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Sex Determination in Marsupials: Evidence for a Marsupial-Eutherian [and Discussion] Dichotomy

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Sex determination in marsupials: evidence for a marsupial–eutherian dichotomy

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[Plate 1]

In this paper, we review briefly the current state of knowledge about sexual differentiation in eutherian mammals, and then describe the situation in detail in two marsupial species: the North American opossum and the tammar wallaby.

The conventional explanation for the genesis of all male somatic sexual dimorphisms in mammals is that they are a consequence of the systemic action of testicular hormones. In the absence of testes, the embryo will develop a female phenotype.

We present evidence for the tammar wallaby that calls into question the universal applicability of this hormonal theory of mammalian sexual differentiation. We have shown that extensive somatic sexual dimorphisms precede by many days the first morphological evidence of testicular formation, which does not occur until around the third day of pouch life. Male foetuses, and pouch young on the day of birth, already have a well-developed gubernaculum and processus vaginalis, paired scrotal anlagen, and a complete absence of mammary anlagen, whereas female foetuses and newborn pouch young have a poorly developed gubernaculum and processus vaginalis, no scrotal anlagen, and well-developed mammary anlagen. Because it seems unlikely that the male gonad could begin hormone secretion until after the Sertoli and Leydig cells are developed, our results strongly suggest that some sexually dimorphic somatic characteristics develop autonomously, depending on their genotype rather than the hormonal environment to which they are exposed.

We have been able to confirm the hormonal independence of the scrotum, pouch and mammary gland by administering testosterone propionate daily by mouth to female pouch young from the day of birth; although the Wolffian duct was hyperstimulated, there was no sign of scrotal development, or pouch or mammary inhibition. When male pouch young were treated with oestradiol benzoate in a similar fashion, there was hyperstimulation of the Müllerian duct and inhibition of testicular migration and development, but no sign of scrotal inhibition or pouch or mammary development. Our results in the tammar wallaby are consistent with the earlier studies on the opossum, whose significance was not appreciated at the time.

Further evidence in support of this hormonal independence comes from earlier studies of spontaneously occurring intersexes in several species of marsupial, including the opossum and the tammar wallaby. An XXY individual had intra-abdominal testes and complete masculinization of the male reproductive tract internally, but externally there was a pouch and mammary glands and no scrotum. A similar picture was found in two XY individuals. On the other hand, an XO individual had hypoplastic ovaries, normal development of the female reproductive tract internally, and an empty scrotum. Thus the scrotum can develop in the absence of a testis, whereas the pouch and mammary glands can develop in the presence of one.

These results suggest a fundamental dichotomy between marsupials and eutherians

in their sex-determining mechanisms. Although both subclasses probably require a Y-linked gene or genes for testis determination, marsupials appear to use other X-linked genes to control the development of structures such as the scrotum, pouch and mammary glands. In eutherians, on the other hand, scrotal and mammary development appears to be entirely under hormonal control. The lack of any genetic interchange between the X and the Y during meiosis in marsupials has presumably resulted in a much greater degree of genetic isolation of one sex chromosome from the other than is the case in eutherians, and the small size of the marsupial Y suggests that marsupials may have progressed further than eutherians in capture of genetic material by the X from the ancestral Y. Marsupials seem destined to play a vital role in the years to come in the mapping of sex-linked genes and determining their modes of action. Clearly they have much to tell us about the evolution of sex-determining mechanisms in all mammals.

Introduction

The conventional view of mammalian sexual differentiation has been that a gene or genes on the Y chromosome causes the indifferent gonad to develop into a testis, which then secretes two classes of hormone which are responsible respectively for masculinizing the Wolffian duct derivatives and the external genitalia to form the male reproductive tract, and bringing about atrophy of the Müllerian duct derivatives. In the absence of a Y chromosome, the indifferent gonad develops into an ovary which is endocrinologically quiescent to begin with, so that the Wolffian duct derivatives atrophy, the male external genitalia fail to develop, and the Müllerian ducts persist to form the female reproductive tract. All somatic sexual dimorphisms have therefore been assumed to be a consequence of gonadal hormone action (Ohno 1967; Jost *et al.* 1973).

