Localization of Murine X and Autosomal Sequences Homologous to the Human Y Located Testis-Determining Region

Michael Mitchell,* Dominique Simon,† Nabeel Affara,‡ Malcolm Ferguson-Smith,‡ Philip Avner* and Colin Bishop*[1]

**Unité d'Immunogénétique Humaine and †Unité de Génétique des Mammifères, Institut Pasteur, 75724 Paris Cedex 15, France, and ‡Department of Pathology, University of Cambridge, Cambridge CB2 8PQ, England*

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ABSTRACT

Recently a candidate gene for the primary testis-determining factor (*TDF*) encoding a zinc finger protein (*ZFY*) has been cloned from the human *Y* chromosome. A highly homologous *X*-linked copy has also been identified. Using this human sequence it is possible to identify two *Y* loci, an *X* and an autosomal locus in the mouse (*Zfy-1*, *Zfy-2*, *Zfx* and *Zfa*, respectively). Surprisingly *ZFY* is more homologous to the mouse *X* and autosomal sequences than it is to either of the *Y*-linked loci. Both *Zfy-1* and *Zfy-2* are present in the *Sxr* region of the *Y* but *Zfy-2* is absent in the *Sxr* deletion variant Sxr^b (or *Sxr″*) suggesting it is not necessary for male determination. Extensive backcross analyses map *Zfa* to mouse chromosome *10* and *Zfx* to a 5-cM interval between anonymous *X* probe MDXS120 and the tabby locus (*Ta*). We also show that the mouse androgen receptor locus (*m-AR*) believed to underlie the testicular feminization mutation (*Tfm*) shows complete linkage to *Zfx*. Comparative mapping indicates that in man these genes lie in separate conserved DNA segments.

THE mammalian *Y* chromosome plays a dominant role in controlling primary sex determination by inducing the bipotential fetal gonad to develop along the testicular pathway even when multiple copies of the *X* are present (JACOBS and STRONG 1959; RUSSELL and CHU 1961). This suggests that the *Y* encodes one or more specific sex-determining genes which have been named *TDF* (Testis-Determining Factor) in man and *Tdy* (Testis determining on the *Y*) in mouse. Recently PAGE *et al.* (1987) have cloned a candidate gene for *TDF* from the human *Y* chromosome. *TDF* was precisely mapped to interval 1A2 of the human *Y* chromosome using random DNA probes to analyze the breakpoints in $(Y^+)XX$ males and *XY* females. From this 140-kb interval a highly conserved gene encoding at least 13 zinc finger domains was isolated and has been termed *ZFY* (PAGE 1988). An *X*-linked homolog termed *ZFX* which maps to *X*p21–22.3 was also detected. Such zinc finger proteins probably regulate transcription by binding to specific DNA sequences (KLUG and RHODES 1987) and hence it appears to be a strong candidate for *TDF* itself. In the mouse *ZFY* detects two homologs termed *Zfy-1* and *Zfy-2* (PAGE 1988) both of which map to the sex-determining region of the *Y*. In addition both an *X*-linked and an autosomal homolog (*Zfx* and *Zfa*, respectively) can be found.

Definitive proof that *ZFY* is the testis-determining gene could be obtained by inserting the gene into female mouse embryos using transgenic technology. It would be expected that with the true testes determinant sex reversal would be observed. In addition the use of the mouse model to study the exact mode of action of such a gene will be essential. As a consequence the characterization of the *ZFY* homologies in the mouse is of considerable importance. We therefore present here an analysis of the *ZFY* homologs detected in the mouse including the localization of both *Zfx* and *Zfa* using extensive backcross analysis. Further analysis with the recently cloned (LUBAHN *et al.* 1988) human androgen receptor cDNA (*h-AR*) revealed that the mouse homolog (*m-AR*) was inseparable by recombination from *Zfx*.

