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Genetics

Genetic basis of X–Y chromosome dissociation and male sterility in interspecific hybrids

(X–Y pairing region divergence/mechanisms of speciation/meiotic arrest/genetics of male fertility)

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ABSTRACT **A high frequency of X–Y chromosome dissociation (95%) was found at first meiotic metaphase (MI) in spermatocytes of interspecific hybrids between laboratory mice, C57BL/6J (BL/6) and *Mus spretus*, compared with an X–Y dissociation frequency of only 3–4% in parental mice. The X–Y dissociation in F_1 hybrids occurred before diakinesis rather than as a precocious dissociation at MI. The high X–Y dissociation was accompanied by spermatogenic breakdown after MI, resulting in male sterility. All F_1 males were sterile and approximately half of the backcross males from fertile F_1 females crossed with either BL/6 or *M. spretus* males were sterile. Male sterility was highly correlated with X–Y dissociation in both backcrosses. All of the mice with high X–Y dissociation were sterile and all of the males with low X–Y dissociation were fertile or subfertile. This correlation suggested that genetic divergence of the X–Y pairing region could contribute to the male sterile phenotype such that the BL/6 X chromosome would not pair with the *M. spretus* Y chromosome. The segregation of species-type alleles of amelogenin ($Amel^b$ and $Amel^s$), a distal X chromosome locus adjacent to the X–Y pairing region, was followed in backcross males that were analyzed for X–Y dissociation and sterility (we have used *Amel* as the designation for the mouse amelogenin locus; the current designation for this locus is *Amg*). A 95% concordance between $Amel^b$ with fertility and $Amel^s$ with sterility was observed in backcrosses with BL/6, whereas the converse was observed in the backcross to *M. spretus*. These results imply that X–Y pairing plays an important role in male fertility and suggest that genetic divergence in X–Y pairing region between *Mus* species can contribute to the reproductive barriers between species and the process of speciation.**

The X and Y chromosomes are normally associated end-to-end during the first meiotic metaphase (MI) of spermatocytes in the male house mouse (1–4). However, a variable frequency of X and Y chromosome dissociation (0–50%) at MI of spermatocytes has been reported in various laboratory mouse strains and *Mus musculus* subspecies stocks (5–8). In addition to the variable within-strain dissociation, a high frequency of X–Y dissociation (50–90%) has been observed at MI of spermatocytes in interstrain hybrids or intersubspecies hybrids between laboratory mice and several Asian *M. musculus* subspecies that were genetically divergent from laboratory mice (7–9). In these cases, the observed X–Y dissociation involved a precocious dissociation of chromosomes that were once paired at pachytene, and there was no plausible correlation between the precocious X–Y dissociation with nondisjunction or fertility (8, 9). A genetic analysis of the high X–Y dissociation in F_1 hybrids between laboratory mice and Japanese wild mice, *Mus molossinus*, suggested that at least one heritable factor controlling the end-to-end association of the sex chromosome at MI was located on the common region of X and Y chromosomes (10). The genetic factor had no influence on fertility of the intersubspecies hybrids, although their testis weight was significantly reduced.

Our laboratory as well as others have used crosses between laboratory strains of mice and *Mus spretus* for genetic analysis (11–13). *M. spretus* is an aboriginal species from the western Mediterranean region, including southern France, Spain, and Morocco, that is highly divergent from laboratory strains (*Mus domesticus*) (14, 15). The geographical range of *M. domesticus* and *M. spretus* overlap but there is no evidence that they interbreed in the wild (15). *M. spretus* males will interbreed with females of laboratory strains of house mice under laboratory conditions to produce viable hybrid progeny. The F_1 females are fertile and they can be backcrossed to males of either species. By contrast, F_1 hybrid males are sterile.

We have examined the testes of hybrids between laboratory mice, C57BL/6JRos (BL/6) and *M. spretus*, to determine whether the patterns of X–Y dissociation observed in crosses of more closely related *Mus* species are present in these hybrids. A high frequency of X–Y dissociation was observed in spermatocytes of interspecific hybrids between laboratory mice, BL/6, and European wild mice, *M. spretus*. These F_1 males are sterile with small testis sizes. We have asked whether male sterility segregates in backcross male progeny and whether the X–Y chromosome dissociation trait was concordant with the segregation of sterility. The high X–Y dissociation and male sterility segregated in a coordinate fashion in backcross progeny and to some extent these features were accompanied by small testis size. We present evidence that genetic divergence in the X–Y pairing region between the different *Mus* species is a primary reason for the X–Y dissociation and that dissociation of X and Y during meiosis leads to disruption of spermatogenesis.

