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Role of Mammalian Y Chromosome in Sex Determination [and Discussion]

P. S. Burgoyne, U. Wolf and M. W. J. Ferguson

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Role of mammalian Y chromosome in sex determination

By P. S. Burgoyne

MRC Mammalian Development Unit, Wolfson House, 4 Stephenson Way, London NW1 2HE, U.K.

It has long been assumed that the mammalian Y chromosome either encodes, or controls the production of, a diffusible testis-determining molecule, exposure of the embryonic gonad to this molecule being all that is required to divert it along the testicular pathway. My recent finding that Sertoli cells in XX↔XY chimeric mouse testes are exclusively XY has led me to propose a new model in which the Y acts cell-autonomously to bring about Sertoli-cell differentiation. I have suggested that all other aspects of foetal testicular development are triggered by the Sertoli cells without further Y-chromosome involvement. This model thus equates mammalian sex determination with Sertoli-cell determination. Examples of natural and experimentally induced sex reversal are discussed in the context of this model.

Introduction

More than four decades have passed since Jost (1947, 1953) did an elegant series of experiments on rabbit foetuses, from which he concluded that the development of male characteristics was brought about by two testicular secretions: testosterone and a Müllerian inhibitor. As a consequence of this work, the term 'sex determination', as applied to mammals, has come to be used interchangeably with 'testis determination'. Although there are certain male characteristics of the tammar wallaby (and presumably of marsupials in general) that are independent of testicular development (O *et al.* 1988; Renfree & Short, this symposium), in the eutherian mammals one can still equate 'sex determination' with 'testis determination'.

What then is meant by testis determination? In the absence of a Y chromosome the mammalian embryonic gonad develops as an ovary, whereas in the presence of the Y the embryonic gonad is diverted to form a testis. Testis determination is the process, initiated by the Y chromosome, by which the embryonic gonad becomes 'locked in' to the testicular pathway. It is inherent to the concept of testis determination that, if the process were to be interrupted, the gonad would develop as an ovary (as distinct from an abnormal testis, or ovotestis). A testis-determining gene can be defined as any gene that is involved in this 'locking in' process. Complete inactivation of such a gene would result in ovarian development, although partial activity could result in the development of ovotestes.

What distinguishes early testicular development from early ovarian development?

Before considering how the Y acts to bring about testis determination, it is necessary to have a basic understanding of the differences between early ovarian and testicular development. An important point, emphasized by Jost in this symposium, is that the differentiation of testicular cell types occurs earlier than the differentiation of ovarian cell types: during the initial stages of sex differentiation the female gonad is recognized solely by the absence of testicular cell types

and organization. There are thought to be three gonad-specific cell lineages in the embryonic gonad, each of which has to be diverted to a new fate in the developing testis. Firstly, there is the germ-cell lineage, destined to form meiotic oocytes in the foetal ovary, but which is diverted in the developing testis to form prospermatogonia arrested in the G1 phase of the mitotic cycle. Secondly, there is a 'supporting-cell' lineage which forms the follicles enveloping the oocytes in the ovary, but which is diverted to form the Sertoli cells that surround the germ cells in the foetal testis and are the source of Jost's 'Müllerian inhibitor'. Thirdly, there is a 'steroid-cell' lineage which contributes to the interstitial tissue of the foetal ovary, and ultimately to the oestrogen-secreting theca cells of the mature ovary, but which is diverted to form testosterone-secreting Leydig cells in the foetal testis. In addition to these lineages that form sex-specific cell types, there are vascular and connective tissue contributions to the developing gonads which adopt a more complex architecture in the foetal testis than the foetal ovary. The vascular and connective tissue framework of the foetal testis, of which the tunica albuginea is an integral part, provides the pathways for testosterone export.

How does the Y act to bring about testis determination?