Recent research has begun to fill in the fine details of this general conceptual framework. Page *et al.* (1987) have succeeded in cloning a 230 kilobase segment of the human Y chromosome which contains some or all of the testis-determining gene, and it now seems certain that the H-Y antigen is not involved in the process of testis determination (Simpson *et al.* 1987). The first morphological evidence of transformation of the indifferent gonad into a testis is the appearance of primordial Sertoli cells, which later aggregate to form the seminiferous cords (Magre 1985). These Sertoli cells appear to develop autonomously, under the influence of a Y-linked testis-determining gene (Burgoyne *et al.* 1988). They subsequently produce Müllerian-inhibiting substance (MIS), elsewhere referred to as anti-Müllerian hormone (AMH), a 144 kDa glycoprotein dimer of 575 amino acids, which has recently been isolated and sequenced with its regulatory gene (Cate *et al.* 1986). We have identified MIS activity by bioassay in the testes of newborn tammar wallabies from the time of first testicular formation, and have shown that it is undetectable in ovaries at comparable stages of development (Hutson *et al.* 1988). In addition to causing regression of the Müllerian ducts, MIS appears to inhibit the mitotic division of female but not of male primordial germ cells, and in culture it can bring about a transformation of cells of the indifferent female gonadal blastema into Sertoli cells; MIS is therefore probably responsible for the ovarian arrest and subsequent testicular transformation in the freemartin (Vigier *et al.* 1987). There is also growing evidence to suggest that MIS may play a role in the transabdominal migration of the testes to the internal inguinal ring, although subsequent descent into the scrotum appears to be androgen dependent (Hutson & Donahoe 1986; Hutson *et al.* 1988).

The female (XX) germ cells of eutherian mammals also appear to be under some degree of

autonomous genetic control because they are incapable of producing normal spermatozoa even when in a testis (Short 1972; Burgoyne 1987). However, male germ cells can undergo oogenesis in an ovarian environment, and this subject will be discussed at length elsewhere in this symposium.

The Virginia opossum, *Didelphis virginiana*

A great deal of the early work on mammalian sexual differentiation was done in this species by workers in North America during the 1930s and 1940s (see review by Renfree *et al.* 1987; Short *et al.* 1988). These pioneering investigators established that in this marsupial, where the young weigh a mere 125 mg at birth following a 13-day gestation, the whole of sexual differentiation takes place after birth, during pouch life. First, the indifferent gonad develops into a testis on the third to fourth day; the ovary does not become recognizable as such until about the seventh day. It is claimed that nipples and mammary anlagen are present in both sexes at birth, but they subsequently regress in the male. The scrotum first becomes apparent in males on about day 10, and is said to form by fusion of the caudal ends of the developing pouch folds. The rest of the pouch then regresses in males, and persists in females, so it has been suggested that the pouch and scrotum share a partial homology.

The Wolffian duct starts to differentiate into the male reproductive tract from day 20 onwards, whereas in females it starts to regress at this time. The Müllerian duct begins to involute in males at around day 20 also, whereas in females it differentiates into the female reproductive tract. The testes begin their trans-abdominal migration soon after day 14 and enter the inguinal canal at about day 30, with testicular descent into the scrotum being complete by about day 80. Prostatic buds start to develop from the urogenital sinus of males at around day 16, coincident with the time at which the phallus of the male starts to enlarge more than that of the female, to form the characteristic bifid glans penis of the opossum.

There is nothing in the above description that is in any way out of keeping with the sequence of events that would be encountered in a eutherian mammal except, of course, the development of the pouch. However, two incidental observations whose significance was not appreciated by these early investigators should have alerted us to a major difference from the eutherian plan.

In 1925, Carl Hartman described an intersex opossum that had essentially a male external phenotype, except that the scrotum was empty, and there were some pouch rudiments still visible; nipples and mammary glands were absent (Hartman & League 1925). Internally, the animal had a normal, although infantile, female reproductive tract and no Wolffian duct derivatives. The gonads appeared to be hypoplastic ovaries containing numerous small cystic follicles, such as are sometimes found in normal females, but there were no oocytes present. Apart from the presence of the empty scrotum, the absence of mammary glands and the rudimentary pouch, the only other indications of masculinization were a normal male phallus, and the large size of the animal (1.75 kg) with a typical male-type head.

In retrospect, the remarkable feature of this animal is that internally it had a normal female phenotype, whereas externally it was male. The absence of Wolffian duct derivatives, and the persistence of Müllerian ducts, is proof that there could not have been a functional testis present during the first month of life when the internal and external genitalia was being formed. So how could the scrotum develop, and the pouch and mammary glands undergo atrophy in the absence of androgen? The answer to that question will gradually become apparent from the

discussion that follows. The one unexplained feature is the presence of a normal penis; perhaps the sterile ovaries eventually produced sufficient androgen to masculinize the phallus later in life.

The second observation comes from the results of the work of R. K. Burns on androgen and oestrogen administration to newborn opossum pouch young, although its significance completely escaped him at the time (Burns 1961). In one of his illustrations (see figure 1), he

