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Species Differences in *TSIX/Tsix* Reveal the Roles of These Genes in X-Chromosome Inactivation

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Transcriptional silencing of the human inactive X chromosome is induced by the *XIST* gene within the human X-inactivation center. The *XIST* allele must be turned off on one X chromosome to maintain its activity in cells of both sexes. In the mouse placenta, where X inactivation is *imprinted* (the paternal X chromosome is always inactive), the maternal *Xist* allele is repressed by a *cis*-acting antisense transcript, encoded by the *Tsix* gene. However, it remains to be seen whether this antisense transcript protects the future active X chromosome during *random* inactivation in the embryo proper. We recently identified the human *TSIX* gene and showed that it lacks key regulatory elements needed for the imprinting function of murine *Tsix*. Now, using RNA FISH for cellular localization of transcripts in human fetal cells, we show that human *TSIX* antisense transcripts are unable to repress *XIST*. In fact, *TSIX* is transcribed *only* from the inactive X chromosome and is coexpressed with *XIST*. Also, *TSIX* is not maternally imprinted in placental tissues, and its transcription persists in placental and fetal tissues, throughout embryogenesis. Therefore, the repression of *Xist* by mouse *Tsix* has no counterpart in humans, and *TSIX* is not the gene that protects the active X chromosome from random inactivation. Because human *TSIX* cannot imprint X inactivation in the placenta, it serves as a mutant for mouse *Tsix*, providing insights into features responsible for antisense activity in imprinted X inactivation.

Introduction

As a result of X inactivation, only one X chromosome is transcriptionally active in diploid somatic cells of both sexes (Lyon 1962); additional X chromosomes are inactivated. Transcriptional silencing is initiated during embryogenesis and can be recapitulated in murine embryonic stem (ES) cells (Martin et al. 1978; Lee et al. 1996). The key control region on the X chromosome is called the "X inactivation center" (*XIC* human, *Xic* mouse). It contains the X-inactive specific transcript gene (*XIST* human, *Xist* mouse [MIM 314670]) (Brockdorff et al. 1992; Brown et al. 1992), which programs the X chromosome for inactivation. The accumulation of *XIST* transcripts at the critical stage of embryonic development is essential for the cascade of events that ultimately silences the chromosome from which it is transcribed (Penny et al. 1996; Marahrens et al. 1997). The noncoding *XIST* RNA binds to the future inactive X (Beletskii et al. 2001), to propagate transcriptional silencing by the inducing of changes in chromatin (Jeppesen and Turner 1993; Wutz and Jaenisch 2000).

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Having no functional X chromosome—or more than one (Takagi and Abe 1990)—is lethal for diploid cells of both sexes; therefore, one—and only one—X chromosome must be protected against *XIST*-induced inactivation. This is done by repressing the *XIST* allele on one X chromosome. In males, the single *XIST* allele is turned off. In females, the *XIST* allele is repressed on only one X chromosome, designating it the active X. The other X chromosome(s) in the cell is inactivated via the *XIST*-induced inactivating signal(s). The choice of parental *XIST* allele to repress is usually random in eutherian mammals. However, in mouse placenta, X inactivation is imprinted (the paternal X chromosome is inactive; the maternal X chromosome is active) (Takagi and Sasaki 1975); the maternal *Xist* allele is always turned off, so that the maternal X chromosome is protected from inactivation (Lee 2000).

The prime candidate for the gene that represses the murine *Xist* allele in *cis* is the *Tsix* gene (MIM 300181), because (1) it encodes an antisense transcript that overlaps the entire *Xist* locus (Lee et al. 1999) and (2) its promoter includes a CpG island that is methylated on the active X chromosome (Debrand et al. 1999). Like *Xist, Tsix* lacks open reading frames, and the RNA remains in the nucleus; however, unlike *Xist,* the *Tsix* RNA is associated with the future *active* X chromosome (Lee et al. 1999). *Tsix* seems to be an antagonist of *Xist* in the mouse placenta, where X inactivation is imprinted.

