Variability of Sequence Surrounding the Xist Gene in Rodents Suggests Taxon-Specific Regulation of X Chromosome Inactivation

Alexander I. Shevchenko1,2, Anastasia A. Malakhova1,2, Eugeny A. Elisaphenko1,2, Nina A. Mazurok1,2, Tatyana B. Nesterova3, Neil Brockdorff3, Suren M. Zakian1,2,4

1 Institute of Cytology and Genetics, Russian Academy of Sciences, Siberian Branch, Novosibirsk, Russian Federation, 2 Institute of Chemical Biology and Fundamental Medicine, Russian Academy of Sciences, Siberian Branch, Novosibirsk, Russian Federation, 3 Department of Biochemistry, University of Oxford, Oxford, United Kingdom, 4 Research Center of Clinical and Experimental Medicine, Siberian Branch, Russian Academy of Medical Sciences, Novosibirsk, Russian Federation

Abstract

One of the two X chromosomes in female mammalian cells is subject to inactivation (XCI) initiated by the Xist gene. In this study, we examined in rodents (voles and rat) the conservation of the microsatellite region DXPas34, the Tsix gene (antisense counterpart of Xist), and enhancer Xite that have been shown to flank Xist and regulate XCI in mouse. We have found that mouse regions of the Tsix gene major promoter and minisatellite repeat DXPas34 are conserved among rodents. We have also shown that in voles and rat the region homologous to the mouse Tsix major promoter, initiates antisense to Xist transcription and terminates around the Xist gene start site as is observed with mouse Tsix. A conservation of Tsix expression pattern in voles, rat and mice suggests a crucial role of the antisense transcription in regulation of Xist and Xic in rodents. Most surprisingly, we have found that voles lack the regions homologous to the regulatory element Xite, which is instead replaced with the Sic1a3 gene that is not associated with the X-inactivation centre in any other eutherians studied. Furthermore, we have not identified any transcription that could have the same functions as murine Xite in voles. Overall, our data show that not all the functional elements surrounding Xist in mice are well conserved even within rodents, thereby suggesting that the regulation of XCI may be at least partially taxon-specific.

Citation: Shevchenko AI, Malakhova AA, Elisaphenko EA, Mazurok NA, Nesterova TB, et al. (2011) Variability of Sequence Surrounding the Xist Gene in Rodents Suggests Taxon-Specific Regulation of X Chromosome Inactivation. PLoS ONE 6(8): e22771. doi:10.1371/journal.pone.0022771

Editor: Brian P. Chadwick, Florida State University, United States of America

Received April 16, 2011; Accepted June 29, 2011; Published August 3, 2011

Copyright: © 2011 Shevchenko et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Russian Foundation for Basic Research (grant no. 11-04-00799-a and 11-04-00847a, http://www.rfbr.ru/; the Wellcome Trust (grant no. 081385, http://www.wellcome.ac.uk/funding/index.htm). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: zakian@bionet.nsc.ru

Introduction

X chromosome inactivation (XCI) is a developmentally regulated process, which results in heterochromatization and transcriptional silencing of one of the two X chromosomes in eutherian females [1]. Imprinted XCI occurs on the paternal X chromosome (Xp) in the preimplantation embryo of some eutherians (for example, rodents) and is further maintained in the placenta [2,3]. Random XCI takes place on either the paternal or maternal X chromosome in the preimplantation embryo of some eutherian females [1]. Imprinted XCI occurs on the paternal X chromosome (Xp) in the preimplantation embryo of some eutherians (for example, rodents) and is further maintained in the placenta [2,3]. Random XCI takes place on either the paternal or maternal X chromosome in the preimplantation embryo of some eutherian females [1].

A complex X-linked locus termed the X-inactivation centre (Xic) governs both imprinted and random XCI (reviewed in [4]). It has been shown that the initiation of XCI and propagation of silencing are mainly provided by the Xist gene which produces a 17 kb nuclear RNA associated with the inactive X chromosome [5–8]. This is the only functional element of the Xic that has been identified in all eutherian studied [9–11]. The studies of the Xic in mice have detected multiple elements in surrounding Xist with roles at different stages of XCI. Two non-coding nuclear RNA genes Enox (Jpx) and Phx are localized 5′ to Xist [12,13]. Both genes are positive regulators of Xist [14,15]. The microsatellite region DXPas34, the Tsix gene (the antisense counterpart to Xist), and enhancer Xite, a 37 kb bipartite counting element have been mapped 3′ to Xist (reviewed in [16,17]). As is demonstrated, these elements in mice regulate Xist expression during imprinted and random XCI and are involved in the mechanisms underlying the counting of X chromosome number per diploid set of autosomes and the choice of the X chromosome to be inactivated during random XCI. However, these regulatory elements have not been definitively identified in other eutherians.

In this study, we intended to find any conserved elements surrounding Xist in rodents (mouse, rat, and common voles). Mouse and rat represent Muridae rodents. These two species diverged from a common ancestor 2 million years ago [18,19]. Voles are Arvicolidae rodents, which diverged from Muridae lineage 15–25 million years ago. We have earlier identified and described the nucleotide sequences of Xist in four common vole species [20]. In this work, we have extended our analysis of vole Xic further downstream of Xist and compared the region 3′ to Xist in vole, mouse, and rat. We have also determined all transcription upstream, downstream, and across Xist in vole, as such transcription has been suggested to regulate Xist expression and be involved in counting and choice function of Xic [21].
We have found that the vole sequences downstream of Xist have homology to the minisatellite DXPas34 region and the Tsix major promoter of mouse Xic. We have demonstrated that this putative Tsix promoter identified in voles is a site of origin for transcription antisense to Xist, displaying a very similar expression pattern to mouse Tsix. Conservation of the Tsix major promoter sequences and the pattern of Tsix expression between vole, mouse and rat suggest a crucial role for antisense transcription in the regulation of Xist expression and XIC in rodents. However, we have found that the region containing the Tsix minor promoter and the regulatory element Xite in mice is replaced with the Slc7a3 gene and its surrounding sequence in voles. This allows us to suggest that both the nucleotide sequences of these elements and the transcription associated with them are not absolutely necessary for XCI even in rodents.

