

Inactivation of the *Zfx* gene on the mouse X chromosome

(Searle's translocation/reverse transcription-PCR/allele-specific expression)

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ABSTRACT *ZFX*, an X chromosome-linked gene encoding a zinc-finger protein, has previously been shown to escape X inactivation in humans. Here we report studies of the inactivation status of the homolog, *Zfx*, on the mouse X chromosome. We took advantage of both the preferential inactivation of the normal X chromosome in females carrying the T(X;16)16H translocation and the high degree of nucleotide sequence variation between the *Mus musculus* and *Mus spretus* genomes. An *EcoRV* restriction fragment difference between *M. musculus* and *M. spretus* was detected after amplification of *Zfx* transcripts using the reverse transcription-polymerase chain reaction. Using this allelic variation, we assessed expression of the two *Zfx* genes in females carrying the T(X;16)16H translocation (from *M. musculus*) and an intact X chromosome (from *M. spretus*). Such females exhibit *Zfx* transcription from the active *M. musculus* chromosome but not from the inactive *M. spretus* chromosome. These results indicate that the mouse *Zfx* gene is subject to X inactivation.

Mammalian X chromosome inactivation is a dosage compensation phenomenon that results in the same level of expression of most X chromosome-linked genes in males and females (1, 2). However, some X chromosome-linked loci have been shown to escape inactivation in humans and in mice. At first this escape from X chromosome inactivation appeared simply to reflect the position of the locus relative to the pseudoautosomal region of the chromosome. Genes that have been shown to escape inactivation in humans were found to be located within (*MIC2*, which encodes a cell surface antigen; ref. 3) or near (*XG*, which encodes the Xg blood group; refs. 4–6) the pseudoautosomal region. In addition, the steroid sulfatase gene (*STS*), also located near the pseudoautosomal region, partially escapes X inactivation in humans (7, 8), whereas in mice the *Sfs* gene is located within the pseudoautosomal region and completely escapes X inactivation (9, 10).

More recently, genes located farther away from the pseudoautosomal region of the human X chromosome have also been shown to escape X inactivation. These genes include *ZFX* (which encodes a zinc-finger protein) in Xp21.3–22.1 (11–15), *AIS9* in Xp11.3 (16, 17), and *RPS4X* (which encodes the ribosomal protein S4) in Xq13.1 (18). Clearly, escape from X inactivation is not limited to the most distal portion of the short arm of the human X chromosome. Little is known of the inactivation status of the corresponding genes in mouse.

Genetic variation between inbred strains of the laboratory mouse and interfertile *Mus* species has been readily identified as allelic differences at almost every locus examined (19, 20). This genetic variation can be used to follow the allelic

expression of X chromosome genes in heterozygous combinations. Female mice carrying the Searle's translocation, T(X;16)16H (21) (Fig. 1), exhibit a nonrandom X chromosome inactivation pattern in adult tissue (22). The normal X chromosome is inactivated in all or most of the cells in tissues from adult balanced translocation carriers (22–28). This seemingly preferential X chromosome inactivation results, at least in part, from the selective death of cells with incorrect dosage for certain critical genes on chromosomes X and 16 (25, 29). We have exploited the genetic variation between inbred strains and *Mus spretus* and the nonrandomness of X chromosome inactivation in mice carrying the Searle's translocation to analyze the expression of the *Zfx* gene when it is carried on the inactive X chromosome (Fig. 1). Using the reverse transcription (RT)-PCR method, we show that the *Zfx* gene undergoes X chromosome inactivation in mouse.

MATERIALS AND METHODS

Tissue Samples. T(X;16)16H mice were bred at Roswell Park Memorial Institute. Females carrying the balanced translocation were crossed with outbred *M. spretus* males. Among the resulting female F₁ progeny are balanced translocation carriers that can be distinguished from normal mice by isozyme expression of the enzymes phosphoglycerate kinase and hypoxanthine phosphoribosyltransferase (30). These mice have a balanced translocation from T(X;16)16H and one normal X chromosome derived from *M. spretus*. The strain background of the T(X;16)16H mice is an outbred laboratory strain, including C57BL/6J. Animals were killed at 3 months of age, and organs (kidneys, liver, submaxillary gland, spleen, and brain) were removed and quick-frozen on dry ice for nucleic acid preparation.