When *Tsix* RNA is eliminated from the maternal X chromosome by induced recombinant-based deletions that alter the *Xic,* the *Xist* allele on that chromosome is not repressed; this leads, in some cells, to inactivation of both X chromosomes (Lee and Lu 1999; Lee 2000; Sado et al. 2001). On the basis of such observations, Lee (2000) and Sado et al. (2001) have proposed that *Tsix* is the factor that protects the maternal X chromosome from *imprinted* X inactivation in placental tissues (by interacting with the *Xist* transcript on that chromosome). On the basis of deletion-induced skewing of X inactivation, Lee and her colleagues have further proposed that *Tsix* has a role in *random* inactivation as well, in that it chooses the active X chromosome (Boumil and Lee 2001; Stavropoulos et al. 2001), and that it is, along with the transcription factor CTCF, the regulatable epigenetic switch for X inactivation (Chao et al. 2002). It should be noted that these induced deletions are not *Tsix*-specific deletions but are genomic deletions that not only alter the *Tsix* transcript but also may alter other, yet-unknown regulatory elements as well. Therefore, although it is likely that murine *Tsix* has a role in imprinted X inactivation, a role for *Tsix* in random inactivation has yet to be established.

We have recently identified the human counterpart of murine *Tsix,* within the human *XIC* (Migeon et al. 2001). Like the mouse gene, *TSIX* is well expressed in embryo-derived cells, initiates downstream of the 3′ end of *XIST,* and produces an untranslated RNA. *TSIX* is transcribed (1) from human *XIC* transgenes in mouse ES cells and (2) in human embryoid body–derived (HED) cells. The *TSIX* RNA is transcribed from the strand opposite that from which *XIST* is transcribed and is, in part, antisense to *XIST,* overlapping the *XIST* locus at its 3′ end.

However, the human *TSIX* gene differs from its murine counterpart in that it lacks the CpG island that is essential for the function of murine *Tsix* (Lee 2000; Sado et al. 2001) (fig. 1). The antisense *TSIX* transcript is abbreviated; it overlaps only exons 5–8 of the *XIST* transcript, so that exons 1–4 and the *XIST* promoter are not covered (Migeon et al. 2001) (fig. 1). Such differences suggest that *TSIX* is unable to function like the murine gene—a supposition that is consistent with the fact that X inactivation is *random,* not imprinted—in human placental tissues (Migeon and Do 1979; Migeon et al. 1985).

On the basis of RT-PCR assays, both *XIST* and *TSIX* have been shown to be transcribed in ES cells carrying six copies of the human *XIC* transgene (Migeon et al. 2001). Since both transcripts originate from the transgene—and, hence, form the same chromosome—it seemed likely that *TSIX* did not repress *XIST* expression. Yet, the possibility that *XIST* is transcribed in some cells and that *TSIX* is transcribed in others could not be excluded. Furthermore, we did not know the

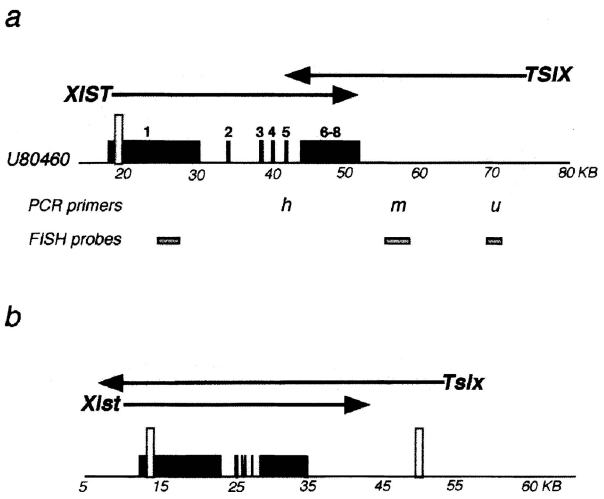

Figure 1 Partial maps of (*a*) human and (*b*) mouse *XIC* regions, comparing locations of *XIST* and *TSIX* transcripts and of CpG islands. Blackened boxes indicate *XIST* exons; unblackened boxes indicate CpG islands; and arrows show the transcripts and the direction of transcription. *a,* Human *XIC.* The relevant 18–80 kb of the U80460 DNA sequence (GenBank) is shown. *h, m,* and *u* are primer sets, and the gray-shaded boxes indicate the FISH probes. *b,* Mouse *XIC.* The relevant 60 kb is shown.

location oftranscripts in human cells. RNA FISH analysis permits us to determine the origin of these RNA transcripts at the cellular level. By simultaneously hybridizing probes for human *XIST* and *TSIX* to RNA in interphase nuclei from human fetal cells, we could see (1) which X chromosome(s)—active, inactive, or both—was the source of the *TSIX* transcript and (2) whether one transcript had an effect on the other.