Results

Comparative Study of the Sequences 3’ to Xist in Voles, Mouse and Rat

We have earlier identified and described the nucleotide sequences of Xist in four common vole species [20]. In this study, we have extended our analysis further downstream of Xist, as it is known that this region in mouse comprises the DXPas34, Tsix, and Xite regulatory elements, which act at different steps of XCI (reviewed in [16,17]). Several clones were isolated from phage genomic libraries for each vole species, and the sequence contig extending over 24 kb downstream of the vole Xist built (Fig. 1).

Comparative sequence analysis shows that sequences 3’ to Xist of the four vole species display an overall similarity.

Then we compared the mouse and rat Xic sequences 3’ to Xist up to the Tsix gene from the UCSC Genome Bioinformatics database and found that this region of the two rodent species displayed a high similarity. In the rat sequence, we have identified all the regulatory elements mapped in the mouse Xic region (Fig. S2).

Comparison of the vole sequence 3’ to Xist with the corresponding mouse and rat regions detected homology to the mouse minisatellite DXPas34 region, the major promoter of mouse Tsix, and some sequences surrounding these elements (Fig. 1, Fig. S3).

We have not found any significant homology to the mouse minor Tsix promoter and the associated exon or Xite within the vole region studied. Surprisingly, we located the vole ortholog of the mouse Slc7a3 gene in close proximity (5 kb) to the putative vole Tsix major promoter (Fig. 1, Fig. S3). Then we examined in more detail the similarities and differences found in the region 3’ to Xist between vole and two other rodent species.

DXPas34

DXPas34 in mice has been described as a block of minisatellite repeats with a monomer of 34 bp [22]. The repeats are CpG-rich, and each monomer contains a binding site for the CTCF protein, known to be involved in XCI regulation [23–25]. However, our more comprehensive analysis of mouse DXPas34 using the Tandem Repeat Finder program revealed three blocks of repeats composed of monomers of 34, 31, and 30 bp (Mus-34, Mus-31, and Mus-30) respectively (Fig. 2A). Two blocks of repeats composed of the 31- and 32-bp monomers (Rn-31 and Rn-32) have been found in rat (Fig. 2A). Three blocks of tandem repeats were detected in the four studied vole species. The first block comprises the monomers with an average length of 70 bp (Mc-70); the second, of 48 bp (Mc-48); and the third, of 34 bp (Mc-34) (Fig. 2A). The vole species differ in the copy number of monomers in the blocks. The similarity of vole monomers within blocks varies from 53 to 89 %. We managed to identify a motif that retained a high degree of conservation in all monomer types of mouse, rat, and voles (Fig. 2C). This motif may be necessary for binding of a protein factor involved in XCI regulation.

The CpG dinucleotide content and the number of binding sites for CTCF factor in the DXPas34 region in rodents displays considerable variation (Fig. 2A). The corresponding region of rat appeared the richest in CpG content; however, it contained considerably smaller number of CTCF binding sites compared with mouse. The least CpG content and number of CTCF binding sites was observed in voles (Fig. 2A). Note that this region in M. arvalis completely lacked CTCF binding sites and almost lacked CpG dinucleotides. Note also that the M. arvalis X chromosome is predominantly active in the cells of interspecific hybrids obtained by reciprocal crosses of this species with the three remaining vole species [26].

Tsix Major Promoter and Associated Exon

The 100-bp region located upstream of the main Tsix transcription start site, identified in mouse by RACE experiments [27] is the most conserved between rodent species (Fig. S3). Presumably, this region represents a basal Tsix gene promoter, which contains the sites necessary for initiation and regulation of its expression. The sequence immediately adjacent to the vole region similar to the Tsix promoter has a homology to mouse Tsix exon and contains a CpG island in three vole species (except M. arvalis). As CpG islands in this region of the mouse female inactive X chromosome are hypermethylated [28–30], we decided to clarify the methylation status of CpG dinucleotides in the corresponding vole sequence. We digested the genomic DNA isolated from M. arvalis and M. rossiaemerialis with the restriction endonuclease HpaII, sensitive to methylation, and assayed it by Southern blot hybridization (Fig. 3). The genomic region was not methylated on the only active male X and, consequently, its DNA was completely digested with HpaII. In females, carrying one active and one inactive X chromosome, in addition to the HpaII-digested fraction, a fraction inaccessible for digestion with HpaII due to the presence of methylated DNA on the inactive X, was also detected. Thus, at least individual CpG dinucleotides in this region of the vole female inactive X chromosome are hypermethylated.

Pseudo NIF3L1BP1

Immediately adjacent to the major promoter of Tsix, we have found several regions homologous to the NIF3L1BP1 gene in voles, mouse, and rat (Fig. 1, Fig. S3). They are likely to represent the remains of an ancient pseudogene the parts of which have been separated by an intensive integration of mobile elements. Pseudo NIF3L1BP1 is the last element common to the vole, mouse, and rat regions 3’ to Xist. In voles, pseudo NIF3L1BP1 is located at the boundary of the rearrangement that led to Slc7a3 embedding.