MATERIALS AND METHODS

Southern blot analysis: Approximately 15 μg of DNA were digested with the appropriate restriction enzyme (Pharmacia), separated on 0.8% agarose gels then transferred to GeneScreen membranes (New England Nuclear) using 0.5 N NaOH/1.5 M NaCl. DNA was then fixed to the membrane by baking at 80° for 2 hr followed by UV irradiation. Probe DNA was labeled with ^{32}P to a specific activity of approximately 10^9 cpm/μg by random priming (FEINBERG and VOGELSTEIN 1984). Hybridizations were carried out overnight in 0.5 M phosphate buffer/7% SDS at 68° (CHURCH and GILBERT 1984). Washings were performed in 1 × SSC/0.1% SDS, 55° (nonstringent) or 0.1 × SSC/0.1% SDS, 65° (stringent), 1 × SSC = 0.15 M NaCl/0.015 M sodium citrate. Blots were dehybridized for reuse by washing in 10 mM Tris (pH 8), 1 mM EDTA (pH 8), 0.1% SDS at 80°. The human zinc finger containing sequence was obtained by screening the Livermore human

[1] To whom correspondence should be addressed.

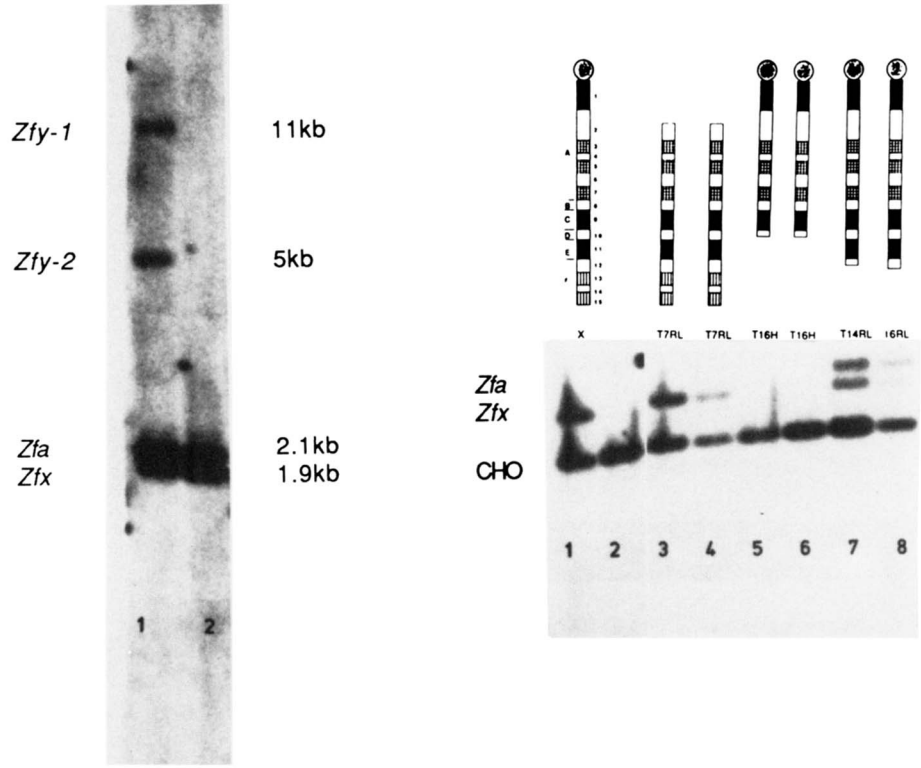

FIGURE 1.—(Left panel) Result of probing *Eco*RI digested male (lane 1) and female (lane 2) C57BL/6Pas DNA with the human *ZFY* probe pCMPXY1. Under nonstringent conditions, four loci can be identified. *Zfy-1* and *Zfy-2* represent the *Y* homologs, *Zfx* the *X* homolog and *Zfa* the autosomal homolog. Under stringent conditions only the *Zfa* and *Zfx* loci can be detected. (Right panel) Result of probing *Eco*RI-digested DNA from the *X* chromosome somatic cell hybrid deletion panel (AVNER *et al.* 1987) with pCMPXY1.

flow sorted *Y* chromosome library with a consensus oligonucleotide (30 mer) based on published amino acid sequence data (PAGE *et al.* 1987). The 1.3-kb *Hin*dIII fragment identified was sequenced and found to be identical to that described. It was then subcloned into the *Hin*dIII site of Bluscript (Stratagene) and designated pCMPXY1. The human androgen receptor probe is a 700-bp *Eco*RI/*Hin*dIII fragment of the cDNA clone pCMVAR of LUBAHN *et al.* (1988) which was kindly supplied by E. WILSON.