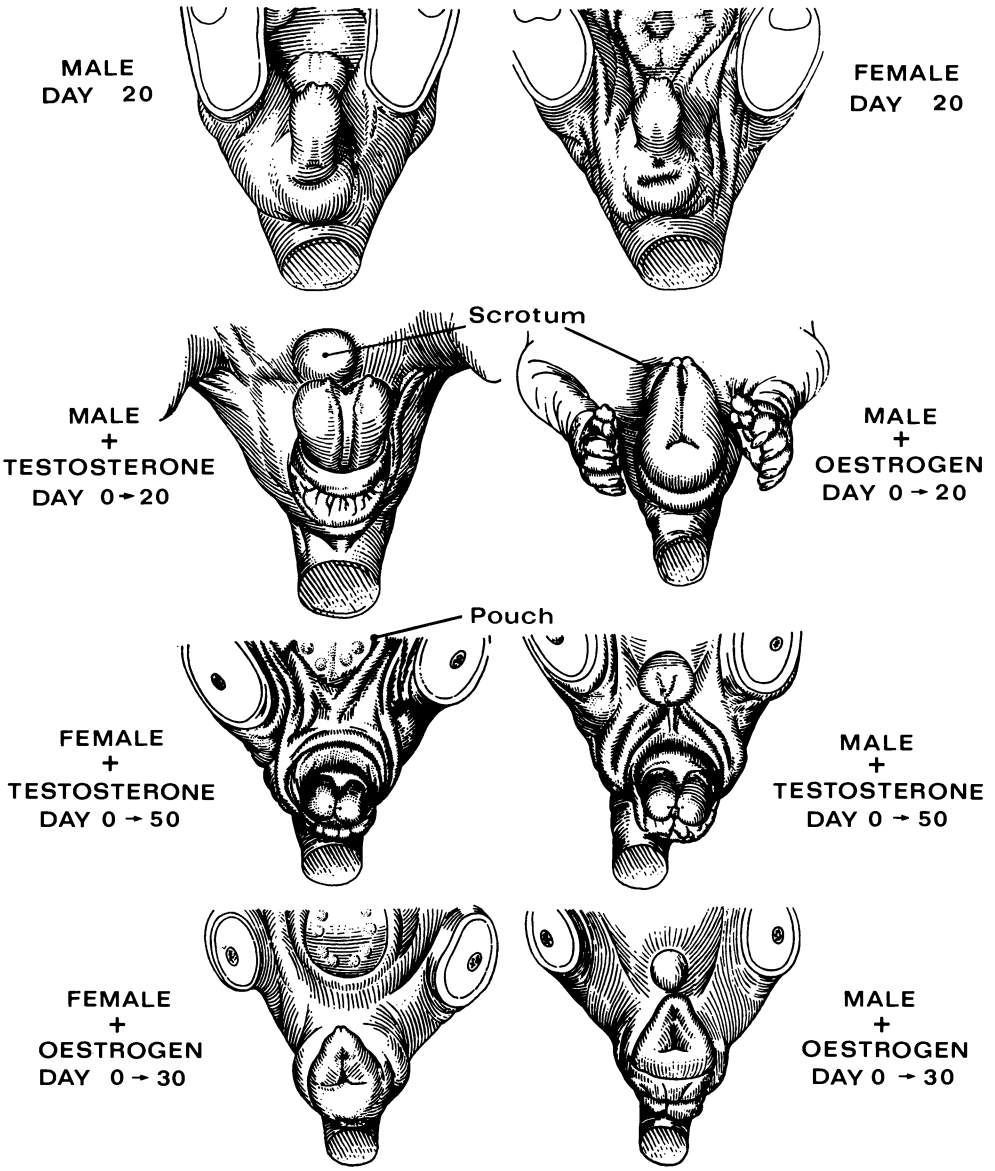

FIGURE 1. The effects of sex hormones on the external genitalia of young opossums (*Didelphis virginiana*). In the normal day-20 pouch young the phallus is slightly larger in the male, but otherwise similar. Sex is readily distinguished at this age by the scrotal sac in males and the presence of pouch folds and mammary rudiments in the female. When young are given testosterone propionate from birth to 20 or 50 days the phallus enlarges to form the penis in both males and females, but in the female the pouch and mammary anlagen appear unchanged and no scrotum develops. When young are given oestradiol dipropionate from birth to 20 or 30 days, the phallus assumes the typical female form regardless of sex. However, the scrotum is unchanged in the males. From Burns (1961).

clearly shows how testosterone propionate treatment of a newborn female from the day of birth for 50 days stimulated the development of a male phallus, but was completely without effect on the normal development of the pouch and mammary glands, and failed to induce scrotal development. Similarly, oestradiol dipropionate given for the first 30 days of life to a normal male suppressed penile development but failed to inhibit scrotal development, or induce pouch or mammary development. The conclusion should have been obvious: the mammary gland, pouch and scrotum develop independently of the steroidal environment. But Burns came to the conclusion that 'the hormones [androgen and oestrogen] produce genitalia of typical male or female form, regardless of the sex of the subject'. If he included scrotum, pouch and mammary gland in his definition of genitalia, then he was wrong.