Slc7a3

Unlike mouse and rat, Slc7a3 was found in voles at distance 5 kb from the region similar to the pseudogene NIF3L1BP1 (Fig. 1, Fig. S3). Slc7a3 is not located within the Xic in any other eutherians studied. In all other eutherians, the Xic region downstream of Xist is flanked with the protein-coding genes Tsx, Chic1, and Cdx4. In mice, Slc7a3 is located 2.4 Mb upstream of the 5’ boundary of Xist. We have found that vole Slc7a3 has the same exon-intron structure as mouse Slc7a3 and consists of 13 exons. The presence of the sequences homologous to promoter and the surrounding of this gene in mouse, similarity of the exon-intron structure, and absence of stop codons and framenshifts in the coding region allowed us to assume that we had identified functional Slc7a3, brought close to vole Xic as a result of a chromosome rearrangement.
Having discovered the rearrangement in the vole Xic region, we decided to determine the localizations of Xist, Slc7a3, Chic1, and Cdx4 on the M. rossiaemeridionalis metaphase chromosomes. Despite Chic1 and Cdx4 having been obviously removed from the close proximity of Xist in voles, they were nevertheless located within the same cytogenetic band on the vole X chromosome as Xist and Slc7a3 (Fig. S4).

**Bipartite Counting Element**

The bipartite counting element is defined as a 37 kb region 3’ to Xist independent of DXPas34 and Tsix. Deletion of the region in XY and XO embryonic stem (ES) cells results in aberrant inactivation of the only X chromosome [31–33], and insertion of certain sequences from the region into autosomes in XX ES cells interferes with normal counting process and blocks XCI [34]. In voles, the distance between 3’ of Xist and the nearest protein-coding gene Slc7a3 is only 15 kb, of which only 4 kb displays similarity to the sequences of the mouse 37-kb bipartite counting element. This similarity is detectable across a region of about 3 kb adjacent to DXPas34 and over 1 kb upstream of the major Tsix transcription start site (Fig. S3).

**Comparison of 3’ Xist Region in Rodents and Other Mammalian Species**

We have compared the region 3’ to Xist in rodents and other mammals and found no sequences homologous to Tsix.
DXPas34, and Xite in primates (human and chimpanzee), ungulates (bovine), and carnivores (dog) (data not shown). Thus, the sequences homologous to Xic functional elements found in the mouse 3' to Xist region are detectable only in the order Rodentia. However, we have identified four areas of homology between mouse and human 3' to Xist regions (R1 – R4) (Fig. 4), three of which (R1-R3) have been previously reported [35]. We found that human R3 and R4 are separated by species-specific transposable elements, whereas an insertion of DXPas34 with the 5' region of Tsix occurred between mouse R3 and R4.

Previously, within a transposable element 3' to Xist in human, several short dispersed regions of similarity to DXPas34 were identified [36]. Nevertheless, the results of our comparative analysis show that the homology in human is not between R3 and R4 (Fig. 4). Taken together these facts suggest that microsatellite repeats and adjacent sequences could have emerged from a mobile element, but that a transposon with homology to DXPas34 found in human is probably not the genuine ancestor of the region.

Overall Transcription Upstream, Downstream, and Across the Xist Gene in Voles

We analyzed the transcriptional activity at Xist and adjacent regions using strand-specific RT–PCR.

First, we decided to confirm the transcriptional activity of the Slc7a3 sequence detected in voles. It has been found that this gene, as expected, is transcribed antisense to Xist and ubiquitously expressed in fetal tissues of both males and females (Fig. 1V, amplicon 14).

We have not identified any transcription in the intergenic region between 3' end of Slc7a3 and the putative transcription start site of vole Tsix (Fig. 1B). Thus, we have found in voles neither the sequences similar to Xite nor the intergenic antisense to Xist transcription that could have the same functions as Xite in mouse.

Antisense to Xist transcription was detected between the putative vole Tsix promoter and Xist promoter in the embryo and placenta of both males and females. Transcription in vole is identical to the transcription of the mouse Tsix (Fig. 1B).

Xist expression was revealed only in female tissues. It fits the vole Xist transcription unit reported previously (Fig. 1B).
Differences in the Xic nucleotide sequences between the vole species allow us to determine the allele-specific profile for Slc7a3, Tsix, and Enox (Jpx) expressions. For this purpose, we used 12.5 dpc vole XX and XY embryos and placentas of M. rassiaemeridionalis and M. arvalis.

Figure 3. CpG Dinucleotide Methylation at 5’ Region of Vole Tsix. DNA methylation was detected by digestion of genomic DNA with the methyl-sensitive endonuclease HpaII and subsequent blot-hybridization. Genomic DNA of adult M. arvalis (Ma) and M. rassiaemeridionalis (Mr) male (m) and female (f) was isolated from liver. DNAs were pretreated with the HindIII endonuclease, and then digested with HpaII. doi:10.1371/journal.pone.0022771.g003

The 3’ RACE primers for Tsix were designed for the regions +1200 bp relatively to Xist transcription start site. For M. arvalis and M. rassiaemeridionalis, 5’ RACE products obtained by RACE were subcloned and sequenced. Analysis of the sequences of 3’ RACE products demonstrated that Tsix transcription in voles terminated at multiple sites encompassing the Tsix transcription start site (Fig. 5). The most distant Tsix transcription termination site was detected at position –1279 bp from the Tsix transcription start site.

The Tsix gene-specific primers for 5’ RACE were designed for the region +1200 bp relatively to Xist transcription start site. For M. arvalis and M. rassiaemeridionalis, 5’ RACE clones with a length of 600 bp were obtained (Fig. 5). The first 134 bp in the clones correspond to the sequence located immediately after the primer. The next 110 bp correspond to the sequence of Xist exon 1, located at a distance of 6 kb from the first homologous region. The remaining 450 bp are identical to the newly determined genomic sequence localized 12 kb downstream of Xist exon 8. This fragment is homologous to the mouse exon located downstream of the major Tsix start site. In addition, we found clones with regions of continuous 600–800-bp homology to Xist exon 1 detected after the 134-bp region; these homology regions started 1.5 kb downstream of the 5’ boundary of the 110-bp fragment. Presumably, these clones were truncated at the 5’ end due to a premature termination of reverse transcription. To obtain the complete structure of the Tsix transcript isoform, we carried out

Exon–Intronic Structure and Boundaries of the Vole Tsix and Enox Genes

Figure 4. Comparison of 3’ to Xist Region in Mouse and Human by Reciprocal Percent Identity Plots. R1 – R4, areas of 3’ to Xist homology in mouse (m) and human (h) marked with red frames. Black frame shows a fragment within human transposable element which has several short dispersed regions of similarity to monomer of DXPas34 [36]. Arrowheads and arrowheaded rectangles represent different transposable elements. doi:10.1371/journal.pone.0022771.g004

Transcription neither sense nor antisense to Xist was detected in the intergenic region starting 2 kb upstream of the Xist promoter and continuing up to the CpG island near the Enox (Jpx) gene regions (Fig. 1V, amplicons 6 to 4). We did however identify transcription both sense and antisense relative to Xist transcription in this region because of the bidirectional activity of Tsix, Xist, and Enox (Jpx) promoters [21].

