Sex chimaerism, fertility and sex determination in the mouse

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Summary

Adult intraspecific mouse chimaeras, derived by introducing male embryonal stem cells into unsexed host blastocysts, were examined to determine whether gonadal sex was correlated with the sex chromosome composition of particular cell lineages. The fertility of XX<-XY and XY<XY male chimaeras was also compared. The distribution of XX and XY cells in 34 XX<-XY ovaries, testes and ovotestes was determined by in situ hybridisation using a Y-chromosome-specific probe. Both XX and XY cells were found in all gonadal somatic tissues but Sertoli cells were predominantly XY and granulosa cells predominantly XX. The sex chromosome composition of the tunica albuginea and testicular surface epithelium could not, in general, be fully resolved, owing to diminished hybridisation efficiency in these tissues, but the ovarian surface epithelium (which like the testicular surface epithelium derives from the coelomic epithelium) was predominantly XX. These findings show that the claim that Sertoli cells were exclusively XY, on which some previous models of gonadal sex determination were based, was incorrect, and indicate instead that in the mechanism of Sertoli cell determination there is a step in which XX cells can be recruited. However, it remains to be established whether the sex chromosome constitution of the coelomic epithelium lineage plays a causal role in gonadal sex determination.

Male chimaeras with XX<XY testes were either sterile or less fertile than chimaeras with testes composed entirely of XY cells. This impaired fertility was associated with the loss of XY germ cells in atrophic seminiferous tubules. Since this progressive lesion was correlated with a high proportion of XX Leydig cells, we suggest that XX Leydig cells are functionally defective, and unable to support spermatogenesis.

Key words: sex chimaerism, mouse testis/ovotestis/ovary, fertility, sex determination, DNA–DNA in situ hybridisation, mouse Y chromosome probe.

Introduction

A major question in reproductive biology is the origin and nature of the signals involved in diverting the early embryonic gonad to testis or ovary. In mammals the Y chromosome encodes the testis-determining gene(s), designated Tdy in mice, and it is the absence of the Y chromosome rather than the presence of two X chromosomes that determines ovary development (reviews Goodfellow and Darling, 1988; McLaren and Ferguson-Smith, 1988; Wachtel, 1989). Tdy can almost certainly be identified with Sry, a gene which maps to the minimum sex-determining region of the mouse Y chromosome and is deleted in a line of sex-reversed female mice carrying a mutant Y chromosome (Gubbay et al. 1990), is conserved in a range of mammalian species (Sinclair et al. 1990), is expressed in the foetus only in gonadal tissue during the time at which testes begin to form (Koopman et al. 1990) and is the only gene known to be present on a 14 kb DNA fragment capable of causing testis determination when introduced as a transgene into XX mouse embryos (Koopman et al. 1991). Organ culture experiments have established that from day 11.5pc, the control of sexual differentiation in mice is an inherent property of the genital ridge, the precursor of the gonad (see McLaren, 1984; McLaren and Buehr, 1990). Germ cells are not required for primary sex determination (reviewed
McLaren, 1984, 1987, 1991), or for foetal Sry expression (Koopman et al. 1990), implying a role in sex determination for the somatic cells of the gonad. It is not, however, known in which gonadal somatic cell type(s) Sry is expressed.

There are two major gonadal somatic cell lineages: the supporting cell lineage (Sertoli cells in the testis and granulosa cells in the ovary) and the steroidogenic lineage (Leydig cells in the testis and theca cells in the ovary). The origin of these cell types is presently the subject of considerable debate, and cell lineage relationships between the different somatic tissues of the ovary and testis are also poorly understood (reviews Byskov, 1986; Byskov and Hoyer, 1988). Jost (1970) proposed that testis development is the active gene-directed dominant event where Tdy functions to switch gonadal differentiation from the ‘default’ ovarian pathway (reviewed by Jost and Magre, 1988).

Ovotestes are rare in adult XX→XY chimaeric mice and, in apparently balanced strain combinations, most XX→XY chimaeras (70–80%) develop as phenotypic males with testes (reviews McLaren, 1984; Bradbury, 1987). These observations and the report of a fertile mouse in which the somatic cells of the testis were 95% XX (Mintz, 1969) led to the widespread belief that the initial stages of testis organisation are orchestrated by a locally diffusible testis-organising molecule controlled by the Tdy gene on the Y chromosome but capable of affecting XX and XY cells alike.

In recent years, however, the involvement of a male-determining diffusible factor has been challenged. Burgoyne et al. (1988a) reported that the Sertoli cells were exclusively XY in XX→XY chimaeric mouse testes. They proposed that Sertoli cell differentiation is due to cell-autonomous Tdy expression in an initially bipotential supporting cell lineage, and that subsequent steps in testis differentiation including Leydig cell determination are the consequence of Sertoli cell activity (‘cell-autonomous Y action’ model). Bradbury (1987) found that most foetal XX→XY mouse chimaeras develop as hermaphrodites but there is a subsequent transition from foetal ovotestis to postnatal testis. Granulosa cell survival is dependent on oocytes and Vigier et al. (1987) reported that Mullerian inhibiting substance (MIS) produced by Sertoli cells kills foetal oocytes. These findings led Burgoyne et al. (1988a) to propose that the presence of testes in most adult XX→XY mouse chimaeras could be due to the regression of ovarian tissue following the elimination of meiotic oocytes in foetal life (due to Sertoli cell action) rather than to the recruitment of XX cells by a diffusible testis organiser.

The ‘cell-autonomous Y action’ model predicts that granulosa cells should be XX but Burgoyne et al. (1988b) later identified XY granulosa cells in a BALB/c (XY)→F1 (CBA/Ca×C57BL/6) (XX) chimaeric mouse ovary. However, the failure to detect XY granulosa cells in one follicle analysed from a chimaera derived from inbred strains (BALB/c (XX)→CBA(XY)) led Burgoyne et al. (1988b) to propose that XY cells form granulosa cells rather than Sertoli cells when a developmental mismatch occurs due to chimaeric strain imbalance such that the ovary-determining signal (of the crossbred component) pre-empts Tdy activity (‘timing mismatch’ model).

