Further examination of the *Xist* promoter-switch hypothesis in X inactivation: Evidence against the existence and function of a P_0 promoter

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The onset of X inactivation coincides with accumulation of *Xist* RNA along the future inactive X chromosome. A recent hypothesis proposed that accumulation is initiated by a promoter switch within *Xist*. In this hypothesis, an upstream promoter (P_0) produces an unstable transcript, while the known downstream promoter (P_1) produces a stable RNA. To test this hypothesis, we examined expression and half-life of *Xist* RNA produced from an *Xist* transgene lacking P_0 but retaining P_1. We confirm the previous finding that P_0 is dispensable for *Xist* expression in undifferentiated cells and that P_1 can be used in both undifferentiated and differentiated cells. Herein, we show that *Xist* RNA initiated at P_1 is unstable and does not accumulate. Further analysis indicates that the transcriptional boundary at P_0 does not represent the 5′ end of a distinct *Xist* isoform. Instead, P_0 is an artifact of cross-amplification caused by a pseudogene of the highly expressed ribosomal protein S12 gene *Rps12*. Using strand-specific techniques, we find that transcription upstream of P_1 originates from the DNA strand opposite *Xist* and represents the 3′ end of the antisense *Tsix* RNA. Thus, these data do not support the existence of a P_0 promoter and suggest that mechanisms other than switching of functionally distinct promoters control the up-regulation of *Xist*.

In mammals, dosage compensation of X-linked genes is achieved by the transcriptional silencing of a single X chromosome during early female development (1). The *Xist* gene resides within the X inactivation center (*Xic*), a master control region for X inactivation, and is a current focus for understanding the early events that lead to X inactivation. *Xist* encodes a large untranslated RNA (2, 3), which is localized to the nucleus (4) and is required for X inactivation (5). Before differentiation, *Xist* is expressed at low levels from all X chromosomes in male and female cells (6–9). During differentiation, *Xist* RNA becomes abundantly expressed from the future inactive X in females and is silenced on future active female X and male X chromosomes. At this time, *Xist* RNA accumulates along and completely coats the inactive X in cis, an event that correlates with chromosome silencing. In the prevailing view, *Xist* up-regulation results from increased RNA half-life at the onset of cellular differentiation (8, 9).

Johnston *et al.* (10) recently proposed that a developmentally regulated *Xist* promoter switch is responsible for this change in RNA half-life. The study suggests that two functionally distinct promoters are present within the *Xist* gene. The P_1 (and P_2) promoter is used in differentiated female cells for production of a stable transcript that coats the inactive X chromosome. According to the hypothesis, a second promoter, P_0, lies 6.6 kilobases (kb) upstream of P_1 and produces unstable *Xist* transcripts in undifferentiated cells that have not undergone X inactivation. The model proposes that a switch from P_0 to P_1 during cell differentiation occurs on a single X chromosome in female cells, resulting in the production of stable *Xist* transcripts and initiation of X inactivation in cis.

The model makes several testable predictions. First, if the P_0 promoter is responsible for *Xist* transcription in undifferentiated cells, deleting the P_0 promoter would lead to loss of *Xist* expression. Second, *Xist* expression from P_1 would produce an intrinsically stable transcript. Third, the extended half-life of *Xist* RNA initiated from P_1 would enable RNA accumulation in cis. We have tested these predictions by using a truncated *Xist* transgene lacking P_0. We find that P_0 is not required for *Xist* expression in undifferentiated cells. Furthermore, P_1-directed transcription does not produce stable *Xist* transcripts or lead to accumulation of *Xist* RNA in undifferentiated cells. These results argue against functionally distinct promoters and prompted us to investigate the nature of the P_0 transcript further. To our surprise, the reported location of P_0 coincides with a ribosomal protein S12 pseudogene. Moreover, we find that transcription upstream of the *Xist* P_1 promoter is antisense to *Xist* and represents the 3′ end of the *Tsix* gene. These data provide an alternative explanation for observations relating to P_0 and suggest that promoter switching does not underlie the dynamic regulation of *Xist* at the onset of X inactivation.

Materials and Methods

All sequence information in this report is based on the conventional *Xist* numbering scheme (3). The *Rps12* pseudogene (*pS12X*) is referenced as *Rps12-ps1* in the mouse database.