Because the testicular cell types appear earlier than the corresponding ovarian cell types, the Y is acting to pre-empt ovarian development. The most widely accepted view of how the Y achieves this has assumed that there is only one gene on the Y involved in testis determination. In the mouse, this gene has been given the label *Tdy*, and in man, the label *TDF*. For simplicity's sake, in what follows I shall refer to the gene as '*Tdy*', irrespective of species. *Tdy* has been assumed to either encode, or control the production of, a diffusible testis-determining molecule. Under this model, an embryonic XX gonad exposed to this molecule would be diverted along the testicular pathway. Wachtel *et al.* (1975) proposed that the minor histocompatibility antigen H-Y was the long-sought testis-determining molecule, but the discovery of H-Y-negative male mice (McLaren *et al.* 1984; Simpson *et al.* 1986) showed that this was incorrect. Burgoyne *et al.* (1986) questioned the view that there was a diffusible testis-determining molecule, suggesting that the Y was in fact acting at the level of single cells (cell autonomously) in diverting the supporting-cell lineage to form Sertoli cells. Subsequently Burgoyne *et al.* (1988) analysed the sex chromosomal constitution of the cell types in postnatal testes of XX↔XY mouse chimeras, and found that although the Leydig cells and connective tissue elements included both XX and XY cells, the Sertoli cells and germ cells were exclusively XY-derived. The failure of testicular XX germ cells was already known to be due to the presence of two X chromosomes, rather than to the absence of *Tdy*. Thus XX*Sxr* germ cells (*Sxr* includes *Tdy*) are also absent from adult testes of 'sex-reversed' mice although they do enter the testicular pathway as prospermatogonia (reviewed by Burgoyne 1987). However, the failure to find any XX Sertoli cells led the authors to conclude that *Tdy* did indeed act cell autonomously in bringing about Sertoli-cell differentiation. They went on to suggest that diversion of all the other components of the embryonic gonad to the testicular pathway was directed by the Sertoli cells, and did not involve further Y-chromosome activity. This model thus equates sex determination with Sertoli-cell determination. The essential feature of this model is that at no point in the testis-determination pathway is a diffusible testis-determining molecule produced which is capable of diverting an embryonic XX gonad to form a testis (figure 1).

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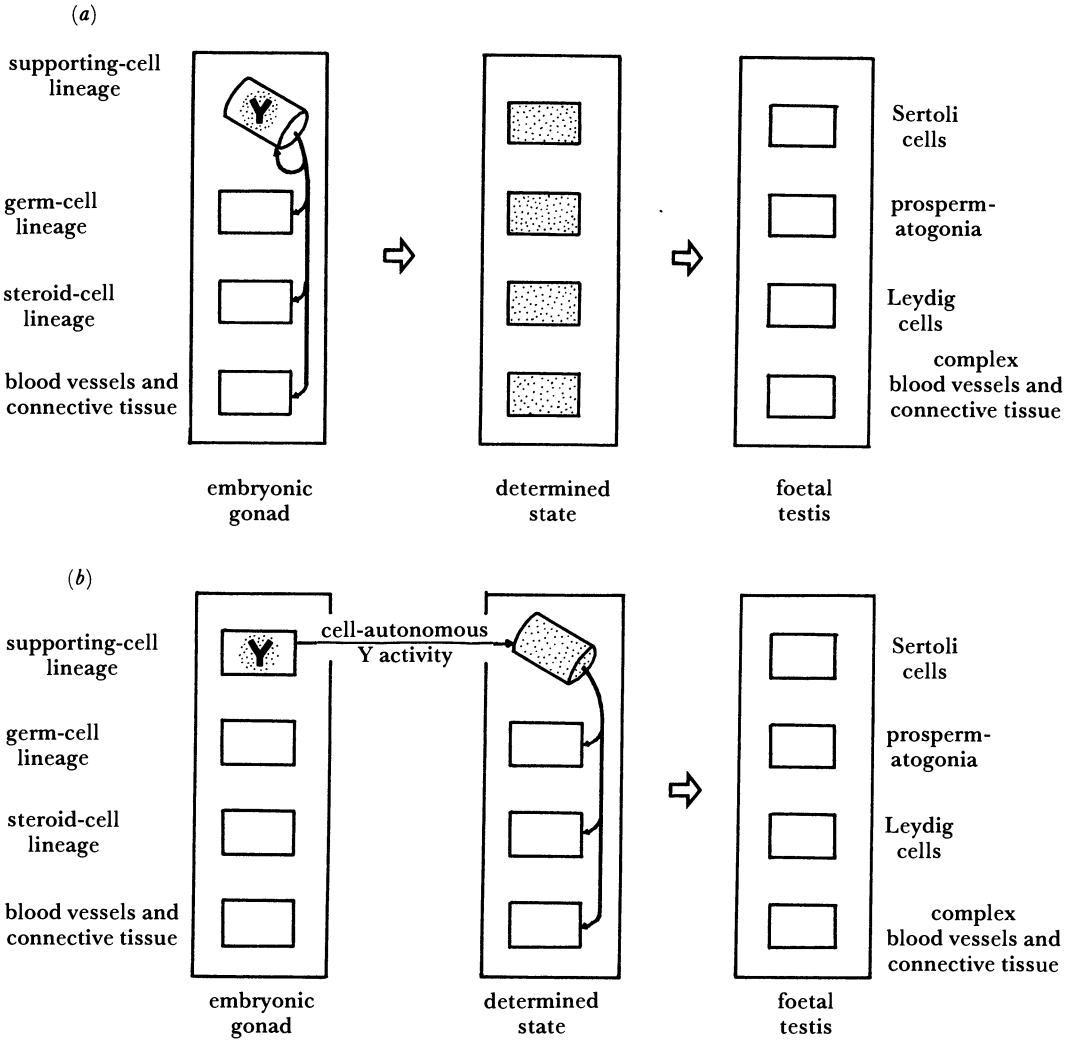

