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 Sts 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), AF59 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 Mus 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 Mus 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-μl reaction mixture (10 mM Tris-HCl, pH 8.3/50 mM KCl/5 mM MgCl2) containing 160 pg of oligo(dT) as primers, 1 μg 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 μl 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.

Abbreviation: RT, reverse transcription.

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PCR oligonucleotide primers were selected for the mouse Zfx gene and for the mouse hypoxanthine phosphoribosyltransferase 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'-CAGTGTGCATCCAGGATGTC-3' (nucleotides 185–204) and 5'-TCGTTGTCCATAGTCAGTCC-3' (reverse complement of nucleotides 770–789). The primers used for PCR amplification of Hprt were 5'-AAATGTGTATTCTCAGGGA-3' (nucleotides 188–207) and 5'-CAACATCAGGTAGATGTC-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× 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× 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 × M. spretus F1 females translocation carriers (designated hereafter as F1 translocation carrier females) resembles that of the C57BL/6J parent (Fig. 2A, lanes 2–7). These results are consistent with the Zfx

![Fig. 1. Schematic representation of chromosomes X and 16 in F1 translocation carrier females resulting from the T(X;16)16H × M. spretus cross. Depicted below are the expected allelic products of EcoRV digestion of RT–PCR-amplified Zfx transcripts.](image-url)

![Fig. 2. (A) EcoRV digests of RT–PCR products of amplification of Zfx transcripts from kidney RNAs of six F1 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 × M. spretus F1 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 × M. spretus F1 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.](image-url)
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 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 (13, 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.

![Fig. 3. EcoRV 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).](image-url)
(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 ZFY 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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