Tsix, a gene antisense to *Xist* at the X-inactivation centre

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In mammals, dosage compensation is achieved by X inactivation[1] and is regulated in *cis* by the X-inactivation centre[2] (*Xic*) and *Xist* (refs 3–5). The *Xic* controls X-chromosome counting, choice of X to inactivate and initiation of silencing. *Xic* action culminates in a change in *Xist* RNA property from a scarce, unstable RNA (refs 6,7) to highly expressed *Xist* RNA that coats the future inactive X (ref. 8). Deleting a 65-kb region downstream of *Xist* results in constitutive *Xist* expression and X inactivation, implying the presence of a *cis*-regulatory element[9]. In this region, we now report the discovery of a gene antisense to *Xist*. *Tsix* is a 40-kb RNA originating 15 kb downstream of *Xist* and transcribed across the *Xist* locus. *Tsix* sequence is conserved at the human *XIC*. *Tsix* RNA has no conserved ORFs, is seen exclusively in the nucleus and is localized at *Xic*. Before the onset of X inactivation, *Tsix* is expressed from both X chromosomes. At the onset of X inactivation, *Tsix* expression becomes monoallelic, is associated with the future active X and persists until *Xist* is turned off. *Tsix* is not found on the inactive X once cells enter the X-inactivation pathway. *Tsix* has features suggesting a role in regulating the early steps of X inactivation, but not the silencing step.

We found evidence for an RNA antisense to *Xist* when examining *Xist* expression in embryonic stem (ES) cells using strand-specific probes by RNA fluorescence *in situ* hybridization (FISH). The antisense signal was reminiscent of *Xist* RNA in that it was pinpoint, localized to the *Xic* and found in male, female and transgenic ES nuclei (>95%, n>2,000; Fig. 1). Antisense signals were consistently brighter than sense signals, suggesting either greater abundance or accessibility of the antisense RNA. We named this novel transcript *Tsix* in reference to its antisense orientation.

We further characterized *Tsix* in ES cells, a developmental model for X inactivation[10]. To map the extent of antisense transcription, we generated strand-specific probes across a 60-kb region (Fig. 2). In the antisense ('as') orientation, probes 1as–11as gave strong signals (>95%, n>2,000 nuclei), whereas probes 12as and 13as gave none. In the sense ('s') orientation, probes 3s–8s yielded robust signals (>95%, n>2,000 nuclei), whereas probe 9s gave faint signals in 20–30% of cells (n>2,000 nuclei). The alternatively spliced exon 7 (ref. 11) of *Xist* lies within probe 9, so the faint signal may represent its use in some cells. The size and intensity of sense and antisense signals suggested that *Xist* and *Tsix* may be expressed at comparably low levels. Analysis of male, female and transgenic ES cells gave identical results. Thus, antisense transcription spans 40 kb, including all of *Xist*, and sense transcription agrees with the published *Xist* map[4].

Strand-specific RT-PCR confirmed the map of *Tsix* (Fig. 3*a*,*b*). RT-PCR of randomly primed cDNA was used to determine whether a transcript of either sense or antisense orientation existed at 19 positions. No RT-PCR product was detected at positions 1, 18, 19 (Fig. 3) or regions beyond (data not shown). At positions 2–17, where RT-PCR gave products, strand-specific cDNA was used to determine RNA orientation. In the sense orientation (*Xist*), a PCR product was detected at positions 5–12. In the antisense orientation (*Tsix*), a PCR product was seen at positions 2–17. Analyses of male, female and transgenic ES cells revealed identical results and were consistent with RNA FISH data.

To map the 5′ end of *Tsix*, we performed 5′-RACE, sequenced the products and found transcription start sites approximately 14,940 bp and 14,990 bp downstream of *Xist* in a CpG island[12]

