.

The addition-attrition hypothesis predicts that in different lineages the sex chromosomes could have received independent autosomal additions, and the X and Y could have diverged to different extents. No such variation is observed in different eutherians, but variation is observed among the three major mammal groups, and even within marsupials. Monotreme sex chromosomes, being larger than eutherian, must include genes that are autosomal in eutherians as well as the conserved suite of XCR genes. In marsupials, on the other hand, marsupial sex chromosomes have completely differentiated, so no further addition and recombination can take place. However, there is evidence of a unique addition in kangaroo and wallaby species, in which both X and Y chromosomes, or just the X, contain a nucleolar organizer (42). This suggests that an autosomal NOR-bearing region was added to an ancient PAR of one sex chromosome, recombined onto the other, then degraded on the Y in most species.

Thus comparisons of the gene content of eutherian, marsupial, and monotreme sex chromosomes have allowed us to identify original and added regions of the Y in all three lineages. The conserved region of the mammalian Y chromosome appears to be a degraded relic of an ancestral X, and even the recently added regions of the eutherian Y have largely been degraded. Only the PAR remains.

Evolution of the Pseudoautosomal Region

Studies of aberrant sex chromosomes in mouse and man imply that X-Y pairing and recombination are vital for correct meiotic segregation, and that disruptions to the PAR lead to male infertility (11). How general is this rule?

The marsupial X and Y pair at the tips but do not form synaptonemal complex or undergo recombination (86), yet segregation is regular. The observation that marsupials show no X-Y homologous pairing immediately challenges the hypothesis that the PAR plays a universally critical role.

Even if pairing were necessary for proper segregation, how critical is the gene content of the PAR to this function? The hypothesis of cyclical addition and attrition to the sex chromosomes predicts that the PAR could have quite different gene contents in different mammal groups either because of different recent additions, or because of internal rearrangements that produce different terminal regions that are the last to be differentiated in different lineages (32). Not surprisingly, genes within and near the human PAR are autosomal in marsupials (33; R Toder, personal communication). Indeed, there is no evidence for the conservation of the PAR even between mouse and man, although both may be derived from the same addition (31). Recent work suggests that the

mouse PAR is composed largely of GC-rich repetitive sequences and contains only an atypically GC-rich *Sts* gene (21, 83). This suggests that even the PAR is ultimately degraded. Perhaps, as a last holdout against complete X-Y differentiation, its base composition is selected for pairing rather than for gene content. The present PAR in eutherian mammals therefore represents a relic of the latest addition to the sex chromosomes, and its function, if any, does not depend on its gene content.

It was recently suggested that sterility in interpecies hybrids may be the result of mispairing between a diverged PAR of the X and Y derived from different species. This could account for the generality of Haldane's Rule (38), which states that if, in an interspecific hybrid, one sex is absent, rare, or sterile, that sex is always the heterogametic sex. Here marsupials show real perversity, for one of the few rules they do obey is Haldane's Rule. In crosses between species of marsupials, it is always the heterogametic sex (male) that is infertile. Thus rearrangements within the PAR cannot provide a general explanation for Haldane's Rule, since marsupials have no PAR.

Thus comparative mapping of the marsupial and monotreme Y chromosomes tells us that the pseudoautosomal region need not be a constant, or even a necessary part of sex chromosomes, and that divergence within the PAR cannot provide a general explanation of infertility of male interspecies hybrids.

X CHROMOSOME INACTIVATION AND GENOMIC IMPRINTING

Dosage compensation for X-borne genes between XY male and XX female occurs via inactivation of one of the two X chromosomes in somatic cells of female mammals. The Lyon hypothesis (56) proposes that one or other X chromosome becomes genetically inactive and cytologically heterochromatic at an early stage of embryogenesis. This change is stably inherited in somatic cells, giving rise to a mosaic phenotype in females heterozygous for a sex-linked trait. Inactivation emanates from an inactivation center, which exerts a spreading effect in *cis* even over translocated autosomal material. Many studies of human and mouse X-linked gene function have confirmed the major tenets of this hypothesis, although some modifications have had to be proposed. For instance, genes with copies on the Y as well as the X escape inactivation, presumably because these are (or were until recently) paired by active partners on the Y and have no need of inactivation.

X chromosome inactivation is a spectacular example of gene repression on a grand scale, and its molecular basis has been enthusiastically studied. Inactivation represents transcriptional repression (34), which is associated with

interphase condensation, delayed DNA synthesis, differential DNA methylation and acetylation, and an altered chromatin conformation, which interact in a highly stable multistep regulation system (27, 28, 47, 66). Recently, the XIST gene was cloned from the inactivation center region on the human and mouse X (5–7). It is transcribed only from the inactive X, prior to the time of inactivation (51) and is essential for inactivation in *cis* (76). XIST is expressed, but the giant transcript is not translated and its action is still quite unclear.

During the past few years, it has become apparent that certain autosomal regions share at least some of the attributes of the inactive X. Several groups of genes in mouse, and their homologues in humans, have been discovered to be imprinted according to their parental origin, so that they are expressed from only the maternal, or only from the paternal allele (70, 93). Imprinting represents transcriptional repression, is tissue and stage specific, and is accompanied by differential replication time and methylation. Among other suggestions is the idea that genomic imprinting functions to limit the effects of growth factors in the embryo, or in the extraembryonic membranes, in which there is differential parental investment (37).

The study of inactivation in distantly related mammals has revealed variation that has necessitated revisions of the Lyon hypothesis, and now offers a wider view of the mechanism and evolution—and even the role—of X inactivation and genomic imprinting.

X Inactivation in Marsupials and Monotremes

X chromosome inactivation certainly occurs in marsupials and possibly monotremes, but the phenotype and mechanism are qualitatively different from that in human and mouse (reviewed in 18). One X in kangaroo females replicates late, and isozyme studies showed that only one allele is expressed in kangaroos heterozygous for X-linked traits. However, inactivation is not random, but affects only the paternal allele (81, 85). It appears to be less stable, for loci on the inactive opossum X are readily reactivated in culture (62).

Nor is X inactivation complete in marsupials. Different loci on the paternal X are inactive in different tissues. This incomplete, tissue-specific inactivation may represent a piecemeal inactivation (97) or differential spreading from an X inactivation center (29). Observations of asynchronous replication of part of the X in platypus and echidna lymphocytes, but not fibroblasts, suggest that partial and tissue-specific inactivation also occurs in monotremes (109).

Thus there are major differences in X inactivation in distantly related mammals. Again, it is tempting to brand the marsupial system as an oddity. However, paternal, incomplete, and unstable inactivation were subsequently described in eutherian extraembryonic tissue. Paternal X inactivation was found to occur

in the early differentiating extraembryonic tissues of the mouse, and instability may be a feature of X inactivation in human extraembryonic membranes (reviewed in 26). The occurrence of paternal and less stable inactivation in eutherian extraembryonic tissues, and of partial and tissue-specific inactivation in monotremes as well as marsupials, exposes the hyperstable eutherian system as the oddity.