McLaren (1987) proposed an alternative model to account for the differentiation of XY granulosa cells, viz., that a product of the supporting cell lineage produced as a consequence of Tdy activity has to reach a certain threshold concentration in order to complete commitment of XY cells in this lineage to the Sertoli cell pathway, and with too few XY supporting cells this threshold is not reached, thus allowing XY cells to differentiate as granulosa cells. This ‘threshold’ model implies that Sertoli cell differentiation not only requires cell-autonomous Tdy expression but some other external signal, itself controlled in some way by Tdy sequences. However, both the ‘timing mismatch’ and ‘threshold’ models are based on the analysis of only cumulus granulosa cells from one ovary. Furthermore, both are developed from the ‘cell-autonomous Y action’ model, which was based on the claim that Sertoli cells were exclusively XY (Burgoyne et al. 1988a). The presence of rare XX Sertoli cells could not, however, be rigorously excluded. Using Y chromosome-directed in situ hybridisation Singh et al. (1987a, b) reported unlabelled cells in seminiferous tubules from a mouse which may have been either a spontaneous XX→XY chimaera or an XX/XY mosaic. It was assumed that these cells were not Sertoli cells, but interpretation of the data is complicated by unsatisfactory testis histology and by the use of a mouse Y chromosome probe, M34, which is not totally Y chromosome-specific (Singh et al. 1988; C. E. Patek, A. R. Clarke, K. W. Jones and M. L. Hooper, Manuscript in preparation).

Here we present an in situ hybridisation analysis of 34 XX→XY chimaeric mouse gonads using a Y chromosome-specific probe, pM34–2/0.6t (Ansell et al. 1991) and re-examine the claim that Sertoli cells are exclusively XY. We also for the first time examine the overall fate of XY cells in ovaries and ovotestes, and relate the colonisation pattern in chimaeric testes to fertility. This study was made possible by the availability of XX→XY chimaeras described by Ansell et al. (1991). The XY cells in these chimaeras are derived from embryonal stem (ES) cells deficient in HPRT (hypoxanthine guanine phosphoribosyl transferase, EC2.4.2.8). This does not affect the interpretation of the results since HPRT-deficiency is without effect on fertility in the mouse and results in little or no selective disadvantage in contribution to somatic lineages in the adult testis (Ansell et al. 1991). Because these chimaeras were used for breeding to transmit the defective hprt allele to their offspring, only adult gonads were examined. While conclusions about fetal development must be drawn with care in the light of intervening processes such as cell death, sampling and selection, the present work provides a test of the validity of previous interpretations of the sex chromosome constitution of adult chimaeric gonads, on which are based the ‘cell-autonomous Y action’, ‘threshold’ and ‘timing mismatch’ models of gonadal sex determination.
Materials and methods

Chimaeras

Intraspecific mouse chimaeras were produced by injecting male ES cells (line E14TG2a) derived from strain 129/Ola into unsexed F2(C57BL/6JLac×CBA/CaLac) host blastocysts as described previously (Hooper et al. 1987). Methods used for assessment of fertility of male chimaeras are described by Ansell et al. (1991).

DNA–DNA in situ hybridisation

From each chimaera, one gonad was processed for cryostat sectioning and the other for paraffin wax sectioning, and 30 sections were analysed from each comprising 3 sets of serial sections at approximately 1/4, 1/2 and 3/4 depth. Two DNA probes were employed: pM34−2/0.6t, a mouse Y chromosome-specific repetitive DNA probe (Ansell et al. 1991) and pMR196, which recognises a repetitive sequence present in Mus musculus (Siracusa et al. 1983). The labelling of the probes and the analysis of cryostat and paraffin wax sections by DNA–DNA in situ hybridisation were performed as described by Ansell et al. (1991).

Isozyme analysis

The glucose phosphate isomerase isozymes of freeze/thawed testis samples were analysed as described by Ansell et al. (1991).

Results

(1) Sex ratio distortion, sex chromosome constitution and fertility in chimaeras

The classification of the chimaeras used in this study has been described (see Fig. 1 of Ansell et al. 1991). 71 of 92 chimaeras (77%) were externally male. On the basis of breeding and in situ hybridisation studies, 43 chimaeras were identified as XY<->XY and 35 as XX<->XY, with 14 animals (40%) in the latter group being externally male. Of the 14 XX<->XY male chimaeras, 6 were fertile, transmitting only ES cell-derived markers to their offspring (‘total transmitters’), while 8 were sterile. Of the latter group, six proved on autopsy to be true males, i.e. possessing bilateral testes, and two to be hermaphrodites, each possessing an ovary and contralateral ovoid testis. One total transmitter with an XY<->XY constitution was also identified (Ansell et al. 1991).

Table 1. Relationship between transmission pattern and fertility of chimaeric male mice

<table>
<thead>
<tr>
<th>Transmission pattern</th>
<th>Number of chimaeras</th>
<th>Number of plugs</th>
<th>Number of litters</th>
<th>Number of pups born per litter (mean±s.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total transmitter⁴</td>
<td>7</td>
<td>119</td>
<td>41</td>
<td>6.68±0.63</td>
</tr>
<tr>
<td>Partial transmitter⁵</td>
<td>17</td>
<td>224</td>
<td>145</td>
<td>10.26±0.45</td>
</tr>
<tr>
<td>Non-transmitter⁶</td>
<td>17</td>
<td>206</td>
<td>139</td>
<td>11.43±0.38</td>
</tr>
</tbody>
</table>

Comparison of partial transmitters with non-transmitters: ratio of liveborn litters to mating plugs, χ²ₓ = 0.349, P>0.05; mean number of pups per litter, χ²ₓ = 38.38, P<0.001; pups per litter, fₒ = 5.93, P<0.001.

(2) In situ hybridisation analysis of testes

When wax and cryostat sections of control, non-chimaeric mouse testes were analysed by in situ hybridisation using the mouse Y chromosome-specific repetitive DNA probe, pM34−2/0.6t (Ansell et al. 1991), hybridisation due to the Y chromosome (Y-body signal) was visible in a proportion of cells. In diploid cell types this proportion was similar to that observed in brain (spermatogonia 82%; Sertoli cells 72%; Leydig cells 73%; cf. brain 76% (Ansell et al. 1991)). In cell types expected to contain subpopulations lacking the Y chromosome, less than half the cells showed a Y-body signal (spermatocytes – no distinction made between primary and secondary – 35%; spermatids 40%). Spermatozoa failed to give a signal in most sections, as did the squamous cells of the tunica albuginea including its outer layer, presumed to be the testicular surface epithelium, i.e. the tunica vaginalis visceral.

When the testes of the six XX<->XY total transmitters were examined by in situ hybridisation, in both testes of five animals, Y body signal was present in substantially fewer cells than in control testes. This was particularly marked in certain cell types (see below) and indicated the presence of both XX and XY cells. The remaining animal, no. 45, (Table 2) showed a hybridisation pattern in both testes which was indistinguishable from that of control testes, indicating that the testes had been populated exclusively by XY cells of ES cell origin. As expected, the hybridisation pattern in the testis of the XY<->XY total transmitter, no. 34, was also indis-
Prominent vacuoles were found in all atrophic seminiferous interstitial tissue was found next to severely atrophic disorganised in many tubules with spermatogonia and tubules (Fig. 1A,B). The seminiferous epithelium was seminiferous tubules, and an increased amount of the seminiferous epithelium, and the degree of compared with 10mm for XY testes. All XX<XY testes from total-transmitters were composed of an apparently random mixture of normal and atrophic testes from total-transmitters (Table 2). XX<XY more pronounced in testes from sterile chimaeras than amounts of spermatozoa in the epididymides and was degenerative change was correlated with reduced testes exhibited focal degenerative lesions involving loss of an XY testis.

