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Cell-autonomous action of the testis-determining gene: Sertoli cells are exclusively XY in XX↔XY chimaeric mouse testes

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Summary

The distribution of XX and XY cells in XX↔XY chimaeric mouse testes was analysed by enzyme marker analysis of separated testicular tissues and by *in situ* DNA marker analysis of air-dried testicular cells and testis sections. XX cells contributed to the Leydig cells, the peritubular cells and the vascularized connective tissue of the tunica albuginea. The Sertoli cells, on the other hand, appeared to be exclusively XY. These results indicate that during the development of the testis, Sertoli cell differentiation is triggered by cell-autonomous activity of the Y chromosomal testis-determining gene *Tdy*. Subsequent steps in testis differentiation may be a consequence of Sertoli cell activity.

Key words: testis determination, XX↔XYchimaeras, Sertoli cells, Leydig cells.

Introduction

Although on average half of the chimaeras produced by aggregating pairs of preimplantation mouse embryos should be XX↔XY combinations, hermaphroditism is rare and the majority of adult XX↔XY chimaeras are fertile males (McLaren, 1984). This finding lent support to the widely held view (probably originating from Witschi, 1934, 1967) that the initial stages of testicular organogenesis are orchestrated by a locally diffusible testis-organizing molecule, controlled by the Y chromosome, but capable of affecting XX and XY cells alike. In 1975, it was proposed that this testis-organizing molecule was the transplantation antigen H-Y (Wachtel *et al.* 1975), but this subsequently proved not to be the case (McLaren *et al.* 1984; Simpson *et al.* 1986). The role of male-specific antigens in testis development has been discussed recently by Wiberg (1987).

Burgoyne *et al.* (1986) have in fact questioned the very existence of a diffusible testis organizer, at least with respect to testis cord formation. They showed that embryonic XX gonadal tissue cocultured or cografted (under the kidney capsule) with developing testes, did not divert to the testicular pathway; or, more precisely, the germ cells and somatic cells of the XX gonad did not organize into testis cords. However, a connective tissue sheath resembling a tunica albuginea did form around some XX grafts. Similar findings were reported by Ożdżeński *et al.* (1976).

However, the developing testis does produce a locally acting factor which effectively eliminates fetal meiotic oocytes from contiguous ovarian grafts, and this is followed by regression of the ovarian tissue (Macintyre *et al.* 1960; Ożdżeński *et al.* 1976; Burgoyne *et al.* 1986). Recently, Vigier *et al.* (1987) showed that the factor responsible is the 'anti-Müllerian hormone' (AMH), alternatively known as Müllerian-inhibiting substance (MIS). Thus the presence of testes in the majority of XX↔XY chimaeras could be due to the regression of any ovarian tissue following the elimination of meiotic oocytes in fetal life, rather than to recruitment of the XX cells by a diffusible testis organizer. Indeed, evidence for a transition from fetal ovotestes to postnatal testes has been obtained from studies of XO/XY mosaics (Whitten *et al.* 1979; Eicher *et al.* 1980). Burgoyne *et al.* (1986) concluded their discussion as follows: 'The fact that differentiating XY testis cords do not induce testis cords in contiguous embryonic XX gonads suggests that the Y acts cell autonomously. That is to say that Sertoli cells can never be XX; something which might be testable using the testes of adult XX↔XY chimeras'. The present paper describes the results of an analysis of the distribution of XX and XY cells in XX↔XY chimaeric testes.

Materials and methods

(A) *Analysis of separated testicular tissues*

Chimaeras were produced by aggregating BALB/c embryos with embryos from a CBA strain which was homozygous for the T6 translocation marker (Ford *et al.* 1956) and in which the normal mouse Y had been replaced by a metacentric variant (Winking, 1978). These markers facilitated subsequent sex chromosomal analysis. BALB/c expresses the glucose phosphate isomerase electrophoretic variant GPI-1A while CBA expresses GPI-1B.

