

# Inactivation of the *Rps4* Gene on the Mouse X Chromosome

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The human *RPS4X* and *RPS4Y* genes, located on the X and Y chromosomes, appear to encode isoforms of ribosomal protein S4. Haploinsufficiency of these genes may contribute to the human phenotype known as Turner syndrome. Although *RPS4X* maps near the X-inactivation center, the gene is expressed on inactive human X chromosomes. We cloned *Rps4*, the mouse homolog of *RPS4X*. Exploiting allelic variation in *Rps4*, we examined transcription of the gene from active and inactive mouse X chromosomes *in vivo*, in female mice carrying an X-autosome translocation. We report that mouse *Rps4*, unlike human *RPS4X*, is subject to X inactivation. This finding may explain, at least in part, why the phenotypic consequences of X monosomy are less severe in mice than in humans. © 1991

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## INTRODUCTION

Most X-linked mammalian genes are dosage compensated via X inactivation, the transcriptional silencing of all but one X chromosome per cell. A few genes, however, have been found to escape X inactivation. Among these are the human *ZFX* and *RPS4X* genes (Fisher *et al.*, 1990; Schneider-Gädicke *et al.*, 1989). *ZFX* encodes a putative DNA-binding protein whose function is unknown; *RPS4X* encodes ribosomal protein S4, a component of the small subunit. Recently, using a novel assay we showed that in the mouse, in contrast to the human, *Zfx* undergoes X inactivation (Adler *et al.*, 1991). We used the same experimental strategy to determine the inactivation status of mouse *Rps4*.

The tissues of female mice are normally mosaic with respect to inactivation of paternally or mater-

nally derived X chromosomes. However, in females carrying Searle's reciprocal translocation of chromosomes X and 16 (Searle, 1962), hereafter designated "T16H," the translocated X is active and the intact X is inactive in adult tissues (Lyon *et al.*, 1964). This is apparently the result of selection during embryogenesis against cells with abnormal levels of expression of genes on chromosomes X and 16 (Tagaki, 1980). Using T16H females, one can assess a gene's X-inactivation status by determining whether the allele on the intact (inactive) X is expressed.

The assay requires the ability to distinguish allelic products of the intact and translocated X chromosomes. As with *Zfx*, we took advantage of the high degree of genetic variation between inbred strains of laboratory mice and *Mus spretus* to identify nucleotide sequence polymorphisms in *Rps4* alleles. Exploiting restriction site variations and allele-specific oligonucleotide hybridization, we show that *Rps4*, like *Zfx*, is subject to X inactivation in the mouse.

## MATERIALS AND METHODS

### Characterization of Mouse *Rps4* cDNA

A  $\lambda$ gt10 testis cDNA library from mouse strain FVB (Mardon and Page, 1989) was screened by hybridization with human *RPS4Y* cDNA pDP1278 (Fisher *et al.*, 1990). Positives were subcloned as *EcoRI* fragments into the vector Bluescript (Stratagene). The largest cDNA subclone, pDP1340, contained a 0.9-kb insert; its nucleotide sequence was determined by the chain termination method using T7 DNA polymerase and [<sup>35</sup>S]dATP.

### Tissue Samples, Nucleic Acid Preparation, Reverse Transcription Polymerase Chain Reaction (RT-PCR)

*M. spretus* males were crossed to laboratory (C3H and C57BL/6 interbred) females carrying Searle's

Sequence data from this article have been deposited with the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under Accession No. M73436.

translocation. Female progeny (F1) were typed as either chromosomally normal (XX) or balanced translocation carriers (T16H) by X-linked isoenzyme analysis (Adler *et al.*, 1991; Krumlauf *et al.*, 1986). RNAs were prepared from tissues as previously described (Adler *et al.*, 1991) and treated with DNase prior to RT-PCR. *Rps4* transcripts were amplified by RT-PCR using primers TGCTGGATAAGTTGACTGGC (coding region, sense) and AGCATGTC-TCTAGAGACCA (3' untranslated region, antisense) (Fig. 2b). Conditions were as previously described (Adler *et al.*, 1991), except that the PCR annealing step was performed at 60°C. Control reactions omitting reverse transcriptase yielded no product. The RT-PCR product of *M. spretus* parental RNA was gel-purified and directly sequenced (Casanova *et al.*, 1990).