FIGURE 1. (*a*) Conventional model. In this model, Y-chromosome activity in the supporting cell lineage leads to the production of a diffusible testis-determining molecule which acts on all components of the embryonic gonad, including the supporting cells, and is responsible for 'locking' the embryonic gonad into the testicular pathway. Note that under this model an embryonic XX gonad exposed to the testis-determining molecule would develop as a testis. (*b*) Cell-autonomous Y-action model. In this model there is a Y-directed cell-autonomous step in the supporting cells which 'locks' them in to Sertoli-cell differentiation. Only after this cell-autonomous determination step does the supporting cell lineage interact with other components of the embryonic gonad to 'lock' them in to the testicular pathway. Note that there is no diffusible molecule produced that is capable of triggering the differentiation of Sertoli cells in an embryonic XX gonad.

Recently, a candidate testis-determining sequence has been cloned from the human Y chromosome (Page *et al.* 1987). The deduced protein sequence proved to be typical of a 'zinc finger' protein. This would be expected to bind to DNA or RNA, suggesting a cell-autonomous action for *Tdy*. Although this is consistent with the model we have proposed, it does not rule out the production of a diffusible testis-determining molecule in response to the zinc-finger protein 'trigger'. The findings of Page *et al.* (1987) are thus compatible with either of the models in figure 1.

Sex reversal

There are several instances of naturally occurring or experimentally induced sex reversal, where the fate of the supporting-cell lineage appears to be at odds with the sex-chromosome complement (i.e. XY follicle cells or XX Sertoli cells). Do these instances of sex reversal contribute to our understanding of the normal process of sex determination, and can they be accommodated by our model?

XY follicle cells

First, I want to consider examples where the formation of XY follicle cells occurs in association with a genetic defect. I am excluding those cases where loss or inactivation of *Tdy* is thought to be involved, as in many XY women (Disteche *et al.* 1986) and in some recently identified fertile XY female mice (R. Lovell-Badge and E. Robertson, personal communication). There is evidence from three mammalian species that an X-linked mutation can cause a failure of testis determination. In the wood lemming, a proportion of the females caught in the wild are XY (Fredga *et al.* 1976). These females are fertile, and it has been established that the condition is inherited in an X-linked fashion (Fredga *et al.* 1977). The mutant X has a visibly different banding pattern from the normal X, suggesting a chromosome rearrangement (Herbst *et al.* 1978). In the horse also there are pedigrees suggesting an X-linked mutation associated with the production of XY mares (Kent *et al.* 1986), although the infertility of the XY mares rules out formal proof of X-linkage. Similar pedigrees that include several XY women have also been reported (Simpson *et al.* 1981). The X-linked gene that has been mutated in these examples is clearly a testis-determining gene, according to the definition I gave earlier, and I shall refer to it in what follows as *Tdx*. It is fascinating that Page *et al.* (1987) have identified DNA sequences on the human X that appear to be similar, if not identical, to the candidate testis-determining sequences that they have found on the Y. Related X-linked sequences have been found in all other mammals tested, suggesting that these X-linked sequences may be *Tdx*. The similarity of the X and Y sequences has rekindled interest in a suggestion that the mammalian XX/XY sex-determining system operates through a gene dosage difference (Chandra 1985), XY providing a double '*Td*' dose (*Tdx* and *Tdy*), and XX providing a single '*Td*' dose, one of the two copies of *Tdx* being X-inactivated. Before leaving this topic, it is worth pointing out that a *Tdx* mutation has never been found in the mouse, despite extensive breeding tests with X-linked markers (Russell 1976). However, Page *et al.* (1987) have reported the mouse to be unusual in having two copies of the putative testis-determining sequence on the Y, raising the possibility that the mouse *Tdx* is redundant.