Mouse strains: Two panels of interspecific backcross mice between *Mus mus domesticus* and *Mus spretus* were used for mapping purposes. For mapping the *X* located sequence, 100 backcross mice (males and females) belonging to a (B6CBARI/Pas × SPE/Pas) × B6CBARI/Pas backcross panel were used (B6CBARI/Pas is a recombinant inbred strain constructed using C57BL/6Pas and CBA/Pas parentals). This panel has been previously used to build up a high resolution map of the mouse *X* chromosome and has been characterized for approximately 40 *X*-linked markers (AMAR *et al.* 1988). For mapping the autosomal sequence a backcross between (C57BL/6Pas × SPE/Pas) × C57BL/6Pas was used. This panel comprises 75 male mice which have previously been typed for approximately 80 dispersed autosomal markers.

RESULTS

The result of screening *Eco*RI digested C57BL/6Pas mouse DNA with pCMPXY1 (nonstringent washing conditions) are shown in Figure 1 (left panel). Two *Y*-specific bands of 11 kb (*Zfy-1*) and 5 kb (*Zfy-2*) can be found in male DNA. In addition two bands of 2.1 kb and 1.9 kb present in both male and female DNA can be detected. Although difficult to determine from Figure 1 the lower 1.9 kb band appears twice as intense in female compared to male DNA suggesting an *X* location, whereas the upper 2.1-kb band appears of equal intensity in both indicating an autosomal location. When washed under stringent conditions (0.1 × SSC, 65°) both the *Y* located bands disappear, whereas the *X* and autosomal sequences remain (data not shown).

Preliminary mapping of these bands using an *X* chromosome somatic hybrid deletion panel (AVNER *et al.* 1987) is shown in Figure 1 (right panel). Above each track is shown that part of the mouse *X* chromosome retained in the hybrid with the name of the translocation breakpoint from which it was derived; T7RL = T(*X*;*4*)7RL, T16H = T(*X*;*16*)16H, T14RL = T(*X*;*2*)14RL, T6RL = T(*X*;*7*)6RL. The names of the hybrids are given below. Lane 1 = VI/6, 2 = Chinese hamster control, 3 = G13n28, 4 = G13n23, 5 = B48c, 6 = B20c12, 7 = E11, 8 = N15. All hybrids contain multiple undefined autosomes except for VI/6 which is known to contain only chromosome *16* in addition to the entire *X*. This accounts for the appearance of the 2.1-kb autosomal band in the hybrids E11 and N15. These data cannot formally exclude that the 1.9-kb band maps to chromosome *16*. However, the presence of an *X*-linked homology was confirmed by the subsequent linkage analysis making it reasonable to assume that the 1.9-kb *Eco*RI band is *X* linked. Hence *Zfx* can be placed between the T(*X*;*16*)16H and T14RI breakpoints in the central region of the *X* chromosome. It can be seen that the 1.9-kb band maps to the central region of the *X* between the breakpoints defined by the somatic cell

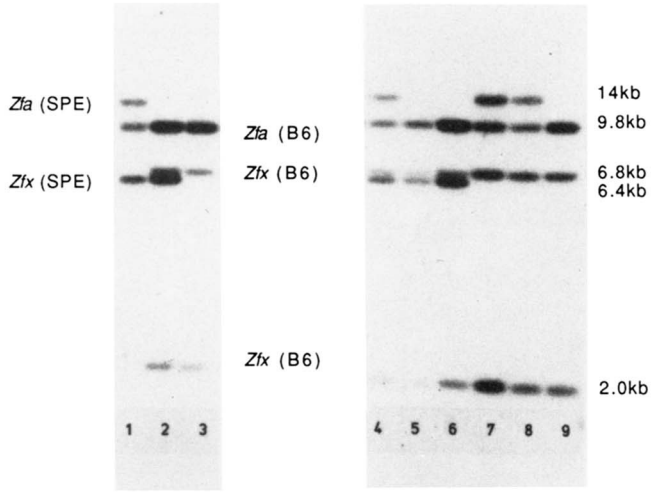

FIGURE 2.—Linkage analysis of the *Zfx* locus using the (B6CBARI × SPE/Pas) × B6CBARI backcross panel. pCMPXY1 is shown hybridized to *Pst*I digested panel DNA. The alleles are marked with SPE for *M. spretus* and B6 for B6CBARI. The filters were washed under stringent conditions.