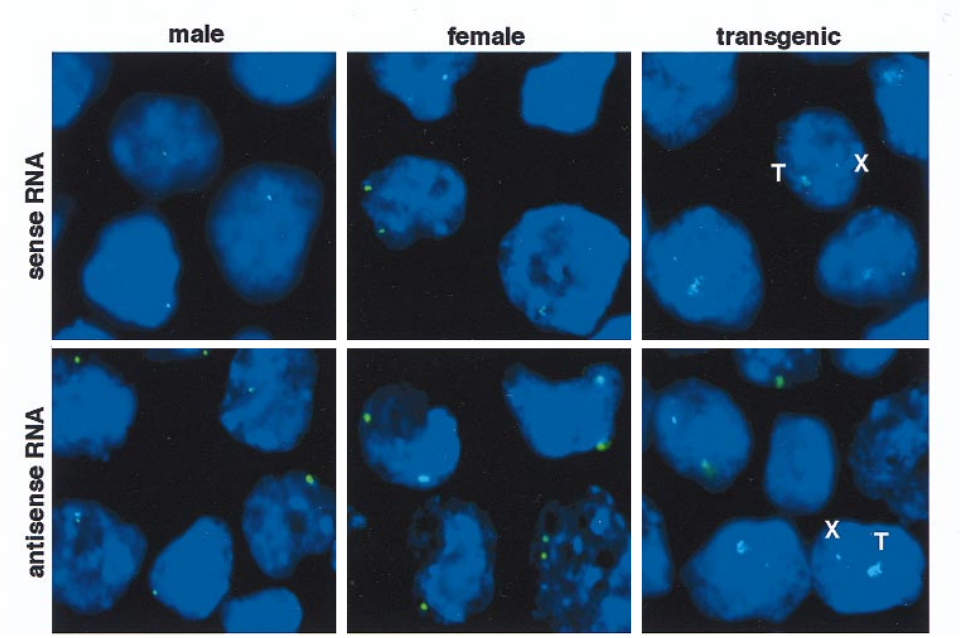

Fig. 1 Strand-specific FISH probes reveal an antisense RNA crossing the *Xist* locus. RNA FISH using fluorescein-labelled (green) strand-specific probes (derived from *Xist* bp 4,512–14,458) in male, female and transgenic ES cells. The female line is a mosaic of 40 XX (cells with 2 dots) and 39 XO cells (single dots). In 116.6, X represents the X-linked locus, whereas T is derived from the 20-copy transgenic locus (determined by RNA/DNA FISH). All nuclei were counterstained with DAPI (blue).

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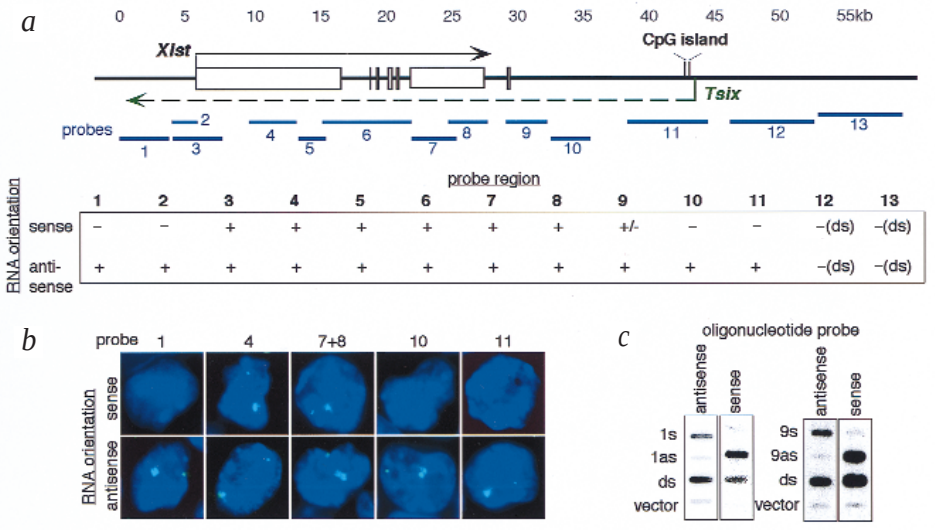

Fig. 2 Mapping *Tsix* by RNA FISH. ***a***, Strand-specific probes across a 60-kb *Xic* region were generated and gel-purified to eliminate double-stranded DNA contamination. Probes were labelled with fluorescein (green) and hybridized to undenatured ES cells (blue). The chart is a compilation of results from male, female and transgenic experiments, all of which gave identical results. Probes 1–11 were strand-specific, whereas 12 and 13 were double-stranded (ds). ***b***, Representative RNA FISH results of (*a*). Transgenic nuclei are shown because the large transgenic RNA clusters are easier to visualize. Cells were prepared without Triton X-100 to visualize cytoplasmic RNAs. ***c***, Slot blot hybridization of single-stranded phage DNA was carried out to verify strand-specificity of templates used to make FISH probes. In the examples shown, sense (1s, 9s) and antisense (1as, 9as) phage DNA templates were hybridized to end-labelled, strand-specific oligonucleotides. Positive control, double-stranded (ds) replicative forms of phage DNA; negative control, pGEM11Zf(+) vector.