Comparison of Group 2 with Group 3: litters per plug, χ² = 3.568, P = 0.06; pups per litter χ² = 1.210, P = 0.05). Comparison of Group 1 with Groups 2 and 3 combined: litters per plug, χ² = 6.900, P = 0.01; pups per litter, χ² = 2.801, P = 0.05.

Sex chromosome constitution of chimaeras determined by analysis of brain sections by DNA–DNA in situ hybridisation using the mouse Y-chromosome-specific repetitive DNA probe, pM34-2/0.6t.

Sex chromosome constitution of both testes determined by in situ hybridisation using pM34-2/0.6t.

Normal testes, and epididymides rich in spermatozoa.

Atrophic lesions found in 5–20% of seminiferous tubules and epididymides contain 10–30% less spermatozoa than controls.

Atrophic lesions found in 50–80% of seminiferous tubules and epididymides contain 40–60% less spermatozoa than controls.

Small testes composed entirely of atrophic seminiferous tubules, trace amounts of spermatozoa found in epididymides but not in testes.

Normal testes, and epididymides rich in spermatozoa; the failure of this chimaera to sire progeny is unexplained (see text).

Testes either XY (ES cell-derived) or XY<XY.

ES cell-derived testes.

tinguishable from that of control testes. When analysed in a similar way five of the six sterile male (non-hermaphrodite) chimaeras proved to have testes containing both XX and XY cells, while the remaining animal, no. 53, showed a hybridisation pattern typical of an XY testis.

All XY testes, including those of male no. 53, were of normal size and histological appearance and the associated epididymides were rich in sperm. The failure of male no. 53 to sire progeny is unexplained, but only a small number of mating plugs was produced (Table 2). In contrast, all XX<XY testes were smaller, measuring only 1–5 mm in maximum anteroposterior length, as compared with 10 mm for XY testes. All XX<XY testes exhibited focal degenerative lesions involving loss of the seminiferous epithelium, and the degree of degenerative change was correlated with reduced amounts of spermatozoa in the epididymides and was more pronounced in testes from sterile chimaeras than in those from total-transmitters (Table 2). XX<XY testes from total-transmitters were composed of an apparently random mixture of normal and atrophic seminiferous tubules, and an increased amount of interstitial tissue was found next to severely atrophic tubules (Fig. 1A,B). The seminiferous epithelium was disorganised in many tubules with spermagonia and spermatocytes mixed with spermatozoa in the lumina. Prominent vacuoles were found in all atrophic seminiferous tubules, and some tubules contained only Sertoli cells and spermagonia arranged next to the basal lamina (Fig. 1C).

Two main types of severely atrophic seminiferous tubules were found in all XX<XY testes: Sertoli cell only (SCO) tubules, containing only Sertoli cells, and Sertoli cell enriched (SCE) tubules, containing Sertoli cells next to the basal lamina and aggregates of strongly basophilic cells either in the centre of the lumen or displaced to one side (Fig. 1D). The morphology of these cells and their association with loss of seminiferous epithelium suggest that they are degenerating germ cells, but it is also possible that they are displaced Sertoli cells, (cf. Chung, 1974). Very few Sertoli cells were found in some atrophic tubules, indicating a loss of Sertoli cells as well as germ cells (Fig. 1D). TUBules in XX<XY testes from sterile chimaeras were entirely of the SCO and SCE types, but trace amounts of spermatozoa were found in the epididymides of at least three of these animals, indicating that the testes had not been completely aspermatogenic.

In situ analysis of XX<XY testes showed that most cells within the seminiferous tubules were XY but most interstitial cells were XX (Fig. 2A and Table 3: compare the non-chimaeric testis shown in Fig. 2B). The proportion of germ cells and Sertoli cells with Y-body signal in most normal and atrophic seminiferous tubules from XX<XY testes was similar to that of control

Table 2. Relationship between fertility and the sex chromosome constitution and histology of testes in sterile and total-transmitter chimaeric male mice

<table>
<thead>
<tr>
<th>Chimaera</th>
<th>Sex chromosome constitution:</th>
<th>Number of plugs</th>
<th>Number of litters</th>
<th>Mean no. of pups born per litter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1c</td>
<td>XY&lt;XY</td>
<td>XY&lt;XY</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Group 2d</td>
<td>XX&lt;XY</td>
<td>XX&lt;XY</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>Group 3b</td>
<td>XX&lt;XY</td>
<td>XX&lt;XY</td>
<td>21</td>
<td>8</td>
</tr>
<tr>
<td>Sterile</td>
<td>XX&lt;XY</td>
<td>XX&lt;XY</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>Group 4c</td>
<td>XX&lt;XY</td>
<td>XX&lt;XY</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Group 5e</td>
<td>XX&lt;XY</td>
<td>XX&lt;XY</td>
<td>14</td>
<td>2</td>
</tr>
</tbody>
</table>

Comparison of Group 2 with Group 3: litters per plug, χ² = 3.568, P = 0.05; pups per litter χ² = 1.210, P = 0.05. Comparison of Group 1 with Groups 2 and 3 combined: litters per plug, χ² = 6.900, P = 0.01; pups per litter, χ² = 2.801, P = 0.05.
Fig. 1. Atrophic changes in XX<->XY testes from sterile and total-transmitter mouse chimaeras. Sections stained with haematoxylin and eosin. (A) Cryostat section of testis from total-transmitter chimaera composed of an apparently random mixture of normal and atrophic seminiferous tubules, the latter including Sertoli cell only (sco) and Sertoli cell enriched (sce) tubules with cell aggregates (possibly degenerate Sertoli cells and/or germ cells) in the lumina. Loss of the seminiferous epithelium is associated with a local increase in the amount of interstitial tissue. (B) PW (paraffin wax) section of testis from total-transmitter chimaera showing a selection of atrophic seminiferous tubules, some with spermatozoa, but the seminiferous epithelium is in the process of being displaced into the lumen. (C) PW section of atrophic seminiferous tubule with numerous vacuoles (v). Note Sertoli cells (s) with distinct nucleoli and strongly basophilic spermatogonia (sg) arranged next to the basal lamina, but no other germ cell stages present. (D) PW section showing sce and sco seminiferous tubule, the latter with very few Sertoli cells. The interstitial tissue (it) is mainly composed of Leydig cells. Atrophic tubules, like normal tubules, are lined by a single layer of elongate peritubular myoid cells, (arrows). Scale bar in A and B, 100 μm; in C and D, 20 μm.