Eight overtly chimaeric males were killed 12–15 days *post coitum*, samples of liver, kidney, spleen and adrenal were frozen in liquid nitrogen for subsequent GPI analysis, a sample of bone marrow was obtained for subsequent chromosome analysis and the testes were removed for tissue separation. The tunica albuginea was stripped from each testis with watchmakers' forceps and frozen in liquid nitrogen. The testis tubules were teased apart in a drop of Hank's balanced salt solution (BSS) and incubated for 15 min in 2 ml of a 0·1 % solution of collagenase (Type 1A, Sigma) in BSS at 31°C on a roller (5 revs min^{-1}). The resulting tubule fragments were gently pipetted up and down and then allowed to settle. The supernatant ('Leydig' fraction) was removed, the cells pelleted (5 min, 1000 revs min^{-1}), the pellet washed in BSS and frozen in liquid nitrogen. This 'Leydig' fraction is quite heavily contaminated with germ cells and Sertoli cells. In the meantime, the tubule fragments were again incubated for 15 min in 0·1 % collagenase and then were washed twice in BSS. This was followed by two 15 min incubations in 0·1 % hyaluronidase to aid removal of the peritubular cells (Tung *et al.* 1984) and two washes in 1 % bovine serum albumin (BSA) in BSS. The tubule fragments were then washed and resuspended in 0·04 % EGTA in BSS lacking calcium and magnesium, pipetted vigorously up and down 50 times to produce small Sertoli cell/germ cell aggregates, and washed in 1 % BSA in BSS (Tung *et al.* 1984). The aggregates were resuspended in a drop of 1 % BSA and were layered onto two 25 mm round 'Thermanox' coverslips (Lux Scientific Corporation) in 50 mm Petri dishes containing Hepes-buffered minimum essential medium [MEM] (Flow Laboratories) supplemented with nonessential amino acids, glutamine, fungizone, penicillin and streptomycin. The dishes were incubated in humidified air at 31°C for 6 days with a medium change after 3 days. The Sertoli cells form a monolayer during this period while the germ cells detach and die. Finally, the coverslips were rinsed in Hepes MEM, one was fixed in 3:1 methanol acetic acid and stained with Giemsa in order to assess the purity of the culture, while the other coverslip was frozen in liquid nitrogen, thawed and a single drop of Hepes MEM was moved over the coverslip with a Pasteur pipette to recover released cellular constituents for subsequent GPI analysis.

A further three chimaeras, which had been identified as XX↔XY by chromosomal analysis of spleen biopsies, were killed as adults and the 'Leydig' fraction obtained by collagenase treatment as before. Pure Leydig cell samples for GPI analysis were then collected by mouth pipette (Leydig cells are clearly distinguishable under phase optics by their bright yellowish appearance — Schumacher *et al.* 1978).

GPI electrophoresis was carried out as described by McLaren & Buehr (1981) with the modifications described by Buehr & McLaren (1985). Quantification of the gels was carried out using a Sigma FTR20 scanning densitometer, minor trailing bands being ignored. A trial using blood samples had shown a good correlation ($r = 0{\cdot}95$) between sample concentration over a tenfold range and the relative GPI activity estimated by densitometry.

(B) In situ *hybridization of a Bkm-related probe to air-dried testicular cells*

One testis of an adult XX↔XY chimaera (identified by spleen biopsy) was removed under 'Avertin' anaesthesia and air-dried slides of testicular cells were prepared as described by Evans *et al.* (1964). The slides were stained in Giemsa, photographed and then destained in methanol followed by ethanol. GPI analysis was carried out on samples of liver, kidney, spleen and adrenal taken at autopsy.

The *Pst*1/*Bam*H1 fragment of the Bkm-related *Drosophila* DNA clone 2(8) (Singh *et al.* 1984) was subcloned into the plasmid vector pSP64 and transcribed to yield a 580-base RNA probe labelled with ^{35}S-UTP (New England Nuclear) to a specific activity of 2×10^8 disints min^{-1} μg^{-1}. The probe was hydrolysed in alkali to an average length of 100 bases (Cox *et al.* 1984), heat denatured and used at 120 $\mu g\,ml^{-1}$ in a hybridization mixture containing 50 % formamide, 10 % dextran sulphate, 10 mM-dithiothreitol, 10 mM-Tris pH 8·0, 0·3 M-NaCl, 1 mM-EDTA, 0·5 mg ml^{-1} tRNA and 1× Denhardt's solution.