(Fig. 3*f*,*g*). These results agreed with primer extension analysis, which yielded a product of approximately 600 bp with a primer positioned at +14,392 bp (Fig. 3*e*). To determine if *Tsix* was one continuous RNA, we performed long-range reverse transcription with subsequent PCR at downstream positions (Fig. 3*c*). An RT-PCR product would be detected only if cDNA extended from the primer to the amplified region. With extension from primer I, specific RT-PCR products were observed up to 9 kb downstream of *Xist* and were not observed at random locations upstream (position a, Fig. 3*c*; other positions, data not shown). Using primer II, specific products were detected up to 7 kb into *Xist* exon 1. Our results linked upstream and downstream antisense transcription with that crossing *Xist*. Together with the lack of gaps detected by FISH and RT-PCR mapping, this argued that *Tsix* is a continuous 40-kb antisense RNA that spans all of *Xist*. RNA slot blot analysis indicated that *Xist* and *Tsix* were expressed at comparably low levels in undifferentiated ES cells (Fig. 3*d*; *Tsix* at relative levels of 1.0, 1.7 and 7.5 in male, female and transgenic cells, respectively; *Xist* at 1.0, 1.7 and 5.5). Northern analysis failed to detect *Tsix* RNA in ES cells.

We questioned whether some developmental features previously ascribed to *Xist* might belong to *Tsix*, because *Xist* had his-