Burns made another important discovery that has been widely acknowledged as an odd exception to the general rule that steroid hormones are incapable of sex-reversing the development of the mammalian (i.e. eutherian) gonad, but his observations have never been adequately explained, nor fully exploited. He showed that if newborn male opossums are treated with a relatively 'low' dose (0.2–0.3 $\mu g\ d^{-1}$) of oestradiol dipropionate from the day of birth for about 30 days, this will cause regression of the developing seminiferous tubules, suppression of interstitial tissue differentiation, and persistence of the germinal epithelium which subsequently gives rise to a proliferation of secondary sex cords. This new cortical zone contains a variable number of germ cells which have all the morphological characteristics of oocytes in meiotic prophase; they are often surrounded by cells that look like the early follicular cells of primordial follicles.

On the face of it, Burns had succeeded in producing almost complete gonadal sex reversal in a mammal with a steroid hormone, albeit at a grossly pharmacological dose. Unfortunately, the animals, 46 in all, were killed at the end of hormone treatment, so we do not know what would have happened to the gonads if the animals had been allowed to reach puberty. Could germ cells which were genetically XY really be transformed into functional oocytes that were capable of inducing normal follicular development up to the point of ovulation? These experiments, if repeated and extended, could shed some fascinating new light on the plasticity of germ-cell development in mammals.

In an attempt to repeat the work of Burns, Fadem & Tesoriero (1986) studied a related species, the pouchless gray opossum *Monodelphis domestica*, native to South America. They treated newborn pouch young of both sexes (average mass 100 mg) with testosterone propionate (20 μg) or oestradiol benzoate (1 μg) once or twice during the first week of life, and killed the animals at 22 weeks of age. They noted that it was only the oestradiol treatment of males that produced any significant effects, and then only if the animals were treated twice, on day 1 and day 3. They found that testicular development was inhibited by this relatively enormous dose of hormone (equivalent to 10 $mg\ kg^{-1} \times 2$), so that at autopsy the gonads were vestigial, and no longer recognizable morphologically as either male- or female-like. Not surprisingly, no germ cells were visible. No comments were made about the presence or absence of mammary glands in any of the animals, but one highly significant finding was that empty scrotal sacs were found in all the oestrogenized males, even though there was no phallic development, and the internal genitalia were entirely female in appearance. As in Burns' *Didelphis* study, this confirms that the development of the scrotum appears to be androgen-independent in this species.

Very recently, E. S. Robinson (personal communication) has been able to examine an

intersex *Monodelphis* that had a 17,XO karyotype ($2n = 18$). This animal, which was stunted in appearance, had small, undifferentiated gonads with no detectable germ cells, a completely normal but hypoplastic female reproductive tract internally, and no phallic development, and yet it had a well-developed scrotum. It is not yet known whether any mammary glands were present. Once again, this animal provides beautiful confirmation of the androgen-independence of the scrotum, and suggests that a Y chromosome is not necessary for scrotal development.

Sex determination and differentiation in the tammar wallaby, *Macropus eugenii*

We have recently summarized the ontogeny of sexual differentiation in this species (Renfree *et al.* 1987). Our interest in sex determination and differentiation in the tammar was aroused through the incidental observation by one of us (M.B.R.) that foetuses could apparently be sexed in late gestation by the presence or absence of scrotal anlagen, at a time when the gonads were still at the indifferent stage of development. Alcorn (1975) had also observed scrotal and mammary anlagen in male and female tammars respectively immediately before and after birth, but did not comment on the significance of this. Our curiosity aroused, we decided to undertake a systematic investigation of sexual differentiation in this species.

We and our co-workers Dr Shaw and Dr O began by collecting 15 pouch young on the day of birth (mass at birth approximately 450 mg), recording their head length, body mass, and making a careful note of whether or not scrotal anlagen could be seen with the naked eye. Tissue was taken for karyotyping to determine genetic sex, and the hindquarters were fixed in formalin, embedded and serially sectioned (O *et al.* 1988). Our suspicions were fully confirmed. As Alcorn (1975) had previously claimed, it is possible to be 100% correct in assigning sex by visual inspection; scrotal bulges, lying on either side of the midline just anterior to the genital tubercle, were only present in males (figure 2). We could detect no histological or volumetric difference in gonadal structure or size between males and females; the first evidence of testicular differentiation does not become apparent microscopically until the third day of pouch life, although scanning electron microscopy suggests that there may be a difference in shape of the male and female gonads before this (O *et al.* 1988; Renfree *et al.* 1987).

The serial sections revealed clear sex differences not only in the presence or absence of scrotal anlagen but also in the size and shape of the gubernaculum and processus vaginalis (well developed in males, poorly developed in females) (see figure 3, plate 1), and in the presence or absence of mammary and pouch anlagen. Dr Shaw and Dr O quantified the degree of development of the Wolffian and Müllerian ducts, and found that they were identical in males and females, providing no evidence for the onset of any gonadal hormone secretion which, it could be argued, might precede morphological differentiation of the gonads.