The air-dried testicular cells were treated with 70 % formamide in 2×SSC (SSC = 0·15 M-NaCl, 0·015 M-sodium citrate, pH 7·0) at 70°C for 10 min and quenched in 0·1×SSC at 4°C before incubation with hybridization mixture for 4 days at 42°C. Slides were then treated with 20 $\mu g\,ml^{-1}$ RNase A at 37°C, and washed in 2×SSC at 20°C and 0·1×SSC at 37°C for 30 min each. Slides were dehydrated, dipped in 50 % Ilford G5 emulsion and exposed for 3 days at 4°C. The signal was visualized by development in 20 % Ilford Phenisol for 10 min at 20°C and the slides stained in Giemsa. Sertoli cells, pachytene spermatocytes and sperm were identified and marked on the photographs taken before hybridization. These cells were then identified under the microscope and scored for the presence of hybridization.

Table 1. *GPI analysis of separated testicular tissues and nontesticular tissue samples*

Chimaera type	Mouse No.	GPI A activity as % of total activity*						
		Sertoli†	Leydig‡	Tunica	Adrenal	Kidney	Liver	Spleen
XY BALB↔XY CBA (GPI A) (GPI B)	4	48	57	75	81	74	60	89
	9	35	38	58	58	61	59	43
	11	25	35	36	45	47	46	36
XX BALB↔XY CBA (GPI A) (GPI B)	6	13	38	–	56	60	51	54
	7	12	43	55	47	56	54	43
	10	9	38	50	52	56	61	45
XX CBA↔XY BALB (GPI B) (GPI A)	5	88	–	36	40	48	47	41
	8	84	66	54	51	57	54	44

* $\frac{\text{Area under GPI A peak}}{\text{Area under GPI A \& B peaks}} \times 100$, the areas being obtained from the densitometer traces.
† Contaminated with peritubular cells – see text.
‡ Contaminated with Sertoli cells and germ cells.

(C) In situ *hybridization of* Mus musculus *and* Mus caroli *probes to chimaeric* Mus musculus↔Mus caroli *testis sections*

Chimaeras were made by injecting immunosurgically derived inner cell masses from *M. caroli* blastocysts into *M. musculus* blastocysts (CD1 random-bred albino from Charles River) as previously described (Rossant & Chapman, 1983).

Unilateral orchidectomy was performed on three adult *M. musculus*↔*M. caroli* males which were 'balanced' chimaeras as judged by coat pigmentation and the testes fixed in ethanol: acetic acid (3:1) overnight. After rinsing in ethanol, the samples were embedded in ester wax (BDH 1960) and sectioned at 7 μm. Alternate sections were hybridized with biotin-labelled nick-translated plasmids containing 1400 bp of the *M. musculus* major satellite DNA sequence (C. Davis, unpublished data) or a 900 bp fragment of the *M. caroli* major satellite sequence (G. Fraser & J. Rossant, unpublished data). Conditions of hybridization were essentially as previously described (Rossant *et al.* 1986). Hybridization was visualized by binding of streptavidin–biotin–horseradish peroxidase complex (Enzo Biochem) to the biotinylated DNA. Horseradish peroxidase activity was revealed by diaminobenzidine staining followed by silver enhancement (Enhance, Amersham).

When the chimaeras were killed, a sample of bone marrow was obtained for sex chromosome analysis and samples of somatic tissues for GPI analysis.

Results

(A) *Analysis of separated testicular tissues*

The results of the GPI analysis for the eight prepuberal chimaeras (three XY↔XY, five XX↔XY) are given in Table 1 with examples of the GPI gels for testicular tissues in Fig. 1. The results for the XX↔XY chimaeras, expressed as percentages of XX cells in the various tissues, are summarized in