Material and Methods

Cell Lines

Human.—The HED cells were two 46,XX cell lines originating from female primordial germ cells at 5 and 11 wk after fertilization (and hereafter designated "LVEC" and "SDEC," respectively). These fibroblast cultures, established by Dr. John Gearhart, are no longer totipotent but transcribe some genes expressed early in development (Shamblott et al. 2001). Our use of these HED cells was approved by the Johns Hopkins University committee concerned with patents and by Geron, Inc., according to the licensing agreement.

The somatic cells of human fetal origin were fibroblasts derived from discarded products of conception, at various gestational stages. Aliquots of frozen cells obtained for previous studies were the source of proliferating cells for the present study. Specimens are described in table 1 and include (1) chorionic villi cultures of normal newborn placentas (five female and one male), (2)

Table 1

***TSIX* Expression in Human Somatic Cells, on the Basis of RNA FISH and RT-PCR Analysis**

Source and Age[a]	Reference Line[b]	FISH Signal [No. of Signals per Cell] of[c] (%)		Status with *TSIX* Primer Set[d,e]		
		XIST	*TSIX*	*h*	*m*	*u*
Embryoid bodies:						
5 wk	LVEC, XX	72	40	+	+	+
11 wk	SDEC, XX	76	32	+	+	+
Fetal fibroblasts:						
5–8 wk	ET-2, XX	92	86	+	+	+
	K-19, XX	74	52	+	+	+
	Fe18, XX	82	36	+	+	+
	K-1, XY	0	0	−	−	−
Hydatidiform moles:						
10 wk	K1248, XpXp	88	68	+	+	Trace
Chorionic villi:[f]						
Birth	CV-5, XX	78	42	+	+	+
	CV-4, XY	0	0	−	−	−
	PG, XXX	82 [2]	64 [2]	+	+	+
Postnatal fibroblasts:						
Birth	TW-1, XX	62	40	ND	ND	ND
2 mo	TW-2, XX	94	32	+	Trace	+
	Clone 1[g]	92	46	ND	ND	ND
	Clone 2[h]	86	30	ND	ND	ND
2 years	HJ, XXXXY	72 [3]	6 Trace [3]	Trace	−	−
4 years	TaW, XX	80	26 Trace	+	Trace	−
4 years	CW, XX	79	0	ND	Trace	−
8 years	AB, XX	84	22 Trace	+	Trace	Trace
20 years	PS, XX	86	0	−	−	−
24 years	CC, XX	90	0	−	−	−
25 years	MF, XX	82	0	ND	−	−

[a] Age is from conception, as estimated from LMP and fetal measurements.
[b] Includes sex-chromosome complement of specimen, X and Y; Xp = paternal X chromosome.
[c] Performed with *XIST*- and *TSIX*-specific probes simultaneously. For most postnatal fibroblasts, the *TSIX* signals were present in trace amounts. A few cells had a *TSIX* signal in the absence of an *XIST* signal (average 0.3% [range 0%–8%]).
[d] RT-PCR primers are as shown in figure 1. Strand-specific priming was used for *XIST* exon 5 primer set *h*; primer sets *m* and *u* are *TSIX* specific.
[e] + = Present; − = absent; ND = not done.
[f] From full-term placentas.
[g] Inactive maternal X chromosome.
[h] Inactive paternal X chromosome.

fetal fibroblast cultures from 5–18 wk gestation (six female and one male), and (3) fibroblasts from a hydatidiform mole (K1248), with two copies of the identical paternal X chromosome (Jacobs et al. 1980). Also analyzed were fibroblasts obtained, with Institutional Review Board approval, from individuals ranging, in postnatal age, from 2 mo to 25 years (eight females and one 49,4XY male). Clones originating from single cells were obtained by cell dilution (Migeon et al. 1985), and parental origin of the active X chromosome was determined on the basis of parental X-chromosome markers.