hybrids derived from the *X*-autosome translocations T(*X*;*16*)16H and T14RL. The 2.1-kb band was absent from hybrid clone VI/6 which contains the complete mouse *X* showing it to be autosomally located. The mouse *X* locus has been termed *Zfx* (PAGE 1988) and the previously unreported autosomal locus has been termed *Zfa* (this publication).

The segregation of a restriction fragment length polymorphism (RFLP) at the *Zfx* locus was then examined in 100 mice from an interspecies backcross (B6CBARI/*M. spretus*) allowing a more precise localization. Figure 2 shows the polymorphic profile used. DNAs were digested with *Pst*I and probed with pCMPXY1 (stringent washing conditions). The C57BL/6 form of *Zfx* is split into two bands of 2.0 kb and 6.8 kb, the *M. spretus* form giving only one band at 6.4 kb. Mouse 1 is male and its single *X* chromosome is *M. spretus* derived in this region—note the absence of both C57BL/6-derived bands. Mice 2–9 are all female being either heterozygous (Nos. 2, 4, 5 and 6) or homozygous (Nos. 3, 7, 8 and 9) for the C57BL/6 allele. In addition a polymorphism for the *Zfa* locus can be seen to segregate independently of *Zfx*, although it could not be used for mapping purposes as this panel has not been characterized for autosomal loci. The *X* linkages were then computed with respect to 20 known markers (AMAR *et al.* 1988) giving the results shown in Table 1. The most likely locus order given is based on the computed linkage data and a detailed analysis of individual recombination breakpoints found in the pedigrees. It can be seen that *Zfx* maps to the 5.3-cM (±2.1) interval defined by anonymous DNA probe MDXS120 proximally and the Tabby coat color mutation (*Ta*) distally. As it is known that testicular feminization (*Tfm*) (LYONS and HAWKES 1970), a mutation believed to involve the androgen receptor leading to loss of responsiveness to testosterone, maps to this interval, we decided to use the recently cloned human androgen receptor (*h-AR*) (CHANG, KOKONTIS and LIAO 1988; LUBAHN *et al.* 1988) as a further marker in this region. A 700 bp *Eco*RI/*Hin*dIII fragment of the *h-AR* clone, pCMVAR (kindly supplied by Dr. E. Wilson), was used to screen the *X* chromosome somatic cell hybrid deletion panel and was found to map to the same deletion interval as *Zfx* (data not shown). Segregation analysis was then examined on the backcross panel using a *Pst*I generated RFLP as shown in Figure 3. No recombination was found between the *Zfx* and *m-AR* loci in the 81 mice typed for both markers showing them to be very closely linked (Table 1).

TABLE 1

Linkage analysis of *Zfx* to 20 *X* chromosome marker loci

Locus	Probe	Effective no. mice[a]	Percent recombination	SD
Centromere				
Otc	MN152	92	27.2	4.6
DXPas7	M2C	74	25.7	5.1
Syn-1	E2	95	26.3	4.5
SXPas3	p66	49	18.4	5.5
DXPas4	p87	51	19.6	5.6
DXPas5	p100	91	20.9	4.3
Hprt	HPT5	97	15.5	3.7
DXPas6	pc11	61	11.5	4.1
GdX-P	pG28	97	9.3	3.0
DXPas21	pHS7	84	8.3	3.0
DXPas8	pSt-14	57	12.5	4.4
Dmd	DIR87	92	6.5	2.6
	MDXS120	87	3.5	2.0
Zfx	**pCMPXY1**			
m-AR	**pCMVAR**	81	0	—[b]
Ta		79	3.9	2.2
Pgk-1	BE05:65	92	7.6	2.8
DXPas2	p52	61	6.6	3.2
DXPas20	cX52.5	96	21.9	4.2
Plp	P-23	83	20.5	4.4
DXPas1	p45	93	31.2	4.8
	pL9	74	31.1	5.4
Telomere				