testes. The minimum proportion of XY Sertoli cells seen in a section of a normal seminiferous tubule was 40%; in such tubules Y-body signal was still found in the expected proportion of germ cells (Fig. 2C). Most spermatogonia were also XY in atrophic seminiferous tubules in which the germ cells were in the process of being sloughed-off from the basal lamina into the lumen (Fig. 2D). Most recognisable Sertoli cells in SCE tubules (arranged next to the basal lamina) were also XY as were the cells aggregated in the centre of the lumina (Fig. 2E,F). Most Sertoli cells in SCO tubules were XY (not shown). However, in a minority of SCO tubules from sterile and total-transmitter chimaeras only 5–50% of the Sertoli cells were labelled (Fig. 2G,H). No such phenomenon was seen in control testes, where a minimum of 60% of Sertoli cells in individual tubule sections were labelled. In XX<->XY testes, labelled and unlabelled Sertoli cells were morphologically indistinguishable, both having all the characteristic features of normal adult Sertoli cells (Fig. 2H). Overall, more than 90% of Sertoli cells were XY in XX<->XY testes from sterile and total-transmitter chimaeras. The mean percentage of XY Sertoli cells was significantly greater than that of XY brain cells in these chimaeras ($t_{22}$=8.75, $P<0.001$) but no significant difference was found between the mean percentage of XY Sertoli cells in testes from groups 2–4 (Table 3).

Leydig cells contained a significantly lower proportion of XY cells than brain ($t_{22}$=4.97, $P<0.001$) and the mean percentage of XY Leydig cells was significantly different between testes in groups 2–4 (group 2 versus group 3, $t_5=3.79$, $P<0.05$; group 3 versus group 4, $t_5=7.51$, $P<0.001$; Table 3), i.e. a low proportion of XY Leydig cells was associated with a high proportion of atrophic tubules and with impaired fertility (Table 2). In XX<->XY testes from total-transmitters, XY Leydig cells were found adjacent to atrophic seminiferous tubules but no difference was found in the
proportion of XY Leydig cells adjacent to normal tubules and tubules in which the seminiferous epithelium was in the process of being lost.

In three partial-transmitter and three non-transmitter chimaeras, the contribution of ES cells to testes was quantified by glucose phosphate isomerase (GPI) assay (data not shown). In the case of one non-transmitter both testes were entirely host blastocyst-derived, while in testes from the remaining five animals the proportion of the ES cell-derived GPI-1A isozyme ranged from 17 to 90 %, with strong correlation between left and right testes. All ten XY→XY testes were histologically normal at age 14–18 months.

(3) Analysis of ovaries
A total of 30 ovaries from 18 chimaeras were analysed and these included ovaries from three hermaphrodites
Fig. 2. Sections of XX-XY testes from sterile and total-transmitter mouse chimaeras and of control, non-chimaeric mouse testes hybridised in situ with the 3H-labelled mouse Y-chromosome-specific repetitive DNA probe pM34-2/0.6t. Sections stained with haematoxylin and eosin. (A) Cryostat section of testis from total-transmitter chimaera showing Y-body signal in most cells within the seminiferous tubule (st), but most interstitial cells (it) are unlabelled. (B) Testis from control, non-chimaeric mouse showing Y-body signal in most interstitial cells. (C, D) PW sections of testis from total-transmitter chimaera showing normal seminiferous tubule with Y-body signal in spermatogonia (sg) and spermatocytes (sc) but not in Sertoli cells (s) or Leydig cells (lc); sz, spermatozoa. (D) PW section of atrophic seminiferous tubules present in testes from total-transmitter chimaeras showing displacement of XY germ cells (with Y-body signal) from the basal lamina into the lumen. Most germ cells shown are spermatogonia. Solid arrows indicate unlabelled Sertoli cells. Pt, peritubular cells. Large open arrow indicates direction of lumen. (E, F) Cryostat section of SCE seminiferous tubule from total-transmitter chimaera showing Y-body signal in most Sertoli cells and/or germ cells. In cryostat sections, Sertoli cells were identified by their strongly basophilic nucleolus within the paler, irregularly-shaped nucleus. Y-body signal is also present in most cells aggregated in the lumen (ca; possibly degenerate Sertoli cells and/or germ cells). M, myoid cell; lc, Leydig cells. Large arrow indicates rare XY Leydig cell. E and F show the same SCE tubule but at different magnifications. (G) Cryostat section of SCO tubule showing Y-body signal in only one (large arrow) of at least 14 Sertoli cells; unlabelled Sertoli cells are indicated by small arrows. Y-body signal also shown in some myoid cells (m) and Leydig cells (some indicated by arrowheads). (H) PW section showing part of an SCO tubule with Y-body signal in only one (large arrow) of nine Sertoli cells; unlabelled Sertoli cells are indicated by small arrows. The characteristic tripartite structure of the adult Sertoli cell nucleolus (cf. Mirre and Knibiehler, 1982) is clearly visible consisting of one (s1) but usually two (s2) heterochromatic bodies (h) next to the nucleolus (n). Y-body signal is located over one of the two heterochromatic bodies. Scale bar in A–H, 20 μm.

Table 3. Sex chromosome constitution of testes, ovotestes (testicular poles) and non-gonadal tissues of adult XX-XY chimaeric male mice

<table>
<thead>
<tr>
<th>Classification of testes</th>
<th>Chimaera number</th>
<th>Leydig cells</th>
<th>Sertoli cells</th>
<th>Blood</th>
<th>Brain</th>
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<tr>
<td>Group 2</td>
<td>7</td>
<td>15</td>
<td>90</td>
<td>36</td>
<td>48</td>
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<td></td>
<td>48</td>
<td>18</td>
<td>96</td>
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<td>55</td>
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<td>93</td>
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<td>38</td>
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<td>Group 3</td>
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<td>90</td>
<td>ND</td>
<td>ND</td>
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<td></td>
<td>70</td>
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<td>91</td>
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<td>Group 4</td>
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<td>Hermaphrodites</td>
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<td>74 (O/T)</td>
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<td>94</td>
<td>ND</td>
<td>61</td>
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*For definition of groups 2–4 see Table 2.

**Determined by in situ hybridisation using probe pM34-2/0.6t. For Leydig and Sertoli cells, 500 randomly chosen cells were scored from 10 sections and the values shown are corrected for failure to routinely detect Y-body signal in 28% of Sertoli cells or 27% of Leydig cells in control testis sections. In the case of testes (group 2–4), the mean value of both gonads is shown. Data for brain (cortex) are from Anseli et al. (1991).