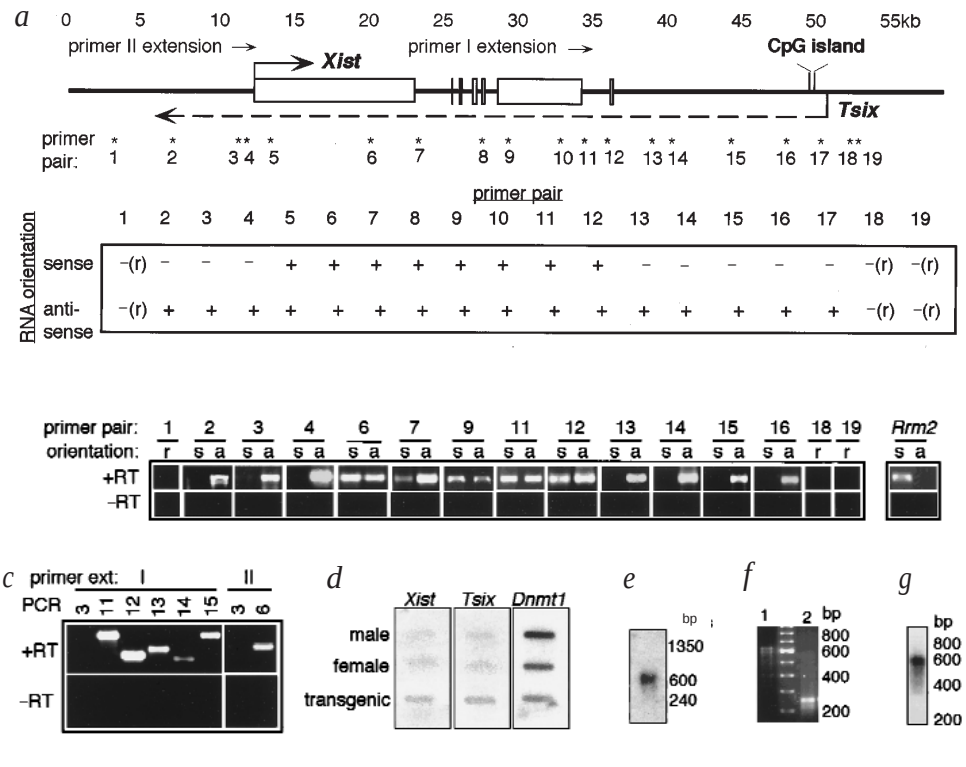

Fig. 3 *Tsix* begins 15 kb downstream of *Xist* and spans 40 kb of sequence. ***a***, RT-PCR was performed at 19 densely spaced intervals to confirm the map of *Tsix*. The chart is compiled from results of male, female and transgenic ES cells, all of which gave identical results. Randomly primed cDNA was first used to determine whether a transcript of either orientation existed at each position. In such instances, strand-specific cDNA was subsequently tested to determine orientation (positions 2–17). For sense transcript, an antisense primer (for example, 4as) was used to prime cDNA synthesis; for antisense transcripts, a sense primer (for example, 4s) was used. First strand cDNA was then amplified with paired sense and antisense primers (for example, 4s+4as). ***b***, Representative strand-specific RT-PCR at select positions shown in (*a*). s, sense; as, antisense; r, random-primed (double-stranded). Positive control, *Rrm2*, a housekeeping gene. ***c***, Long-range reverse transcription: first strand *Tsix* cDNA was synthesized from primers I (4s) and II (11s) and amplified with primer pairs at indicated positions. ***d***, RNA slot blot analysis of *Tsix*, *Xist* and DNA methyltransferase (*Dnmt1*) was performed as described[22]. Total RNA from undifferentiated male, female and transgenic ES cells was slot blotted, hybridized to 10^7 cpm of strand-specific probe and exposed to film overnight; probe 4as (Fig. 2*a*) was used for *Tsix* and probe 4s was used for *Xist*. Since probes were of the same size and from the same region, an approximation of relative *Xist* and *Tsix* levels was achieved. Results were quantitated by phosphorimaging with normalization to *Dnmt1* values. ***e***, Primer extension was performed to map the 5′ end of *Tsix*. A Southern blot of primer extension product hybridized to a nested primer probe is shown. ***f***, 5′-RACE after first round PCR (lane 1) and nested PCR (lane 2). ***g***, Hybridization of first round RACE products to a nested primer probe to demonstrate specificity.

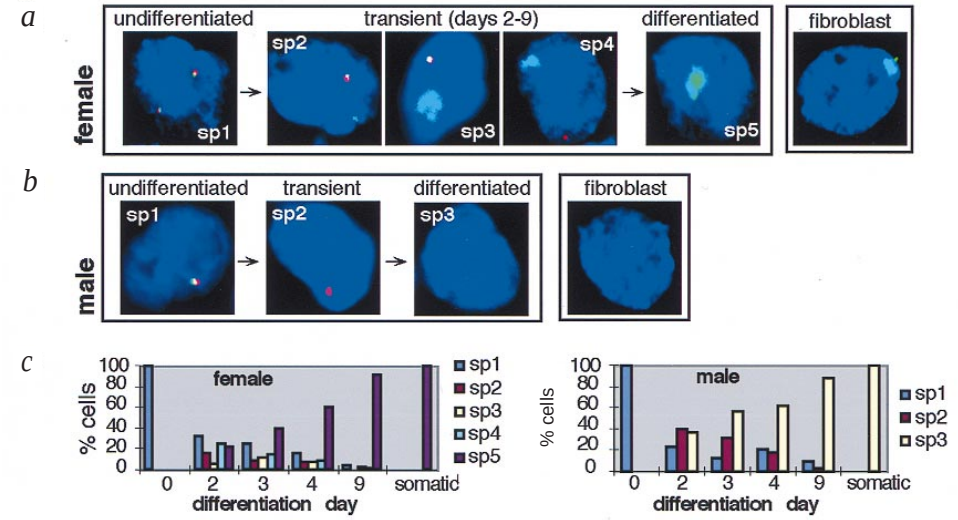

Fig. 4 *Tsix* is dynamically regulated during female and male EB differentiation. *Xist* and *Tsix* RNAs were simultaneously visualized by differential labelling of sense (probe 7s+8s, green, *Xist*) and antisense (probes 7as+8as or 11as, red, *Tsix*) probes. Experiments were performed 2–4 times by scoring 100–300 nuclei. Since female X chromosomes are unstable in culture, only those cells with inferred 40XX constitution were scored. 40XX cells were identified by simultaneous RNA/DNA FISH using an X-specific *Zfx* probe. Approximately 30% were XX and 70% were XO (data not shown). ***a***, Patterns of *Tsix* and *Xist* expression in female ES cells, embryoid bodies and fibroblasts. ***b***, Patterns of *Tsix* and *Xist* expression in male ES, EB and fibroblasts. ***c***, Relative proportions of each pattern during differentiation. sp, species.