MATERIALS AND METHODS

A laboratory inbred strain BL/6, a feral-derived stock *M. spretus*, and their F_1 hybrid and backcross male mice were used for the present study. *M. spretus* were derived from mice originally trapped in eastern Spain and maintained in outbred colony in our laboratory since 1979 (16). F_1 hybrids were obtained by mating BL/6 females with *M. spretus* males. The resulting F_1 females were backcrossed to either BL/6 or *M. spretus* males. The mice used in this study were mature males ranging in age from 9 to 12 weeks in BL/6, *M. spretus*, and F_1 hybrids and 10 to 14 weeks in backcross mice. The right testis was used for chromosomal preparation, the

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Abbreviation: MI, first meiotic metaphase.

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Table 1. Mean testis weights in groups of male mice 75–80 days of age (n = 10) and frequency of X–Y and autosomal dissociation (n = 6) in BL/6, *M. spretus*, and their F_1 hybrids

Mice	Body weight, g	Testis weight, mg	Relative testis weight*	Frequency of dissociation†	
				X–Y	Autosomes
BL/6	26.3 ± 2.3	203.0 ± 14.7	7.7 ± 0.6	3.0 ± 1.3	1.8 ± 0.9
M. spretus	14.9 ± 0.5	192.2 ± 33.2	12.9 ± 2.0	4.3 ± 1.6	1.7 ± 0.9
(BL/6 × *M. spretus*)F_1	19.0 ± 1.6	92.0 ± 11.7	4.8 ± 0.5	95.0 ± 2.7	23.0 ± 7.1

Values are given as mean ± SD.
*(Testis weight/body weight) × 10^{-3}.
†Six hundred cells observed per male.

left testis was for histological analysis, and the kidney was for DNA extraction.

Chromosomal preparations were made by the air-drying method of Imai *et al.* (8) without colchicine treatment. One-hundred spermatocytes at MI were observed from each individual to estimate the frequency of dissociated X and Y chromosomes. In histological analysis, the testis was fixed in 10% buffered formalin solution, and the tissue sections were stained with hematoxylin/eosin or Berg's method (17).

High molecular weight DNA was prepared from the kidneys of backcross males and digested with the restriction endonuclease *Eco*RI. The resulting fragments were separated by electrophoresis on 0.8% agarose gels and transferred to a nitrocellulose membrane (18). The membrane was hybridized with mouse amelogenin cDNA, which was provided by Malcolm L. Snead (University of Southern California, Los Angeles) (19, 20). The probe was radiolabeled by the random priming method (21), using high specific activity [^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq), and was carried out following the method of Chapman *et al.* (22).

RESULTS

Spermatogenesis in F_1 Hybrids. Testis size and weight is a sensitive indicator of male fertility and we used it as an initial measure of the sterility of hybrid males compared with the parental genotypes (Table 1). We observed that the testes of F_1 hybrids were significantly smaller than those of the parental mice, BL/6 and *M. spretus* (t = 17.7 and 8.6, respectively; $P < 0.001$). The body sizes of the laboratory strain, BL/6, were nearly twice that observed for *M. spretus*, 26.3 g vs. 15.0 g, respectively. Hybrids were intermediate to the parental strains. However, the weights of the testes were essentially the same in both genotypes. Thus, the weight of the testis did not correspond to the size of male but, given the variability of testis sizes within parental genotypes, we also calculated relative testis weight of hybrid males compared to those observed in parental genotypes (9). The relative testis weight as well as absolute testis weight was decreased in F_1 hybrids compared with those of BL/6 and *M. spretus* (t = 11.1 and 11.7, respectively; $P < 0.001$).