Nucleic Acid Preparation. DNA was prepared by phenol/chloroform extraction. RNA was prepared by the acid guanidinium thiocyanate/phenol/chloroform procedure (31). Concentrations were determined by A₂₆₀ for RNA and by using a fluorescent method for DNA (32).

RT-PCR. RT-PCR was carried out following the method of Kawasaki *et al.* (33). First-strand synthesis was carried out in a 20- μ l reaction mixture (10 mM Tris-HCl, pH 8.3/50 mM KCl/5 mM MgCl₂) containing 160 pg of oligo(dT) as primers, 1 μ g of total RNA as template, 200 units of Moloney murine leukemia virus reverse transcriptase, and each dNTP at 1 mM. The reaction was carried out at 42°C for 60 min followed by incubation at 95°C for 10 min and chilling on ice for 5 min. PCR amplification was then carried out after the addition of 80 μ l of 10 mM Tris-HCl and 50 mM KCl (pH 8.3) containing 160 pg of each gene-specific primer and 2.5 units of *Taq* polymerase.

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Abbreviation: RT, reverse transcription.

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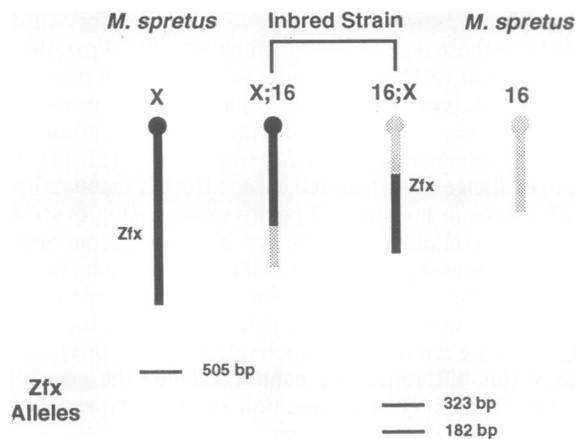


FIG. 1. Schematic representation of chromosomes X and 16 in F_1 translocation carrier females resulting from the $T(X;16)16H \times M. spretus$ cross. Depicted below are the expected allelic products of *EcoRV* digestion of RT-PCR-amplified *Zfx* transcripts.

PCR oligonucleotide primers were selected for the mouse *Zfx* gene and for the mouse hypoxanthine phosphoribosyl-transferase gene (*Hprt*) by computer analysis of the DNA sequence of each gene (*Hprt*, ref. 34; *Zfx*, ref. 35). The complete cDNA sequence for the *M. spretus* allele of *Hprt* was provided by G. Johnson (San Diego State University) (36). To eliminate the possibility of amplifying the *Zfa* gene, an expressed retroposon derived from the *Zfx* gene (37), the upstream primer was selected from a 100-base-pair (bp) *Zfx* cDNA sequence not present in the *Zfa* gene. The following primers were used in PCR amplification of *Zfx*: 5'-CAGTTGTCATCCAGGATGTC-3' (nucleotides 185–204) and 5'-TCGTTGTCATAGTCAGTCC-3' (reverse complement of nucleotides 770–789). The primers used for PCR amplification of *Hprt* were 5'-AAGTGTATTTCCTCATGGA-3' (nucleotides 188–207) and 5'-CAACATCAACAGGACTCCTC-3' (reverse complement of nucleotides 772–791). For both genes, the primers flank a genomic DNA fragment that contains an intron; thus possible DNA contamination of the RNA preparation would produce a PCR fragment of larger size than that of the corresponding cDNA fragment. A control PCR amplification of RNA samples omitting the RT step yielded no products, confirming the absence of DNA amplification in these samples. PCR conditions were 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for *Zfx*; and 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for *Hprt*. After 30 cycles, the extension was continued for 5 min at 72°C before termination by chilling on ice. Control PCR amplification of purified *Zfx* cDNA (pDP1115) and *Hprt* cDNA (pHPT5; no. 37424, from the American Type Culture Collection/National Institutes of Health Repository) yielded the appropriate-sized products (see Fig. 2, lane 13).