Mouse.—The ES cells with the human *XIC* transgene (ES-10) were derived from J1 ES cells (Migeon et al. 1999). ES-10 has six copies of the 480-kb transgene that includes the human *XIST* locus, ~70 kb flanking the 3′ end of *XIST,* and ~380 kb flanking 5′ *XIST* inserted into mouse chromosome 11. The ES*ch*-10 somatic cells were fibroblasts from an 18-d-old stillborn XY chimeric mouse 949-1, carrying the ES-10 transgene (Migeon et al. 1999).

RNA FISH

The procedure was as described elsewhere (Clemson et al. 1996). In brief, interphase cells on slides were permeabilized, fixed in paraformaldehyde, and dehydrated. Then, without denaturation, the slides were hybridized, for 3 h, with labeled probes, along with human COT-1 and salmon sperm DNA. The DNA probes were labeled, by the nick-translation kit (Vysis), with either biotin-11-

dUTP or digoxygenin-16-dUTP (Roche), and the signals were detected with either avidin/biotinylated anti-avidin or anti-digoxygenin-rhodamine, respectively, with DAPI as the counterstain.

FISH Probes

The probes were long-range PCR products that were cloned into the pCR2.1-TOPO vector (Topo TA kit; Invitrogen): All probes come from nonoverlapping segments of the *XIST* and *TSIX* genes. *XIST* exon 1 is specific for *XIST* and contains the ~2.5-kb DNA segment of nt 24658–27215 in the PAC U80460 sequence (GenBank). *TSIX*-specific probes are *TSIX-70* (~1.7 kb; nt 69086–70779) and *TSIX-57* (2.9 kb; nt 55348–58274), both in U80460.

RT-PCR

RNA was obtained from ES cells before and after differentiation into embryoid bodies. Total RNA was isolated with TRIZOL (Invitrogen) and was treated with DNaseI. To screen for transcripts, RT-PCR was performed (1μg of RNA; 35 cycles [Migeon et al. 1999]). The cDNA was generated by random priming, for sequences outside the *XIST* transcription unit, whereas strand-specific priming was used for the portion of *TSIX* that overlaps *XIST* (Migeon et al. 2001).

Primers

Sequence data for *TSIX* and the *XIC* transgene are provided in the GenBank entry for PAC 92E23, which has been sequenced in two parts, U80459 and U80460. The locations of primers used for RNA analysis are shown in figure 1 and are based on the U80460 sequence: for primer set *h,* the start of the antisense primer is at nt 41838 (136-bp product); for primer set *m,* the forward primer starts at nt 57793 (482-bp product), and, for primer set *u,* the start is at nt 70420 (377-bp product. For strand-specific RT-PCR, first-strand cDNA was synthesized by either sense (*XIST*) or antisense (*TSIX*) primers, as described elsewhere (Migeon et al. 2001).

Results

TSIX *Coexpressed with* XIST *from the Inactive X Chromosome in ES Cells*

Figure 1*a* shows the location of *TSIX* and *XIST* transcripts in the GenBank U80460 sequence, as well as the FISH probes used for these studies. These probes were gene specific, since they were homologous to the nonoverlapping segments of *XIST* and *TSIX*. When hybridized independently, the two *TSIX* probes, *TSIX-57* and *TSIX-70,* gave similar signals, but only the studies with *TSIX-70* are shown. The results of RNA FISH, using simultaneous hybridization of digoxygenin-labeled *XIST* and of biotin-labeled *TSIX* probes, were the same for mouse ES cells carrying the ES-10 human transgene, ES-10, and for human female HED cells of germ-cell origin (LVEC). (fig. 2*a*). In each case, there was only one *XIST* signal (red) and one *TSIX* signal (green) per cell. The *XIST* signal was large, resembling that in human adult female cells (fig. 2*a,* "CC" row). The *TSIX* signal was also substantial, indicating many molecules per nucleus. It was as well focused as the signal of *XIST;* however, it was usually smaller than the signal of *XIST* (fig. 2, "*TSIX*" and "Merge" lanes), irrespective of the nature of the fluorochromes used. When the *TSIX* and *XIST* images were merged, the two signals always coincided (yellow signals denote overlap), as would be expected if the two RNA samples originated from the same *XIC.* Both signals in mouse ES-10 cells were seen before and after differentiation into embryoid bodies and came from the mouse chromosome 11 carrying the human *XIC* transgene. In the human cells, both signals came from the inactive X chromosome, visualized as a Barr body in DAPI-stained interphase cells (not shown). The frequency of *TSIX* signals was usually less than that for *XIST* signals (table 1), reflecting some variability in *TSIX* expression. Rare (frequency <5%) cells had a single *TSIX* signal in the absence of *XIST.* Figure 2*a* ("CC" row) shows that *TSIX* was not detectable in somatic cells from an adult female.