[a] Effective no. of mice is the number of mice typed for both *Zfx* and any given locus. *Zfx* was placed in the 5.3 ± 2.1-cM interval between MDXS120 and *Ta* by linkage and an analysis of individual pedigrees. [See AMAR *et al.* (1988) for a description of the markers used.]

[b] Maximum distance between *m-AR* and *Zfx* can be calculated at 3.6 cM (95% confidence interval) according to binomial distribution.

The same procedure was followed in order to map the autosomal sequence in the C57BL/6Pas × *M. spretus* backcross mice (Figure 4). DNAs were digested with *Eco*RI and probed with pCMPXY1 (nonstringent washing conditions—note the presence of the two C57BL/6Pas-derived *Zfy* loci). The C57BL6-derived *Zfa* allele is 2.1 kb; that from *M. spretus* being 6.1 kb. Again the linkages were computed against all previ-

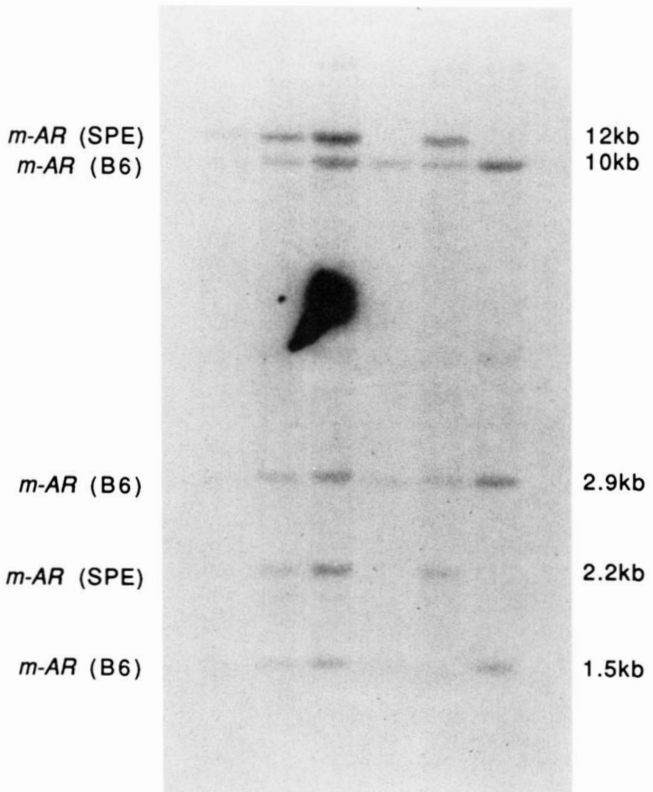

FIGURE 3.—Linkage analysis of the *m-AR* locus using the same filters as in Figure 2 after removal of the pCMPXY1 by dehybridization. A 700-bp *Eco*RI/*Hin*dIII fragment from the human androgen receptor cDNA clone pCMVAR was hybridized to the *Pst*I-digested panel DNA. Two *M. spretus*-derived bands, indicated by SPE, could be identified at 12 and 2.2 kb, the C57BL/6 alleles, indicated by B6, being at 10, 2.9 and 1.5 kb. Washings were nonstringent, being carried out in 2 × SSC at 55°.