To rule out the possibility that early gonadal hormone secretion might nevertheless be the cause of these sexual dimorphisms in scrotum, gubernaculum, processus vaginalis, pouch and mammary gland anlagen, we collected 19 embryos during the last 6 days of pregnancy; all the above-mentioned structures were sexually dimorphic as early as 5 days pre-partum.

We then did a series of hormone administration experiments, giving oestradiol benzoate orally at a dosage rate of 2 mg kg^{-1} d^{-1} (1 μg per pouch young per day) to newborn males for 25 days starting on the day of birth, and testosterone propionate to newborn females at a dosage rate of 40 mg kg^{-1} d^{-1} (20 μg per pouch young per day) using an identical protocol

FIGURE 3. Scanning electron micrograph of the internal genitalia of a male tammar pouch young aged 11 days *post partum*. Note the rounded-up testis, and prominent gubernaculum entering the processus vaginalis at the internal inguinal ring. The mesonephros is still large at this age, and the mesonephric duct (Wolffian duct) within the urogenital cord runs into the urogenital sinus near the base of the bladder (here shown cut off at the base for clarity).

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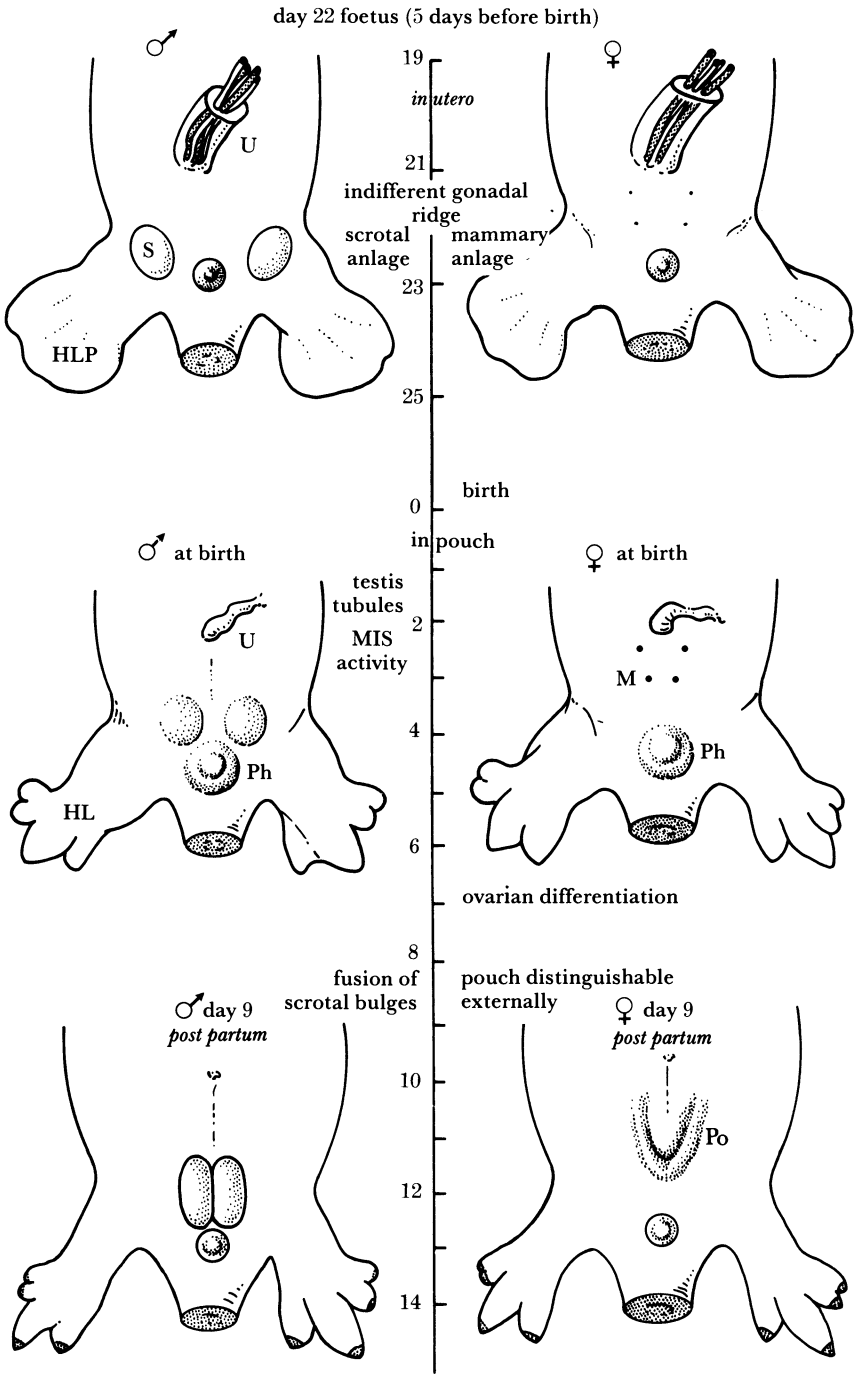