†Determined in whole peripheral blood by GPI analysis (% GPI-1A).

‡Not determined.

Chimaeras nos 40 and 74 are externally male hermaphrodites and each possess an ovary and an ovotestis (O/T; ovotestes nos 3 and 2 respectively, see section 4). Ovotestis no. 1 from the externally female chimaera (no. 82) is not included since the testicular pole was degenerate and histology poor.

§Leydig cells were extremely rare, and the interstitial tissue was mainly composed of pleomorphic epithelioid cells of which <10% were XY.
Table 4. Sex chromosome constitution of ovaries, ovotestes (ovarian poles) and non-gonadal tissues of adult XX-->XY chimaeric mice

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<td>38</td>
</tr>
</tbody>
</table>

% XY cells in

* All chimaeras are externally female (aged 12-16 months) except for nos 40 and 74 which are externally male (aged 14 and 17 months respectively). Chimaeras nos 82, 74 and 40 are hermaphrodites and each possesses an ovary and ovotestis (ovotestes nos 1, 2 and 3 respectively; see section 4).

† Group 1: All follicular stages present but few primordial follicles; average numbers of antral stage follicles and corpora lutea per section both greater than 4. Group 2: All follicular stages present except primordial follicles, average of 3 antral stage follicles per section, but all Graafian follicles were degenerate and cystic follicles were present; no corpora lutea. Group 3: ovaries containing a single enlarged follicular cyst and no corpora lutea; Group 4: ovaries composed of connective stromal tissue; no follicles or corpora lutea. Group 5: ovaries composed of connective stromal tissue; no follicles or corpora lutea.

‡ Determined by in situ hybridisation using probe pM34-2/0.6t. 500 cells were scored from 10 sections and the values shown are mean of 4 brains quoted.

§ Mean values.

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<thead>
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§ Mean values.

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- A and B refer to gonads with no distinction made between left and right. All gonads are ovaries except for 3 ovotestes (O/T).

- Determined by GIPI analysis (% GIPI-1A). Blood samples were whole peripheral blood and brain samples from occipital cortex.

- Mean values.

- Grossly abnormal composition, comprising cells with strongly basophilic small round nuclei (as in haemorrhagic follicle, see note 1), 8% XY; pleiomorphic epithelioid cells, 0% XY, and giant cells showing lipofuscin and amyloid degeneration, 1% XY.

- One brain not examined, therefore mean of 4 brains quoted.

- Cell lineages absent.

- Not determined.

Associated with normal oocytes, and XX and XY granulosa cells were present in both the mural and cumulus cell populations in the same follicle. The difference between the mean percentage of XY cells in the surface epithelium and granulosa cell lineage was of borderline significance (t22=2.04, P<0.05). Although XY cells were consistently scarce in both lineages, the proportions were not correlated (r23=0.002, P>0.05).

XY thecal cells were identified in all XX-->XY ovaries. The mean number of XY cells in the thecal cell lineage was significantly greater than the number present in the ovarian surface epithelium (t23=8.51, P<0.001) and granulosa cell lineages (t23=7.22, P<0.001) but less than that found in the ovarian stroma (t23=2.79, P<0.05), which was itself less than that found in the blood (t23=2.493, P<0.05). Thecal cells in individual follicles, including atretic and cystic ones, were either exclusively or predominantly (70%) XX. No difference in sex chromosome composition was noted between theca interna and theca externa cells or between the different cell types of the theca interna including the polyhedral, presumed steroid secreting (reviewed Gore-Langton and Armstrong, 1988) and the spindle-shaped cells (Fig. 3F). The proportions of XY cells in theca and stroma were strongly correlated (r23=0.923, P<0.01).

Granulosa cells of atretic and cystic follicles were mainly XX, as in normal follicles, and no XY granulosa cells were detected in the few primordial follicles present in these mature ovaries. No atretic or cystic follicles contained more XY than XX thecal cells. The proportion of XY granulosa-lutein cells (Fig. 3G) and XY granulosa cells was similar. In some corpora lutea the large polygonal granulosa-lutein cells were XX but
Fig. 3. Cryostat sections (unless otherwise indicated) of XX→XY ovaries hybridised in situ with pM34-2/0.6t. Stained with haematoxylin and eosin. (A) Y-body signal in cells of the tunica albuginea ovarii (tao) but not in the ovarian surface epithelium (ose), shown here in oblique section. (B) Y-body signal in cells of the tunica albuginea ovarii (tao) and in one cell (arrow) in the ovarian surface epithelium (ose). (C) Y-body signal in cells of the ovarian stroma. (D) Y-body signal in a granulosa cell (arrow) of the cumulus oophorus. (E) PW section showing Y-body signal in most granulosa cells of a follicle but only in some (arrow) theca cells (tcs). (F) Y-body signal in theca interna and theca externa cells (some indicated by large and small arrows respectively) but not in granulosa cells (gcs). (G) Y-body signal in granulosa-lutein cells. (H) Y-body signal in ovarian stroma (os) and in presumed theca-lutein cells (tlcs) but not in the large polygonal granulosa-lutein cells (gls). Scale bar in A–H, 20 μm.

the smaller pleomorphic peripheral lutein cells were a mixture of XX and XY cells with XX cells predominant (Fig. 3H).

The failure to detect Y-body signal in most granulosa/granulosa-lutein cells, surface epithelial cells and theca/theca-lutein cells was not due to poor hybridisation technique since strong signal was found in >95% of these cell types when hybridised with pMR196, which recognises a repetitive DNA sequence present in Mus musculus (Siracusa et al. 1983). No signal was found in negative control ovarian sections from Mus caroli when hybridised with pMR196.

(4) Analysis of ovotestes

Three ovotestes were examined: in each case the contralateral gonad was an XX→XY ovary (see section 3).

Ovotestis no. 1 with attached Fallopian tube was identified in an externally female chimera and was composed of caudal and cranial ovarian poles separated by a degenerate testicular region (Fig. 4A). The contralateral ovary contained numerous healthy follicles and corpora lutea. Ovotestes nos 2 and 3 with attached poorly developed epididymides, lacking spermatozoa, were identified in sterile externally male chimaeras. The contralateral ovaries contained numerous atretic antral follicles and no corpora lutea. Ovotestis no. 2 was small, 1 mm in length, and composed entirely of atrophic SCO and SCE seminiferous tubules as found in XX→XY testes but was
C. E. Patek and others

classified as an ovotestis since it was enclosed by an ovarian surface epithelium (Fig. 4B,C). Ovotestis no. 3 was predominantly ovarian with a small testicular pole (Fig. 4D).