Comparisons of the molecular mechanism of X inactivation in distantly related mammals may help to unravel the individual steps of the complex eutherian X inactivation mechanism. Late DNA replication is an invariable feature of the inactive X in all marsupial tissues, but condensed sex chromatin has not regularly been observed at interphase (18). Searches for DNA methylation differences at the promotors of X-borne genes have been unsuccessful, although internal methylation differences have been described (50, 77) similar to those, uncorrelated with activity, in human and mouse genes. Overmethylation of the active X was also indicated by in situ nick translation of kangaroo chromosomes with methyl-sensitive enzymes (55). One X is underacetylated (MJ Wakefield, unpublished). Attempts to detect a marsupial XIST homologue have been unsuccessful to date.

Thus DNA synthesis changes, condensation, and acetylation may be fundamental to X inactivation, but DNA methylation is likely to be a more recent change, perhaps associated with stable, random inactivation. If no XIST homologue exists, it will be hard to argue that XIST action is fundamental for X inactivation.

It is quite wrong to consider the marsupial X-inactivation system as some kind of imperfect early model of inactivation, which has been much improved in higher mammals. Eutherians and marsupials have been evolving independently for exactly the same time, and one system cannot be said to be more primitive than the other (nor is one mammal higher than the other!). More likely is that the incomplete X inactivation in marsupials serves a purpose in regulating dosage of an X-linked gene critical for marsupial sexual differentiation. Complete hyperstable inactivation may have evolved relatively recently in eutherians with the loss of this independent sexual differentiation pathway. Random inactivation, which bestows the protection of mosaicism on heterozygotes for disadvantageous traits, may have evolved to accompany differentiation within the inner cell mass, unique to the eutherian embryo. Again, it is eutherians that are unusual.

Genomic Imprinting in Marsupials and Monotremes?

Paternal X inactivation in marsupials provided the first evidence that genomic imprinting occurs in mammals (19). Does autosomal imprinting also occur in marsupials, and is there any evidence of an evolutionary link with X inactivation? If genomic imprinting arose as a result of an arms race between paternal

and maternal genomes, it might be expected to be apparent only in placental mammals, since extraembryonic membranes are underdeveloped in marsupials, and monotremes, after all, lay eggs.

Four genes imprinted in mouse and human have now been cloned from marsupials. It is not yet known whether they are expressed from one or both alleles. They map in the same autosomal clusters as in mouse and human (94), so they must have been located on separate autosomes for at least 150 million years. An evolutionary link with the X, if there is one, must be more ancient. It will be important to clone and map imprinted genes in monotremes and study their expression.

All these data are consistent with the hypothesis of an ancestral X-inactivation system that was paternal, unstable, incomplete, and tissue specific. Its mechanism involved late DNA synthesis, chromatin condensation, and acetylation, but not methylation. So far, there is no direct evidence for a link with autosomal imprinting in eutherian mammals.

THE ROLE OF Y-BORNE GENES IN SEXUAL DIFFERENTIATION

The hypothesis that the Y chromosome has been highly degraded predicts that only genes serving some selectable function have been preserved. Since half the population has no Y, these genes can hardly exert a function vital for survival. Thus the only selectable function open to a gene on the Y is a role in male determination or differentiation.

The sex-determining function of the eutherian Y is paramount. The development of testis triggers all the other male dimorphisms. Phenotypes of sex chromosome aneuploids clearly showed that the Y chromosome is male determining, and deletion mapping localized the "testis-determining factor" TDF to the short arm of the Y. A frenetic search for TDF ensued, culminating in the cloning of the *SRY* gene. Comparisons between the sequence and location of candidate sex-determining and differentiation genes in distantly related mammals have had an important part in the identification of TDF, and the discovery of variant *SRY* genes and exploration of its action raises questions about how it determines testis. Once more, the unusual features of sex determination in marsupials leave us with very broad questions about how sex determination evolved in mammals and how it works.

Search for the Testis-Determining Factor

Many years of painstaking deletion analysis, using DNA from "sex reversed" humans with fragments of a Y chromosome, mapped the testis-determining factor (TDF) within the distal region of the small short arm of the human Y.

The analysis of two patients narrowed the search to a small region of human Yp, and the cloning of a zinc finger gene *ZFY* from this region was greeted with much excitement, in the expectation that this gene was the long-sought TDF (74). Indeed, *ZFY* fulfilled many of the expectations of a gene thought to trigger a cascade of genetic changes. It was highly conserved between mammals and coded for a zinc finger protein containing motifs similar to those of known transcription factors. Unexpectedly, *ZFY* detected a homologue *ZFX*, which mapped to the short arm of the X chromosome.

The first indication that *ZFY* was not TDF came from the discovery that its homologues were autosomal in marsupials, mapping within clusters of other XRA genes on human Xp (88). *ZFY* mapped within the same clusters on monotreme autosomes (99). The detection of two marsupial *ZFX*/Y homologues was initially a puzzle, but recent cloning and sequencing make it clear that only one is closely homologous overall to *ZFX*/Y (C Frost, unpublished). The best explanation is that the *ZFX*/Y gene was originally autosomal and was moved to the X in eutherians along with its neighbors within the XRA. The failure of the *ZFY* gene to pass the "marsupial test" made it a very unlikely candidate for a universal mammalian testis-determining gene.

The conclusion that *ZFY* was the wrong gene was not immediately accepted. Again, the suspicion was that "marsupials are weird." Indeed, marsupial sex determination is not wholly a function of the Y chromosome, although the Y does determine testis. However, the conclusion that *ZFY* was not TDF was soon supported by the finding that its expression was not confined to the appropriate tissue. In mouse, *ZFY* is transcribed only in adult testis but is confined largely within the germ cells (52), not the somatic tissue in which TDF is active (10). Human *ZFY* is widely expressed. Ultimately, more refined mapping excluded *ZFY* from a role in sex determination, since several males were found to have fragments of the Y chromosome that did not include *ZFY* (75). This defined a new minimum sex-determining region of the human Y just proximal to the pseudoautosomal region.

A year later, the *SRY* gene was cloned from this region (87). The finding of *SRY* mutations in many human XY females supported the proposition that this gene represented TDF (41). The identity of *SRY* as the testis-determining factor was irrefutably demonstrated by the male development of XX mice transgenic for mouse *SRY* (53). Furthermore, a related gene was cloned from marsupials and shown to map to the Y (24). *SRY* had passed the marsupial test.

SRY Action and Testis Determination

Although it is accepted that *SRY* is the mammalian testis–determining factor—at least in man and mouse—it is far from clear how it acts. Marsupials offer challenges to our interpretation of a TDF that acts as a simple transcriptional activator.

It was expected that molecular and biochemical studies of *SRY* would clarify how this gene triggers testis development. Human, mouse—and kangaroo—*SRY* is a small, intronless gene. It codes for a protein with homology to an 80–amino acid DNA binding region (HMG box) shared by a wide group of proteins, including the high mobility group. *SRY* protein binds to DNA at a preferred 6-base consensus target sequence (39). The box region of *SRY* and other HMG proteins introduces specific bends into DNA, which might bring other sequences, or proteins bound to them, into juxtaposition required for activity (22). This idea is supported by the finding that proteins made by mutant *SRY* genes show anomalous binding and/or bending of DNA. It is not yet clear how binding to such a small target could be specific, nor how bending promotes associations of sequences or proteins to which they are bound.