FIGURE 2. The ontogeny of scrotum and pouch in the foetal and neonatal tammar wallaby (*Macropus eugenii*) related to time of gonadal differentiation. Scrotal bulges and mammary anlagen are first observed in male and female foetuses respectively 5 days before birth (day 22; birth at day 26.5). In the males, the two scrotal anlagen gradually move to the midline where they fuse into a single scrotal sac by day 8 *post partum*. Pouch folds are just distinguishable at this time. Testis differentiation is first discernable on the third day *post partum* (d2) by histology and by Müllerian inhibiting substance (MIS) activity but the ovary lags behind this and is differentiated by about day 7. HLP, hind-limb paddle; HL, hind limb; M, mammary anlagen Ph, phallus; Po, pouch; S, scrotal anlagen: U, umbilicus.

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(Shaw *et al.* 1988). At the end of the treatment period the pouch young were killed, and subsequently serially sectioned.

The oestrogen treatment significantly retarded testicular development and trans-abdominal migration, and impeded development of the gubernaculum and processus vaginalis, but the gonads were still obviously testicular in appearance, and there was no evidence of the type of sex-reversal described by Burns in the opossum. The Müllerian ducts had persisted, and the oestrogen had caused greater stimulation than that seen in control females. However, there was no sign of scrotal inhibition, or mammary or pouch development.

The androgen-treated females showed normal ovarian development, normal Müllerian development, stimulation of the Wolffian duct and prostatic buds, but no sign of scrotal development, or inhibition of the pouch or mammary glands. This is therefore in agreement with our other observations; the development of the mammary gland, pouch and scrotum does not seem to be under hormonal control. However, the gubernaculum and processus vaginalis do appear to be under some degree of hormonal control, in addition to the genetic control described above.

Further confirmatory evidence as to the hormonal independence of these various structures comes from the studies of H. Tyndale-Biscoe, S. McConnell and L. Hinds (personal communication). They did a series of gonadal transplants in tammars on the tenth day of pouch life, grafting testes into female pouch young which were then reared to adulthood, along with the castrated male donors. At autopsy, the female recipients of the testicular grafts were of normal female body mass, had a normal pouch with four teats and mammary glands, and had failed to develop a scrotum. There was a well-developed penis, prostate, and bulbo-urethral gland and a vas deferens, co-existing with normal uteri, vaginae, and ovaries with Graafian follicles but no corpora lutea. The castrated male donors, on the other hand, had achieved a normal male body mass, and had an empty scrotum, no pouch or mammary glands, and no penile development. It would be interesting if body size was yet another characteristic that could be added to the list of sexually dimorphic characteristics that are under genetic rather than hormonal control; one is reminded of Hartman & League's intersex opossum with a female reproductive tract but male body size referred to above.

In the light of the foregoing information, it is now possible to interpret the two intersex tammar wallabies described by Sharman *et al.* (1970). One animal was Klinefelter-like, with a 17,XXY karyotype (normal, $2n = 16$). However, externally it had a pouch with four well-developed teats and mammary glands and no scrotum, whereas internally it had intra-abdominal testes and a completely normal male reproductive tract, and a normal penis. It is not difficult now to understand how the pouch and mammary gland could develop normally in the face of so much androgen, but it is more difficult to see why no scrotum had formed.

The second animal had a 15,XO karyotype, and presents a most confusing picture. The gonads were in the ovarian position, but the right gonad had seminiferous tubules present, whereas the left gonad was clearly ovarian, although lacking primordial follicles. Internally the tract was like that of a normal female, but externally it seemed as if there was a hemi-pouch with two teats and two mammary glands on the ovarian side, and a hemi-scrotum on the testicular side. Perhaps this animal was a chromosomal mosaic, which might have accounted for the bilateral gynandromorphic effect; such an asymmetry would be very difficult to explain on hormonal grounds, but it is possible that the pouch and scrotal tissues could have differed in their genetic makeup.

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Sharman *et al.* (1970) also described two other intersexes, a euro (*Macropus robustus*), and a brush-tailed possum (*Trichosurus vulpecula*), both of which had normal male karyotypes, intra-abdominal testes and normal male reproductive tracts internally, but externally they had normal pouches and mammary glands, and no scrotum. At present, these animals defy explanation genetically, although they confirm the hormonal independence of pouch, mammary gland and scrotum.

We can conclude that because scrotal, mammary and pouch development can occur independently of the presence or absence of a Y chromosome, these structures are more likely to be controlled by X-linked genes. Cooper *et al.* (1977) also hinted that the presence or absence of the Y cannot be wholly responsible for sex-determination in marsupials. Whether we are dealing with a simple X-dosage effect – one X for a scrotum, two for a pouch and mammary glands – remains to be determined, although the two XY intersexes with pouches and mammary glands but no scrotum would argue against such an explanation. However, D. W. Cooper (personal communication) suggests that if there is a small region of the X which must be expressed in double dose to produce a pouch and a mammary gland, then it is possible that these two animals carried a duplication of this region which would not have been detected except by G banding.