Histological sections of testes were examined to determine the relative abundance of different stages of spermatogenesis and spermiogenesis in F_1 hybrids using hematoxylin/eosin and Berg's stain (not shown) (Fig. 1). Spermatogenesis in hybrids was relatively normal up to MI, but extensive spermatogenic breakdown occurred after that stage. The extent of the breakdown was variable among hybrid individuals: 5 of 10 F_1 males had a rare occurrence of spermiogenic cells and no spermatozoa (azospermia). The remaining 5 of 10 mice had only a small number of spermatozoa and spermiogenic cells that had passed through secondary spermatocytes, and, of these, the shape of almost all spermatozoa was abnormal (Fig. 2 *L2–L6*). Collectively, the data indicate that spermatogenesis was primarily impaired at MI. A limited number of spermatocytes progressed beyond MI to make differentiated sperm and these were probably dysfunctional.

X–Y Chromosome Dissociation in F_1 Hybrid Mice. The spermatocytes of sterile males were analyzed cytogenetically to ascertain whether chromosomes were normally paired in meiotic prophase and metaphase I of meiosis (MI), especially the X and Y chromosomes. A low frequency of X–Y chromosome dissociation at MI (3–4%) was observed in the primary spermatocytes of BL/6 and *M. spretus* (Fig. 2 *a* and *b*; Table 1). By contrast, even though the sex vesicle was generally formed at pachytene stage in spermatocytes of the F_1 hybrids (Fig. 2*g*), the X and Y chromosomes were dissociated in most of the F_1 spermatocytes at late diakinesis (Table 1; Fig. 2 *c* and *d*). The X–Y association was examined in a total of 200 spermatocytes at early diakinesis in four F_1 hybrid males (50 cells in each mouse) to determine if X–Y dissociation occurs before MI. The X and Y chromosomes

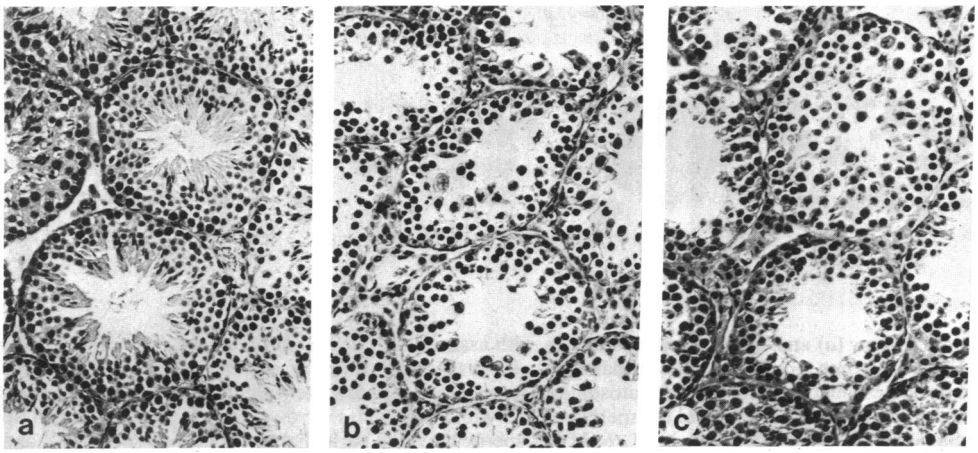

FIG. 1. Cross sections of testes stained with hematoxylin/eosin. (*a*) *M. spretus* (fertile). (*b*) F_1 hybrid (sterile). (*c*) Oligospermia backcross mouse with high frequency of X–Y chromosome dissociation (sterile). (×350.)

were dissociated in most of the cells at early diakinesis (88.5% ± 3.6%, Fig. 2*i*), suggesting that X–Y dissociation in F_1 hybrid spermatocytes started before early diakinesis. These results indicate that X–Y chromosome pairing was disrupted in F_1 hybrid males and that the disruption may have occurred early in meiotic prophase, possibly in the process of X–Y pairing itself.

We also asked whether there was evidence that the normal pairing of autosomes was disrupted in these hybrids. Some autosomal pairs as well as X and Y chromosomes were dissociated frequently in F_1 hybrids, with an average number of dissociated autosomal pairs of 0.268 ± 0.079 per cell (Table 1). Generally, it was the smaller autosomes that were dissociated and they often showed terminal association at MI (Fig. 2*d*). Overall, the occurrence of autosomal dissociation was variable and was not a consistent property of the hybrid cells compared to the X–Y dissociation.