Allele-Specific Profile of Sense and Antisense Transcription in the Vole Xic

placentas displayed double peaks at the polymorphic positions, thereby confirming the presence of both parental alleles in the hybrid placentas (Fig. 1C). Sequencing of the PCR products obtained from the cDNA of hybrid XX placentas demonstrated that only Xist was expressed from the inactive X chromosome, whereas the remaining genes Slc7a3, Tsix, and Enox (Jpx) were expressed from the active X chromosome.

Figure 5. Sequence Comparison of 600-bp Fragment with Mouse Xist Sequence. The 3’ RACE products obtained by RACE were subcloned and sequenced. Analysis of the sequences of 3’ RACE products demonstrated that Tsix transcription in voles terminated at multiple sites encompassing the Tsix transcription start site (Fig. 5). The most distant Tsix transcription termination site was detected at position –1279 bp from the Tsix transcription start site.

To determine the exon–intron structure and transcription boundaries for Tsix and Enox in voles, we used 5’ and 3’ RACE and strand-specific RT–PCR. The RNA for 5’ and 3’ RACE was isolated from 12.5 dpc XX and XY embryos and placentas of M. rassiaemeridionalis and M. arvalis.
Vole Tsix Gene Obtained from RACE Experiments and Strand-Specific RT-PCR.

Genomic map of vole Xist and Tsix are shown; exons of Xist and Tsix are indicated by orange and indigo boxes, respectively. Clones obtained by RACE and RT-PCR are aligned under the map. Clones, 3a_n of M. arvalis, and 3r_n of M. rossiaemeridionalis, were isolated by 3’RACE. Clones, 5a_n of M. arvalis, and 5r_n M. rossiaemeridionalis, were isolated by 5’RACE. Rt1 represents a strand-specific RT-PCR product obtained from M. rossiaemeridionalis placenta using primers Bt11 – SNTR (Table S2).

doi:10.1371/journal.pone.0022771.g005

Transcription Antisense to Xist in Rat

We searched for the mRNA and EST antisense to rat Xist at the Blat server (http://genome.ucsc.edu/cgi-bin/hgBlat). Two spliced RNAs were detected (Fig. S5). EST [GenBank:CF978550] has three exons. The first exon is within the block of tandem repeats DXPas34 and does not coincide with any known mouse or vole

Enox

We performed both 5’ and 3’ RACE to identify boundaries of Enox (Jpx) and its antisense counterpart. However, only 3’ RACE for Enox (Jpx) and 5’ RACE for its antisense transcript was successful. Gene-specific primers were designed within region 2 for 3’ RACE of Enox (Jpx) and within region 3 for 5’ RACE of transcript antisense to Enox (Jpx) (Fig. 1A, Table S2). All clones obtained in 3’ and 5’ RACE were identical to the genomic DNA sequences located immediately after the primers. The nucleotide sequences of the 5’ and 3’ RACE clones were deposited with Gene Bank (accession numbers [GenBank:JF519003], [GenBank: JF519004]). We found that vole Enox (Jpx) terminated 840 bp downstream from the region homologous to the mouse Enox (Jpx) promoter, while the antisense transcription start site was mapped within the Enox (Jpx) transcription unit 342 bp before its 3’ end. Thus, in vole, both Enox (Jpx) and its antisense counterpart represent a single exon transcription unit, which taking into account RT-PCR data (Fig. 1A,B) spans about 1500–2000 kb.

Figure 6. Northern Blot Analysis of Tsix Expression in Vole and Rat 14 dpc Male Embryos. About 5 μg of poly(A)+RNA from each species was hybridized with the species- and strand-specific probe corresponding to the exon located after the major Tsix start site in mouse [27].

doi:10.1371/journal.pone.0022771.g006
The second exon coincides with vole exon C and the third, with the terminal Tsix exon of vole and mouse. This RNA seems to be a spliced variant of the rat Tsix transcript.

The other RNA, [GenBank:AY539944], comprises ten exons and contains a native open reading frame encoding a hypothetic protein Lrrm [30,39,40], the transcription of which has been detected in the rat liver. The exons of this gene are localized in the region homologous to the Xite regulatory element, in Tsix exon downstream of the major promoter and in Xist introns (Fig. S5A). Neither vole nor mouse retains the native open reading frame of the rat Lrrm gene.

Short unspliced RNA immediately downstream region homologous to the Enox (Jpx) transcriptional start site was also identified EST [GenBank:CK839650, GenBank:BE109826].

Using strand-specific mRNA from 14 dpc rat placentas and embryos, we confirmed the exon-intronic structure of Tsix and the transcription corresponding to Enox (Jpx) identified by EST analysis (Fig. S5A). The nucleotide sequences of cDNA obtained in the experiments are deposited with the Gene Bank under accession numbers [GenBank:JF519002], [GenBank:JF519001], [GenBank:JF519000]. We also confirmed by RT-PCR that in rat Tsix, transcription passed through the Xist gene promoter. Northern blot hybridization with a strand-specific probe representing the exon which follows the major Tsix start site revealed in rat a high molecular weight transcript, presumably corresponding to unspliced Tsix and two spliced isoforms of ~6 kb and ~1.8 kb (Fig. 6).