*In situ* analysis revealed that all three ovotestes were XX^XY (Tables 3 and 4). The patterns of distribution of XY cells in the three ovotestes were similar to each other and to those seen in ovaries and testes. Sertoli cells were predominantly XY in ovotestes nos. 2 and 3 (no. 1 was uninformative) while granulosa cells and thecal cells were predominantly XX and a high proportion of stromal cells were XY in ovotestis no. 1, the only informative case. Ovarian surface epithelium cells were predominantly (ovotestis no. 1) or exclusively (ovotestes nos. 2 and 3) XX (Fig. 4E). In contrast about 60% of cells were labelled in the tunica albuginea and testicular surface epithelium in ovotestes nos. 1 (Fig. 4F) and 3. This difference between the sex chromosome compositions of the ovarian and testicular surface epithelia was clearly evident at the junctions of the ovarian and testicular poles in both gonads (Fig. 4G,H).

**Discussion**

**Sex chimeraism in the testis**

The difference in breeding performance between partial and total transmitters (Table 1) cannot be explained solely by differences in the genetic constitution of offspring, since total transmitters with XY testes showed significantly better performance than
Fig. 4. Sections of ovotestes from externally male and female mouse chimaeras: (A–D) not subjected to in situ hybridisation, (E–H) hybridised in situ with pM34-2/0.6t. (A,F,H) cryostat sections; remainder wax sections. Stained with haematoxylin and eosin. (A) Ovotestis no. 1 from an externally female chimaera (no. 82) autoposited at 12 months, showing ovarian polles (ov) with atretic antral follicles and no corpora lutea, and a degenerate central testicular region (te) composed of atrophic seminiferous tubules. The interstitial tissue is composed of pleomorphic epithelioid cells with no recognisable Leydig cells. The gonad is bordered by an ovarian surface epithelium except on one side where a tunica alboginea (ta) enclosed by a testicular surface epithelium is found adjacent to the testicular region. (B) Ovotestis no. 2 from a sterile externally male chimaera (no. 74) autoposited at 10.6 months, composed entirely of atrophic Sertoli cell only (sco) and Sertoli cell enriched (sec) seminiferous tubules, the latter with cell aggregates in the centre of the lumen which are possibly degenerate Sertoli cells and/or germ cells. The interstitial tissue is largely composed of pleomorphic epithelioid cells with rare Leydig cells, and no increase in the amount of interstitial tissue is seen adjacent to atrophic seminiferous tubules. The gonad is enclosed by an ovarian surface epithelium. (C) As B, showing ovarian surface epithelium (ose) and thickened tunica alboginea ovarii (tao); see, Sertoli cell enriched seminiferous tubule. (D) Ovotestis no. 3 from a sterile externally male chimaera (no. 40) autoposited at 14 months, showing ovarian pole (ov) enclosed by an ovarian surface epithelium and testicular pole (te) enclosed by a tunica alboginea (ta) with an outer testicular surface epithelium. The testicular pole is composed of atrophic SCO and SCE seminiferous tubules with rare Leydig cells. A single haemorrhagic follicle (f) with no theca cell layer is present in the ovarian pole. (E) Ovotestis no. 2 showing Y-body signal in Sertoli cells (large arrows) and in some cells of the tunica alboginea ovarii (small arrow) but not in cells of the ovarian surface epithelium (ose); sco, Sertoli cell only seminiferous tubule. (F) Testicular region of ovotestis no. 1 showing Y-body signal in cells of the tunica alboginea (large arrow) and in the outermost cell layer, presumed to be the testicular surface epithelium (small arrows) but not in the interstitial tissue (it). (G) Ovotestis no. 3 showing junction of the ovarian and testicular poles with Y-body signal in the testicular surface epithelium (arrows) but not in the ovarian surface epithelium (ose). (H) Ovotestis no. 1 showing junction of the tunica alboginea (ta) and ovarian surface epithelium (ose, outlined by broken line) with Y-body signal only in cells of the tunica alboginea and in the testicular surface epithelium (large arrows), which overlaps the ovarian surface epithelium at the junction. The feature in the ovarian surface epithelium indicated by the small arrow is debris and not autoradiographic signal. Scale bar in A, D, 500 µm; in B, C, 100 µm; in E–H, 20 µm.

Total transmitters with XX<->XY testes (Table 2) and both classes transmit only ES cell-derived markers. We conclude, in confirmation of a previous report based on aggregation chimaeras (Onishi and Mikami, 1985), that most sex chimaeras have reduced fertility, and we further identify the subfertile sex chimaeras as those with XX<->XY testes.

The impaired fertility of these chimaeras was associated with atrophy of seminiferous tubules. The detection of normal and atrophic seminiferous tubules in testes from total-transmitter chimaeras and of a broad spectrum of degenerative change ranging from tubules with disorganised germinal epithelia but with spermatozoa, to SCO and SCE tubules suggests that the lesion, which involves a general disruption of spermatogenesis, is progressive in nature. The fact that XY<->XY testes of the same strain combination were histologically normal even in somewhat older mice rules out the possibility that the degenerative lesions seen in the XX<->XY testes are a consequence of HPRT-deficiency (see Ansell et al., 1991), the use of ES cells, age (see Takano and Abe, 1987) or chimaeric strain combination, and indicates that they result from sex chimaerism. The normality of XY testes from XX<->XY chimaeras further suggests that the degenerative lesions in XX<->XY testes are due specifically to sex chimaerism in the testis (Table 2).

The reason for the impaired fertility of adult XX<->XY testes is uncertain. It could be due in part to germ cell depletion following the death of any XX germ cells as T1 prospermatogonia during perinatal development (see Burgoyne, 1991; Palmer and Burgoyne, 1991a). While the consequent presence of too few XY germ cells in certain tubules could disrupt spermatogenesis, it is difficult to envisage how an early loss of XX germ cells would cause a progressive disruption of spermatogenesis in 6–12 month-old adult testes as found here. Further, a loss of XX germ cells cannot account for the finding that all seminiferous tubules showing initial signs of germinal disruption contained a full complement of XY germ cells. Finally, the normal histological appearance of adult XX<->XY testes from chimaeric mouse strain combinations known to favour a high proportion (54–82 %) of XX T1 prospermatogonia (Palmer and Burgoyne, 1991a) also suggests that the degenerative lesions that we observe are not simply due to an earlier loss of XX germ cells. They are also unlikely to be due to the presence of XX Sertoli cells, since no difference was found between the sex chromosome composition of Sertoli cells in normal and atrophic seminiferous tubules, and most cells, including recognisable Sertoli cells, were XY in severely atrophic SCO and SCE tubules. This conclusion is supported by the finding by Palmer and Burgoyne (1991a) of histologically normal XX<->XY testes with a very similar proportion of XX Sertoli cells to that found here. The extent of degenerative change and impaired fertility in all testes was however strongly correlated with the proportion of XX Leydig cells. It is possible that, as suggested by Singh et al. (1987a,b), a high proportion of XX Leydig cells results in a disruption in spermatogenesis. This may be due to a deficiency in testosterone production by XX Leydig cells, since there is evidence that Y-chromosomal alleles can influence testosterone levels (Jutley and Stewart, 1985).