Cytogenetic Properties of Degenerate Meioses. The degenerated late metaphase, as shown in Fig. 2 *j* and *k*, was observed frequently in hybrids. The dissociated sex chromosomes and autosomes appear to be decondensed compared with the normally paired autosomes. Cells with degenerated prophase were also observed but they were less frequent than cells with degenerated MI (data not shown). These results suggest that spermatogenic breakdown occurred at two stages, early prophase and MI, and that the main cause of F_1 hybrid sterility was degeneration at the later stage after first meiotic metaphase.

Backcross Analysis. The genetic basis of X–Y dissociation and male sterility was determined by analyzing the segregation of these traits in backcross males derived from either BL/6 or *M. spretus* males (Fig. 3; Table 2). The distribution of X–Y dissociation showed a significant bimodal pattern. There were 30/64 backcrosses from the BL/6 mating (BSB) that had X–Y dissociation (Fig. 2*e*) in 80% or more of their meiotic cells, whereas 34/64 had X–Y dissociation of 29% or less, with most of them <10%. A similar pattern was observed in the backcross males from *M. spretus* (BSS) with 18/59 males with >90% dissociation and 41/59 with 29% or less X–Y dissociation. The proportion of backcross males with high X–Y dissociation (X//Y) significantly differed from 50% in BSS but not in the BSB.

The backcross males with >80% X–Y dissociation were classified as high X//Y in both of the backcrosses and those males with 30% or less dissociation were classified as low X//Y (Table 2). Using these classifications, we examined the relative testis sizes of these groups to determine whether there was correlation between low testis size and high X//Y. All 30 of the BSB males with high X//Y had testis weights that were from the lowest portion of the distribution and the modal testis size of this group did not differ from that of F_1 hybrids. By contrast, nearly a third, 11/34, of the males with low X//Y had testis sizes that were similar to those of the high X//Y group, whereas the remaining 23/34 had testis sizes distributed around the sizes observed in BL/6 parental mice. The distribution of testis weights in the BSS males was similar to that observed in the BL/6 cross. The males with high X//Y had the lowest testis weights. Among the low X//Y males, 9/41 had small testis weights, whereas 32/41 had testis weights that were similar to parental males. These results indicate that high X//Y is associated with low testis weights but that there may be an additional factor(s) segregating that results in decreased testis size.