#### Electrophoresis, Restriction Analysis, and Oligonucleotide Hybridizations

PCR products were analyzed by restriction digestion and agarose gel electrophoresis as previously described (Adler *et al.*, 1991). Digests were transferred to GeneScreen Plus filters for hybridization. Allele-specific oligonucleotides spanning the 2-bp insertion/deletion (Fig. 2) were chosen [GAGACCATCACCA (*M. spretus*) and AGACCATTTACCCA (C57BL/6)] and were labeled using [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase. Filters were hybridized for 2 h in 6× SSC, 10× Denhardt's, 0.5% SDS with 5 × 10<sup>5</sup> cpm/ml of probe at 47°C (*M. spretus* oligonucleotide) or 37°C (C57BL/6 oligonucleotide), and then washed three times for 10 min each wash in 6× SSC, 1% SDS at 47°C (*M. spretus* oligonucleotide) or 42°C (C57BL/6 oligonucleotide).

## RESULTS

### Mouse *Rps4* cDNA Sequence

We cloned mouse cDNA pDP1340 by cross-hybridization with human *RPS4Y*. Nucleotide sequencing of pDP1340 revealed a long open reading frame (Fig. 1). The predicted protein is identical in length and primary structure to human *RPS4X* (Fisher *et al.*, 1990) and to rat ribosomal protein S4 (Wool *et al.*, 1990), but differs from human *RPS4Y* at 19 of 263 amino acid residues. The genomic locus corresponding to cDNA pDP1340 was cloned, sequenced, and found to map to the mouse X chromosome (R. Hamvas, A.Z., and co-workers, submitted). As with human *RPS4X* (Fisher *et al.*, 1990), the mouse gene maps between the *Ccg-1* and *Pgk-1* genes, near the X-inactivation center (Keer *et al.*, 1990). Thus, nucleotide sequencing and comparative genetic mapping demonstrate that the

pDP1340 gene, which we refer to as *Rps4*, is the mouse homolog of human *RPS4X*.

### Allelic Variation

We amplified a 757-bp portion of the *M. spretus* *Rps4* transcript by reverse transcription polymerase chain reaction and compared its sequence with that of FVB cDNA pDP1340 (Fig. 2). The sequences differed by two single nucleotide substitutions within the *Rps4* coding region and by a 2-bp insertion/deletion immediately following the stop codon. One base-pair substitution, an A to G transition at nucleotide 393, is silent. The other, a C to A transversion at nucleotide 538, results in substitution of methionine for leucine at amino acid 180 in the predicted translation product. The nucleotide substitutions also create unique recognition sites in the *M. spretus* sequence for the restriction enzymes *Bsp1286I* and *AflIII*. The *Rps4* alleles of other inbred laboratory strains examined, e.g., C57BL/6J (Fig. 3), are like that of FVB.

### X Inactivation

RT-PCR amplification of *Rps4* transcripts from *M. spretus*, C57BL/6J, and XX and T16H F1 females yielded products of the same size as a control PCR with cDNA pDP1340 (Fig. 3a, lane 14). As expected, products from *M. spretus* were cleaved by *Bsp1286I* (lanes 6, 7), while those from C57BL/6J were not (lanes 8, 9). A portion of the RT-PCR product from each XX F1 female was cleaved, consistent with the expected mosaicism for X inactivation (lanes 10–13). In contrast, none of the product from the T16H F1 females was cleaved (lanes 2–5), indicating the absence of *Rps4* transcription on the intact, inactive *M. spretus* X chromosome. Similar results were obtained using *AflIII* (not shown). Analysis of transcripts from *Hprt*, an X-linked gene known to undergo inactivation (Adler *et al.*, 1991; Epstein *et al.*, 1978), confirmed that X inactivation was mosaic in the XX F1 females and that the *M. spretus* X chromosome was inactive in the T16H F1 females (Fig. 3d).

To confirm the presence of *M. spretus* transcripts in the XX F1 females and their absence in the T16H F1 females, we examined the *Rps4* RT-PCR products by hybridization with allele-specific oligonucleotides (Figs. 3b and 3c). The *M. spretus* and C57BL/6 oligonucleotides detected the expected 419-bp *M. spretus* restriction fragment (Fig. 3b, lanes 6, 7) and 759-bp C57BL/6J product (Fig. 3c, lanes 8, 9), as well as several RT-PCR artifacts (see below). Regardless of these artifacts, the probes were highly allele-specific and showed only faint cross-hybridization (Figs. 3b and 3c, lanes 6–9, 14). As expected, the C57BL/6 probe hybridized strongly to RT-PCR products from all F1 females (Fig. 3c, lanes 2–5, 10–13). The critical