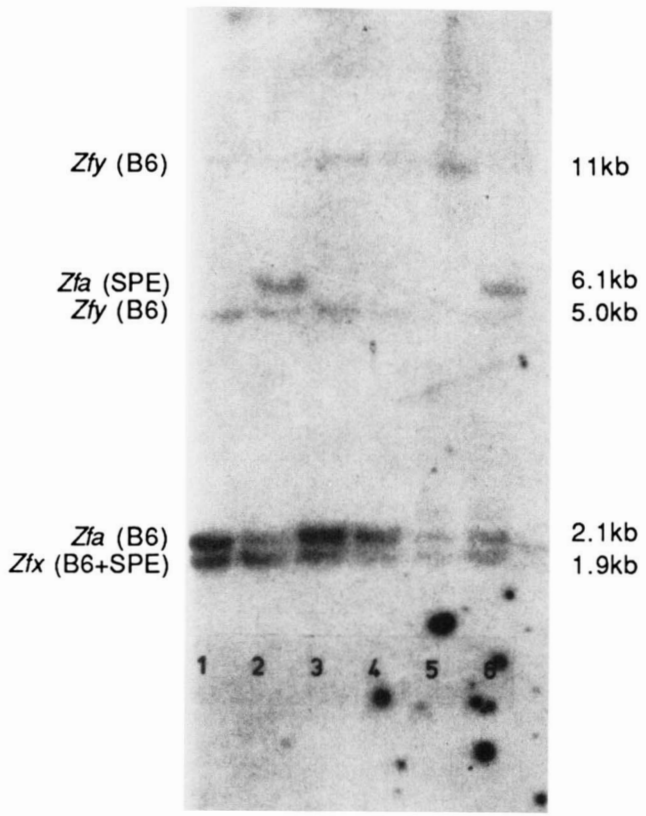

FIGURE 4.—Linkage analysis of the *Zfa* locus using the (C57BL/6Pas × SPE/Pas) × C57BL/6Pas backcross panel. pCMPXY1 was hybridized to *Eco*RI-digested panel DNA. The *M. spretus* alleles are indicated by SPE and the C57BL/6Pas alleles by B6.

ously typed markers (ROBERTS *et al.* 1985; BODE *et al.* 1988). Significant linkage was found only with the chromosome 10 markers c-*myb* (homolog of avian myoblastosis virus v-*myb*) (SAKAGUCHI *et al.* 1984) and *Pah* (phenylalanine hydroxylase) (BODE *et al.* 1988) (Table 2). Analysis of these data gives a most likely locus order of *Myb-Zfa-Pah* although the data are insufficient to establish an orientation with respect to the centromere. This gene order is based on minimizing double recombination events. Hence the gene order *myb-Zfa-Pah* requires only two double recombinants, whereas the gene orders *Pah-myb-Zfa* or *Zfa-Pah-myb* would require 12 and 8 double recombinants, respectively.

TABLE 2

Crosswise recombination percentages between chromosome *10* markers

	Myb[a]	*Zfa*	*Pah*[a]
Myb	—	25.5 ± 5.9 (55)[b]	32.8 ± 5.7 (67)[b]
Zfa		—	17.9 ± 5.1 (56)[b]
Pah			—

[a] BODE *et al.* (1988) previously reported the linkage of *Myb* and *Pah* to chromosome *10*.
[b] Numbers of animals typed are given in parentheses.

DISCUSSION

The data presented here clearly show that in the mouse the human zinc finger domain of *ZFY* as well as detecting two *Y* loci, *Zfy-1* and *Zfy-2* (PAGE *et al.* 1987), also detects an *X* homolog (*Zfx*) and an autosomal homolog (*Zfa*). Both *Y* loci have been shown to be present in the sex-determining *Sxr* region (PAGE *et al.* 1987). This suggests they are physically closely linked as this region is located on the minute *Y* chromosome short arm (ROBERTS *et al.* 1988; MCLAREN *et al.* 1988). In the *Sxr* variant Sxr^b (or *Sxr'*) (MCLAREN *et al.* 1984), which has been shown to be a deletion variant of the *Sxr* region (WEITH, MICHOT and BISHOP 1987; ROBERTS *et al.* 1988), only *Zfy-1* is present (PAGE 1988) showing that the two loci can be separated. The fact that $XXSxr^b$ mice, which have deleted *Zfy-2*, are male shows that this locus is unnecessary for male determination. A surprising finding was that on increasing the stringency of washing, the *Y*-located bands disappeared, whereas the *X* and autosomal bands remained even at high stringency (0.1 × SSC, 65°). This indicates that *Zfx* and *Zfa* are more homologous to each other and to the human *Y* finger se-