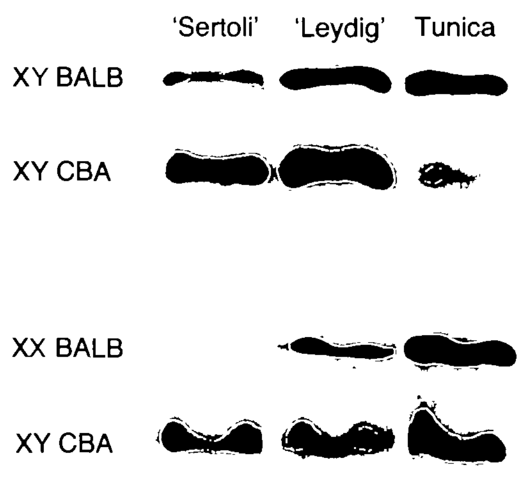

Fig. 1. GPI gels for separated testicular tissues from XY↔XY chimaera 9 (top panel), and from XX↔XY chimaera 10 (bottom panel). In the XX↔XY chimaera the GPI A band (XX) appears to be missing from the 'Sertoli cell' fraction. (When the fixed gel was scanned with the densitometer, a minor XX peak was detected.)

Table 2. All five XX↔XY chimaeras were 'balanced' as judged from the proportion of XX cells in nontesticular tissues, which ranged from 48–56 %. The proportion of XX cells in the tunica albuginea is similar to that in the nontesticular tissues, but in the Leydig and Sertoli cell fractions, XY cells predominate. In view of the contamination of the Leydig cell fraction with germ cells, which in XX↔XY postnatal testes are known to be XY derived (see Burgoyne, 1978), the true proportion of XX Leydig cells is likely to be greater than the results suggest. The results for the purified Leydig cell samples from the three adult XX↔XY chimaeras (Table 3) confirm the presence of XX Leydig cells. In spite of the purification, two of

Table 2. *A comparison of the contribution of XX cells to the testicular and nontesticular tissues of the XX↔XY chimaeras*

Mouse No.	% GPI activity in XX component Sertoli	Leydig	Tunica	Nontesticular* tissues
5	12	–	64	56
6	13	38	–	55
7	12	43	55	50
8	16	34	46	48
10	9	38	50	54
Mean	12	38	54	52

* Mean of adrenal, kidney, liver and spleen values.

Table 3. *The extent of the XX contribution in pure Leydig cell samples from XX↔XY chimaeras*

Mouse No.	% GPI activity in XX component Leydig	Tunica	Nontesticular* tissues
13	25	53	59
14	51	–	54
15	22	58	42

* Mean of adrenal, kidney, liver and spleen values.

the three Leydig cell samples still show a markedly greater proportion of XY cells than do the nontesticular tissues. Germ cell contamination is not a problem with the Sertoli cell fraction (the germ cells die in culture), so the marked predominance of the XY component in this fraction must be a true reflection of the Sertoli cell population. The minor XX component could be due to contamination with peritubular cells (Tung *et al.* 1984), since an examination of pelleted and sectioned tubule fragments following enzyme treatment confirmed that a small minority of fragments still had a peritubular cell layer. Furthermore, Dr Irving Fritz (Toronto) kindly examined the fixed culture samples from chimaera 5 and confirmed that there was contamination with a second cell type. The question as to whether there are any XX Sertoli cells was therefore addressed by using *in situ* DNA markers.

(B) *'Bkm' probe analysis of air-dried testicular cells*

Bkm (Banded Krait minor satellite)-related DNA in the mouse is concentrated on the Y chromosome, so that Bkm-related probes can be used for distinguishing XX and XY cells. The chimaera used for 'Bkm' probe analysis had approximately 38 % XX cells as judged by GPI analysis of somatic tissues. The results of the probe analysis are given in Table 4, with

Table 4. *Bkm probe analysis of air-dried testicular cells from an XX↔XY chimaera*

Cell type	+	?	–
Sertoli cells	141	0	0
Pachytene spermatocytes	30	0	0
Sperm	133*	52†	79

* Dense cluster of grains.
† Above background but not clustered.