Electrophoresis and Restriction Analysis. The products of PCR amplifications were examined on 1.2% agarose gels in $1 \times$ TBE (0.089 M Tris borate/0.089 M boric acid/20 mM EDTA, pH 8.3) prior to restriction analysis. The PCR products were then diluted 1:3 in restriction buffer and digested with *EcoRV* for *Zfx* and with *Mae* II for *Hprt* allelic analysis. Analytical gels were 1% NuSieve GTG agarose/1% SeaKem GTG agarose (FMC) in $1 \times$ TBE.

RESULTS

We identified a restriction site variation in the mouse *Zfx* gene that distinguishes the laboratory mouse C57BL/6J from *M. spretus*. This genetic difference can be used to discriminate between products of RT-PCR amplification of RNA from the two species. Employing primers from within the

coding region of the *Zfx* message, we amplified a product of 505 bp from total cellular RNA of either species. Upon digestion with *EcoRV*, the C57BL/6J-derived product yielded bands of 182 and 323 bp, consistent with the presence of a single *EcoRV* site (at nucleotide 366), as predicted from the known sequence (Fig. 2A, lane 8). In contrast, the *M. spretus* product is not digested by *EcoRV*, indicating that the restriction site present in C57BL/6J is absent in *M. spretus* (Fig. 2A, lane 9).

The restriction pattern of the RT-PCR product from each kidney sample of six $T(X;16)16H \times M. spretus$ F_1 female translocation carriers (designated hereafter as F_1 translocation carrier females) resembles that of the C57BL/6J parent (Fig. 2A, lanes 2–7). These results are consistent with the *Zfx*

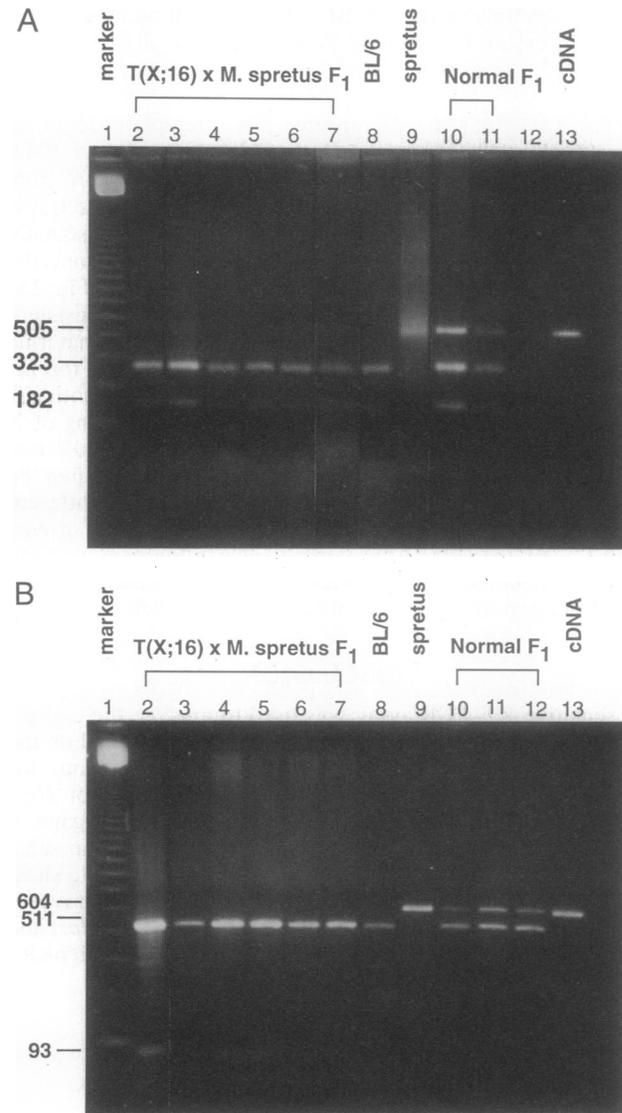


FIG. 2. (A) *EcoRV* digests of RT-PCR products of amplification of *Zfx* transcripts from kidney RNAs of six F_1 translocation carrier females (lanes 2–7, mice 1–6), a C57BL/6J male (lane 8), a *M. spretus* male (lane 9), and two C57BL/6J \times *M. spretus* F_1 females (lanes 10 and 11). Lane 12, no sample; lane 13, undigested control products of amplification of *Zfx* cDNA (pDP1115). The 182-bp fragment present in lanes 2–8 and 10 and 11 is faint due to its small size. (B) *Mae* II digests of RT-PCR products of amplification of *Hprt* transcripts, from the same RNAs as described in A, except that products from an additional C57BL/6J \times *M. spretus* F_1 female are in lane 12. Lane 13, undigested control products of amplification of *Hprt* cDNA (pHPT5). The 93-bp fragment present in lanes 2–8 and 10–12 is faint due to its small size.