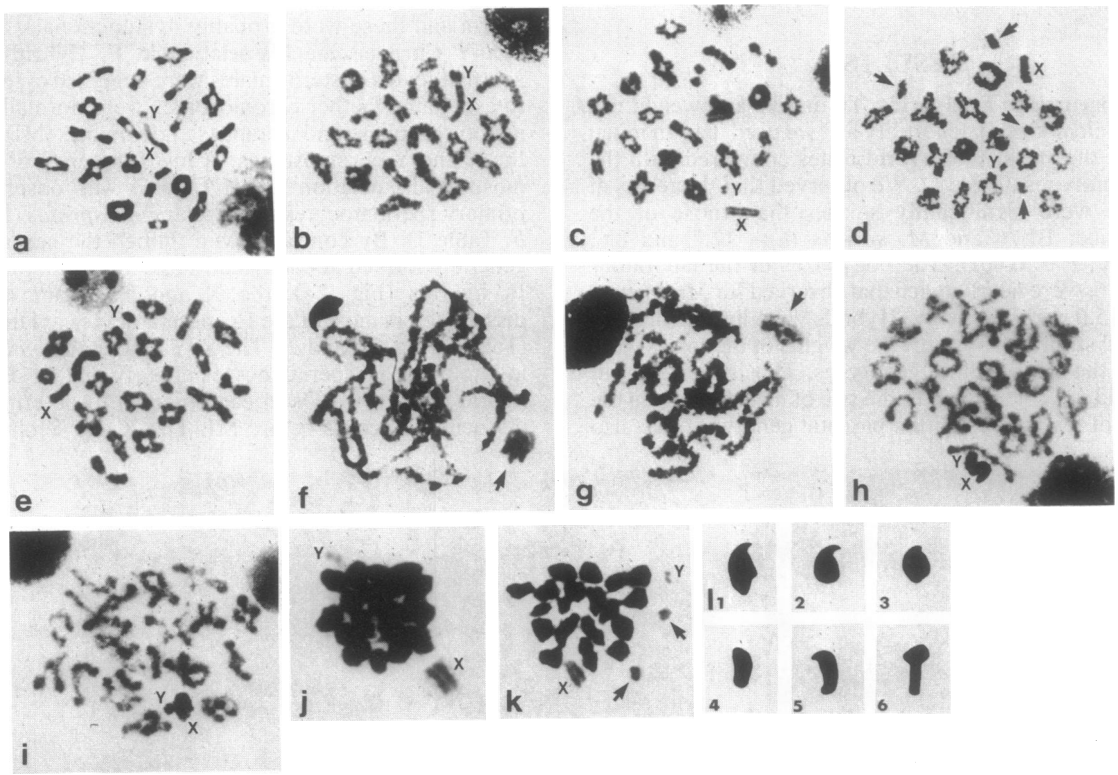

FIG. 2. (*a* and *b*) *M. spretus* (*a*) and BL/6 (*b*) spermatocytes with associated X and Y chromosomes. *M. spretus* Y chromosome is much smaller than BL/6 Y chromosome. (*c*) F_1 hybrid spermatocyte with dissociated X and Y chromosomes. (*d*) F_1 hybrid spermatocyte with dissociated X–Y chromosomes and two pairs of small autosomes (arrows). (*e*) Spermatocyte with dissociated X–Y chromosomes in a backcross mouse by BL/6. (*f* and *g*) BL/6 (*f*) and F_1 hybrid (*g*) spermatocytes with sex vesicles (arrows) at pachytene. (*h* and *i*) Spermatocyte at early diakinesis with associated X and Y chromosomes in BL/6 (*h*) and spermatocyte at early diakinesis with dissociated X and Y chromosomes in F_1 hybrid (*i*). (*j* and *k*) Degenerated spermatocytes at MI stage with dissociated X and Y chromosomes (*j*) and with dissociated X–Y chromosomes and one pair of small autosomes (arrows) (*k*) in F_1 hybrids. Both dissociated sex chromosomes and autosomes are decondensed. (*l1–l6*) Normal sperm head in BL/6 (*l1*) and abnormal sperm heads in F_1 hybrids (*l2–l6*).

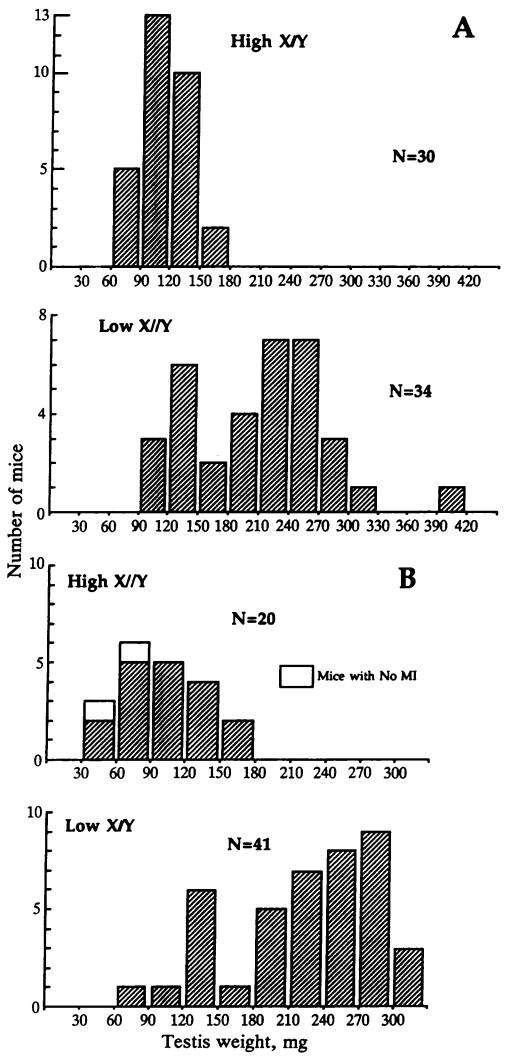

FIG. 3. Distribution pattern of testis weight in backcross mice with high and low frequency of X–Y dissociation (High X//Y and Low X//Y, respectively). Vertical lines show number of mice, and horizontal lines show testis weight. (*a*) (BL/6 × *M. spretus*)F_1 × BL/6. (*b*) (BL/6 × *M. spretus*)F_1 × *M. spretus*. High X//Y and Low X//Y show 80–100% and 0–29% X–Y dissociation, respectively.