examples of the probe hybridization in Fig. 2. In Sertoli cells, the centromeric regions of all the chromosomes are clustered together in a small number (usually 2) of heterochromatic blocks adjacent to the nucleolus (Hsu *et al.* 1971). Consequently 'Bkm', which hybridizes predominantly to the centromeric end of the mouse Y, is located by *in situ* hybridization to one of the heterochromatic blocks (Fig. 2A). Of the 141 Sertoli cells scored, all had a cluster of grains over one block of heterochromatin and, on this basis, were scored as positive, although, in three cells, the cluster of grains was not obviously greater than nearby background clusters. As a positive control, 30 pachytene spermatocytes were scored, and all showed the expected hybridization to the 'sex vesicle' which marks the position of the XY pair (Fig. 2B). Of the sperm heads scored, half were clearly positive with a dense cluster of grains and were presumably Y-bearing, while the remainder lacked a dense cluster of grains and were presumably X-bearing (Fig. 2C,D). Nevertheless, many of these presumed X-bearing sperm clearly had a grain density above the general background. This may have been due to nonspecific binding, or may reflect the fact that Bkm-related sequences, although concentrated on the mouse Y, also occur on other chromosomes (Jones & Singh, 1981; Singh *et al.* 1981).

(C) In situ *DNA probe analysis of* M. musculus↔ M. caroli *testis sections*

In situ hybridization to the testes of three male *M. musculus*↔*M. caroli* chimaeras revealed that two of the males showed a pattern of hybridization consistent with an XX↔XY genotype, namely, spermatogenic cells derived entirely from one genotype (*M. caroli*). Chromosomal analysis confirmed the XX↔XY genotype of male 1, but, in male 2, the quality of the chromosome spreads was not good enough for unequivocal sexing. From the GPI analysis of nontesticular tissues, there were approximately 40 % *M. musculus* (XX) cells in male 1, and approximately 65 % *M. musculus* (presumed XX) cells in male 2. The third male showed spermatogenic cells of both genotypes and was therefore an XY↔XY chimaera.