allele on the translocated X chromosome (C57BL/6J) being active and the *Zfx* allele on the normal X chromosome (*M. spretus*) being inactive. Additional tissue samples (liver, spleen, brain, and submaxillary gland) from the F₁ translocation carrier females also showed the C57BL/6J bands and not the *M. spretus* bands (Fig. 3), confirming the inactivation of *Zfx* on the inactive X chromosome.

As a control, the expression of the *Hprt* gene, which is known to be inactivated (38–40), was also examined. The *Hprt* allele from *M. spretus* is distinguishable from that of C57BL/6J or C3H/HeJ by the presence of a *Mae* II site in the inbred strains only. Accordingly, the parental RT-PCRs yielded two fragments (511 bp and 93 bp) for C57BL/6J (Fig. 2B, lane 8) and one fragment (604 bp) for *M. spretus* (Fig. 2B, lane 9). In the F₁ translocation carrier females, we found expression only of the C57BL/6J allele with no evidence of any expression from the *M. spretus* inactive allele (Fig. 2B, lanes 2–7), as expected for a gene that is subject to inactivation.

To prove that the *M. spretus* RNA template could be amplified in the presence of the laboratory mouse RNA template, RT-PCR was carried out by using RNA from C57BL/6J × *M. spretus* F₁ females (not carrying the translocation). The restriction pattern from these chromosomally normal F₁ mice has all three bands expected from the presence of both parental alleles of *Zfx* and of *Hprt* (Fig. 2A, lanes 10 and 11 and Fig. 2B, lanes 10–12), which is consistent with mosaicism for inactivation of the paternal and maternal X chromosomes. However, the intensity of the *M. spretus* band is lower than that of the C57BL/6J bands for both *Zfx* and *Hprt*. This suggests that there may be a skewing of X inactivation in these normal F₁ mice, with the inbred strain allele being expressed in a larger proportion of cells than the *M. spretus* allele, which may reflect the presence of different *Xce* alleles (X-inactivation controlling element) in *M. spretus* and C57BL/6J (41). Alternatively, the differences in band intensities could reflect differences in the efficiencies of amplification of *M. spretus* and C57BL/6J alleles.

DISCUSSION

A sensitive RT-PCR assay was used to analyze the expression of the *M. spretus* alleles of *Zfx* and *Hprt* carried on the intact, inactive X chromosome of mice heterozygous for Searle's translocation. No *M. spretus* transcripts of *Hprt* were detected in the RNA isolated from several organs of translocation carrier females. Similar analyses of the same RNA samples for the *M. spretus* *Zfx* allele also failed to show expression of the gene when it was on the inactive X chromosome. By contrast, the *M. spretus* alleles of *Hprt* and *Zfx* were detected in RNA extracted from normal XX females

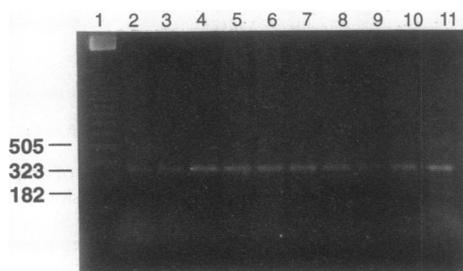


FIG. 3. *Eco*RV digests of RT-PCR products of amplification of *Zfx* transcripts from the following tissues of six F₁ translocation carrier females: liver and spleen of mouse 3 (lanes 2 and 3), liver and spleen of mouse 4 (lanes 4 and 5), liver and spleen of mouse 5 (lanes 6 and 7), spleen of mouse 6 (lane 8), brain and submaxillary gland of mouse 2 (lanes 9 and 10), and submaxillary glands of mouse 1 (lane 11). Lane 1, size markers (in bp).

that were hybrids between *M. spretus* and laboratory strains. Based upon these results, we conclude that, like *Hprt*, the *Zfx* gene is subject to X chromosome inactivation in mouse.