A major puzzle is the poor homology between human, mouse, and marsupial *SRY* sequences, unexpected in a gene so critical for reproduction. Homology between the *SRY* genes is only moderate within the box, and completely absent outside it (24). This suggests that the only activity of *SRY* is to be found within the box itself, unlike related genes, which also show homology within activation domains. Thus *SRY* might act, not by activating transcription of other genes, but by intefering with the binding of other related proteins to the target.

Expression analysis of *SRY* is equally puzzling; indeed, *SRY* would have been rejected as a candidate TDF on the basis of its inconsistent expression patterns! In mouse, *Sry* transcription occurs where and when it is expected; in the genital ridge within a narrow window of embryonic development (48, 54), although transcripts (circular and probably inactive) are present in adult testis (12), and there is evidence of at least limited transcription at very early stages (111). However, *SRY* is widely expressed in man (16), and almost ubiquitously in marsupial embryos and adults (40). At least marsupials are in good (human) company here, and it is the specific expression in mouse that is the oddity. Does *SRY* have functions other than testis determination in humans and marsupials? Perhaps, as in *Drosophila*, the gene retains an ancient function that predated its recruitment into the sex-determining pathway.

Evolution of the SRY Gene

The discovery of a close relative of *SRY* on the marsupial X chromosome changed our perception of the evolution of the sex-determining function of the *SRY* gene. It was observed initially that the human *SRY* probe detected several related sequences in the genomes of other mammals (36, 87), and this was used to define a family of autosomal "*SOX*" genes (for *SRY*-like HMG box containing). This family includes more than 20 representatives, all highly conserved between species within and outside of the box. *SOX* genes may have important general functions in development (for instance of central nervous system) in both sexes (17).

The relevance of these *SOX* genes to sex determination was made clear with the discovery that *SRY* detected a closely related *SOX* gene on the marsupial X chromosome (25). This gene appeared to be the homologue of mouse *Sox*3 (36), which was subsequently found to lie on the X chromosome in eutherian mammals. It was more closely related to the human, mouse, and marsupial *SRY* genes than they were to each other. Thus the *SRY* gene, like other cloned genes on the Y, has a related sequence on the X. It was proposed that this *SOX*3 gene was present on the original mammalian proto-X and Y, and that the Y-linked allele, once isolated from recombination, took on a testis-determining function as the *SRY* gene. Since no sex-specific SRY homologues are evident in other vertebrates, this must have happened at some stage after the emergence of mammals but before the divergence of marsupials and eutherians. This timing can be refined by determining whether there is a sex-specific SRY homologue in monotremes. The stripped down box of the *SRY* gene could be a degraded version of *SOX*3, which may act to repress a related gene (*SOX*3?) in the pathway that shares its DNA binding domain (32). Thus *SRY* may act, not directly as an activator, but as a repressor of a repressor (60), or even more indirectly (30), perhaps through other sex genes (e.g. Sox9).

The hypothesis that *SRY* diverged rather recently from *SOX*3 and may have a very indirect effect on the gonadogenesis pathway implies that *SRY* took over from another sex-determining gene on the original X and Y chromosome. It is not hard to imagine how a new gene could superimpose its function on sex determination, by interacting with the old gene or its target, extending the chain of command, or short-circuiting it. Among candidates for an original mammalian sex determiner is the *DAX*1 gene on the short arm of the human X; when present in two active copies in XdupY females, *DAX*1 acts to suppress male determination (68, 110). Again the "marsupial test" may be applied to determine whether *DAX*1 was a part of the original X. It was not. Marsupial *DAX*1 lies on an autosome near it neighbors on human Xp, so was originally autosomal (A Pask, R Toder, SA Wilcox, G Camerino, JAM Graves, submitted). *SOX*3 is a more likely candidate, because it lies within the conserved original X chromosome.

SRY is thus a typical Y-borne gene, which may have diverged quite recently from an X-borne homologue, and has changed rapidly in evolution. Whereas *SOX*3 specifies almost identical products in human, mouse, and marsupials, *SRY* has evolved rapidly in rodents and primates (95, 105) and is almost unrecognizable between more distantly related mammals (24). In one group of marsupials, *SRY* has a de novo intron (RW O'Neill, FE Brennan, ML Delbridge, JAM Graves, submitted). *SRY* is amplified in several mouse and rat species (69), and a recent report demonstrates that *SRY* is absent from species of mole

vole that accomplish sex determination without the benefit of a Y chromosome (49). Evidently, recent and radical changes in the sex-determining system are apparent even among rodents. Thus marsupials inform us that *SRY* is only indirectly involved in sex determination, and weird rodents tell us that *SRY* is not really necessary at all!

SRY and Speciation

The sequence of the *SRY* gene seems surprisingly variable between species for a gene—even one that codes for a repressor—that apparently fulfills a critical role in reproduction. These variations in sequence may mean that *SRY* cannot function in a different genetic background; for instance, a large proportion of interspecific backcross mice with a *Mus poschiavinus* Y chromosome on a *M. musculus* background develop as intersexes. It is therefore possible that *SRY* variation is implicated in speciation.

This hypothesis was suggested by comparisons of *SRY* sequence between closely related mammals. In most genes with an important function, selection acts to preserve the amino acid sequence, so that synonymous base changes outnumber nonsynonymous. However, a high rate of nonsynonymous change was noted among sequences of primate *SRY* genes, and the suggestion was made that variation in this gene results in low hybrid fertility and presents a reproductive barrier between diverging populations (105). Comparisons between *SRY* sequences of Old World mouse and rat species also detected a high rate of nonsynonymous changes (95). It was suggested that the *SRY* gene is under positive directional selection, leading to reproductive isolation between two speciating groups. It has further been suggested that *SRY* is an example of a "selfish" Y-borne gene with a male advantage or female disadvantage, which evolves rapidly in response to selection for suppressors in the rest of the genome, in an intergenomic conflict that is the basis for Haldane's Rule (45).

However, a recent comparison of marsupial *SRY* sequences does not support the hypothesis. This study takes advantage of the astonishing diversity and recent origin of the rock wallaby (*Petrogale*) complex, in which 15 different species are widely distributed around Australia and offshore islands (20). These hybridize readily in nature, producing F1 that are invariably male sterile in line with Haldane's Rule. However, in this recently diverged and rapidly speciating complex, *SRY* sequences were not correspondingly diverse, suggesting that the radiation of *Petrogale* species occurred in the absence of *SRY* variation (RW O'Neill, MDB Eldridge, RH Crozier, JAM Graves, submitted). Nor was the rate of nonsynonymous substitution significantly high, contradicting the hypothesis of positive directional selection.

A better explanation of Haldane's Rule may be found in considering interactions between homologous genes on the Y and X that diverged from allele pairs.