-28 TGCAGCATTATTTCAGGAAGGCGCACCC

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1 ATGGCTGGTGGTCCCAAGAAACACTGAAGGGGTAGCTGCTCCAAAACATTTGGATGCTGGATAAGTTGACTGGGGTGTGTTGCTCTCGTCCATCCACTGGTCCACAAAGCTGAGGGAA
1 M A R G P K K H L K R V A A P K H W M L D K L T G V F A P R P S T G P H K L R E

121 TGCTGCTCTCATCATTTTCTAAGGAACAGACTTAAAGTATGCOCTGACTGGAGATGAAGTAAAAAGATTTCATGCCAGGATTCATTAAAGATTGATGGGAAGTCAGGAOOGATATA
41 C L P L I I F L R N R L K Y A L T G D E V K K I C M Q R F I K I D G K V R T D I

241 ACCTACCTGCTGGGTTTATGGAATGTCATCAGCATTTGACAAGAOCGGAGAGAATTCOCTGCTGATCTATGACACCAAGGGTGGCTTTGCTGTTCATOGTATTACACGGAGGGAGGOCAG
81 T Y P A G F M D V I S I D K T G E N F R L I Y D T K G R F A V H R I T P E E A K

361 TACAAGTTGTGCAAAAGTGAAGAGATCTTTGTAGGCACAAAAGGAATCOOGCAOCTGGTGAOCCATGATGCTGCTACTATTGGGTACCOCTGATCOOCTCATCAAGGTGAOAGCAOCCATT
121 Y K L C K V R K I F V G T K G I P H L V T H D A R T I R Y P D P L I K V N D T I

481 CAGATTGATTGGAGACAGGCAAAATACTGACTTTCATCAAGTTTGACACTGGGAOCTGTGATGGTACTGGAGGTGCTAACTTGGGAAGAAATGGTGTAAATCAOACAACAGAGAGAGA
161 Q I D L E T G K I T D F I K F D T G N L C M V T G G A N L G R I G V I T N R E R

601 CATOCCGGCTCTTTTGTATGTTGTCATGTGAAGAATGCAATGGCAACAGCTTTGCCACTGGCTGTGCCAACATTTTGTATTGGCAAGGGTAAACAACCATGGATCTCTCTTCOOCAGA
201 H P G S F D V V H V K D A N G N S F A T R L S N I F V I G K G N K P W I S L P R

721 GGAAAAGGAATCOGCOCTCAOCCATTGCTGAAGAGAGACAAGAGGCTTTGGGCOCAACAGAGCAGTGGGTGAAATGGTCTCTAGGAGACATGCTGGAAAAGTGTGTGTACAAGCOCTTTCT
241 G K G I R L T I A E E R D K R L A A K Q S S G *

841 AGGCAACATACATGCTAGATTAAACAGCATGGTGAACACTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 923
    
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FIG. 1. Nucleotide sequence of mouse *Rps4* cDNA pDP1340 and the predicted amino acid sequence of the encoded protein. The complete nucleotide sequence of the cDNA insert of plasmid pDP1340 is shown. The predicted 263-amino-acid sequence is given below the corresponding nucleotide sequence. Numbering of nucleotides and amino acids begins with the first in-frame AUG codon. There is a 5' untranslated region of 28 nucleotides.

finding was that the *M. spretus* probe hybridized strongly to products only from XX F1 females (Fig. 3b; compare lanes 10–13 with lanes 2–5). We conclude that in the T16H females there is little or no tran-

scription of *Rps4* on the inactivated *M. spretus* X chromosome; mouse *Rps4* is subject to X inactivation.

Two types of RT-PCR artifacts were detected by hybridization. One type included abundant small