That the *Zfx* gene is inactivated in mice is in contrast to the situation in humans where there is no evidence of attenuation of *ZFX* expression on inactive X chromosomes (11, 14). Two lines of evidence demonstrated escape from X inactivation of the *ZFX* gene in humans: (i) positive expression of *ZFX* in hybrid cells containing an inactive X chromosome and (ii) increased expression of *ZFX* in cell lines containing multiple X chromosomes. The methodology used in these *in vitro* experiments is quite different from the one used in the present study, which examines the inactivation of *Zfx* *in vivo*. Because of this difference, we cannot exclude the possibility that there is a modest attenuation of *ZFX* expression on inactive human X chromosomes.

What determines whether a gene escapes or is subject to X chromosome inactivation? Relevant factors may include the gene's location with respect to the pseudoautosomal region and the inactivation center, the evolution of the X chromosome, the existence and expression of a Y-related homolog, and finally cis-acting regulatory sequences within or in proximity of the gene. Genes located within the pseudoautosomal region escape X inactivation (*MIC2*, ref. 3; *Sts* in mouse, refs. 9 and 10), as might be expected, since these genes have identical counterparts on the Y chromosome. In addition, proximity to the pseudoautosomal region may modify the X chromosome inactivation pattern by a position effect, which may be the case for genes such as *STS* (7, 8) and *XG* in humans (4). Since the human *ZFX* gene is separated from the pseudoautosomal region by the phosphoribosyl pyrophosphate synthetase 2 gene (*PRPS2*) (42), which appears to be subject to inactivation (L. Shapiro, personal communication), the different locations of human *ZFX* and mouse *Zfx* with respect to the pseudoautosomal region are not sufficient to explain their different patterns of inactivation in humans and mice. The proximity of the mouse *Zfx* gene to the inactivation center (15, 43, 44) may render it more susceptible to inactivation as compared to the human *ZFX* gene, which is located far from the inactivation center (12, 13, 15). The case of the human *RPS4X* gene, however, demonstrates that proximity to the X chromosome inactivation center does not ensure that a gene will be subject to X chromosome inactivation (18).

Escape from X chromosome inactivation may also be related in part to the evolution of the X chromosome. Most genes located on the short arm of the human X chromosome, including *ZFX*, are autosomal in marsupials and monotremes (45, 46). Although it is not known which of the marsupial, monotreme, or eutherian X chromosomes is more "primitive," one possibility is that the onset or maintenance of X inactivation of genes located on the human X chromosome short arm might be incomplete. The mouse X chromosome may have further evolved and be more completely and stably inactivated, as compared to the human X chromosome.

Both in mice and in humans, the *Zfx/ZFX* genes have Y homologs. However, the X and Y chromosome-linked genes are characterized by different patterns of expression between the two species. In humans, the *ZFX* and homologous *ZFY* genes are both widely, if not ubiquitously, expressed and may thus have closely related functions (11, 14). In contrast, in mouse, the expression of the *Zfy-1* and *Zfy-2* genes is largely restricted to the adult testis (47–49), whereas the *Zfx* gene is expressed in many embryonic and adult tissues (35, 37). It should be noted that for the non-pseudoautosomal genes shown to escape X inactivation in human, except for the *AIS9* gene, related sequences located on the Y chromosome have been reported. Both the *ZFX* and *RPS4X* genes have functional homologs on the short arm of the Y chromosome

(18, 50), whereas an *STS*-derived pseudogene is located on the long arm of the Y chromosome (8).

Finally there may be differences in regulatory sequences of the *ZFX* and *Zfx* genes. These sequences may affect the primary onset of X inactivation during embryogenesis or modify the stability of epigenetic changes associated with X chromosome inactivation. Analysis of the genomic structure and sequence of the *Zfx* locus in mice and the *ZFX* locus in humans may reveal sequence motifs associated with genes that escape or with genes that undergo X chromosome inactivation.

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