Persistence of TSIX *Expression in Somatic Cells*

Before X inactivation, murine *Tsix* is transcribed, to a modest extent, from both X chromosomes; at the time of inactivation, the allele on the inactive X chromosome is repressed, whereas that on the future active X chromosome is up-regulated transiently, disappearing from ES cells 2–9 d after their differentiation into embryoid bodies and from chimeras 12–15 d after conception (Lee et al. 1999; Sado et al. 2001). However, the human *TSIX* signal was present in 56% of cells from a chimeric mouse fetus 18 d after conception (data not shown).

TSIX expression also persists in human fetal somatic cells long after X inactivation is established. Figure 2*b* shows the monoallelic expression of *TSIX* in 46,XX fetal specimens (ET-2 and Fe18) with gestational age 5–8 wk. Characteristically, the *TSIX* signal was associated with an *XIST* signal at the same location. Most (74%–92%) cells had an *XIST* signal, and many (36%–86%) also had a *TSIX* signal. This signal varied in size, like that for *XIST,* but, in merged images, it usually did not cover the entire *XIST* signal (see fig. 2, "Merge" lane). Both signals overlie the sex chromatin body.

In addition, *TSIX* was expressed from the inactive X chromosome in female placental tissues at term (table 1 and fig. 2*b,* "PG" row). As would be expected for an