Autosomal genes have also been implicated in testis determination, both in man (de la Chapelle 1987) and mouse (reviewed by Eicher & Washburn 1986). The most interesting of these, T-associated sex reversal (*Tas*) in mice, is described in detail by Eicher elsewhere in this symposium, and appears to have the right credentials for a testis-determining gene. The other genes referred to by Eicher & Washburn are less well understood, and are not necessarily in the testis-determining pathway. *Tda-1*, for example, is defined by the finding that a *Mus domesticus* Y chromosome is inefficient in testis determination when on a C57BL/6 inbred background – adult XY individuals may be males, females or true hermaphrodites – although it functions normally on other inbred backgrounds. This could be because of the C57BL *Tda-1* allele responding poorly to the signal from the foreign Y. However, Eicher & Washburn (1987) have suggested that the *M. domesticus* Y is late-acting, so that *Tda-1* expression, rather

than being reduced, may simply be too late in some cases to pre-empt ovarian development. In this latter scenario, *Tda-1* could instead be an ovary-determining gene, with C57BL/6 having an early acting *Tda-1* allele.

The preceding examples of XY follicle cells, although they point to there being several steps in the testis-determination process, do not pose any problems for the model we have proposed. However, Ford *et al.* (1974) reported obtaining XY mitotic cells (which they presumed to be follicle cells) from the ovaries of two XX↔XY female chimeras. Because of our finding that Sertoli cells in XX↔XY male chimeras are exclusively XY, we felt that the existence of XY follicle cells in XX↔XY female chimeras needed confirmation. Our first approach was to isolate follicles from an XX, CBA↔XY, BALB/c female chimera, separate the follicle cells from the oocytes, and then assay the follicle cells from single follicles for GPI-A (BALB/c) and GPI-B (CBA) activity. The results showed that approximately one third of the follicles included cells of both GPI types. To rule out the possibility that these GPI-A-positive cells were XO (the Y could be lost by nondisjunction) we did *in situ* hybridization to sections of XX↔XY chimeric ovaries by using a Bkm-related probe which hybridizes predominantly to the mouse Y (Singh *et al.* 1984). This confirmed the presence of XY cells in some follicles (P. S. Burgoyne, M. Buehr, P. Koopman and A. McLaren, unpublished data).

If follicle cells are derived from the supporting-cell lineage, and the Y acts in this lineage to bring about Sertoli-cell differentiation, how can one account for these XY follicle cells? McLaren (1987) suggested 'that the presence of *Tdy* sequences in the genome of a supporting cell is a necessary but not a sufficient condition to determine its development as a Sertoli cell. There needs also to be an inducing signal that has to reach a certain threshold intensity, reflecting perhaps a minimum ratio of XY to XX cells'. In terms of our model, it seems to me that this 'inducing signal' must come from the supporting-cell lineage because it is produced as a consequence of *Tdy* activity. Moreover, it cannot be sufficient by itself to bring about Sertoli-cell differentiation from the supporting-cell lineage, because it does not meet the criterion of cell-autonomous action. McLaren (1987) has envisaged this 'inducing signal' acting back on the supporting-cell lineage to close off the option for follicle-cell differentiation (a restriction of potency). An alternative explanation for the formation of XY follicle cells in XX↔XY females can be derived from Eicher & Washburn's (1986) hypothesis for explaining XY sex reversal. Briefly, if the XY component of the XX↔XY chimera has a late-acting *Tdy* allele, then *Tdy* action could be pre-empted by the ovary-determining programme of the XX component. The recruitment of XY cells to form follicle cells would require that a follicle cell 'inducing signal' was produced by the XX component. An attractive possibility is that this 'inducing signal' comes from early meiotic oocytes.

XX Sertoli cells

Let us now look at the other side of the coin and consider examples where XX supporting cells have formed Sertoli cells. First, I shall deal with examples where there is evidence for a genetic defect. I am excluding those cases where Y chromosomal material (presumed to include *Tdy*) has been shown to be present, such as in XX*Sxr* male mice (Singh & Jones 1982) and in most XX men (Affara *et al.* 1986; Vergnaud *et al.* 1986). There are human pedigrees that include several true hermaphrodites, sometimes also including XX males (de la Chapelle 1987). The pattern of inheritance suggests an autosomal recessive. The recessive nature of the defect certainly argues against the involvement of *Tdy*; indeed, all XX true hermaphrodites

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and some XX males appear to lack Y chromosomal sequences (Vergnaud *et al.* 1986; Waibel *et al.* 1987).