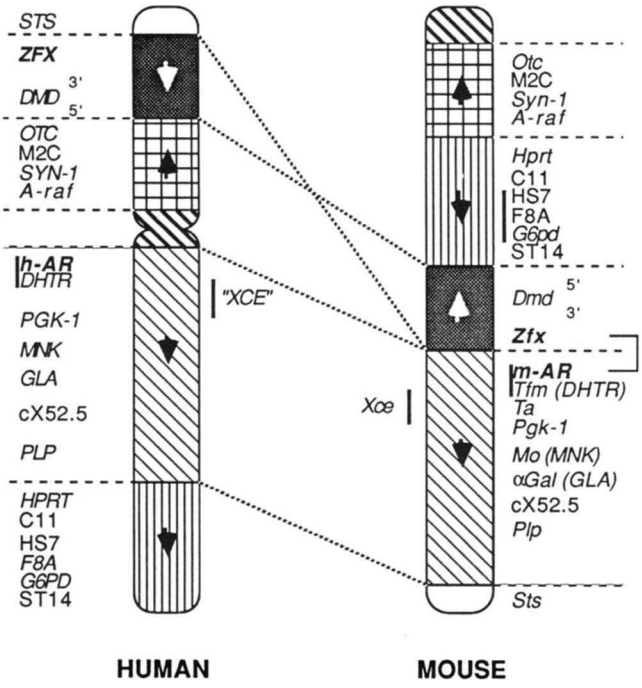

FIGURE 5.—Comparative maps of the *X* chromosomes of mouse and man. The chromosomes are represented schematically divided into six segments within which the loci and their relative order is observed to be conserved between man and mouse. Corresponding segments between the two species have the same shading and their relative orientation is indicated with an arrow. The loci defining these segments are shown at the side of the chromosomes and where necessary the human locus is shown bracketed beside the corresponding mouse locus. The relative order of the *Xce*, *Mo* and *Gal* loci in the mouse are inferred as they have not been typed in the mouse cross. Loci as yet unseparated by recombination are bracketed together. The approximate mapping positions of the *X* inactivation centers (*Xce* and "*XCE*") are shown on the opposite sides of the respective chromosomes. The corresponding boundaries of the conserved segments to which it is proposed *m-AR/h-AR* and *Zfx/ZFX* map are linked by dotted lines between the two chromosomes. The order of *m-AR* and *Zfx* presented is based on its consistency with the current six segments of conservation. The opposite order would require a further two such segments to be postulated (for further details see AMAR *et al.* 1988).

quence than they are to *Zfy-1* and *Zfy-2*. This taken together with the presence of an autosomal locus shows that the murine and human systems are arranged in a significantly different way. It remains to be seen whether this indicates a dissimilarity in the mechanisms of testis determination of mouse and man or merely reflects nonfunctional DNA rearrangements which have occurred after the separation of man and mouse from a common ancestor. We are presently investigating the exact sequence relationships between these zinc finger domains in the mouse.

Although the existence of the *X* homolog has been clearly established in all mammalian species studied (PAGE *et al.* 1987), no evidence for an autosomal homolog has been put forward, suggesting that the mouse may be unique in this respect. The linkage of *Zfa* to *Myb* and *Pah* places the autosomal homolog on chromosome *10*. Due to the lack of markers on this chromosome it is not possible to pinpoint their physical location. Preliminary *in situ* data suggest they may share the same chromosomal band as the Steel (*Sl*) mutation. In the mouse several autosomal loci have been postulated to play a role in primary sex determination (EICHER and WASHBURN 1986). These include *Tas* (*T*-associated sex-reversal), *Tda*-1, and *Tda*-2 (testis determining autosomal-1 and -2). Only *Tas* has been unequivocally mapped to chromosome *17* within the deletions defined by the T^{Hp} (*T-Hairpin*) and T^{Orl} (*T-Orleans*) mutations. Hence *Zfa*, if indeed it represents a functional locus and not merely an *X*-derived pseudogene, does not correspond to this locus.