torically been examined by double-stranded probes. Using differentially labelled strand-specific probes, we studied the temporal and spatial expression patterns of *Xist* and *Tsix* in ES cells in which X inactivation can be induced by differentiation into embryoid bodies[10] (EB). Time-course analysis of EB cells revealed a dynamic relationship correlating with early events of X inactivation (Fig. 4). Before differentiation in females, *Tsix* and *Xist* were biallelically expressed at low levels (species 1). During differentiation, transient expression patterns appeared in three forms: species 2, retaining low level *Xist* and *Tsix* on one X but lacking *Tsix* on the other X; species 3, with high level *Xist* but lacking *Tsix* on one X and low level *Xist* and *Tsix* on the other X; and species 4, with high level *Xist* and no *Tsix* on one X and low level *Tsix* on the other X. From days 2–9, the transient species increased sharply in number as the undifferentiated species 1 diminished, but began to decline as species 5 became dominant. In species 5, high levels of *Xist* RNA are expressed on one X and no *Tsix* is expressed. Species 5 was the somatic pattern of *Xist* and *Tsix* expression, as it was the only pattern observed in differentiated cells. Although *Xist* RNA was upregulated on the future inactive X, we did not detect *Tsix* by RNA FISH (Fig. 4*a*) or RT-PCR (data not shown) in cells of day 12.5 embryos, fibroblasts and adult tissue including brain, skeletal muscle, heart, kidney, liver and adrenal glands (data not shown). In male ES cells, *Xist* and *Tsix* RNA were also expressed in more than 95% of undifferentiated cells (Fig. 4*b*). Upon differentiation, we observed three species: species 1, in which *Xist* and *Tsix* persisted (undifferentiated form); species 2, expressing only *Tsix* (transient); and species 3, expressing neither. Species 2 was an apparent intermediate, appearing on day 2 and disappearing with time. Species 3 was the somatic form, as it became the only pattern observed in fibroblasts (Fig. 4*b*) and adult tissue (data not shown).