Discussion

Variability of Transcription of Xist Surrounded in Rodents

In this study we identified in vole and rat 5’ to Xist transcription corresponding to the mouse Enox (Jpx) gene. We also found in vole transcription antisense to Enox (Jpx) which was previously described in mouse [21]. However, the transcription boundary and its start site in mouse and vole differ both for Enox (Jpx) and its antisense transcript. Vole Enox (Jpx) transcript is not spliced and the transcription unit spans about 2 kb from the initiation site, while mouse Enox (Jpx) is transcribed through tens of kb and gives spliced mRNA containing up to 5 exons [12,13]. It should be noted that vole Enox (Jpx) transcription originates from the species-specific CpG-rich region and obviously transcriptional regulation of the gene differs in vole and mouse. Thus, we can propose that the regulation of XCI by transcription associated with Enox (Jpx) is not the same in vole and mouse. It is intriguing that Enox (Jpx) transcription is more similar in mouse and human [12,13] than in mouse and vole. Finally, it should be also noted that Enox (Jpx) is not completely silenced on the inactive X chromosome during random inactivation and is biallelically expressed in female mice [13,15]. However, Enox (Jpx) appears to be expressed only on the active X chromosome in vole during imprinted inactivation. The difference in Enox (Jpx) expression between vole and mouse could be ascribed in equal degree both to species-specific features and different function of the gene during imprinted and random inactivation. Further studies are needed to clarify these issues.

Our results demonstrate that in both rat and vole there is expression antisense to Xist which corresponds to mouse Tsix. It starts from the region homologous to the mouse Tsix major promoter and ends around Xist transcription start site. The vole Tsix gene has an expression pattern similar to that of mouse Tsix. Both vole and rat Tsix transcripts cover the Xist promoter, which, as has been demonstrated for mouse, is obligatory for Tsix functioning in XCI [41–43]. Thus, it is most likely that similar to the mouse Tsix, the vole and rat Tsix is able to regulate Xist expression. However, this requires additional confirmation. Similar to mouse the vole and rat Tsix RNA undergoes, at least in part, alternative splicing. The terminal exons of the Tsix genes in mouse, rat and vole are identical, whereas the remaining exons differ between rodent species. It has been shown that many exons in the noncoding RNA genes of Xic, such as Xist and Enox (Jpx), originated from mobile elements of various classes [9]. In this work, we have found another example of how a part of a species-specific SINE is present in spliced mature RNA of vole Tsix and represents one of its exons. This example illustrates the idea that integration of mobile elements into noncoding RNA genes of Xic continues in contemporary eutherian species and also supports the assumption that the emergence of exons from mobile elements is a general way of evolution and rearrangement of genes encoding large nuclear regulatory RNAs. However, the differences in the exon-intronic structures of the rodent Tsix genes together with the data on the absence of the differences in the ratio of spliced and unspliced Tsix transcripts [37] and the absence of XCI abnormalities caused by mutations of mouse Tsix splicing sites [44] confirms the earlier assumption that Tsix splicing is not necessary for its normal function.

It should be noted that transcription in the region of the Tsix major promoter is bidirectional in mouse [36], however, we only identified transcription antisense to Xist in vole. Xite is present and expressed in mouse [35], but not in vole. Tissue-specific protein-coding gene Lrrm is transcribed through Xite, Tsix, and Xist in rat [38–40], but is not even found in mouse and vole. Thus, comparative analysis data has shown that many transcripts that surround the Xist gene in rodents are taxon-specific. The taxon-specific transcripts either are able to influence XCI (as Xite), or are not absolutely linked with it (as Lrrm).

Conservation of the Xic 3’ to Xist Elements in Rodents and Other Eutherians

Comparison of the vole, mouse, and rat sequences has demonstrated that the most conserved of all the known regulatory elements downstream of Xist in rodents are the regions of Tsix’s major promoter and minisatellite repeats. Their conservation and necessity in rodents suggests that they are absolutely essential for XCI in this mammalian order. Note that the region of the Tsix major promoter is the most conserved in rodents. However, targeted deletions of the Tsix promoter in mice do not impair Tsix function in random XCI [36].

As the sequences homologous to mouse DXPas34 and Tsix major promoter are only detected in vole and rat, but not in others mammals, we could propose that this region emerged de novo in Rodentia, most likely as a result of the transposition and subsequent amplification of the same sequences which led to the formation of minisatellite repeat arrays. Note that transcription antisense to Xist is also not well conserved in eutherians. The transcript antisense to Xist is revealed in human, but does not overlap the Xist promoter [45–47]. Moreover, it is coexpressed with Xist from the inactive X chromosome in fetal and neonatal female cells and does not downregulate Xist, suggesting that this species-specific transcription is not linked with XCI. Thus, regulation of inactivation by Tsix may have arisen in the evolution quite recently and apparently is unique to rodents.

As voles lack the regions homologous to the Tsix minor promoter and Xite, we can assume that they are not absolutely necessary for XCI in eutherians. Moreover, as Xite sequences are only detected in mouse and rat, we cannot exclude that this element has emerged and evolved only in Muridae.
In all eutherians, for which whole genome sequences were obtained, Xic 3' to Xist is flanked by the protein-coding genes Tsx, Chic1 and Cd64 [9]. There is a speculation that others besides the known regulatory elements of XCI may lie within the region of these protein-coding genes (reviewed in [16]). The rearrangement detected in the vole Xic region demonstrates that the presence of protein-coding genes Tsx, Chic1, and Cd64 and the sequences linked with them downstream of the 3' Xist boundary are not necessary for XCI. The Xic rearrangement has evolved and, as a result, as little as 15 kb of the sequences downstream of Xist characteristic of this region in mouse appear sufficient for normal imprinted and random XCI regulation in vole.