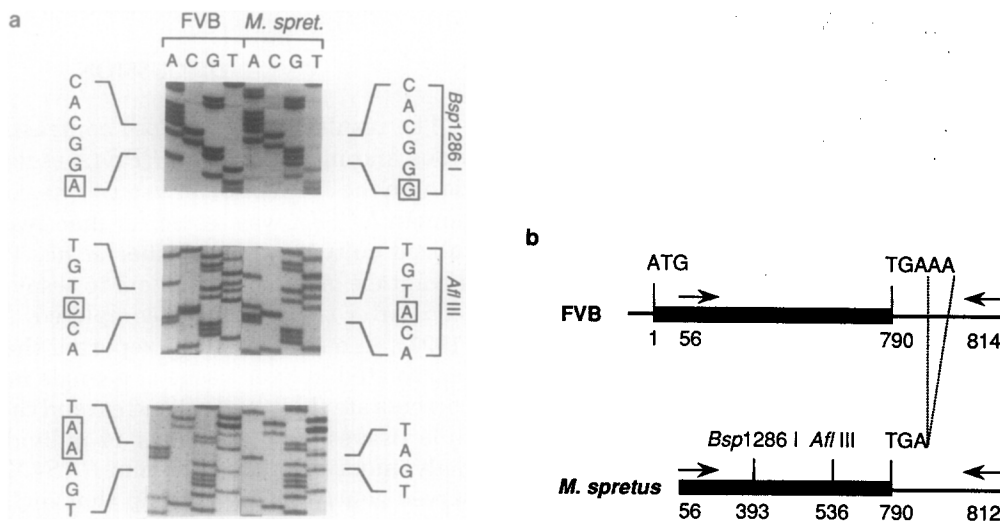
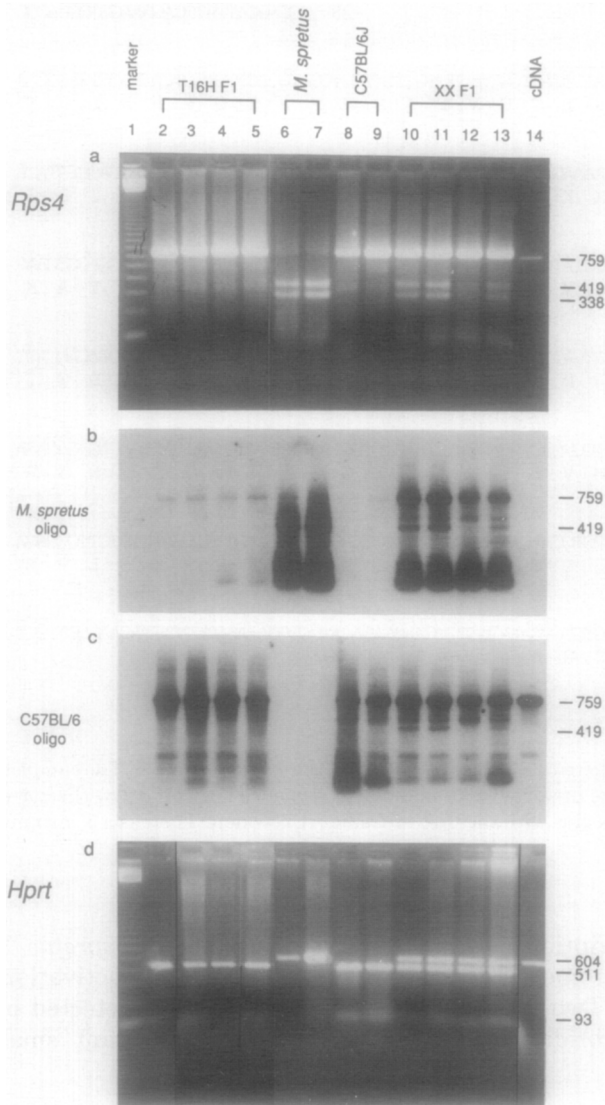


FIG. 2. Allelic variation at *Rps4*. (a) The *M. spretus* *Rps4* RT-PCR product was directly sequenced and compared with the sequence of FVB cDNA pDP1340. Nucleotide sequence polymorphisms are boxed. (b) Schematic representation of sequence variation. Thick lines, translated regions; arrows, RT-PCR primers; dashed lines, 2-bp insertion/deletion. Numbering of positions of primers and restriction sites is as in Fig. 1.



**FIG. 3.** *Rps4* undergoes X inactivation. RT-PCR analysis of (a–c) *Rps4* and (d) *Hprt* transcripts in tissue RNAs from T16H F1 females (lanes 2–5), an *M. spretus* female (lanes 6, 7), a C57BL/6J female (lanes 8, 9), and XX F1 females (lanes 10–13). Similar results were obtained with a fourth T16H F1 female (not shown). RNAs were prepared from kidney (lanes 2, 8, 13), spleen (lanes 3, 5), liver (lanes 4, 7, 9, 11), or brain (lanes 6, 10, 12). RNAs in lanes 2 and 3 derive from the same animal, as do those in 10 and 11 and those in 12 and 13. RT-PCR products were restriction digested with *Bsp1286I* (in the case of *Rps4*) or *MaeII* (*Hprt*) prior to agarose gel electrophoresis. Lane 14: PCR amplifications of (a–c) *Rps4* cDNA pDP1340 or (d) *Hprt* cDNA pHPT5 (American Type Culture Collection). Digests prepared as in a were transferred to nylon membrane and hybridized with oligonucleotides specific to the (b) *M. spretus* or (c) C57BL/6 alleles at *Rps4*.