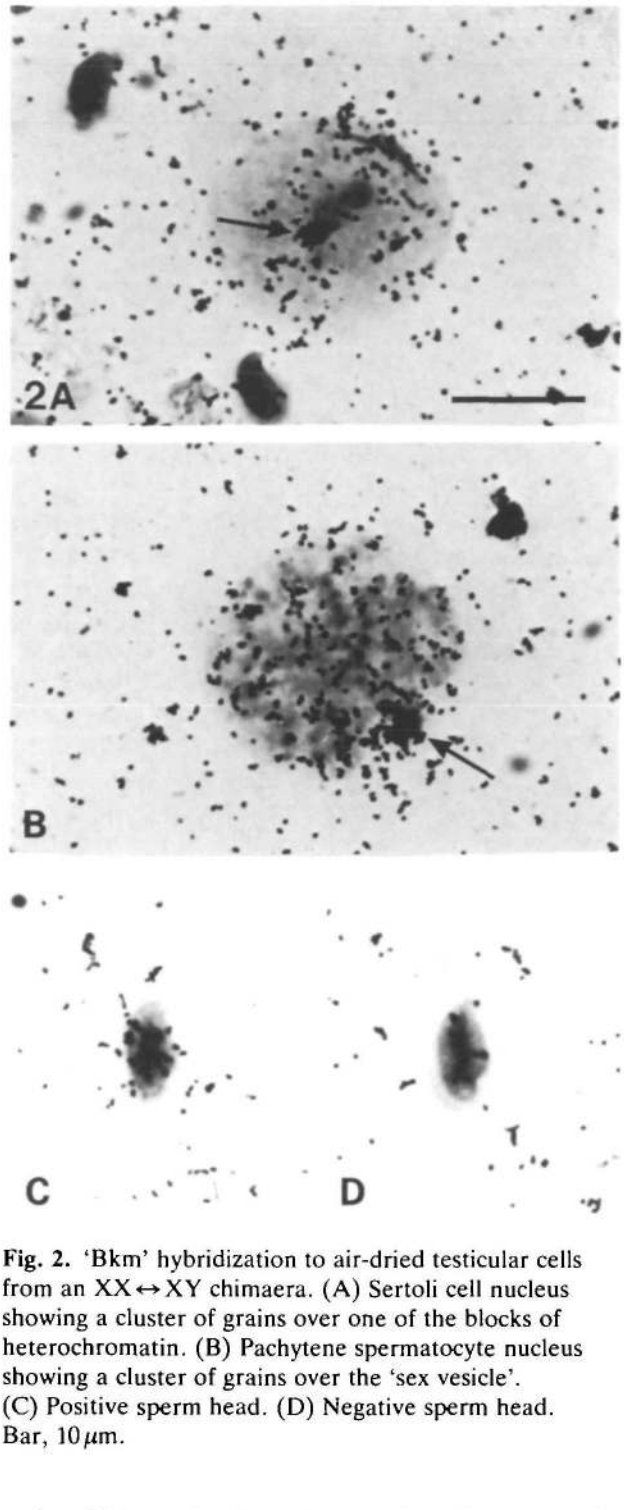

Fig. 2. 'Bkm' hybridization to air-dried testicular cells from an XX↔XY chimaera. (A) Sertoli cell nucleus showing a cluster of grains over one of the blocks of heterochromatin. (B) Pachytene spermatocyte nucleus showing a cluster of grains over the 'sex vesicle'. (C) Positive sperm head. (D) Negative sperm head. Bar, 10 μm.

Careful examination of over 20 sections from the testes of the two XX↔XY males failed to reveal any evidence of *M. musculus* XX cells inside the spermatogenic tubules, indicating that the Sertoli cells as well as the germ cells were *M. caroli* XY in origin (Fig. 3A). Very occasionally, an isolated *M. musculus* cell was identified apparently inside the spermatogenic tubules, but high-power examination revealed all these cases to be cells displaced during sectioning. *M. musculus* (XX) cells were present among the Leydig cells and peritubular cells (Fig. 3A), showing that there was no restriction as to the chromosomal sex of these cells.

Absence of hybridization to Sertoli cells is not a function of inefficient hybridization to these cells, since they hybridize strongly with the *M. caroli* probe (Fig. 3B). Also, Sertoli cells hybridizing with the *M. musculus* probe were present in the XY↔XY chimaera (Fig. 3C). The patches of different genotypes are very large in the spermatogenic cell populations of the XY↔XY chimaera. The implications of this will be discussed elsewhere (J. Rossant and V. Prideaux, in preparation).

Discussion

The present results demonstrate that, in prepuberal and adult XX↔XY chimaeras, the Sertoli cells are exclusively XY, confirming the suggestion made by Burgoyne *et al.* (1986). Both XX and XY cells were present among the Leydig cells, the peritubular cells and the vascularized connective tissue. Singh *et al.* (1987) have recently obtained results consistent with those presented here using *in situ* hybridization of a Y-specific DNA probe to testis sections of an XX/XY male mouse. In previous studies, it has been assumed that the XX contribution detected in XX↔XY chimaeric testes included Sertoli cells (Mintz, 1969; Ohno *et al.* 1978), but, in the light of our results, this assumption was almost certainly incorrect.

It has previously been widely accepted that the Y chromosomal testis-determining gene (*Tdy*) either regulates the production of, or encodes, a diffusible 'testis-determining' molecule to which both XX and XY cells can respond. The present results, combined with the results of grafting experiments (Burgoyne *et al.* 1986), suggest that Sertoli cell differentiation involves cell-autonomous Y activity. A reappraisal of the role of the Y in testis determination is therefore necessary.

Before attempting any reappraisal, it is helpful to summarize the events involved in the diversion of the inherently female gonadal primordium along the testicular pathway. Either directly or indirectly, *Tdy* activity is responsible for changing the fate of three gonad-specific cell lineages: (1) a 'germ cell' lineage which forms prospermatogonia in the fetal testis rather than the meiotic oocytes of the fetal ovary; (2) a 'supporting cell' lineage which forms Sertoli cells in the fetal testis but is destined to form follicle cells in the ovary and (3) a 'steroid cell' lineage which forms Leydig cells in the fetal testis but is destined to form theca cells in the ovary. In addition, *Tdy* must in some way direct the formation of a complex vascularized connective tissue network (of which the tunica albuginea is an integral part) so that testosterone produced by the fetal Leydig cells can be exported from the testis.