In this study we found that not all functional elements flanking Xist in mice were well conserved even within rodents. Non-coding RNA transcripts in the region surrounding Xist can appear, disappear, change their promoters, exon-intron structure and borders. No common conserved elements located 3' to Xist have been identified in eutherians, suggesting that the XIC functional elements responsible for ‘counting’, ‘choice’, regulation of XCI may lie within the region of known regulatory elements of XCI may be at least partially taxon-specific.

Methods
Ethics statement
The study was carried out according to "The Guidelines for Manipulations with Experimental Animals." The study was approved by the Ethical Committee of the Institute of Cytology and Genetics, Novosibirsk, permit number: (order of the Presidium of the Russian Academy of Sciences of April 02, 1980 no. 12000–496).

Screening of phage genomic libraries of common voles from the genus Microtus
The phage genomic libraries of a male Microtus arvalis, female M. rossiaemeridionalis, female M. kingiuron, and female M. transasciatus in the λ DASH II (Stratagene) vector [20] were screened. The amplified sequences downstream the exon 8 of M. rossiaemeridionalis Xist and the protein-coding regions of the mouse genes Cd64, Chic1, Stc1a2, and Stc7a3 were used as probes for screening. The manipulations with the libraries were performed according to the Stratagene recommendations. Hybridization was conducted on Colony/Plaque Screen NEN Life Science Product membranes according to the manufacturer’s instructions.

DNA sequencing and analysis
The recombinant DNA sequences from phage clones were subcloned into plasmid vectors and sequenced using both the universal and specific primers. The sequencing reactions were carried out using a Big Dye Terminator v. 3.1 kit. The reaction products were analyzed in ABI Prism automated sequencers. The sequencing reactions were performed using a Big Dye Terminator v. 3.1 kit. The reaction products were analyzed in ABI Prism automated sequencers. The DNA sequencing and analysis were performed using ABI Prism automated sequencers. The DNA sequences localized 3' to Xist were determined in both strands for each of the four vole species and joined with the earlier obtained sequences containing the vole genes Enox, Jpx and Xist which were deposited with the GENEBANK under accession numbers [GenBank:AJ310129] (M. arvalis), [GenBank:AJ310130] (M. rossiaemeridionalis), [GenBank:AY090554] (M. kingiuron), and [GenBank:AJ310127] (M. transasciatus). Comparative sequence analysis was conducted using the following software packages: BLAST [48], http://www.ncbi.nlm.nih.gov/BLAST for searching for homologous sequences; Tandem Repeat Finder [49] for identifying tandem repeats; RepeatMasker [50] (http://www.repeatmasker.org) for detecting mobile elements; Fasta [51] and CLUSTALX [52] for aligning two and more sequences (the programs and data are available at http://genome.ucsc.edu/, http://www.ensembl.org/, and http://bio.csc.psu.edu/); and PipMaker [33] for conducting genomic analysis of extended loci.

The following sequences of mouse, rat, bovine, dog, chimpanzee, and human, extracted from the corresponding databases of sequenced genomes at the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/), were also used in the comparative analysis: mouse Feb 2006 (mm8) assembly range = chrX:9550937–10040904; gu Feb 2005 (hg18) assembly range = chrX:91358074–91909712; dog May 2005 (canFam2) assembly range = chrX:60100000–60735000; chimpanzee Mar 2006 (panTro2) assembly range = chrX:75165660–75392649; human Mar. 2006 (hg18) assembly range = chrX:72449111–74160153.

RNA and DNA isolation, reverse transcription, and PCR
RNA and DNA were isolated using a Tri Reagent (Sigma) kit from pooled preimplantation vole blastocysts, 12.5 dpc vole embryos and placentas. The cDNA was synthesized using total RNA by SuperScript III (GIBCO-BRL) reverse transcriptase at 50–55°C according to the manufacturer’s instructions.

The strand-specific primers used for reverse transcription and cDNA amplification are listed in Table S1. Each reverse transcription reaction mixture was additionally supplemented with the strand-specific primer BAs to beta actin [21]. A negative control reaction, with the reaction mixture lacking reverse transcriptase, was conducted for each reverse transcription reaction. The sex of 12.5 dpc embryos and placentas was determined using the primers UB1X and UB1Y [21], in which vole giving a PCR products exclusively with male genomic DNA, and the strand-specific RT-PCR, detecting Xist expression exclusively in females (Fig. S1).

5’ and 3’ RACE
The exon-intron structure and boundaries of Tsix were analyzed using SMART RACE kit (Clontech). The gene-specific primers for 5’ and 3’ RACE are listed in Table S2. The amplified cDNA was cloned in plasmid vectors, sequenced, and analyzed by Southern blot hybridization. Vole Tsix cDNA obtained from RACE experiments was registered in GENE BANK under accession numbers [GenBank:JF519007] (M. arvalis), [GenBank:JF519006], [GenBank:JF519005] (M. rossiaemeridionalis).

Northern blot hybridization
About 5 μg of poly(A)+RNA isolated using Oligotex-dT30 (Qiagen) from vole and rat 14 dpc male embryos were run on a 1% agarose gel containing formaldehyde and transferred onto GeneScreen membrane (PerkinElmer). Both vole and rat cDNA amplicons corresponding to the exon located after the major Tsix start site in mouse [27] were obtained. The primer pairs used were gTF TCTTTGTGCGCTTTGGCGTCA - SNTR CTTCGCC- TGGCTGCCCTCGCTCGACTC for vole and Rnt5 TCTAATATGA- CATTGGCCAGAT - Rnt6 GGCTCGGCTTTCCGGACTATC for rat. For probe labeling 50 ng of the amplicons were added in linear PCR with [α-32P]dCTP and strand-specific primers (gTF or Rnt5) producing DNA strand antisense to Tsix transcript. Hybridization was performed in x3 SSC, 0.5% SDS at 65°C overnight. Washing was carried out according to the manufacturer’s instructions.