Two BSS progeny showed complete spermatogenic arrest in which there were many spermatocytes at pachytene but no MI. Oligospermia was observed in about half of the X//Y males (15/30 in the BSB and 12/18 in the BSS), as shown in Fig. 1*c*. Almost all of the spermatozoa of the oligospermia mice (90–93%) were abnormally shaped. The other half of the mice with high X//Y had no spermatozoa.

Table 2. Relationship between X–Y dissociation and genotype of amelogenin in backcross mice by BL/6 (BSB) or *M. spretus* (BSS)

X–Y dissociation, %	BSB			BSS		
		Amel			*Amel*	
	n	B	S	*n*	B	S
High						
90–100	26			18		
80–89	4			0		
Total	30	2*	28	18	17	1*
Low						
20–29	1			2		
10–19	6			11		
0–9	27			28		
Total	34	34	0	41	2*	39

*Number of recombinant mice/total mice = 5/123 (0.041).

Mice with low X//Y were generally divided into two groups: one was a group of mice with smaller testes, ranging from 60 to 179 mg and 30 to 149 mg in BSB and BSS, respectively, whereas the second group had larger testes, ranging from 180 to 419 mg and 150 to 299 mg in BSB and BSS, respectively (Fig. 3). Mice with larger testes had a large number of spermatozoa and they appeared to be similar to fertile males. On the other hand, the spermatogenic status in the mice with smaller testes was variable. Some of them had a large number of spermatozoa, whereas others showed slight oligospermia, in which the number of spermatozoa was less than that of the parental mice but greater than that of sterile backcross mice, which had smaller testes and high X–Y dissociation. Based upon these criteria we judged them to be fertile or subfertile. A few backcross males in the low X//Y class had small numbers of spermatozoa but the fertility of these males could not be determined from testes cell preparations and histological sections.

These results suggest that there were at least two genetic factors controlling testis weight and fertility. A major factor was correlated with fertility and X–Y association. The other factor had a minor effect on fertility but no effect on X–Y association. If X–Y dissociation is due to a genetic difference between BL/6 and *M. spretus* X–Y pairing region, we would expect that the sterile backcross male would be either *M. spretus* X gene allele and BL/6 Y or BL/6 X gene and *M. spretus* Y in the reciprocal backcross. We examined the correlation of X–Y dissociation pattern with segregation of the amelogenin (*Amel*) gene, which was mapped to the most distal portion of the mouse X chromosome proximal to the X–Y pairing region [ref. 22 (we have used *Amel* as the designation for the mouse amelogenin locus; the current designation for this locus is *Amg*)].

The $Amel^b$ allele was observed in 34/34 backcross males with low X//Y in the BL/6 cross, whereas 28/30 with high X//Y had the $Amel^s$ allele. The reciprocal association was observed in the backcross males from *M. spretus* males where 39/41 of the males with low X//Y had the $Amel^s$ allele, whereas 17/18 males with high X//Y had the $Amel^b$ allele. If X//Y is considered as a segregating genetic trait, we observed 5/123 recombinations between *Amel* and this locus or 4.1 ± 0.35 centimorgans (Table 2). These results indicate that the levels of X–Y dissociation are coincident with the segregation of the X–Y pairing region and that interspecific divergence at the X–Y pairing region leads to a disruption of the normal X–Y pairing process in meiotic prophase. These findings and the observations on the fertility of F_1 males and backcross males with high X//Y further suggest that the failure to have normal X–Y pairing leads to a disruption of meiosis primarily at MI.

DISCUSSION

We have examined the testes of BL/6 × *M. spretus* hybrid males and established that they have a high frequency of X–Y dissociation, that they are very small, and that spermatogenesis is largely blocked at MI in spermatocytes. We have also shown that the X–Y dissociation and sterility are coordinately segregating in backcross males. Lastly, we have demonstrated that X–Y dissociation and sterility cosegregate with the amelogenin (*Amel*) locus at the distal end of the X chromosome. These results suggest that genetic divergence in the X–Y pairing region produces sterility in interspecific hybrid males by interfering with the normal process of X–Y pairing.