Studies of the sex chromosomal constitution of testicular cells in male sex chromosome chimaeras and mosaics enable us to define which of these events involve cell autonomous Y activity. From previous studies of XX↔XY chimaeras and XO/XY mosaics (reviewed by Burgoyne, 1987), it is clear that XO and XX germ cells can form prospermatogonia; thus a germ cell does not need a Y chromosome in order to divert to the testicular pathway. Cell autonomous Y activity is, however, subsequently needed for the normal proliferation and survival of male germ cells (Burgoyne *et al.* 1986; Levy & Burgoyne, 1986), but this involves a Y-chromosomal gene (*Spy*) which is distinct from *Tdy*, and need not concern us here. The present study demonstrates that XX cells can also form Leydig cells and peritubular cells and can contribute to the vascularized connective tissue components of the testis. However, our evidence that Sertoli cells in prepuberal and adult XX↔XY chimaeras are exclusively XY, taken together with the finding that fetal testicular tissue does not induce Sertoli cell formation in contiguous XX gonadal primordia (Burgoyne *et al.* 1986), strongly suggests that cell autonomous *Tdy* activity is involved in initiating Sertoli cell differentiation.

How can we reconcile such a direct involvement of the Y chromosome in Sertoli cell differentiation with reports that Sertoli cells can form in XX gonads; namely, in the bovine freemartin gonad (Jost *et al.* 1975), in XX fetal mouse ovaries grafted to male and, to a lesser extent, female host kidneys (Taketo-Hosotani *et al.* 1985; Taketo & Merchant-Larios, 1986) and in ageing rat ovaries (Crumeyrolle-Arias *et al.* 1976; Crumeyrolle-Arias & Ascheim, 1981; Crumeyrolle-Arias *et al.* 1986). In all three cases, Sertoli cells are differentiating in gonads that previously had developed as ovaries, the Sertoli cells being derived either from follicle cells or from the developmentally related intraovarian rete cells (Byskov & Lintern-Moore, 1973). Furthermore, in the case of the fetal mouse ovaries grafted to female hosts and in the ageing rat ovaries, the Sertoli cells form without any Y-chromosomal involvement whatsoever. This 'transdifferentiation' of XX follicle cells into Sertoli cells in these exceptional circumstances tells us that the gene activity that defines the Sertoli cell phenotype does not involve genes on the Y chromosome. This in no way undermines our conclusion that during the normal process of testis development the Y (via *Tdy*) acts cell autonomously to trigger Sertoli cell differentiation.

Since Sertoli cell differentiation is the first step in testis development (Magre & Jost, 1980), it could be that the only direct effect of *Tdy* is to trigger Sertoli cell differentiation, the subsequent steps being directed by the Sertoli cells. In this context, it is intriguing that the germ cells of fetal rat ovaries cultured in the presence of AMH (a Sertoli cell product), show reduced mitotic activity and many fail to enter meiosis (Vigier *et al.* 1987); that is to say, they behave more like prospermatogonia than like oocytes. Also these authors report the formation in the presence of AMH of a structure resembling a tunica albuginea around the ovary. Possibly then, in addition to causing regression of the Müllerian ducts, the AMH produced by the fetal Sertoli cells has a role in testis differentiation. The differentiation of Leydig cells could also be triggered by Sertoli cells, since in XX ovaries grafted into male host kidneys, Leydig cell differentiation (as evidenced by testosterone secretion) follows the appearance of the XX Sertoli cells (Taketo-Hosotani *et al.* 1985).

In summary, we reject the widely held view that the testis-determining gene *Tdy* acts via a diffusible testis-determining molecule to which XX cells can respond. Rather, we suggest that *Tdy* acts cell autonomously to bring about Sertoli cell differentiation and that all subsequent steps in testis differentiation may be a consequence of Sertoli cell activity.