Allelic expression assay
Comparison of the M. arvalis and M. rossiaemeridionalis Xics detected interspecific single nucleotide polymorphisms (SNPs) in
the DNA sequences. The PCR primers (Table S3) were designed to generate the products containing SNPs. RT–PCR and PCR of genomic DNA were performed by standard methods using RNA and DNA samples of 12.5 dpc placentas of interspecific vole hybrids obtained from the crosses M. rossiaemelridionalis × M. arvalis. As voles have imprinted XCI in extraembryonic tissues [26], the majority of placental cells have the inactive X chromosome of paternal origin and the active X chromosome of maternal origin. The PCR products containing SNPs were purified by extraction from agarose gel, and their nucleotide sequences were analyzed in an ABI Prism automated sequencer.

DNA FISH was carried out as previously described [54, 55]. The phage clones containing the sequences of the M. rossiaemelridionalis genes Xist, Slc7a3, Chic1, Cdx4, and Pkgl labeled with biotin and digoxigenin were used as probes.

Supporting Information

Figure S1 Figure illustrating sexing of vole 12.5 dpc placentas and embryos. (A) The sex of 12.5 dpc embryos and placentas was determined using the primers UB1X and UB1Y [21], which produce a PCR product exclusively from vole male genomic DNA. (B) Additionally, the strand-specific RT–PCR, which detects Xist expression exclusively in females, was performed. Strand-specific primer for Xist cDNA synthesis was SDX3, GTGATTAATTCATTCC, and primers NSX19, CCCAGTGCTGGTGAGCTATTCC. Subsequent PCR was performed with primers NSX19, GTGATTAATTCATTCTATCGTGC and MS2X7, TTGTTCAGATATCCGATG. (TIF)

Figure S2 Results of comparison of h`to Xist region in mouse and rat by PIP maker software. (PDF)