The *X*-mapping data unequivocally show that both *Zfx* and *m-AR* map to the central region of the mouse *X*. Using the *X* chromosome somatic hybrid deletion panel they can be placed between the breakpoints of the T(*X*;*16*)16H-derived hybrids B48c and B20c12 and the T14RI-derived hybrid E11. Linkage analysis coupled with an examination of individual recombinant pedigrees places *Zfx* between random probe MDXS120 and *Ta* (Table 1) and is consistent with its physical mapping using this hybrid panel. It has been shown that the T(*X*;*16*)16H breakpoint maps to band X_D and the more distal locus *DXPas2*, defined by anonymous mouse probe 52, has been localized to the X_D/X_E boundary by *in situ* hybridization (AVNER *et al.* 1988). Thus, physically, the *Zfx/m-AR* loci can be assigned to mouse chromosomal band X_D or proximal X_E.

The MDXS120-*Ta* distance based on 113 mice is 5.3 ± 2.1 cM. It is interesting to note that no recombination could be found between *Zfx* and the *m-AR* locus. This latter locus is itself intimately involved in sexual differentiation as a defect in the receptors leads to testicular feminization in *XY* male mice. In addition the mouse *X* inactivation center *Xce* (GARTLER and RIGGS 1983), which has been postulated to be intimately involved in sex determination (CHANDRA 1985; PAGE *et al.* 1987), is tightly linked to *Ta* and hence close to *Zfx* and *m-AR*. Therefore three loci potentially involved in different aspects of sex determination/differentiation can be located in an interval of approximately 3.5 cM on the mouse *X* chromosome.

In the human it has been shown that *ZFX* maps to *X*p21–22.3 (PAGE *et al.* 1987) distal to the *X* chromosome breakpoint of a reciprocal *X*:*21* translocation originally reported by WORTON *et al.* (1984). The *X* chromosome breakpoint of this translocation has been shown to lie within the Duchenne muscular dystrophy (*DMD*) gene (BURGHES *et al.* 1987) which argues that in man *ZFX* maps telomeric to the 3′ end of the *DMD* gene. We have recently analyzed (AMAR *et al.* 1988)

the mouse homologies to a series of probes derived from proximal regions of the short arm of the human *X* chromosome including the *DMD* gene. Based on a single intragenic recombinant mouse, we have suggested an orientation for the corresponding mouse chromosomal segment of centromere-*3'Dmd* 5'-telomere. Analysis of a larger number of intragenic recombinants has, however, produced evidence for the opposite orientation of centromere-*5'Dmd* 3'-telomere (RYDER-COOK *et al.* 1988) and it therefore seems more likely that the latter is the correct polarity. In this study we have mapped *Zfx* to the telomeric side of *Dmd* in the mouse, which in view of these latter data show that, as in man, *Zfx* maps to the 3' side of the murine homolog of the *DMD* gene. It is therefore probable that the *DMD* and *ZFX* mouse homologs have been maintained on a single contiguous fragment of the *X* chromosome throughout the evolutionary period separating man from mouse.

Dosage theories of sex determination in mammals involving *X-Y* homologous genes have recently been proposed (PAGE *et al.* 1987; CHANDRA 1985). Such hypothesis require the inactivation of *X*-linked genes in females and any escape from *X* inactivation would invalidate them. The localization of *ZFX* to *X*p21–22.3 in the human places it close to that region of distal *X*p known to escape *X* inactivation. Thus its inactivation status in females remains uncertain. However, the localization of *Zfx* in the mouse to a region of the *X* known to be fully inactivated argues that *ZFX* will also be inactivated in man. Hence, if the underlying mechanism of sex determination is the same in both man and mouse, the dosage theory remains a real possibility.

Despite the close linkage between *Zfx* and *m-AR* seen in the mouse, in the human these loci are widely separated and reside on different conserved segments. Indeed, the *Zfx/m-AR* interval of less than 3.6 cM accurately defines an evolutionary breakpoint between the mouse and human *X* chromosomes. The comparative mapping data diagrammed in Figure 5 suggest that the *m-AR* locus would map distal to *Zfx* and therefore closer to *Ta* and *Xce* although linkage analysis of a greater number of animals will be needed to confirm this order.

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