If XX Leydig cells are defective, the focal nature of the disruption of spermatogenesis in XX<->XY testes could be a consequence of the fact that tubules at different stages of spermatogenesis differ in their degree of testosterone-dependence (see Zirkin et al. 1992).
Follicles are probably oligoclonal in origin, and that the et al. (1988) that granulosa cells of individual continuously recruited from these lineages. Support the view, based on nuclear morphology the tunica albuginea ovarii and stroma, does not whether all such cells are functional. The scarcity of XY cases, it may therefore be important to generate ES cell markers to all their progeny. The progressive chimaeras formed by injecting XY embryonal stem cells into XX blastocysts are valuable because they transmit chromosome, Y chromosomal genes may be required different studies may be due to variations in the extent of cell selection in different strain combinations.

Thus, we propose that, while Leydig cell differentiation is not dependent on genes located on the Y chromosome, Y chromosomal genes may be required for correct Leydig cell function. In the mouse, male chimaeras formed by injecting XY embryonal stem cells into XX blastocysts are valuable because they transmit ES cell markers to all their progeny. The progressive degenerative lesions that we report here do however reduce their fertility, and it is possible that in other species, particularly those that take longer to reach maturity, they may abolish fertility completely. In these cases, it may therefore be important to generate XY<XY rather than XX<XY chimaeras.

Sex chimaerism in the ovary
In chimaeric ovaries, XY cells were detected in all somatic lineages, but it remains to be determined whether all such cells are functional. The scarcity of XY cells in the ovarian surface epithelium, compared with the tunica albuginea ovarii and stroma, does not support the view, based on nuclear morphology (Gruenwald, 1942), that surface epithelium cells are continuously recruited from these lineages.

The spatial distribution of labelled granulosa cells confirms earlier conclusions based on isozyme analysis (Telfer et al. 1988) that granulosa cells of individual follicles are probably oligoclonal in origin, and that the functionally distinct subsets of mural and cumulus granulosa cells (reviewed by Gore-Langton and Armstrong, 1988) probably originate from a common precursor pool.

Since the proportions of XY cells in the different, functionally distinct populations of thecal cells were similar, these probably arise from a common lineage, and thecal cells of individual follicles are, like granulosa cells, probably oligoclonal in origin. Thecal cells may derive from the granulosa cell lineage or from unspecialised mesenchyme cells in the ovarian stroma (reviewed Gore-Langton and Armstrong, 1988). The latter is supported here by the finding of XY thecal cells but not XY granulosa cells in some ovaries and by the strong correlation between the mean number of XY cells in the thecal and stromal lineages.

The proportion of XY cells was similar in granulosa cells of normal, cystic and atretic follicles and in granulosa-lutein cells. Thus, the scarcity of XY granulosa cells that we observe cannot be due to selection processes operating in the adult against the growth and development of follicles that contain XY granulosa cells. The pattern of hybridisation in corpora lutea suggests, in line with some morphological evidence (reviewed by Niswender and Nett, 1988), that granulosa-lutein and peripheral lutein cell types are developmentally distinct and that, in mice, thecal cells can luteinise and be incorporated into the corpus luteum.

**Sex determination**

The three most recently proposed models of sex determination, viz, the ‘cell-autonomous Y action’, ‘threshold’ and ‘timing mismatch’ models, are based on reports that Sertoli cells are exclusively XY in testes composed of XX and XY cells (see Introduction). However, the proportion of labelled Sertoli cells which we observe in XX<XY testes, and particularly the presence of tubules in which very few Sertoli cells are labelled, demonstrate that these cells are predominantly but not exclusively XY. Palmer and Burgoyne (1991a, b) also report a minor proportion of non-Y-bearing Sertoli cells both in XX<XY chimaeras and in XO/XY and XO/XY/XYY mosaic mice and, in the light of this new information, have reinterpreted previous findings which led to the conclusion that Sertoli cells were exclusively XY (Burgoyne et al. 1988a). The other workers who reached this conclusion (Singh et al. 1987a, b) probably misidentified the unlabelled cells seen in their study (see Introduction). The claim that Sertoli cells are exclusively XY, on which all three models are based, is therefore incorrect. Furthermore, the ‘timing mismatch’ model (Burgoyne et al. 1988b) does not predict the consistent scarcity of XY granulosa cells which we find, and cannot account for the recent report of XY granulosa cells in ovaries of XO/XY and XO/XY/XYY mosaic mice where no strain effects operate (Palmer and Burgoyne, 1991b).

The detection of XX and XO Sertoli cells in testes and ovotestes does not necessarily rule out cell-autonomous mechanisms of Sertoli cell determination.
since it is not known whether XX and XO Sertoli cells are formed at the same time and by the same mechanism as XY Sertoli cells. It is possible that XY Sertoli cells form first by a cell-autonomous mechanism and that XX and XO cells are recruited subsequently. It is, however, more likely that all Sertoli cell types are formed concurrently by a single non-cell-autonomous mechanism, since XX and XY Sertoli cells are both present in chimaeric mouse testes as early as 13.5 days p.c. (Palmer and Burgoyne, 1991a) and both coexist in the same functional seminiferous tubule (this study; Palmer and Burgoyne, 1991a). In order for XX Sertoli cells to be formed in this way it would suffice for one step in the mechanism between the transcription of the Tdy gene and Sertoli cell determination to be capable of recruiting XX cells. This step is unlikely to be the action of the Tdy gene product itself, since the Sry gene, which is almost certain to correspond to Tdy (see Introduction), contains a sequence element that suggests that it codes for a DNA-binding protein and therefore that it acts cell-autonomously (Gubbay et al. 1990). It is therefore more likely to be a subsequent step in the determination process which is a consequence of Tdy expression.

Importantly all studies based on these different mouse model systems agree that most, but not all, Sertoli cells contain a Y chromosome and that most, but not all, granulosa cells lack a Y chromosome. In addition, we report that ovarian surface epithelial cells are predominantly XX both in ovaries and in ovotestes; the sex chromosome constitution of the testicular surface epithelium was generally unresolved due to diminished hybridisation efficiency. These results indicate that the supporting cell lineage may not be the only lineage whose sex chromosome constitution is correlated with gonadal determination, but that this may also apply to the surface epithelium, which is derived from the coelomic epithelium.