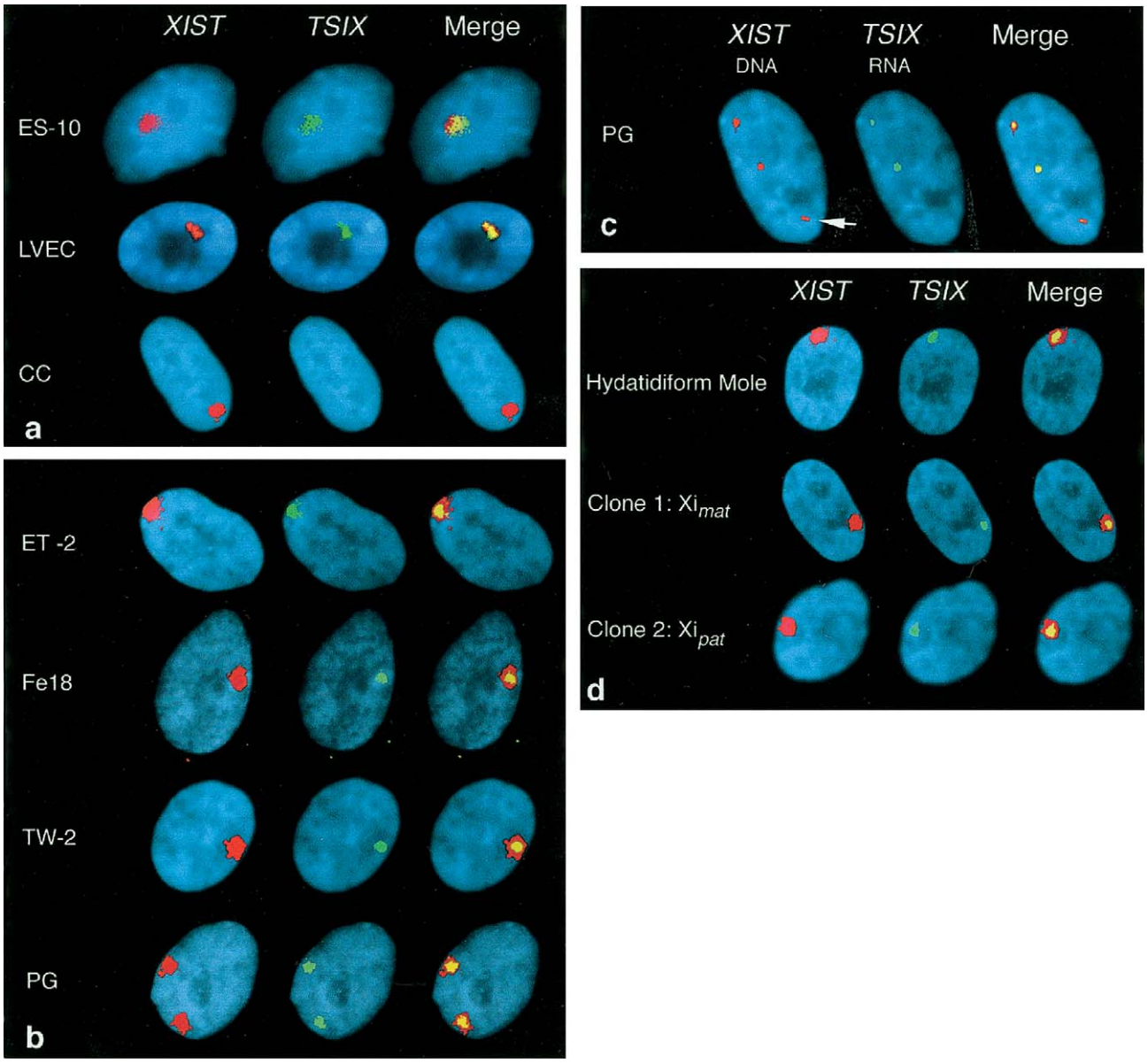

Figure 2 Simultaneous hybridization, in situ, of *XIST*- and *TSIX*-specific probes to nuclear RNA. In panels *a, b,* and *d,* the probes are *XIST* exon 1 (*red*) and *TSIX-70* (*green*); overlapping signals (*yellow*) are seen in merged images. *a, TSIX* and *XIST* coexpressed in mouse ES-10 cells carrying the human transgene (row ES-10), human HED cells (row LVEC), and 46,XX adult human female fibroblasts (row CC). *b,* Persistence of *TSIX* transcripts in human female cells from fetus, placenta, and neonate: 5–6 wk gestation (row ET-2), 6.5–8 wk gestation (row Fe18), 2 mo after birth (row TW-2), and full-term placenta, 47,XXX (row PG). *c, TSIX* expressed from the inactive X chromosome. Sequential *TSIX* RNA/*XIST* DNA hybridization showing three *XIST* alleles (*red*) but only two *TSIX* RNA signals (*green*) in PG (47,XXX) placenta. The arrow indicates the active X chromosome lacking the *TSIX* signal. *d, TSIX* expressed from the human inactive X chromosome, of either parental origin, in hydatidiform mole (K1248, with inactive paternal X chromosome) and in clones 1 and 2, from specimen TW-2, that have either the maternal X chromosome (Xi_{mat}) or the paternal X chromosome (Xi_{pat}) as the inactive X chromosome, respectively.

inactive X-associated transcript, *TSIX* was never seen in male placental cells. The placental cells from a newborn with a 47,XXX karyotype had two *TSIX* and two closely associated *XIST* signals per cell, consistent with the presence of two inactive X chromosomes (fig. 2*b,* "PG" row). Sequential *TSIX* RNA hybridization followed by *XIST* DNA hybridization in these cells showed three X chromosomes but only two *TSIX* RNA signals, confirming that the active X chromosome did not express *TSIX* (fig. 2*c,* "PG" row).

Because *TSIX* was expressed in all the female fetal specimens and in chorionic villi at birth, we examined post-

natal specimens to determine when the *TSIX* transcript disappears. Significant expression persisted in skin fibroblasts of a 2-mo-old female (fig. 2*b,* "TW-2" row). *TSIX* RNA was also present in trace amounts in specimens from 4–8-year-old females and from a 2-year-old 49,XXXXY male, but it was extinguished in young adults (table 1).

TSIX *Expression—Monoallelic but Not Parentally Imprinted*

That *TSIX* was monoallelically expressed and that there were fewer *TSIX* signals than *XIST* signals (table 1) raised the possibility that *TSIX* expression might be imprinted—coming only from the maternal X chromosome, as in the case of mouse placental cells. Therefore, we looked at *TSIX* expression in cells from a hydatidiform mole, a placental tumor, whose chromosomes come exclusively from sperm. Our previous analysis of these 46,XX cells showed that both X chromosomes were of paternal origin and, in fact, had been derived from duplication of a single paternal X chromosome (Jacobs et al. 1980). The single *XIST* signal in these cells showed that one of the identical X chromosomes was transcriptionally inactive. It was associated with a *TSIX* signal in 68% of the cells (table 1 and fig. 2*d*). We also studied two clones obtained from TW-2 skin fibroblasts—one with the maternal inactive X chromosome and the other with the paternal inactive X chromosome, in all the cells (see the "Material and Methods" section). The *TSIX* signal was seen in both clones (fig. 2*d,* "Clone 1:Xi *mat*" and "Clone 2:Xi *pat*" rows), showing that *TSIX* is expressed from an inactive X chromosome of either parental origin.