For at least some of these genes, the Y copy might operate by interacting with (inhibiting?) the X copy. In an F1 hybrid male, the X and Y alleles come from different species and have diverged independently, such that heterospecific interactions are ineffective (34b). Since only genes with male-specific functions can be retained for a long time on the Y, hybrid males will be physically normal but sterile, and females will not be affected.

Thus there is contradictory evidence to implicate *SRY* sequence changes in reproductive isolation. Perhaps a more conservative view is that the rapid sequence evolution is more likely to indicate a lack of functional constraint on regions surrounding the HMG box, so they cannot withstand degradation of Y-borne sequences by drift or hitchhiking on the non-recombining Y.

Spermatogenesis Genes on the Y

As well as its control of male sex determination, the human and mouse Y has independent effects on meiosis and sperm production. Many normal, but sterile men have deletions of the Y chromosome that do not include *SRY*, and XX mice transgenic for *SRY* have testes, but no germcells. It is clear that one or more Y-borne genes have a role in fertility, but it is not yet clear which. Again, comparisons with distantly related mammals can inform us of the evolutionary history of candidate genes and allow us to deduce their past and present functions.

Deletion analysis, positional cloning, and expression pattern have identified several human candidate spermatogenesis genes. Loss of all or part of the long arm of the Y chromosome was associated with azoospermia, and this region was proposed to contain an azoospermia factor AZF (92). Molecular analysis of overlapping deletions in several series of infertile males mapped the putative AZF gene to Yq, just proximal to the heterochromatin (2, 3, 98).

A candidate gene *RBM1* (for *R*NA *B*inding *M*otif, gene 1, previously called *YRRM*) was isolated from a 1–2 Mb region of overlap (57). *RBM1* is present as tandemly repeated copies in two clusters on Yq. Most are probably pseudogenes, but the cloning of two different testis cDNAs suggests that at least two *RBM1* copies are transcribed. It has strong homology to a family of RNA binding proteins (HnRNP) that are involved in RNA trafficking between nucleus and cytoplasm and that may be involved in RNA splice site choice. Unlike HnRNPs, which are widely expressed, *RBM1* expression is testis specific and most prominent in the spermatogonia and/or early spermatocytes lining the walls of the testis tubules (13).

However, the putative role of *RBM1* sequences in spermatogenesis is far from confirmed, and other candidates have been proposed for AZF. Very recently, *DAZ* has been cloned from a region of the human Y just distal to *RBM1* (78), and on the basis of deletion analysis, a strong case has been put forward that

DAZ, rather than *RBM1*, represents the azoospermia factor. Like *RBM1*, *DAZ* is transcribed in testis and codes for a protein with an RNA-recognition motif, albeit a different one. Like *RBM1*, *DAZ* is present in more than one copy on the human Y.

The marsupial test may be applied to distinguishing between rival candidate spermatogenesis genes, on the grounds that genes with such a critical male-specific function will have been conserved, even though most genes on the Y have been degraded. Recent work has demonstrated a male-specific *RBM1* copy, but only an autosomal *DAZ* homologue in marsupials (ML Delbridge, unpublished). This suggests that *RBM1* performs a critical male-specific function conserved in therian mammals, but *DAZ* does not.

At least two different spermatogenesis functions have been mapped to the mouse Y (9). A spermatogenesis factor has been mapped by deletion analysis to the tiny Yp in mouse, and three genes have been cloned from this small region [*Zfy*, *Ube1y*, and *Smcy*, the candidate HY antigen (1, 59, 64)], all considered candidates. The testis-specific expression of *Zfy* in mouse is consistent with a function in germcell differentiation. However, its autosomal location in marsupials implies that any male-specific function must have been recently acquired. Its wide expression in humans, and the 2X-active status of the human *ZFX* gene may indicate that *ZFY* has recently acquired a specialized function only in the rodent lineage.

In contrast, there are *UBE1Y* and *SMCY* homologues on the marsupial Y (1, 65). The conservation of these two genes for 150 MYr against a background of degraded sequences implies that they must be functionally important. *Ube1y* is expressed in testis in the mouse and is conserved in most therian mammals. However, it is absent from the human Y, surprising for a gene critical for spermatogenesis. Surprising, too, is the finding that in both human and mouse, *Smcy* (as well as *Smcx*) is ubiquitously expressed, and *Smcx* is exempt from X inactivation. It is possible that the Y-borne copy of this gene retains its original general function in both sexes.

In comparing Y-borne genes and their function, marsupials and monotremes can hardly be said to break the rules. Indeed, there do not appear to be any rules for genes on the Y chromosome! Distantly related mammals at least provide examples of variation that inform us of the evolutionary history, and therefore the likely function, of candidate sex- determining and spermatogenesis genes, but Y-borne genes, even those with critical male-specific functions, are eventually subject to inactivation and loss.

Role of the Y Chromosome in Sex Determination

The diminished role of the Y chromosome in sex determination and differentiation in marsupials has been established for more than a decade, but is still

little understood. At the least, it may point to genes on the X with some role in sexual differentiation; at the most, it might overturn some fundamental beliefs about the role of the Y chromosome in mammalian sex determination.

The pre-eminent role of the Y chromosome in mammalian sex determination, one of the most fundamental mammalian genetic rules, was established as a result of observations on mammals with aberrant sex chromosome constitutions. In humans, XXY individuals are phenotypically male, and XO are female (23, 46). Indeed, the presence or absence of the Y chromosome appears to determine maleness or femaleness, no matter what the number of X chromosomes, and observations of X aneuploids of other eutherian species confirm this. A dominant effect of the Y is easy to explain in terms of a positive action of *SRY* in triggering the indifferent gonad to differentiate into a testis. Once formed, the embryonic testis rapidly takes control, secreting two powerful hormones that control all other aspects of sexual differentiation, including genitalia, growth, and secondary sexual characteristics like mammary development, hair, voice, even behavior. The phenotype of X aneuploids has been interpreted as a demonstration of the lack of a dosage effect of the X. However, the phenotype of sex chromosome aneuploids may not constitute a rigorous exclusion of this hypothesis, since each possesses only a single active X.

As for most wild animals, few sex chromosome aneuploids have been detected among marsupials, and none in monotremes (regrettably, there is no clinic for sterile kangaroos and platypus). However, the phenotypes of the few XXY and XO marsupials described completely contradict the rule that the Y has a male dominant effect, for they are intersexes (44, 84). Several XXY kangaroos have been described with testicles that were well developed, though undescended and empty of germcells. However, they lacked a scrotum, gubernaculum, and the long cremastor muscle typical of males, and instead possessed a pouch with well-developed mammary glands. The phenotype of XO kangaroos and Tasmanian devils was more variable but generally complementary; they lacked testis and had ovaries or undeveloped gonads. Some lacked pouch and mammary glands and had, instead, an empty scrotum, while a few showed pouch development on one side and scrotum on the other. Evidently, then, the Y chromosome determines the presence of testes in marsupials, but testicular hormones do not control development of scrotum or other male dimorphisms.