A second genetic defect leading to the formation of XX Sertoli cells is found in goats. Once again there is an autosomal recessive pattern of inheritance, and the sex reversal is intriguingly linked with a dominant polling effect (Soller & Angel 1964). Thus heterozygous polled XX goats lack horns and are female, whereas homozygous polled XX goats lack horns and are sex-reversed. The degree of sex reversal of the genital ducts and external genitalia is variable, with only a minority showing complete masculinization (Soller *et al.* 1969). Short (1972) has reported on the gonads of intersex XX goat foetuses, and it seems that the embryonic gonad may be diverted along the testicular pathway from the outset, although sufficient detail on early stages is not yet available.

How can we explain the diversion of the supporting-cell lineage to form Sertoli cells in the absence of a Y chromosome, and why is the degree of masculinization so variable? There are no satisfactory answers to these questions, and it is disappointing that intersex goats have received little attention for over a decade. It is perhaps worth commenting that under the '*Td*' gene dosage model for sex determination, expression of both copies of *Tdx* could lead to the development of testes in XX individuals.

I now want to consider examples where genetically normal XX cells have given rise to Sertoli cells. Into this category falls the classic case of the bovine freemartin, which is a genetically female calf masculinized as a consequence of placental anastomosis between its own placenta and that of a male twin. The solution to the bovine freemartin puzzle is particularly illuminating, because it illustrates how a seemingly watertight case for the involvement of a diffusible molecule in testis determination can prove to be unsound. The most pertinent observations came from Professor Jost's laboratory (Jost *et al.* 1972, 1973, 1975). They were able to show that during the period of testis differentiation in the male cotwin, the freemartin gonad shows no sign of masculinization. Subsequently, during the period when oogonial proliferation and gonadal growth occurs in normal females, the freemartin gonad is severely inhibited and a tunica albuginea develops. As a consequence of this inhibition the freemartin gonad becomes depleted of germ cells and very few enter meiosis. Only after this period of inhibition do cords of Sertoli-like cells develop adjacent to the intra-ovarian rete. These observations clearly demonstrate that the formation of XX Sertoli cells in freemartin gonads is not a case of primary sex reversal, but rather that the Sertoli cells are forming in an abnormal ovary. I shall refer to this as secondary sex reversal.

Parallels to the freemartin story are found in my second example of XX Sertoli cells. Macintyre *et al.* (1960) and Turner (1969) reported the development of 'testis cords' in foetal rat and mouse ovaries that had been cografted with developing testes to kidneys of castrated adult male hosts. Subsequent studies by Ozdzeński *et al.* (1976) and Burgoyne *et al.* (1986) showed that the initial effect of the developing testis on the ovarian cograft is to cause germinal failure and ovarian regression. If Sertoli cells are subsequently formed, then we are once again dealing with an example of secondary sex reversal.

Recently, Vigier *et al.* (1987) have reported that cultured foetal rat ovaries exposed to AMH (anti-Müllerian hormone: Jost's Müllerian inhibitor), show the ovarian inhibition, tunica albuginea formation and subsequent development of Sertoli-cell cords, which characterize the freemartin gonad. This strongly supports an earlier suggestion (Jost *et al.* 1975) that AMH from the male twin (see Vigier *et al.* 1984) is the mediator of the freemartin effect. It also seems

reasonable to attribute the ovarian inhibition and subsequent development of Sertoli-cell cords in the ovaries cografted with developing testes, referred to above, to an effect of AMH originating from the testicular grafts. Similarly, a report of the appearance of 'testicular structures' (i.e. cords) in rotation cultures of newborn rat ovarian cells cultured in medium 'conditioned' by newborn rat testicular cells (Zenzes *et al.* 1978), could also be due to AMH secreted by the testicular (Sertoli) cells.

Foetal mouse ovaries grafted alone to adult male host kidneys may also develop Sertoli-cell cords after a period of ovarian regression (Taketo-Hosotani *et al.* 1985). Suspicions that AMH might again be responsible were allayed by the finding that it also occurred in castrated male hosts (Taketo & Merchant-Larios 1986), and in any case it now seems that adult mammalian testes do not produce detectable amounts of AMH (Tran *et al.* 1987). Although male hosts were at first thought to be necessary to obtain the masculinization of the foetal ovaries, it was later found to occur at a low frequency in female hosts. The frequency could be increased to that in male hosts if the mesonephros (usually present in the graft) was removed before grafting (Taketo-Hosotani & Sinclair-Thompson 1987). A notable feature of these studies was the convincing ultrastructural evidence that the Sertoli cells were derived from 'pre-granulosa cells', that is, cells that would have formed follicles if oocytes had been present. Also, it was shown that peritubular myoid cells and testosterone-secreting 'Leydig cells' differentiated in association with the Sertoli-cell cords.