RT-PCR Analysis of TSIX *Expression*

Many of the same specimens were analyzed by RT-PCR using either primer sets unique to *TSIX* (see fig. 1) or strand-specific priming of the cDNA (fig. 3). The results in table 1 show that *TSIX* RNA is in fetal specimens for as long as 2 mo after birth. *TSIX* RNA was detected neither in adult control cells (human male and female) nor in the fetal or placental cultures established from males (table 1 and data not shown). Some of the postnatal specimens had traces of RT-PCR products, confirming the results of the FISH studies.

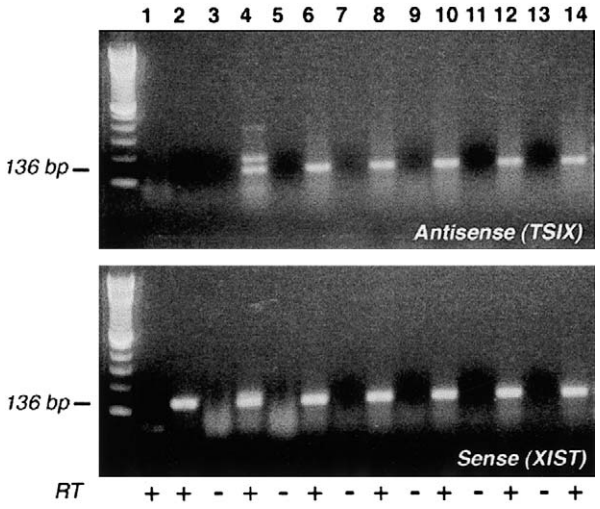

Figure 3 Results of RT-PCR analysis, showing persistence of *TSIX* expression in human female cells of fetal and placental lineages: strand-specific priming of cDNA and amplification with primer set *h,* for *XIST* exon 5 (136-bp product) in the presence (+) or absence (−) of reverse transcriptase (*RT*). *Top,* Antisense (*TSIX*). *Bottom,* Sense (*XIST*). Specimens are from an adult male (*lane 1*), an adult female (*lanes 2* and *3*), mouse ES-10 cells with the transgene (*lanes 4* and *5*), a female HED cell, *LVEC* (*lanes 6* and *7*), placentas CV2 (*lanes 8* and *9*) and CV5 (*lanes 10* and *11*), and fetal fibroblasts at 12 wk (*lanes 12* and *13*) and at 18 wk (*lane 14*). Extra bands for ES-10 are of mouse origin.

Discussion

We have studied the expression of human *TSIX* in cells analogous to those used for the study of the mouse *Tsix* gene, employing similar methods and conditions. The results of our analysis show differences between the two antisense transcript genes. They differ in respect to (1) the chromosome from which the gene is transcribed (*Tsix,* active X chromosome; *TSIX,* inactive X chromosome) and (2) the ability to repress *XIST/Xist* (*Tsix,* yes; *TSIX,* no). Our observations provide compelling evidence that human *TSIX* is not the gene that protects the human active X chromosome against *XIST*-induced inactivation, because it cannot repress the *XIST* allele in *cis*. The *TSIX* locus is coexpressed with *XIST* from one and the same inactivation center in ES-10 cells and from the inactive X chromosome in human germline–derived HED cells (fig. 2*a*) and in fetal somatic cells of females (fig. 2*b*). In addition to its failure to inhibit *XIST* expression, *TSIX* does not prevent inactivation of the chromosome from which it is transcribed; in fact, *TSIX* is expressed uniquely from an *inactive* X chromosome and clearly does not interfere with the silence of this chromosome. In contrast to the tightly regulated control of murine *Tsix,* the human gene is subject to prolonged and variable expression, persisting throughout gestation and even beyond. *TSIX* expression certainly is not harmful to cell viability. Because *TSIX* in normal human fetal cells is transcribed from the inactive X chromosome and not the active one, and because its abundant transcript does not prevent *XIST* expression, it seems that *Tsix* repression of murine *Xist* has no counterpart in humans.