products that were not prominent by ethidium bromide staining (e.g., Fig. 3b, lanes 6, 7, 10–13). These small products, which were present in both parental and progeny samples, were allele-specific. The second type of artifacts was larger and gave less hybridiza-

tion. One, a 419-bp *Bsp1286I* fragment detected by the C57BL/6 probe (Fig. 3c, lanes 10, 11, and 13), represents formation *in vitro* of chimeric molecules (Paabo *et al.*, 1990; Meyerhans *et al.*, 1990). Another, a 759-bp product that hybridized to the *M. spretus* probe but was not cleaved by *Bsp1286I* (Fig. 3b, lanes 10–13), could reflect similar chimeric products, or alternatively, heteroduplex molecules that are not cleaved by the enzyme. These two larger artifacts were present only in samples from progeny with both allelic transcripts, and not in parental samples. The specificity of the RT-PCR assay was also confirmed by direct sequencing of the parental products (Fig. 2a, *M. spretus*, and data not shown, FVB). Therefore, the artifacts do not interfere with our conclusion that in the T16H females the *M. spretus Rps4* allele is X-inactivated.

One might suppose that in the XX F1 females the proportion of allelic transcripts derived from the *M. spretus* X chromosome would be similar for various X-linked genes. We note that, as judged by restriction digestion, the *M. spretus* chromosome appears to contribute a smaller fraction of the transcripts for *Rps4* than for *Hprt* (Figs. 3a and 3d, lanes 10–13). However, the RT-PCR artifacts may confound this assessment. For example, heteroduplexes that are not cleaved by the restriction enzyme would result in underrepresentation of the *M. spretus Rps4* product (and overrepresentation of the *M. spretus Hprt* product). Also, the restriction analysis does not take into account the abundant smaller RT-PCR products. Finally, it is not certain that the allelic products are amplified with equal efficiencies. For these reasons, we do not believe that the intensity of the ethidium bromide-stained bands necessarily reflects the true ratio of allelic transcripts in the XX F1 females.

## DISCUSSION

The result for mouse *Rps4* contrasts with the previous finding that human *RPS4X* escapes X inactivation. In the absence of an *in vivo* experimental system, human *RPS4X* was tested for inactivation in immortalized cultured cells (Fisher *et al.*, 1990). We took advantage of a genetic system to assay mouse *Rps4* *in vivo*. After this work was completed, Ashworth *et al.* (1991), in a similar study, reported that *Rps4* was X-inactivated in liver tissue of a single mouse. Our data corroborate their finding and extend the result to multiple tissues from a number of individuals. Interestingly, mouse *Rps4* and human *RPS4X* map to a conserved genetic linkage group that includes the X-inactivation center (Fisher *et al.*, 1990; R. Hamvas, A.Z., and co-workers, submitted); the species difference in X inactivation cannot be explained by the position of the genes with respect to the inactivation center.

Human *RPS4X* is one of several X-linked genes that escape inactivation and have a homolog on the Y chromosome (Fisher *et al.*, 1990; Schneider-Gädicke *et al.*, 1989). Such Y homologs may serve to equalize amounts of gene product in males and females. This equalization could be especially important for ribosomal protein S4, a stoichiometric component of a ubiquitous multisubunit structure. In mouse, no Y-linked homolog of *Rps4* has been identified (Ashworth *et al.*, 1991; A.Z., unpublished results). Because nucleotide sequence divergence (e.g., silent or neutral substitutions) could explain this failure, it would be premature to conclude that no mouse Y homolog exists. Nonetheless, our results offer a teleologic rationale for the absence of a mouse Y homolog: because mouse *Rps4* is dosage compensated by X inactivation, a Y homolog might confer little or no selective advantage.

One percent of human embryos are monosomic for the X chromosome. Most of these abort spontaneously (Carr, 1965; Hassold, 1986). Those that survive to birth display the Turner phenotype, with short stature, gonadal degeneration, and multiple somatic anatomic abnormalities (Ullrich, 1930; Turner, 1938). XO mice are less severely affected, with only mild intrauterine growth retardation, diminished fertility, and no apparent anatomic defects (Cattanach, 1962; Lyon and Hawker, 1973; Burgoyne *et al.*, 1983). It has been hypothesized that, in humans, haploinsufficiency of the *RPS4* genes contributes to the Turner phenotype (Fisher *et al.*, 1990). Because human *RPS4X* is not dosage compensated, one would predict less expression in X-monosomic than in XX embryos. In contrast, one would predict comparable expression of *Rps4* in XO and XX mouse embryos following X inactivation. It is possible that, at least in part, the phenotypic consequences of X monosomy are less severe in the mouse because mouse *Rps4*, unlike human *RPS4X*, is dosage compensated by X inactivation.

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