The first detailed analysis of genetic basis of X–Y chromosome dissociation in mouse spermatocytes was performed by using the fertile intersubspecies hybrids between laboratory mice and Japanese wild mice, *M. molossinus* (10). These

workers suggested that the high X–Y dissociation in the hybrid was controlled by a genetic factor (*Sxa*) located on the common region of X and Y chromosomes and that this region was responsible for the end-to-end association of sex chromosomes (10). Recently, Imai *et al.* (23) demonstrated the *Sxa* was linked closely to cream (*Crm*), a gene located near the distal end of the X chromosome (recombination value = 4.6%). Our study revealed that X–Y dissociation at MI was much more frequent in the interspecific hybrid (95%) than the intersubspecies hybrid (>50%). Additional studies suggest that the greater amount of dissociation in the *M. spretus* hybrids may be a result of a failure to pair rather than precocious dissociation in diakinesis (unpublished). Furthermore, the high X//Y in the *M. spretus* hybrid was accompanied by extensive disruption of spermatogenesis after the MI stage. These observations suggest that X–Y chromosome association is a prerequisite for the process of spermatogenesis.

The genetic factor controlling X–Y chromosome association and fertility was mapped on about 4 centimorgans, far from *Amel* gene. This result suggests that the genetic regulation of X//Y in *M. spretus* hybrids might be the same as *Sxa* of *M. molossinus*, although there may be some difference in the severity of the phenotype that correlates with the degree of genetic divergence between intersubspecies and interspecies. The partial synapsis of X and Y chromosomes is a common phenomenon among mammals (24–29).

Hybrid male sterility is a phenomenon that was described nearly 70 years ago by Haldane (30), who concluded that hybrid sterility was common to the heterogametic sex. Despite the widespread validity of Haldane's rule, the genetic mechanisms responsible for the occurrence of hybrid male sterility have not been clearly defined in mammals. In principle, hybrid male sterility could be caused either by a genetic imbalance between X chromosome and autosomes in F_1 male hybrids or by a genetic interaction between X and Y chromosomes. Based on mating analysis of *Drosophila melanogaster* subspecies, Coyne (31) suggested this type of hybrid sterility apparently arose from incompatibility between X and Y chromosomes, not from imbalance between sex chromosomes and autosomes. More recently, the specificity of the X–Y pairing in *Drosophila* has been localized to the ribosomal genes on the X and Y chromosomes (32).

The meiotic arrest in hybrids between the different *Mus* species is consistent with X and Y chromosome incompatibility. Our data suggest that the interaction between X and Y chromosomes in the X–Y pairing region is largely responsible for heterogametic sterility in *Mus* species hybrids and that the normal association of X and Y chromosomes was disrupted in meiotic prophase as a consequence of genetic divergence of the X–Y pairing region. We suggest that the failure of X–Y association caused meiotic arrest and spermatogenic breakdown and that these are important elements in the reproductive barriers between genetically divergent populations. Thus, the hybrid male sterility arising from genetic divergence of X–Y pairing region is proposed as a primary mechanism in the process of speciation. Reduced fertility is observed in intersubspecies hybrid males that is correlated with precocious X–Y dissociation. This may be the result of an intermediate level of genetic divergence of the X–Y pairing region (8, 9) between laboratory mice and Asian *M. musculus* subspecies that represents a transitional stage that will eventually lead to reproductive isolation.

It has been suggested that the synapsis in homologous X–Y pairing region may be unsaturated when X–Y pairing is incomplete and that the meiotic pairing site may become "activated," leading to inappropriate expression of genes in the unpaired chromosomes (33). Under these circumstances, inactivation of the X chromosome in meiotic prophase may be impaired in hybrid spermatocytes (34). By comparison, meiotic prophase in oogenesis has two active X chromosomes and meiosis is arrested at the dictyate stage just prior to MI (35). Degenerated spermatocytes were observed at late MI in the sterile hybrid males with dissociated X–Y pairing, which supports this hypothesis. The dissociated sex chromosomes or autosomes were decondensed, whereas the normal autosomes showed a dense, heterochromatic structure (Y.M. and V.M.C., unpublished observations). These observations suggest that the pairing-failed chromosomes were transcriptionally active at late MI stage in the sterile mice but that transcriptional activity generally seemed to be repressed. A more detailed analysis of pairing pattern of X and Y chromosomes at early prophase is necessary to clarify the mechanisms of meiotic arrest and spermatogenic breakdown in the interspecific hybrid male.

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