The conclusion that some sexual dimorphisms are independent of testis determination was also drawn from the observation that the development of scrotal bulges in XY wallabies precedes testis differentiation by some days (71). Scrotum and pouch/mammary gland development in marsupials appear to be mutually exclusive, and controlled by a switch of developmental potential in male or female animals. This switch is independent of the Y chromosome,

and thus of *SRY*. Rather, it appears to be operated by either the parental origins or the numbers of X chromosomes (18). Both these alternatives have exciting implications for the evolution of mammalian sex-determination and genetic control systems.

One possibility is that maternally and paternally derived X chromosomes have different effects on sexual differentiation, as a result of genomic imprinting. Female, but not male, offspring receive an X chromosome from their father as well as their mother. A gene inducing the development of female structures may be inactivated on the maternal X, but active on the paternal X. Alternatively, the switch may be operated by a dosage-sensitive gene on the X chromosome, a single dose of which determines scrotum and two doses of which determine pouch/mammary gland. The idea of dosage-sensitive sexual differentiation is of particular interest, since in other vertebrates and many invertebrates, sex determination is dose dependent. It has even been suggested the dose-dependent action of X-linked genes with homologues on the Y, and regulated by X inactivation, might underlie human sex determination (14), or might at least indicate an ancestral mechanism that has been superseded by the dominant Y-borne *SRY* gene.

Either theory would make sense of the partial inactivation of the marsupial X chromosome in some tissues. A maternally imprinted gene would need to be exempt from paternal X inactivation; likewise, a gene required in double dose must be exempt from inactivation.

Since the switch gene lies on the marsupial X, it is expected to be part of the conserved ancestral mammalian X and is therefore likely to have a human homologue. Are there any candidates for such an ancestral sex differentiating gene? We have already seen that *DAX*1 was originally autosomal and is therefore unlikely to represent the hypothesized switch gene. However, one of several genes on human Xq, mutants of which effect genitalia, may represent the ancestral homologue (18).

Evolution of Sex Determination and Differentiation in Mammals

The hormone-independent control of some male dimorphisms in marsupials suggests that these features probably evolved independently from Y-controlled sex determination. Neither scrotal development nor mammary glands were present in a reptilian ancestor. Monotremes have no scrotum, and the scrotum in marsupials and eutherians may have evolved independently. In marsupials, the scrotum is anterior to the penis, and may be the embryological analogue of the pouch (44). Thus, scrotal and mammary gland development may have come under hormonal control only recently in the eutherian lineage.

At an even more fundamental level, the hypothesis that mammalian sex determination was once subject to a very different control is hard to dismiss, if only because it is qualitatively different from systems in other vertebrates. Reptiles show a great variety of sex chromosome systems, as well as environmental sex determination in the absence of differentiated sex chromosomes. Birds have a ZZ male:ZW female system of female heterogamety, and there is no homology between the conserved region of the mammalian X and the bird Z (30). This implies that XY and ZW sex chromosome pairs evolved independently from different autosomes in a reptilian ancestor. It is likely that the male dominant action of the *SRY* gene evolved relatively recently, and there may yet be traces, in the marsupial or monotreme system, of the genes that predated it.

Comparisons between closely and distantly related mammals, even other vertebrate classes, have therefore forced us to appreciate that genes on the human and mouse Y chromosome that have a putative function in sex determination and differentiation are arbitrary and inconstant. Whereas the genes in the basic pathway of gonad differentiation are likely to be extremely conserved in vertebrates, the control of sex determination in mammals may have undergone drastic changes in recent evolutionary history.

CONCLUSIONS

Marsupials and monotremes, the mammals most distantly related to human and mouse, share essentially the same genome but show major variations in chromosome organization and function. Rules established for the mammalian genome by studies in human and mouse do not always apply, and we must make new and more general laws. Some examples are:

1. Sex differences in recombination rates favor males rather than females, suggesting that sex differences in the distribution of chiasmata are unlikely to reflect some universal selective factor.

2. Conservation of gene arrangements over a very great evolutionary distance suggests that eutherians, not marsupials, break a rule of vertebrate genome conservation.

3. Marsupials have a small (original?) X and Y and no pseudoautosomal region. This casts doubt on the universal importance of a PAR for pairing and sex chromosome segregation.

4. Marsupials and monotremes break Ohno's Law of conservation of the X, but a subset of conserved markers define a smaller ancestral X and a recently added region. Part of the monotreme X has been conserved in the

absence of inactivation, implying that inactivation cannot wholly account for conservation.

5. The eutherian and marsupial Y have a common origin, but the eutherian Y has received additions. The tiny marsupial Y is a good model for a minimal mammalian Y.

6. Features of marsupial X inactivation are shared by eutherian extraembryonic membranes and possibly monotremes, implying that it is the random, hyperstable eutherian system that is exceptional.

7. Application of "the marsupial test" can distinguish between candidate sex-determining and spermatogenesis genes. Thus *SRY*, but not *ZFY*, and *RBM1*, but not *DAZ*, were included on the original mammalian Y and are the more promising candidates.

8. The variation in *SRY* sequence between man, mouse, and kangaroo suggests that this gene acts in an indirect way to trigger testis determination, possibly through interaction with its erstwhile homologue *SOX*3, and Sox9.

9. *SRY* is not subject to directional selection in a rapidly and recently radiating marsupial species complex, so is unlikely to drive speciation.

10. Marsupial observance of Haldane's Rule implies that divergence of the PAR cannot be a general explanation. Rather, male sterility of F1 species hybrids may be due to faulty heterospecific interactions between Y-borne genes and their X-borne homologues.

11. The Y is not paramount in marsupial sex determination. Some sexual dimorphisms are determined, not by the presence or absence of a Y, but by the dosage or parental origin of the X. This suggests that an X-borne switch gene with a role in sexual differentiation has come under hormonal control in eutherians.

Thus it is not always the marsupials and monotremes—considered weird mammals—that are exceptional. In many of these features, it appears that the weird mammals are humans, and particularly mouse, which break more general mammalian, or even vertebrate rules.

ACKNOWLEDGMENTS

I thank my long-time collaborators Professor DW Cooper and Dr. RM Hope, as well as past and present members of JennyTech Laboratories, for stimulating discussions, and Dr. DL Hayman for first drawing my attention to the

genetic value of weird mammals. I am also grateful to Professor Cooper, Dr. L Selwood, and Professor MB Renfree for gifts of marsupial material. The work from my laboratory has been supported over many years by the Australian Research Council and the Australian National Health and Medical Research Council.