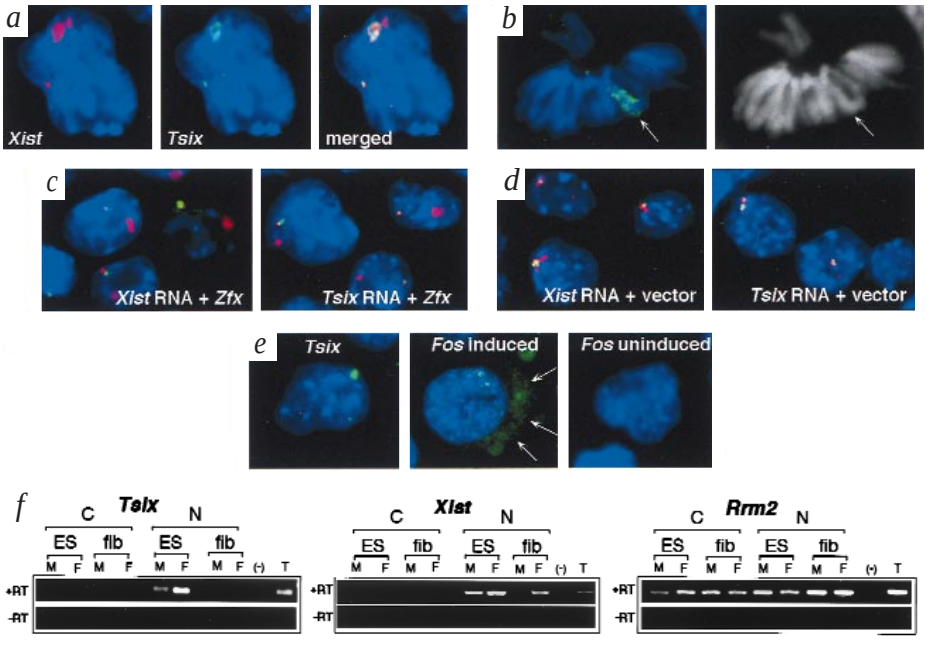

Fig. 5 *Tsix* RNA co-localizes with *Xist* RNA and is exclusively nuclear. ***a***, Two-colour RNA FISH of transgenic undifferentiated ES cells using differentially labelled *Xist* (7s+8s, red) and *Tsix* (7as+8as, green) probes. ***b***, Metaphase chromosomes of a female EB cell showing that only *Xist* (green, 7s+8s) coats the X. *Tsix* probes (red, 7as+8as) gave no signal. The DAPI-stained image is in black and white. Arrows, inactive X. ***c***,***d***, RNA/DNA FISH on denatured transgenic nuclei showed that both *Xist* and *Tsix* RNAs (red) are *cis*-localized to the *Xic*. In (*c*), the small RNA clusters co-localized with *Zfx* (green), a gene tightly linked to the *Xic*. In (*d*), larger *Xist* and *Tsix* RNA clusters co-localized with vector-specific DNA sequences (green; YAC vector), demonstrating that ectopic *Tsix* RNA retained its *cis* property. This co-localization was seen using probes 4as, 7as, 8as and 11as. ***e***, RNA FISH was performed on cells prepared without Triton X-100 to retain cytoplasmic RNAs (ref. 8). *Tsix* probes 7as, 8as and 11as did not reveal cytoplasmic hybridization, but gave nuclear signals. The serum-induced *Fos* expression (green, arrows) was seen in the cytoplasm, whereas the uninduced *Fos* expression was not detected. ***f***, RT-PCR to examine *Xist*, *Tsix* and *Rrm2* expression was performed on nuclear (N) and cytoplasmic (C) fractions of male (M) and female (F) ES cells and fibroblasts (fib) with either primer pairs 16 or 17 (same results). (–), negative water control; T, total female ES RNA.

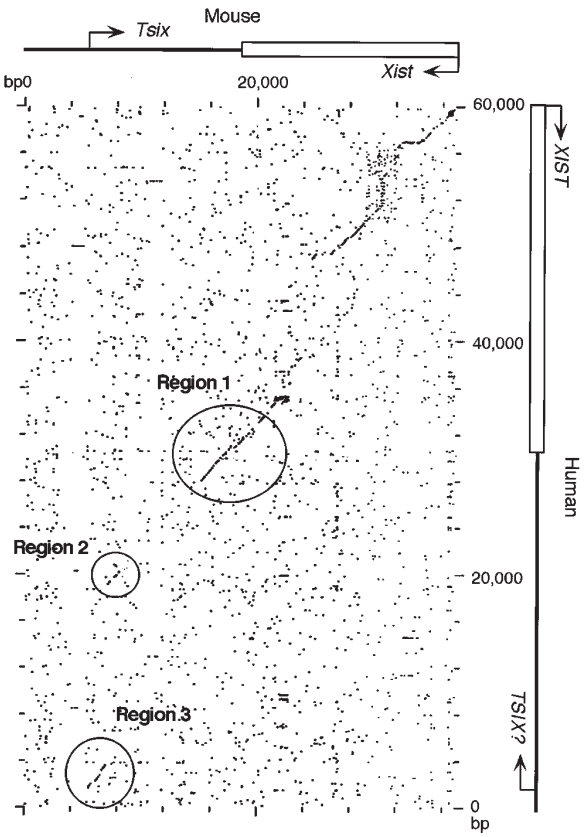

Fig. 6 The mouse *Tsix* sequence is conserved at the human *XIC*. Dotplot analysis of mouse *Xic* and human *XIC* sequences using GCG software. Sliding 21-bp windows and a stringency of 16 in 21 were used so that a 76% nucleotide identity generated a dot. Significant cross-species homology was detected not only in *Xist/XIST* as previously reported[3,4], but also in the 5′ third of *Tsix*, including regions 1, 2 and 3 (circled). Region 1 was 56.7% identical to the human sequence (GCG software penalizing for creating but not for elongating gaps; gap weight of 50, length weight of 0). Regions 2 and 3 were 45.3% identical. No obvious homology lay beyond the CpG island. The identity within the *Xist/XIST* gene body (63.5% from bp 6,200–10,200 of *Xist*) was higher than in regions 1–3. Overall, the conservation within *Xist/XIST* and *Tsix/TSIX* was lower than for average protein-coding exons (β-globin, 82.4% over 444 positions[26]), but was higher than for random X-linked sequences (38.2% at 50–53 kb downstream of *Xist*) and similar to introns of X-linked protein-coding genes (*Tsx/TSX* 50.6% over 1,700 positions).

The dynamic relationship between *Xist* and *Tsix* in female cells is as follows: (i) before the onset of X inactivation, *Xist* and *Tsix* are biallelically co-expressed; (ii) at the onset of X inactivation (differentiation), *Tsix* becomes monoallelic, preceding upregulation of *Xist* RNA on the future inactive X (species 2); (iii) in the monoallelic form, *Tsix* RNA associates only with the future active X; and (iv) once X inactivation is established, *Tsix* is repressed. *Tsix* expression is specific to undifferentiated cells regardless of sex, persists briefly at the onset of X inactivation, appears to associate only with the future active X and disappears after X inactivation is established. This profile suggests that *Tsix* may regulate events at the onset of X inactivation. Its antisense nature raises the possibility that *Tsix* directly blocks *Xist* RNA action on the X chromosome.