Discussion

The effects of steroid treatment on pouch and scrotum in American and Australian marsupials are entirely consistent with one another, and suggest the existence of a fundamental dichotomy between marsupials and eutherians in their sex-determining mechanisms. Tyndale-Biscoe's suggestion (Tyndale-Biscoe & Renfree 1987) that differentiation of the pouch and scrotum is determined by the particular chromosomal constitution of the cells of the anlagen, and that the Jost hypothesis may not apply in its entirety to sexual differentiation in marsupials is amply confirmed by all the recent evidence summarized here. This dichotomy presumably dates back to their divergence in the mid-Cretaceous period around 100 million years ago. Although both subclasses probably require a Y-linked gene or genes for testis determination, and rely on testicular androgens for Wolffian duct, prostatic and penile development, MIS for Müllerian duct inhibition, and a combination of the two hormones for testicular migration and descent into the scrotum, there the similarities end.

In eutherians, the androgen-dependent scrotum of the male is homologous with the vaginal labia of the female, which develop in the absence of androgen. In marsupials, because the rectal and urogenital passages all open into a common urogenital sinus with a single external orifice that is common to both sexes, there is no female counterpart of the scrotum. The scrotum of marsupials is not under the control of testicular hormones, and so it is presumably under autonomous genetic control. Because a scrotum can form in the absence of a Y chromosome, perhaps it is regulated by an X-linked gene, which must be inactivated in some way when two X chromosomes are present: hence the presence of a scrotum in XO or XY individuals, and the absence in XX and XXY individuals.

In most eutherians, mammary glands are present in adult males, but they are functionally inhibited by testicular androgen secretion. In some eutherians, e.g. rats and mice, mammary glands are absent in adult males, having been suppressed by androgen action during foetal life. In marsupials, mammary glands appear to be universally absent in all adult males. Testicular hormones are without effect on normal mammary development, so once again this control

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must be genetically determined. The presence or absence of a Y chromosome appears to be irrelevant for mammary development, so perhaps it also is regulated by an X-linked gene, which normally requires the presence of two X chromosomes for expression. Ancestral marsupials were thought to be pouchless (Tyndale-Biscoe & Renfree 1987) so the pouch may be a more recently derived characteristic. Because mammary gland and pouch development seem to go hand-in-glove, so to speak, the pouch is also likely to be regulated by an X-linked gene.

We already know of a number of fundamental differences between marsupials and eutherians in the behaviour of their sex chromosomes. Marsupials show paternal X inactivation, whereas in eutherians the X inactivation is random (VandeBerg *et al.* 1983). However, Cooper *et al.* (1977) make the interesting observation that X-inactivation is not complete, so an X-linked gene may be active, inactive or partly active, thus allowing the possibility of a double dosage of an X-linked gene or genes to be expressed in females. The marsupial Y is usually much smaller relative to the X than in eutherians, suggesting that marsupials may have progressed further than eutherians in the capture of genetic material by the X from the ancestral Y. This could also explain why the marsupial X and Y lack a homologous pairing segment and synaptonemal complex (Sharp 1982), preventing the obligatory crossover between the X and Y at meiosis that seems to occur in eutherians (Burgoyne 1982). Perhaps it is the small size of the marsupial Y, and this genetic isolation from the X, that has resulted in so many X-linked genes apparently being involved in sexual differentiation, although why marsupials have preferred genes to hormones for producing so many sexual dimorphisms is still a mystery.

The key to further elucidation of the genetic control of marsupial sexual differentiation lies in part in the examination of the karyotype and phenotype of a wider range of intersexes. Presumably some types of eutherian intersex will not be found in marsupials; XX males and XY females are unlikely to occur if there is no X–Y crossover at meiosis; if paternal X inactivation is obligatory, this will mean that all XO individuals can only arise by paternal non-disjunction. Because of the direct genetic control of so many somatic sexual dimorphisms in marsupials, it would seem to be important to karyotype the tissues of the pouch, scrotum and mammary gland as well as blood or bone marrow. Perhaps some of the perplexing marsupial gynandromorphs (Renfree *et al.* 1987) can be explained by tissue mosaicism in these regions.

It is amazing that it has taken us so long to appreciate that many sexually dimorphic characters in mammals may be genetically determined. So forceful and beguiling has been the hormonal theory of sexual differentiation, which dates back to the classical studies of Keller, Tandler and Lillie on the bovine freemartin at the beginning of this century, that nobody has looked for any alternative explanation. It will be interesting to see how many of the characters we have found in marsupials behave in a similar fashion in eutherian mammals. The gubernaculum, for example, the 'rudder of the testis', first described by John Hunter (1762), is clearly under dual genetic and hormonal control in marsupials. If this were true of eutherians also, it could provide a valuable new clue to our understanding of the aetiology of cryptorchidism. There is already a strong suggestion that male mouse embryos grow faster than females at an early stage of development, long before gonadal differentiation (Sellar & Perkin-Cole 1987), and this could possibly lead to the development of a simple, non-invasive method for embryo sexing in *in vitro* fertilization programmes.