Literature Cited

1. Agulnik A, Mitchell MJ, Lerner JL, Woods DR, Bishop CE. 1994. A mouse Y chromosome gene encoded by a region essential for spermatogenesis and expression of male-specific minor histocompatibility antigens. *Hum. Mol. Genet.* 3:873–78
2. Andersson M, Page DC, Pettay D, Subrt I, Turleau C, et al. 1988. Y-autosome translocations and mosaicism in the etiology of 45, X maleness: assignment of fertility factor to distal Yq11. *Hum. Genet.* 79:2–7
3. Bardoni B, Zuffardi O, Guioli S, Ballabio A, Simi P, et al. 1991. A deletion map of the human Yq11 region: implications for the evolution of the Y chromosome and tentative mapping of a locus involved in spermatogenesis. *Genomics* 11:443–51
4. Bennett JH, Hayman DL, Hope RM. 1986. Novel sex differences in linkage values and meiotic chromosome behaviour in a marsupial. *Nature* 323:59–60
5. Borsani G, Tonlorenzi R, Simmler MC, Dandolo L, Arnaud D, et al. 1991. Characterization of a murine gene expressed from the inactive X chromosome. *Nature* 351:325–29
6. Brockdorff N, Ashworth A, Kay G, Cooper P, Smith S, et al. 1991. Conservation of position and exclusive expression of mouse Xist from the inactive X chromosome. *Nature* 351:329–31
7. Brown CJ, Ballabio A, Rupert JL, Lafreniere RG, Grompe M, et al. 1991. A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* 349:38–44
8. Burgoyne P. 1982. Genetic homology and crossing over in the chromosomes of mammals. *Hum. Genet.* 61:85–90
9. Burgoyne P. 1993. Deletion mapping the functions of the mouse Y chromosome. See Ref. 77a, pp. 353–68
10. Burgoyne PS, Buehr M, Koopman P, Rossant J, McLaren A. 1988. Cell-autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XX-XY chimaeric mouse testes. *Development* 102:443–50
11. Burgoyne PS, Mahadevaiah SK, Sutcliffe MJ, Palmer SJ. 1992. Fertility in mice requires X-Y pairing and a Y-chromosomal "spermiogenesis" gene mapping to the long arm. *Cell* 71:391–98
12. Capel B, Swain A, Nicolis S, Hacker A, Walter N, et al. 1993. Circular transcripts of the testis-determining gene *SRY* in adult mouse testis. *Cell* 73:1019–30
13. Chandley AC. 1995. The genetic basis of male infertility. *Reprod. Med. Rev.* 4:1–8
14. Chandra HS. 1985. Is human X chromosome inactivation a sex-determining device? *Proc. Natl. Acad. Sci. USA* 82:6947–49
15. Charlesworth B. 1991. The evolution of sex chromosomes. *Science* 251:1030–33
16. Clepet C, Schafer AJ, Sinclair AH, Palmer MS, Lovell-Badge R, Goodfellow PN. 1993. The human *SRY* transcript. *Hum. Mol. Genet.* 2:2007–12
17. Collignon J, Sockanathan S, Hacker A, Cohen-Tannoudji M, Norris D, et al. 1996. A comparison of the properties of *Sox3* with *Sry* and two related genes, *Sox1* and *Sox2*. *Development* 122:509–20
18. Cooper DW, Johnston PG, Watson JM, Graves JAM. 1993. X-inactivation in marsupials and monotremes. *Dev. Biol.* 4:117–28
19. Cooper DW. 1971. Directed genetic change model for X chromosome inactivation in eutherian mammals. *Nature* 230:292–94
20. Eldridge MDB, Close RL. 1993. Radia-

tion of chromosome shuffles. *Curr. Opin. Genet. Dev.* 3:915–22

21. Ellison JW, Li X, Francke U, Shapiro LJ. 1996. Rapid evolution of human pseudoautosomal genes and their mouse homologs. *Mamm. Genome* 7:25–30
22. Ferrari S, Harley VR, Pontiggia A, Goodfellow PN, Lovell-Badge R, Bianchi ME. 1992. *SRY*, like HMG1, recognises sharp angles in DNA. *EMBO J.* 11:4497–506
23. Ford CE, Jones KW, Polani PE, Almida JC, Briggs JH. 1959. A sex chromosome anomaly in the case of gonadal dysgenesis. *Lancet* i:711–13
24. Foster JW, Brennan FE, Hampikian GK, Goodfellow PN, Sinclair AH, et al. 1992. Evolution of sex determination and the Y chromosome: *SRY*-related sequences in marsupials. *Nature* 359:531–33
25. Foster JW, Graves JAM. 1994. An *SRY*-related sequence on the marsupial X chromosome: implications for the evolution of the mammalian testis-determining gene. *Proc. Natl. Acad. Sci. USA* 91:1927–31
26. Gartler SM, Dyer KA, Goldman MA. 1992. Mammalian X chromosome inactivation. *Mol. Genet. Med.* 2:121–60
27. Gartler SM, Dyer KA, Graves JAM, Rocchi M. 1985. A two-step model for mammalian X-chromosome inactivation. In *Chemistry, Biochemistry and Biology of DNA Methylation*, ed. GL Cantoni, A Razin, pp. 223–35. New York: Liss
28. Graves JAM. 1982. 5-azacytidine-induced re-expression of alleles on the inactive X chromosome in a hybrid mouse cell line. *Exp. Cell Res.* 141:99–105
29. Graves JAM, Dawson GW. 1988. The relationship between position and expression of genes on the kangaroo X chromosome suggests a tissue-specific spread of inactivation from a single control site. *Genet. Res.* 51:103–9
30. Graves JAM. 1995. The origin and function of the mamalian Y chromosome and Y-borne genes–an evolving understanding. *BioEssays* 17:311–20
31. Graves JAM. 1996. Breaking laws and obeying rules. *Nat. Genet.* 12:121
32. Graves JAM. 1996 Evolution of the mammalian Y chromosome and Y-borne genes. In *Gene Families: Structure, Function, Genetics and Evolution*, ed. RS Holmes, HA Lim, pp. 201–11. New Jersey: World Sci. Publ.
33. Graves JAM, Cooper DW, McKenzie LM Hope RM, Watson JM. 1994. Genetic maps of marsupial and monotreme mammals. In *Genetic Maps; Locus Maps of Complex Genomes*, ed. SJ O'Brien, 4:282–91. Cold Spring Harbor: Cold Spring Harbor Press
34. Graves JAM, Gartler SM. 1986. Mammalian X chromosome inactivation: testing the hypothesis of transcriptional control. *Somat. Cell Mol. Genet.* 12:275–80

34a. Graves JAM, Hope RM, Cooper DW, eds. 1990. *Mammals from Pouches and Eggs: Genetics, Breeding and Evolution of Marsupials and Monotremes.* Melbourne: CSIRO

34b. Graves JAM, O'Neill RJW. 1996. Sex chromosome evolution and Haldane's Rule. *J. Hered*. In press