The sex chromosome constitution of a cell type in the adult gonad may reflect either a causal role for the constitution of its progenitors in sex determination, or selection processes which are a consequence of sex determination. Interpretation of our data depends upon an understanding of whether Sertoli cells and granulosa cells develop from a common progenitor, but unfortunately this question remains unresolved (see Byskov and Hoyer, 1988). The existence of a common progenitor is supported by findings of functional and antigenic similarities between granulosa cells and Sertoli cells including the synthesis of MIS (see Burgoyne, 1988; Byskov and Hoyer, 1988; Jost and Magre, 1988; Ueno et al. 1989) and by claims that Sertoli cells can form in ovaries under certain conditions (e.g. Takeko-Hosotani et al. 1985; Behringer et al. 1990; Charpentier and Magre, 1990; Hashimoto et al. 1990; Burgoyne, 1991). In most of these cases, it is believed that they are derived by transdifferentiation from granulosa cells (Takeko-Hosotani et al. 1985). It is also claimed that Sertoli cells can transdifferentiate into granulosa cells (see Ward et al. 1988; McLaren, 1990, 1991). If indeed Sertoli cells and granulosa cells do originate from a common, bipotential lineage and if XX and XY Sertoli cells differentiate simultaneously from the same lineage, then the sex chromosome composition of these cells is readily explained by a simplification of the 'threshold' model proposed by McLaren (1987), in which commitment in the supporting cell lineage to either granulosa cells or Sertoli cells in an area of the developing gonad is determined by whether the proportion of XY cells in the supporting cell progenitors exceeds or falls below a certain threshold, but there is no additional requirement for cell-autonomous effects of Tdy expression. If it is further postulated that commitment in this lineage is the sole causal factor in sex determination, this leads to a model in which gonadal sex is determined entirely by the level of a Tdy-regulated inducing signal reflecting the proportion of XY cells in a bipotential supporting cell lineage. Alternatively, if XY Sertoli cells differentiate before XX Sertoli cells, the differentiation of the former could be explained by the original 'threshold' model (McLaren, 1987) and of the latter by a subsequent recruitment step. These models would require that all other aspects of testis development are the consequence of Sertoli cell activity as has been postulated (Singh et al. 1987a, b; Burgoyne et al. 1988a), and that the sex chromosome composition of the surface epithelium and of its progenitor, the coelomic epithelium plays no causative role in gonadal sex determination.

The above models are not without problems. First, the postulate that all other aspects of gonadal differentiation are a consequence of the determination of the supporting cell lineage is hard to reconcile with the formation of ovotestis no. 2, in which a core of testicular tissue is surrounded by an ovarian surface epithelium. Second, there is as yet no proof that the cell types produced in instances of putative transdifferentiation are in fact true Sertoli or granulosa cells. All instances reported to date appear to be linked with the presence or absence of oocytes in the testis and ovary respectively (Prepin and Hida, 1989; see Hashimoto et al. 1990; Burgoyne, 1991). Prepin and Hida reported that seminiferous-like cords in transformed rat oocytes lack the 'germinostatic' activity normally associated with foetal testes and proposed that germinal failure in the ovary simply causes follicle cells to rearrange into epithelial cords. Finally, there is some doubt that granulosa cells and Sertoli cells derive from the same cell lineage, and recent evidence in mice (Kanai et al. 1989) support some earlier conclusions (see Byskov and Hoyer, 1988) that Sertoli cells derive entirely from the mesonephros but granulosa cells derive from both the coelomic epithelium and mesonephros. This common but not identical origin could account for antigenic similarities between granulosa cells and Sertoli cells (see Jost and Magre, 1988), and would be consistent with our finding that in ovaries and ovotestes the proportions of XY granulosa and ovarian surface epithelial cells were low but uncorrelated, while, in ovotestis no. 2, Sertoli cells were predominantly XY but the (ovarian) surface epithelium XX. Human granulosa cells are also
believed to derive, at least in part, from the coelomic epithelium (see Makabe and Motta, 1989; Wartenberg, 1989).

If granulosa cells but not Sertoli cells are derived partly from the coelomic epithelium, then the sex chromosome constitution of the coelomic epithelium could play a role in gonadal sex determination. The possibility that it is the sole determining factor, and that the sex chromosome constitution of the Sertoli and granulosa cells is a consequence of selection following commitment to testis or ovary, is unlikely since it cannot account for the formation of ovotestis no. 2. However, it is possible that there is a causal role in sex determination for the sex chromosome constitution of both supporting cell progenitors. Indeed, previous investigators have proposed that the nature of the supporting cell lineage, and thus gonadal sex, is determined by interaction between the coelomic epithelium and the mesonephros at the time of formation of the genital ridge (Wartenberg, 1982; McLaren, 1985; see Wartenberg, 1989).

Whatever mechanism determines gonadal sex, it seems necessary to postulate some selection within the supporting cell lineage to explain why the proportion of XY cells that we have detected in the supporting cell lineage of adult gonads never falls between 9% (maximum for granulosa cells) and 90% (minimum for Sertoli cells). Palmer and Burgoyne (1991a) find a higher proportion of XX Sertoli cells in foetal than in adult testes, which may indicate that selection does occur against XX Sertoli cells following testis determination. A similar age-related selection could account for the scarcity of XY granulosa cells found here in mature 12–16 month ovaries (0–9%) compared with newborn ovaries (15–25%; Palmer and Burgoyne, 1991b) and a young adult ovary (25%; Burgoyne et al., 1988a). If selection against XY granulosa cells does occur, it must operate during the first year of life, since we found no evidence of selection against growth or development of follicles containing XY granulosa cells in this study. In view of these considerations, it is unlikely that the sex chromosome constitution of cell types in adult chimaeras is an exact indication of their constitution at the time of commitment. It will therefore be necessary to determine the latter directly in foetal gonads in order to discriminate between the different models of gonadal determination discussed above.

Although the ‘timing mismatch’ model of Burgoyne et al. (1988b) can no longer explain all observations, this does not exclude the possibility that mismatch can occur, and it seems necessary to invoke such a mechanism, as discussed by Palmer and Burgoyne (1991b), to account for the claim of more than 94% XY granulosa cells in both ovaries of an adult interspecific mouse chimaera (Ford et al. 1974). These cells were, however, never positively identified as granulosa cells.

We conclude that the mechanism of Sertoli cell determination in mice involves a step which is capable of recruiting XX cells. Further work is needed to clarify the role of coelomic epithelium and supporting cell lineages in gonadal sex determination and to elucidate the nature of the signals involved.

We are grateful to Mr Alistair McCondochie for technical assistance and Dr John Ansell, Department of Zoology, Edinburgh University, for GPI analyses. C.E.P. was supported by project grant No SP1600 from the Cancer Research Campaign to M.L.H.

References