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In the years to come, marsupials seem likely to play an increasingly important role in elucidating the role of a multiplicity of sex-linked genes on both the X and Y chromosomes that are apparently involved in mammalian sex determination and sexual differentiation.

We thank our colleagues Dr Geoff Shaw and Dr Wai Sum O for their collaboration in many of the experiments summarized in this review. We also thank Dr Hugh Tyndale-Biscoe and Dr Ted Robinson for allowing us to quote their unpublished results, and Professor Des Cooper for helpful comments on the manuscript.

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Discussion

MARY F. LYON, F.R.S. (*M.R.C. Radiobiology Unit, Didcot, U.K.*). The question is in regard to the anomalies in XXY and XO animals. As Professor Short said, marsupials show X-inactivation, and therefore what is the basis of these anomalies? X-inactivation in marsupial embryos has been relatively little studied; does Dr Renfree or Professor Short know whether inactivation could possibly occur later than in eutherians?

I know of one report by Johnston & Robinson (1985) on X-inactivation in kangaroo embryos. They found inactivation in cells of the embryos themselves, but both Xs remained active in some cells of the yolk-sac.

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Johnston, P. G. & Robinson, E. S. 1985 *Genet. Res.* **45**, 205–208.

M. B. RENFREE AND R. V. SHORT. We do not know anything about the precise time-course of X inactivation in marsupials. It's interesting that paternal X inactivation appears to be the rule in marsupials. If this is obligatory rather than facultative, then XO individuals have presumably arisen as a result of paternal non-disjunction.

URSULA MITTWOCH (*University College London, U.K.*). Did Dr Renfree or Professor Short measure the volumes of the gubernaculum in newborn wallabies, and did they obtain a significant difference between males and females?

M. B. RENFREE. We did not measure the total volume of the gubernaculum as between male and female wallabies, but the sex differences in gubernacular length were readily apparent from an examination of serial sections of foetuses and newborn young.

ANNE GROCOCK (*Department of Human Anatomy, University of Oxford, U.K.*). Firstly, does the gubernaculum disappear with the oestrogen treatment? Secondly, why was testosterone used rather than dihydrotestosterone?

R. V. SHORT. The answer to the first question is no. The gubernaculum was inhibited by oestrogen treatment but did not disappear completely. Secondly, in collaboration with Dr Jean

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Wilson we have shown that there is 5-α-reductase activity present in target tissues in the early stages of development, so the testosterone we administered could have been converted to dihydrotestosterone at its site of action.

M. W. J. Ferguson (*Department of Cellular and Structural Biology, University of Manchester, U.K.*). Professor Short referred in the discussion to our ideas about sex and growth in alligators, and commented that he agreed with these as Dr Tyndale-Biscoe in Australia had castrated male wallabies and shown that, as adults, they grew at the same rate as animals with intact testes, therefore suggesting that growth rate was sex-linked and independent of hormones.

I agreed with this interpretation based on alligator data, where the growth rate of animals up to 6 feet (1.83 m) in length was directly related to the temperature they were incubated at as eggs. That this was an egg-incubation effect and not a subsequent sex-determined effect could be demonstrated at temperatures of egg incubation which produce 50:50 sex ratios (31 °C in alligators). Animals resulting from such incubations grew at the same rate, independent of sex, faster than their 30 °C cohorts but slower than their 33 °C cohorts.

I also commented that from a phylogenetic standpoint it was not surprising to see differentiation of sex-associated characteristics e.g. secondary sexual structures in marsupials, eye pigmentation in quails, before the onset of overt embryonic gonadal differentiation and before any hormone release. The alligator data clearly show that several characters, e.g. pigmentation pattern, adult growth rate, preferred thermoregulatory temperature in the embryo, are determined by temperature of egg incubation as well as sex. If genetic sex determination in mammals evolved from temperature-dependent sex determination in reptiles, then clearly some of these temperature-associated characteristics could become controlled by sex genes in mammals. It was likely that there would be variation in different animals as to which characteristics became sex-gene linked, and so wide diversity might be predicted in mammals and birds.

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FIGURE 3. Scanning electron micrograph of the internal genitalia of a male tammar pouch young aged 11 days *post partum*. Note the rounded-up testis, and prominent gubernaculum entering the processus vaginalis at the internal inguinal ring. The mesonephros is still large at this age, and the mesonephric duct (Wolffian duct) within the urogenital cord runs into the urogenital sinus near the base of the bladder (here shown cut off at the base for clarity).