35. Graves JAM, Watson JM. 1991. Mammalian sex chromosomes: evolution of organization and function. *Chromosoma* 101:63–68
36. Gubbay J, Collignon J, Koopman P, Capel B, Economou A, et al. 1990. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* 346:245–50
37. Haig D. 1993. Genetic conflicts in human pregnancy. *Q. Rev. Biol.* 68:495–532
38. Haldane JBS. 1922. Sex ratio and unisexual sterility in hybrid zones. *J. Genet.* 12:101–9
39. Harley VR, Jackson DI, Hextall PJ, Hawkins JR, Berkovitz GD, et al. 1992. DNA binding activity of recombinant *SRY* from normal males and XY females. *Science* 255:453–56
40. Harry JL, Koopman P, Brennan FE, Graves JAM, Renfree MB. 1995. Widespread expression of the testis-determining gene *SRY* in marsupials [erratum *Nat. Genet.* 11;472]. *Nat. Genet.* 11:347–49
41. Hawkins JR, Taylor A, Berta P, Levilliers J, Van der Auwera B, Goodfellow PN. 1992. Mutational analysis of *SRY*: nonsense and missense mutations in XY sex reversal. *Hum. Genet.* 88:471–74
42. Hayman DL. 1990. Marsupial cytogenetics. See Ref. 34a, pp. 189–207
43. Hope RM, Cooper S, Wainwright B. 1990. Globin macromolecular sequences in marsupials and monotremes. See Ref. 34a, pp. 147–72
44. Hughes RL, Pearse AM, Cooper DW, Joss JMP, Johnston PG, Jones MK. 1993. The genetic basis of marsupial gonadogenesis and sexual phenotype. See Ref. 77a, pp. 17–48
45. Hurst LD, Pomiankowski A. 1991. Causes of sex ratio bias may account for unisexual sterility in hybrids: a new

explanation of Haldane's rule and related phenomena [erratum *Genetics* 1991, 129:603]. *Genetics* 128:841–58
46. Jacobs PA, Strong JA. 1959. A case of human intersexuality having a possible XXY sex-determining mechanism. *Nature* 183:302
47. Jeppesen P, Turner BM. 1993. The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell* 74:281–89
48. Jeske YWA, Bowles J, Greenfield A, Koopman P. 1995. Expression of a linear *SRY* transcript in the mouse genital ridge. *Nat. Genet.* 10:480–82
49. Just W, Rau W, Vogel W, Akhverdian M, Fredga K, et al. 1995. Absence of *SRY* in species of the vole *Ellobius*. *Nat. Genet.* 11:117–18
50. Kaslow D, Migeon BR. 1987. DNA methylation stabilizes X chromosome inactivation in eutherians but not in marsupials: evidence for multistep maintenance of mammalian X dosage compensation. *Proc. Natl. Acad. Sci. USA* 84:6210–14
51. Kay GF, Penny GD, Patel D, Ashworth A, Brockdorff N, Rastan S. 1993. Expression of Xist during mouse development suggests a role in the initiation of X chromosome inactivation. *Cell* 72:171–82
52. Koopman P, Gubbay J, Collignon J, Lovell-Badge R. 1989. *ZFY* gene expression patterns are not compatible with a primary role in mouse sex determination. *Nature* 342:940–42
53. Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R. 1991. Male development of chromosomally female mice transgenic for *SRY*. *Nature* 351:117–21
54. Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R. 1990. Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature* 348:450–52
55. Loebel DA, Johnston PG. 1993. Analysis of DNase 1 sensitivity and methylation of active and inactive X chromosomes of kangaroos (*Macropus robustus)* by in situ nick translation. *Chromosoma* 102:81–87
56. Lyon MF. 1961. Gene action in the X-chromosome of the mouse (*Mus musculus* L). *Nature* 190:372–73
57. Ma K, Inglis JD, Sharkey A, Bickmore WA, Hill RE, et al. 1993. A Y chromosome gene family with RNA-binding protein homology: candidates for the azoospermia factor *AZF* controlling human spermatogenesis. *Cell* 75:1287–95
58. Maccarone P, Watson JM, Francis D, Kola I, Graves JAM. 1992. Human chromosome 21 genes map in two conserved autosomal clusters in marsupials and monotremes. *Genomics* 13:1119–24
59. Mardon G, Mosher R, Disteche CM, Nishioka Y, McLaren A, Page DC. 1989. Duplication, deletion, and polymorphism in the sex-determining region of the mouse Y chromosome. *Science* 243:78–80
60. McElreavey KE, Vilain E, Abbas N, Herskowitz I, Fellous M. 1993. A regulatory cascade hypothesis for mammalian sex determination: *SRY* represses a negative regulator of male development. *Proc. Natl. Acad. Sci. USA* 90:3368–72
61. McKenzie LM, Collet C, Cooper DW. 1993. Use of a subspecies cross for efficient development of a linkage map for a marsupial mammal, the tammar wallaby (*Macropus eugenii*). *Cytogenet. Cell Genet.* 64:264–67
62. Migeon BR, de BS, Axelman J. 1989. Frequent derepression of G6PD and HPRT on the marsupial inactive X chromosome associated with cell proliferation in vitro. *Exp. Cell Res.* 182:597–609
63. Migeon BR, Luo S, Stasiowski BA, Jani M, Axelman J, et al. 1993. Deficient transcription of XIST from tiny ring X chromosomes in females with severe phenotypes. *Proc. Natl. Acad. Sci. USA* 90:12025–29
64. Mitchell MJ, Woods DR, Tucker PK, Opp JS, Bishop CE. 1991. Homology of a candidate spermatogenic gene from the mouse Y chromosome to the ubiquitin-activating enzyme E1. *Nature* 354:483–86
65. Mitchell MJ, Woods DR, Wilcox SA, Graves JAM, Bishop CE. 1992. The marsupial Y chromosome encodes a homologue of the mouse Y-linked candidate spermatogenesis gene *UBE1Y*. *Nature* 359:528–31
66. Mohandas T, Sparkes RS, Shapiro LJ. 1981. Reactivation of an inactive human X chromosome: Evidence for X inactivation by DNA methylation. *Science* 211:393–96
67. Murtagh CE. 1977. A unique cytogenetic system in monotremes. *Chromosoma* 65:37–57
68. Muscatelli F, Strom TM, Walker AP, Zanaria E, Recan D, et al. 1994. Mutations in the *DAX–1* gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. *Nature* 372:672–76

69. Nagamine CM, Carlisle C. 1994. Duplication and amplification of the *SRY* locus in Muridae. *Y Chromosome Workshop (Abstr. 24)*
70. Nicholls RD. 1993. Genomic imprinting and uniparental disomy in Angelman and Prader-Willi syndromes: a review. *Am. J. Med. Genet.* 46:16–25
71. O WS, Short RV, Renfree MB, Shaw G. 1988. Primary genetic control of somatic sexual differentiation in a mammal. *Nature* 331:716–17
72. O'Brien SJ, Seuanez HN, Womack JE. 1988. Mammalian genome organization: an evolutionary view. *Annu. Rev. Genet.* 22:323–51
73. Ohno S. 1967. *Sex Chromosomes and Sex Linked Genes.* Berlin: Springer-Verlag
74. Page DC, Mosher R, Simpson EM, Fisher EMC, Mardon G, et al. 1987. The sex determining region of the human Y chromosome encodes a finger protein. *Cell* 51:1091–104
75. Palmer MS, Sinclair AH, Berta P, Ellis NA, Goodfellow PN, et al. 1989. Genetic evidence that *ZFY* is not the testis-determining factor. *Nature* 342:937–39
76. Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N. 1996. Requirement for *Xist* in X chromosome inactivation. *Nature* 379:131–37
77. Piper AA, Bennett AM, Noyce L, Swanton MK, Cooper DW. 1993. Isolation of a clone partially encoding hill kangaroo X-linked hypoxanthine phosphoribosyltransferase: sex differences in methylation in the body of the gene. *Somat. Cell Mol. Genet.* 19:141–59