Localization of *Tsix* RNA is consistent with this hypothesis (Fig. 5). In ES cells, *Tsix* RNA was found only in the nuclear compartment (Fig. 5*e*,*f*) and co-localized with *Xist* RNA to the *Xic* (Fig. 5*a*,*c*,*d*). Co-localization was seen only in undifferentiated ES cells (male, female, transgenic) and at the onset of X inactivation when the two RNAs were coexpressed (Fig. 4). Once X inactivation was established, *Tsix* became undetectable, whereas *Xist* RNA coated the inactive X. Unlike *Xist* RNA, *Tsix* RNA never spread beyond the *Xic* at any time during differentiation (Fig. 5*b*). A role for *Tsix* was further supported by its conservation between mouse *Xic* and human *XIC* (Fig. 6). Using BLAST, GCG, GRAIL and Exon Finder, comparative sequence analyses revealed no conserved ORFs in *Tsix*. Together with its nuclear localization, this suggests that, similar to *Xist*, *Tsix* encodes an untranslated nuclear RNA.

We have identified a second gene in the *Xic*. *Tsix* is not required for the silencing step itself because a 65-kb deletion including *Tsix* sequences results in constitutive X inactivation[9]. Instead, *Tsix* features suggest an earlier role, such as in X-chromosome counting, choice or initiation, a role supported by available genetic evidence. First, *Tsix* lies partially within a 65-kb region containing a putative *Xist* repressor which is hypothesized to be the counting element[9]; targeted deletion of this region leads to constitutive X inactivation in *cis*. Second, *Tsix* is contained within the 80-kb *Xic* region (with *Xist* and 30 kb of downstream sequence), which can recapitulate the regulation of X inactivation in a transgene-based assay[13]. Furthermore, *Tsix* is expressed by all minimal *Xic* transgenes capable of recapitulating X inactivation (J.T.L., unpublished data). Finally, a cosmid transgene carrying *Xist* but lacking the 5′ end of *Tsix* can express *Xist* RNA and silence a linked reporter gene[14], consistent with the existence of a repressor, deletion of which results in constitutive *Xist* expression. At least three modes of *Tsix* action can be envisioned: (i) *Tsix* RNA may base-pair with and mask functional domains in *Xist* RNA; (ii) *Tsix* transcription may alter chromatin structure and thereby modulate *Xist* expression; and (iii) *Tsix* may regulate *Xist* through expression competition[15]. In mammals, antisense genes have also been found for two imprinted loci, *Igf2r* (ref. 16) and *UBE3A* at the Angelman syndrome locus[17]. Thus, antisense regulation may represent a more common mechanism of long-range transcriptional regulation than previously recognized.

Methods

ES cells and culture. ES cells: male J1, 40XY, 129Sv/J (ref. 18); female EL16, 30%-40XX/70%-39X0, 129x(*M.castaneus*×129) (provided by E. Li); and transgenic 116.6, carrying 20 copies of a 450-kb *Xic* transgene on chromosome 12 (ref. 2). EL16 is karyotypically unstable and stochastically loses one X during culture. We maintained ES cells as described[18] and induced differentiation by growing in suspension culture without LIF (ref. 19). Female and male fibroblasts came from ed13.5 or adult 129Sv/J mice. Serum-induction of *Fos* in fibroblasts was performed by growing in DMEM without serum overnight followed by 10–15-min incubation in DMEM+20% FBS.

FISH. FISH probes were labelled as described[2]. Detailed procedures for RNA and DNA FISH have been described[2,20,21]. Small probes were used for detection of RNA (multicopy) and larger probes were used for DNA (single copy) to ensure signal specificity. RNA specificity was confirmed by destruction of signals when samples were pretreated with RNase.