References

1. Lyon MF (1961) Gene action in the X chromosome of the mouse (Mus musculus L.), Nature 190: 372–373.
2. Huynd KD, Lee JT (2003) Inheritance of a pre-inactivated paternal X chromosome in early mouse embryos. Nature 426: 857–862.
3. Okamoto I, Otte AP, Allis CD, Reinberg D, Heald R (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. Science 303: 644–649.
4. Rastan S, Brown SD (1990) The search for the mouse X-chromosome inactivation centre. Genet Res 56: 99–106.
5. Kay GF, Penny GD, Parn D, Ashworth A, Brockdorff N, et al. (1993) Expression of Xist during mouse development suggests a role in the initiation of X chromosome inactivation. Cell 72: 171–182.
6. Marahrens Y, Panning B, Daumen J, Straus W, Jaenisch R (1997) Xist-deficient mice are defective in dosage compensation but not spermatogenesis. Genes Dev 11: 156–166.
7. Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N (1996) Requirement for Xist in X chromosome inactivation. Nature 379: 131–137.
8. Wutz A, Rasmussen TP, Jaenisch R (2002) Chromosomal silencing and localization are mediated by different domains of Xist RNA. Nat Genet 30: 167–174.
9. Elisaphenko EA, Kolesnikov NN, Shevchenko AI, Rogozin IB, Nesterova TB, et al. (2000) A dual origin of the Xist gene from a protein-coding gene and a set of transposable elements. PLoS One 3: e2521.
10. Hore TA, Kaina E, Wakefield MJ, Marshall Graves JA (2007) The region homologous to the X-chromosome inactivation centre has been disrupted in marsupial and monotreme mammals. Chromosome Res 15: 147–161.
11. Yen ZC, Meyer EM, Karalic S, Brown CJ (2007) A cross-species comparison of X-chromosome inactivation in Eutheria. Genomics 90: 453–463.
12. Chuereau C, Prissette M, Bourdet A, Barbe V, Cattolico L, et al. (2002) Comparative sequence analysis of the X-inactivation center region in mouse, human, and bovine. Genome Res 12: 894–906.
13. Johnston CM, Newall AE, Brockdorff N, Nesterova TB (2002) Exon, a novel gene that maps 10 kb upstream of Xist and partially escapes X inactivation. Genomics 80: 236–244.
14. Chuereau C, Chantaltal S, Romito A, Galvani A, Durut L, et al. Fix is a non-coding RNA which affects Xist expression and chromatin structure within the X-inactivation region. Hum Mol Genet 20: 705–718.
15. Tian D, Sun S, Lee JT (2010) The long noncoding RNA, jpx, is a molecular switch for X chromosome inactivation. Cell 141: 390–403.
16. Cleve P, Avner P (2003) Multiple elements within the Xic regulate random X inactivation in mice. Semin Cell Dev Biol 14: 85–92.
17. Payer B, Lee JT (2008) X chromosome dosage compensation: how mammals keep the balance. Annu Rev Genet 42: 733–772.
18. Cazeneve F, Nevo E, Ahlquist JE, Shiieh CG (1989) Relationships of the chromosomal species in the Eurasian mole rats of the Spalax ehrenbergi group as determined by DNA-DNA hybridization, and an estimate of the spalacid-murid divergence time. J Mol Evol 29: 223–232.
19. Jaeger JJ, Tung H, Buftana E, Inorgay R (1985) The first fossil rodent from the Midwest of Nothern Thailand and their bearing on the problems of the origin of the Muridae. Rev Paleobiol. pp 1–7.
20. Nesterova TB, Shibbounyok SY, Elisaphenko EA, Shevchenko AI, Johnston C, et al. (2001) Characterization of the genomic Xist locus in rodents reveals conservation of overall gene structure and tandem repeats but rapid evolution of unique sequence. Genome Res 11: 833–849.
21. Nesterova TB, Johnston CM, Appanah R, Newall AE, Godwin J, et al. (2003) Skewing X chromosome choice by modifying sense transcription across the Xist locus. Genes Dev 17: 2177–2190.
22. Debrand E, Chuereau C, Arnaud D, Avner P, Heard E (1999) Functional analysis of the DXPas34 locus, a 3′ regulator of Xist expression. Mol Cell Biol 19: 8533–8535.
23. Chao W, Huynd KD, Spencer RJ, Davidow LS, Lee JT (2002) CTFCF, a candidate trans-acting factor for X-inactivation choice. Science 295: 345–347.
24. Navarro P, Page DR, Avner P, Rougaille C (2006) T-box-mediated epigenetic switch of a CTCF-flanked region of the Xist promoter determines the Xist transcription program. Genes Dev 20: 2767–2792.
25. Tsai CL, Rowntree RK, Cohen DE, Lee JT (2008) Higher order chromatin structure at the X-inactivation center drives looping. DNA Res 15: 416–425.
26. Zakian SM, Nesterova TB, Chernyakwe OV, Bochkarev MN (1991) Heterochromatin as a factor affecting X inactivation in interspecific female vole hybrids (Microtidae, Rodentia). Genet Res 58: 105–110.
27. Sado T, Wang Z, Sasaki H, Li E (2001) Regulation of imprinted X-chromosome inactivation in mice by Tsix. Development 128: 1275–1286.
28. Boumil RM, Ogawa Y, Sun BK, Huyyn KD, Lee JT (2006) Differential methylation of Xite and CTCF sites in Tsix mirrors the pattern of X-inactivation choice in mice. Mol Cell Biol 26: 2109–2117.
29. Courrier B, Heard E, Avner P (1995) Xce haplotypes show modified methylation in a region of the active X chromosome lying 3′ to Xist. Proc Natl Acad Sci U S A 92: 3531–3535.
30. Prisette M, ElMaazri O, Arnaud D, Walter J, Avner P (2001) Methylation profiles of DXPas34 during the onset of X-inactivation. Hum Mol Genet 10: 31–40.
31. Clerc P, Avner P (1998) Role of the region 3′ to Xist exon 6 in the counting process of X-chromosome inactivation. Nat Genet 19: 249–253.
32. Morey C, Arnaud D, Avner P, Clerc P (2001) Tsix-mediated repression of Xist accumulation is not sufficient for normal random X inactivation. Hum Mol Genet 10: 1403–1411.
33. Morey C, Navarro P, Debrand E, Avner P, Rougeulle C, et al. (2004) The region 3′ to Xist mediates X chromosome counting and H3 Lys-4 dimethylation within the Xist gene. EMBO J 23: 594–604.
34. Lee JT (2005) Regulation of X-chromosome counting by Tsix and Xite sequences. Science 309: 768–771.
35. Lee JT, Davidow LS, Warshawsky D (1999) Tsix, a gene antisense to Xist at the X-inactivation centre. Nat Genet 21: 400–404.
36. Cohen DE, Davidow LS, Erwin JA, Xu N, Warshawsky D, et al. (2007) The DXPas34 repeat regulates random and imprinted X inactivation. Dev Cell 12: 57–71.
37. Shibata S, Lee JT (2003) Characterization and quantitation of differential Tsix transcripts: implications for Tsix function. Hum Mol Genet 12: 125–136.
38. Xu CS, Chang CF, Yuan JY, Li WQ, Han HP, et al. (2005) Expressed genes in regenerating rat liver after partial hepatectomy. World J Gastroenterol 11: 2932–2940.
39. Xu CS, Yuan JY, Li WQ, Han HP, Yang KJ, et al. (2005) Identification of expressed genes in regenerating rat liver in 0-4-8-12 h short interval successive partial hepatectomy. World J Gastroenterol 11: 2296–2303.
40. Xu CS, Zhang AS, Han HP, Yuan JY, Chang CF, et al. (2004) Gene expression differences of regenerating rat liver in a short interval successive partial hepatectomy. World J Gastroenterol 10: 2689–2699.
41. Navarro P, Richard S, Ciaudo C, Avner P, Rougeulle C (2005) Tsix transcription across the Xist gene alters chromatin conformation without affecting Xist transcription: implications for X-chromosome inactivation. Genes Dev 19: 1474–1484.
42. Sado T, Hoki Y, Sasaki H (2005) Tsix silences Xist through modification of chromatin structure. Dev Cell 9: 159–163.
43. Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT (2008) Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science 322: 750–756.
44. Sado T, Hoki Y, Sasaki H (2006) Tsix defective in splicing is competent to establish Xist silencing. Development 133: 4925–4931.
45. Chow JC, Hall LL, Clemson CM, Lawrence JB, Brown CJ (2003) Characterization of expression at the human XIST locus in somatic, embryonal carcinoma, and transgenic cell lines. Genomics 82: 309–322.
46. Migeon BR, Chowdhury AK, Dunston JA, McIntosh I (2003) Identification of TSIX, encoding an RNA antisense to human XIST, reveals differences from its murine counterpart: implications for X inactivation. Am J Hum Genet 69: 951–960.
47. Migeon BR, Lee GH, Chowdhury AK, Carpenter H (2002) Species differences in TSIX/Tsix reveal the roles of these genes in X-chromosome inactivation. Am J Hum Genet 71: 286–293.
48. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
49. Benson G (1999) Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res 27: 573–580.
50. Smir A, Green P RepeatMasker. Available: http://www.repeatmasker.org.
51. Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci U S A 85: 2444–2448.
52. Jeanninouin F, Thompson JD, Goey M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Chutat X. Trends Biochem Sci 23: 403–405.
53. Schwartz S, Zhang Z, Frazer KA, Smir A, Riemer C, et al. (2000) PipMaker—a web server for aligning two genomic DNA sequences. Genome Res 10: 577–586.
54. Dementyeva EV, Stievenchenko AI, Anopriyenko OV, Mazurok NA, Elshaphenko EA, et al. Difference between random and imprinted X inactivation in common voles. Chromosoma 119: 541–552.
55. Nesterova TB, Duhie SM, Mazurok NA, Isachenko AA, Rubtsova NV, et al. (1990) Comparative mapping of X chromosomes in voles species of the genus Microtus. Chromosome Res 6: 41–48.