77a. Reed KC, Graves JAM, eds. 1993. *Sex Chromosomes and Sex Determining Genes.* Chur, Switzerland: Harwood Academic

78. Reijo R, Lee T-Y, Salo P, Alagappan R, Brown LG, et al. 1995. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat. Genet.* 10:383–93
79. Rettenberger G, Klett C, Zechner U, Bruch J, Just W, et al. 1995. ZOO-FISH analysis: cat and human karyotypes closely resemble the putative ancestral mammalian karyotype. *Chromosome Res.* 3:479–86
80. Rettenberger G, Klett C, Zechner U, Kunz J, Vogel W, Hameister H. 1995. Visualization of the conservation of synteny between humans and pigs by heterologous chromosomal painting. *Genomics* 26:372–78
81. Richardson BJ, Czuppon AB, Sharman GB. 1971. Inheritance of glucose–6-phosphate dehydrogenase variation in kangaroos. *Nat. New Biol.* 230:154–55
82. Rofe R, Hayman D. 1985. G-banding evidence for a conserved complement in the Marsupialia. *Cytogenet. Cell Genet.* 39:40–50
83. Salido EC, Li XM, Yen PH, Martin N, Mohandas T, Shapiro LJ. 1994. Cloning of the mouse steroid sulfatase (*Sts*) gene. *Am. J. Hum. Genet.* 55:A138
84. Sharman G, Hughes R, Cooper D. 1990. The chromosomal basis of sex differentiation in marsupials. *Aust. J. Zool.* 37:451–66
85. Sharman GB. 1971. Late DNA replication in the paternally derived X chromosome of female kangaroos. *Nature* 230:231–32
86. Sharp P. 1982. Sex chromosome pairing during male meiosis in marsupials. *Chromosoma* 86:27–47
87. Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, et al. 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* 346:240–44
88. Sinclair AH, Foster JW, Spencer JA, Page DC, Palmer M, et al. 1988. Sequences homologous to *ZFY*, a candidate human sex-determining gene, are autosomal in marsupials. *Nature* 336:780–83
89. Sinclair AH, Graves JAM. 1991. Gene mapping in marsupials and monotremes. VI. Detection of an ancient autosomal gene cluster. *Genomics* 9:581–86
90. Solinas-Toldo S, Lengauer C, Fries R. 1995. Comparative genome map of human and cattle. *Genomics* 27:489–96
91. Spencer JA, Watson JM, Graves JAM. 1991. The X chromosome of marsupials shares a highly conserved region with eutherians. *Genomics* 9:598–604
92. Tiepolo L, Zuffardi O. 1976. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. *Hum. Genet.* 34:119–24
93. Tilghman SM. 1992. Parental imprinting in the mouse. *Harvey Soc. Lect.* 87:69–84. New York: Wiley-Liss
94. Toder R, Wilcox SA, Smithwick M, Graves JAM. 1996. The human/mouse imprinted genes IGF2, H19, SNRPN and ZNF127 map to two conserved autosomal clusters in a marsupial. *Chromosome Res.* 4:295–300
95. Tucker PK, Lundrigan BL. 1993. Rapid evolution of the sex determining locus

in Old World mice and rats. *Nature* 364:715–17
96. van Oorschot RAH, Porter PA, Kammerer CM,VandeBerg JL. 1992. Severely reduced recombination in females of the South American marsupial *Monodelphis domestica*. *Cytogenet. Cell Genet.* 60:64–67
97. VandeBerg JL, Robinson ES, Samollow PB, Johnston PG. 1987. X-linked gene expression and X-chromosome inactivation: marsupials, mouse and man compared. *Isozymes: Curr. Top. Biol. Med. Res.* 15:225–53
98. Vogt P, Chandley AC, Hargreave TB, Keil R, Ma K, Sharkey A. 1992. Microdeletions in interval 6 of the Y chromosome of males with idiopathic sterility point to disruption of AZF, a human spermatogenesis gene. *Hum. Genet.* 89:491–96
98a. Wakefield MJ, Graves JAM. 1996. Comparative vertebrate maps. *Mamm. Genome* 9. In press
99. Watson JM, Frost C, Spencer JA, Graves JAM. 1993. Sequences homologous to the human X and Y-borne zinc finger protein genes (*ZFY*/Y) are autosomal in monotreme mammals. *Genomics* 15:317–22
100. Watson JM, Meyne J, Graves JAM. 1996. Ordered tandem arrangement of chromosomes in the sperm heads of monotreme mammals. *Proc. Natl. Acad. Sci. USA* 93: In press
101. Watson JM, Riggs A, Graves JAM. 1992. Gene mapping studies confirm the homology between the platypus X and echidna X1 chromosomes and identify a conserved ancestral monotreme X chromosome. *Chromosoma* 101:596–601
102. Watson JM, Spencer JA, Graves JAM, Snead ML, Lau EC. 1992. Autosomal localization of the amelogenin gene in monotremes and marsupials: implications for mammalian sex chromosome evolution. *Genomics* 14:785–89
103. Watson JM, Spencer JA, Riggs AD, Graves JAM. 1990. The X chromosome of monotremes shares a highly conserved region with the eutherian and marsupial X chromosomes despite the absence of X chromosome inactivation. *Proc. Natl. Acad. Sci. USA* 87:7125–29
104. Watson JM, Spencer JA, Riggs AD, Graves JAM. 1991. Sex chromosome evolution: platypus gene mapping suggests that part of the human X chromosome was originally autosomal. *Proc. Natl. Acad. Sci. USA* 88:11256–60
105. Whitfield LS, Lovell-Badge R, Goodfellow PN. 1993. Rapid sequence evolution of the mammalian sex-determining gene *SRY*. *Nature* 364:713–15
106. Wilcox SA, Watson JM, Spencer JA, Graves JAM. 1996. Comparative mapping identifies the fusion point of an ancient mammalian X-autosomal rearrangement. *Genomics* 35:66–70
107. Workshop Comparative Gene Mapping Organization. 1996. *Mamm. Genome* 9: In press
108. Wrigley JM, Graves JAM. 1988. Karyotypic conservation in the mammalian order monotremata (subclass Prototheria). *Chromosoma* 96:231–47
109. Wrigley JM, Graves JAM. 1988. Sex chromosome homology and incomplete, tissue-specific X-inactivation suggest that monotremes represent an intermediate stage of mammalian sex chromosome evolution. *J. Hered.* 79:115–18
110. Zanaria E, Muscatelli F, Bardoni B, Strom TM, Guioli S, et al. 1994. An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. *Nature* 372:635–41
111. Zwingman T, Erickson RP, Boyer T, Ao A. 1993. Transcription of sex-determining region genes *SRY* and *ZFY* in the mouse preimplantation embryo. *Proc. Natl. Acad. Sci. USA* 90:814–17

Annual Review of Genetics
Volume 30, 1996

CONTENTS