RT-PCR. For strand-specific RT-PCR, total cellular RNA (10 μg) isolated by RNAzol B reagent (Teltest) was treated with RNase-free DNase I (2 U) for 1 h at 37 °C. Samples were divided into two for +RT and –RT reactions. Strand-specific *Xist* primers (3 pmol) were added to total RNA (0.2–2 μg), heated to 70 °C for 5 min and equilibrated to 50 °C. First strand cDNA was synthesized using either sense or antisense primers with Superscript II reverse transcriptase (200 U; GibcoBRL) for 1 h at 50 °C. Controls without reverse transcriptase were processed in parallel. The enzyme was heat-inactivated at 80 °C for 45 min. First strand cDNA was amplified with paired sense and antisense primers using *Taq* polymerase for 27–32 cycles with an annealing temperature of 52 °C. For long-range reverse transcription, first strand cDNA was synthesized with 11s (0.5 pmol; extension I) or 4s (3

pmol; extension II) and amplified with *Taq* polymerase for 33 cycles. Nuclear and cytoplasmic RNAs were prepared as described[22] and first strand cDNA was made by random priming. RT-PCR of fractionated material was carried out to 30 cycles with either primer pairs 16 or 17.

Probes. The 250-kb BAC-*Zfx* genomic clone[2], vector pYAC4 (ref. 23) and *Fos* probe p302-356pBS(–) (ref. 24) have been described. The following probes are described in relation to +1 bp of the *Xist* cDNA sequence: 1 (–5,216 to –1,652), 2 (–1,652 to –58), 3 (–1,652 to +1,877), 4 (+4,512 to +6,944), 5 (+6,948 to +8,338), 6 (+7,779 to +10,200), 7 (+10,085 to +12,354) and 8 (+11,601 to +14,458). The following are the indicated distance (bp) from the end of *Xist* exon 6: 9 (2,301–5,251), 10 (5,052–6,150), 11 (10,733–15,047), 12 (16,282–21,502) and 13 (21,183–26,942). To generate strand-specific probes, these sequences were cloned into pGEM-Zf plasmids (Promega) and single-stranded phage DNA was isolated as recommended by Promega. Probe template was gel-purified to eliminate contamination by double-stranded plasmid and verified as strand-specific by DNA slot blot hybridization with end-labelled oligonucleotides. The numbers for sense probes are followed by 's' and those for antisense by 'as'.

Primers. The following primer sequences are described in relation to +1 bp of *Xist* cDNA: 1s (–8,534 to –8,514), 1as (–8,183 to –8,202), 2s (–5,470 to –5,452), 2as (–4,872 to –4,891), 3s (–402 to –383), 3as (–58 to –77), 4s (–364 to –344), 4as (–154 to –173), 5s (+272 to +294), 5as (+775 to +755), 6s (+6,760 to +6,779), 6as (+6,944 to +6,926), 7s (+9,407 to +9,426), 7as (164–145 bp of intron 1), 8s (+9,922 to +9,940), 8as (+10,048 to +10,030), 9s (+11,601 to +11,620), 9as (+12,354 to +12,335), 10s (+13,391 to +13,411) and 10as (+14,500 to +14,484). The following span the indicated distance from the end of *Xist* exon 6: 11s (49–66), 11as (301–284), 12s (2,301–2,321), 12as (2,478–2,460), 13s (5,052–5,072), 13as (5,251–5,233), 14s (5,982–6,000), 14as (6,150–6,132), 15s (9,823–9,842), 15as (10,181–10,161), 16s (12,538–12,556), 16as (12,876–12,857), 17s (14,416–14,435), 17as (14,872–14,854), 18s (15,155–15,174), 18as (15,476–15,455), 19s (15,155–15,174) and 19as (16,015–15,994). Rrm2A and Rrm2C were as described[25].

5′ end mapping. For primer extension, total RNA (10 μg) from transgenic ES cells were annealed to a primer at position +14,392 to +14,409 bp (downstream of *Xist*) and extended with Superscript II (GibcoBRL) at 50 °C. The extension product was resolved on a denaturing agarose gel and detected with a nested probe. For 5′-RACE (Marathon kit, Clontech), double-stranded cDNA was synthesized from 116.6 ES total RNA (8 μg) using a primer at +14,392 to +14,409 bp with AMV reverse transcriptase at 42 °C, and RACE linkers were ligated onto cDNA ends. First round PCR products were generated using a primer positioned at +14,416 to +14,435 bp and linker primer ap1 and then probed with a nested primer (+14,677 to +14,659 bp downstream) to confirm specificity. Second round PCR was carried out with nested primers ap2 and nes1 (+14,792 to +14,811 bp downstream). PCR products were cloned and sequenced.

GenBank accession numbers. Mouse *Xic*, L04961, U41394, U41395, U41396, X99946; human XIC, U80460.

Acknowledgements

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