Cords of Sertoli-like cells are also an occasional feature of ageing rat ovaries (Crumeyrolle-Arias *et al.* 1976, 1986), and here again there is evidence that they are derived from their ovarian counterparts, the follicle cells.

What can we learn from these examples of secondary sex reversal? First, because XX cells can form Sertoli cells, it is clear that the pattern of gene activity that defines the Sertoli cell phenotype does not involve genes on the Y chromosome. During normal testis development *Tdy* must act simply as a trigger for Sertoli cell differentiation, and continued expression of *Tdy* is presumably not required. Secondly, it does seem that supporting cells in the ovary in their various guises (pregranulosa cells, follicle cells) retain an ability to transdifferentiate into Sertoli cells. The fact that in all cases this 'transdifferentiation' is associated with germinal failure, makes one wonder whether oocytes have a positive role to play in maintaining ovarian supporting cells in their differentiated state. A third important point is that peritubular cells and Leydig cells form in the masculinized ovarian grafts described by Taketo-Hosotani *et al.* (1985). This implies that the differentiation of these cells is triggered by the Sertoli cells. Finally, the results of Vigier *et al.* (1987) focus attention on a possible autocrine role for AMH in testicular development, in addition to its known role in Müllerian duct regression.

Conclusion

The recent isolation of testis-determining sequences from the Y chromosome (Page *et al.* 1987), together with the earlier cloning of AMH (Cate *et al.* 1986; Picard *et al.* 1986), opens up new molecular approaches to the study of mammalian sex differentiation. We hope the model for testis determination presented here may prove useful in formulating hypotheses which can be tested in the years to come.

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Discussion

U. Wolf (*Institut für Humangenetik und Anthropologie der Universität Freiburg, F.R.G.*). Serological H-Y antigen has been shown to occur as a soluble factor, and to be present in the freemartin gonad (Wachtel *et al.* 1980). Is there evidence against the assumption that this factor is involved in the virilization of the freemartin gonad?

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P. S. Burgoyne. Vigier *et al.* (1987) have demonstrated that the addition of purified bovine AMH to cultures of foetal rat ovaries *in vitro* causes the ovarian inhibition, tunica albuginea formation and development of Sertoli-cell cords which characterize the freemartin gonad. Jost had previously observed that the time of onset of the ovarian inhibition in the freemartin was correlated with the onset of AMH production of the cotwin. Vigier *et al.* (1987) therefore concluded that AMH is responsible for the virilization of the freemartin gonad. (It is of course possible that the 'H-Y' antiserum used by Wachtel *et al.* (1980) was cross reacting with AMH.)

M. W. J. Ferguson (*Department of Cellular and Structural Biology, University of Manchester, U.K.*). Dr Burgoyne's chimeric studies showed very nice exclusion of XX cells from the seminiferous tubules that were exclusively XY cells. This suggests differential cell sorting as a mechanism (as opposed to diffusible factors) and hence the expression of unique cell surface molecules such as cell-adhesion molecules, e.g. N-CAM, L-CAM, uvumorulin, or differential phosphorylation of such cell-adhesion molecules. Therefore has anyone studied the expression of cell-adhesion molecules in male and female gonads at different developmental times? This could be a possible mechanism for the organization of the medulla into a testis, which may in turn affect differentiation by altered cell–cell, cell–matrix interactions. Is the expression of the genes of cell-adhesion molecules regulated by sex genes or Y-specific genes or *TDF* even though the cell-adhesion genes may not themselves be on either an X or Y chromosome?

P. S. Burgoyne. As far as I am aware, there is no information on the expression of cell-adhesion molecules in the developing testis in response to Y-chromosome activity.

I believe the series of events leading to the absence of XX cells in the seminiferous tubules of adult XX↔XY chimeras to be as follows. (1) In the embryonic chimeric gonad, Y-chromosome expression in XY cells of the supporting cell lineage triggers their differentiation into Sertoli cells. (2) These XY Sertoli cells actively aggregate with each other and surround XX and XY germ cells to form testis cords. (The cell interactions presumably involve cell recognition and adhesion molecules.) (3) The Sertoli cells trigger the XX and XY germ cells to form prospermatogonia. (4) The XX prospermatogonia die perinatally because of the double X dosage.

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