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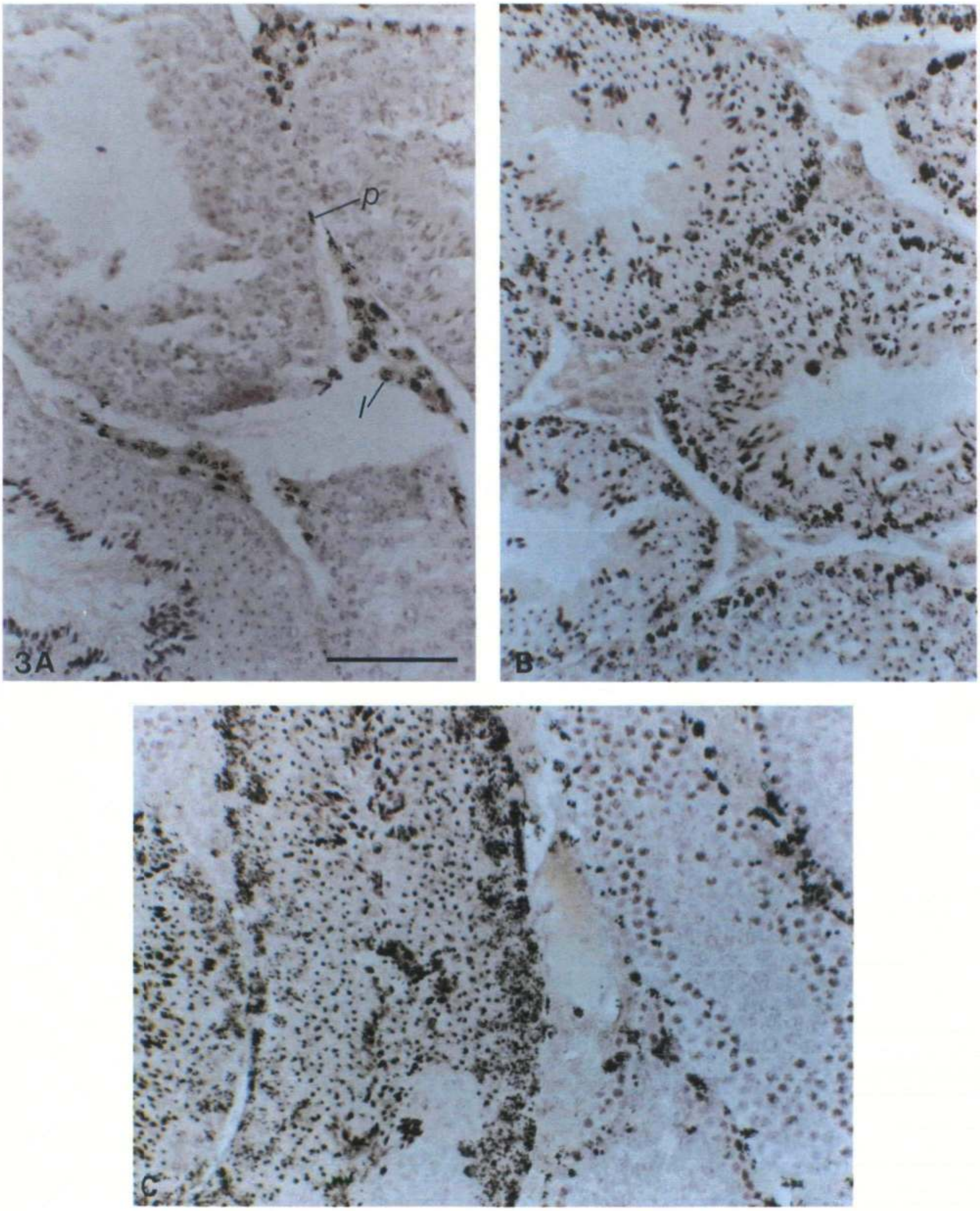

Fig. 3. DNA probe analysis of *M. musculus* ↔ *M. caroli* chimaeric testes. (A) Hybridization of the *M. musculus* major satellite probe to a testis section from XX (*musculus*) ↔ XY (*caroli*) male 1. All the cells within the tubules are unlabelled (XY) while most Leydig cells (*l*) and some peritubular cells (*p*) are labelled (XX). (B) Hybridization of the *M. caroli* major satellite probe to a testis section from male 1 showing the reverse pattern of hybridization. (C) Hybridization of the *M. musculus* probe to a testis section from the XY (*musculus*) ↔ XY (*caroli*) chimaera showing large patches of labelled and unlabelled cells within the tubules. Bar, 100 μm.

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Note added in proof

A testis-determining DNA sequence has now been cloned from the human Y chromosome. It encodes a DNA-binding 'finger' protein. The authors suggest that it acts in a cell autonomous fashion, in agreement with the conclusions we have drawn in the present paper.

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