These observations, considered along with the fact that human *TSIX* lacks the critical elements of murine *Tsix* (i.e., the CpG island and complete overlap with

XIST), strongly suggest that human *TSIX* not only differs from its mouse counterpart but also is a defective gene. Most likely, *TSIX* is an evolutionary vestige of an ancestral gene and never functions in X inactivation in human cells. What remains to be explained is why, during embryogenesis, *TSIX* is monoallelically transcribed exclusively from the inactive X chromosome. *TSIX* expression is not parentally imprinted, because transcripts come from the inactive X chromosome of either parental origin (fig. 2*d*). Perhaps chromatin changes responsible for—or induced by—*XIST* transcription allow the expression of this evolutionary vestige; alternatively, the chromatin changes on the future active X chromosome, responsible for repression of *XIST,* may also inhibit the expression of this ineffective *antisense* transcript. More difficult to explain is the gradual repression of the *TSIX* allele on the inactive X chromosome, after birth. Conceivably, this is due to concomitant changes in *XIST* expression and/or chromatin confirmation of the inactive X chromosome that have not been recognized until now.

Species differences in details of developmental events provide powerful tools for the dissection of these complex processes. Because human *TSIX* is unable to repress *XIST,* it is mutant with respect to its murine counterpart, and it affords insights into mechanisms of *Xist* repression by *Tsix* and of antisense function in parental imprinting. The mechanism most compatible with our observations is that *Tsix* repression of *Xist* in the mouse placenta requires interaction between *Tsix* transcripts and the *Xist* promoter, as well as the presence of the *Tsix* CpG island. The absence of the CpG island in the human *TSIX* gene, considered along with the inactivating effect of similar deletions induced in the murine gene, indicates that this *Tsix* element is essential for antisense activity. On the basis of a survey of imprinted genes on human chromosome 11, Onyango et al. (2000) have suggested that imprinting requires the presence of two CpG islands, one of which is associated with the antisense transcript. That the abbreviated *TSIX* transcript does not overlap the *XIST* promoter or its CpG island undoubtedly contributes to its impotence as an *XIST* inhibitor. Experimental evidence supporting this conclusion comes from studies by Luikenhuis et al. (2001), who have shown that truncation of the *Tsix* transcript, even when the CpG island is intact, interferes with the ability of the mutant *Tsix* to inhibit *Xist* expression in mouse ES cells.

If *TSIX* is unable to repress the *XIST* locus in *cis*, then it cannot be the key gene that protects the active X chromosome against programmed inactivation in human cells. Evidence that mouse *Tsix* may also not be sufficient for *random* X inactivation comes from studies of ES cells with a 65-kb genomic deletion that removes ~15 kb of *Tsix* DNA, eliminating the *Tsix* RNA; targeted site-specific replacement of *Tsix* in these ES cells does not restore random X inactivation (Morey et al. 2001). However, the deletion is large, and restorations may perturb the chromatin in the region. Nonetheless, another candidate (or other candidates) for this function needs to be identified. It is difficult to envision how an X-chromosomal gene could, by itself, regulate the function of an *XIST* allele on only one of the members of the X-chromosome pair. The ultimate regulator of *XIST/Xist* (and of *Tsix*) is probably encoded by an autosomal gene (Lyon 1971; Jacobs and Migeon 1989), is limited in quantity, and is subject to cooperativity. Such a *trans*-blocking factor needs to find only one X chromosome at random, and, once a sufficient number of molecules bind to one X chromosome, the *XIST* allele on that chromosome will be blocked. One might predict that more of this *trans*-blocking factor might protect more than one X chromosome, and this is the case in triploid specimens (i.e., three sets of autosomes and either an XXY or XXX sex-chromosome complement), in which two X chromosomes can remain active (Weaver et al. 1975; Jacobs et al. 1979; Migeon et al. 1979).

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Electronic-Database Information

Accession numbers and URLs for data herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for human *XIC* reference sequence U80460)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *XIST* [MIM 314670] and *TSIX* [MIM 300181])

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