Reverse Transcriptase (RT)–PCR. For strand-specific RT-PCR, 10 μg of total cellular RNA isolated by RNAzol B reagent (Tel-Test, Friendswood, TX) was treated with 2 units of RNase-free DNase I at 37°C for 1 h. DNase was inactivated in the presence of EDTA at 70°C for 10 min. Samples were split in two for +RT and −RT reactions. Strand-specific primer (3 pmol) was annealed to 0.2–2 μg of total RNA at 70°C for 5 min and equilibrated to 50°C. First-strand cDNA was synthesized by using 200 units of Superscript II RT (GIBCO/BRL) for 1 h at 50°C. Reverse transcriptase was omitted for −RT controls. The enzyme was heat inactivated at 80°C for 30 min. The positions of the following primers are relative to the P_1 promoter: 3s (−3,136 to −3,116), 3as (−2,963 to −2,981), 4s (−1,481 to −1,461), 4as (−1,223 to −1,241), 5s (−1,033 to −1,014), and 5as (−794 to −814); "s" denotes the sense primer, whereas "as" denotes the antisense. Primer pairs 2, 6, and 7 correspond to primer pairs 2, 4, and 6, respectively, as previously reported (11). CJ9, CJ10, CJ11, and CJ12 (10), Mix20 and Mx23b (12), and Rrm2A and Rrm2C (13) have been described. Sense RT-PCR at position 1 (see Fig. 5*A*) was performed by priming with CJ9 and amplifying with nested CJ11–CJ12, whereas antisense RT-PCR was performed by priming with CJ12 and amplifying with CJ9–CJ10.

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Abbreviations: kb, kilobase; RT, reverse transcriptase; RACE, rapid amplification of cDNA ends; ES, embryonic stem; RFLP, restriction fragment length polymorphism.

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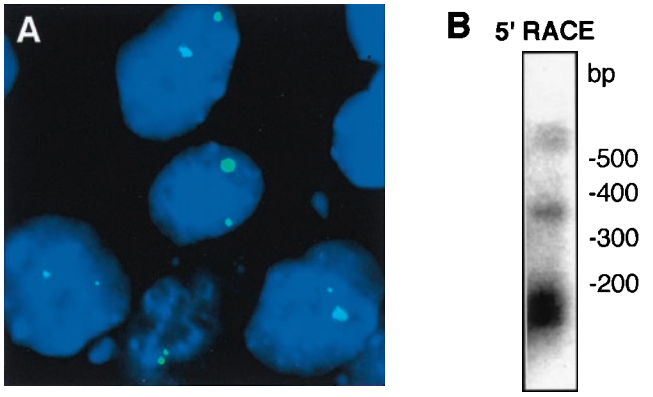

Fig. 1. P_0 is not required for *Xist* expression in transgenic ES cells. (*A*) RNA fluorescence *in situ* hybridization (FISH) was performed with sense-specific exon 1 and 6 *Xist* probes (green). Nuclei shown are from πJL3.9 ES cells, but πJL3.1 yielded identical results. (*B*) RACE (5′) showed that transgenic cells initiated *Xist* expression at P_1 and two other potential start sites at approximately −200 bp and +200 bp relative to P_1. RACE (5′) results were obtained from πJL3.9. Note that, because transgenic cells also carried the endogenous *Xist* gene, detected transcripts may reflect either endogenous or transgenic *Xist* expression.

For all other positions, first-strand cDNA was synthesized with either sense or antisense primers (e.g., 3s) and then amplified with paired sense and antisense primers (e.g., 3s + 3as). *Taq* polymerase PCR was performed for 30–33 cycles with an annealing temperature of 52°C. Fractionated nuclear and cytoplasmic RNAs were prepared as described (14) and random primed to generate first-strand cDNA. RT-PCR of fractionated material was carried out to 30 cycles with primer pairs CJ9–CJ10 (data not shown) or CJ11–CJ12.

5′ End Mapping. For 5′ rapid amplification of cDNA ends (RACE; Marathon kit, CLONTECH), double-stranded cDNA was synthesized from 8 μg of πJL3.9 embryonic stem (ES) total RNA by using primer Xist2 (positioned at +775 to +755 bp downstream of the *Xist* P_1 promoter) with avian myeloblastosis virus RT at 42°C, and RACE linkers were ligated onto cDNA ends. First-round PCR products were generated by using a primer positioned at +378 to +355 bp downstream of the *Xist* P_1 promoter and linker primer ap1, blotted to a nylon membrane and probed with a nested primer, Xist1, positioned at +275 to +295 bp downstream of the *Xist* P_1 promoter, to confirm specificity (Fig. 1*B*). For additional confirmation, second-round PCR was carried out with nested primers ap2 and a primer positioned at +344 to +321 bp downstream of the *Xist* P_1 promoter (data not shown).

Restriction Fragment Length Polymorphism (RFLP) Analysis of CJ11–CJ12 RT-PCR Products. Total RNA (5 μg) isolated with Trizol (GIBCO/BRL) was DNase treated and heat inactivated as described above, precipitated, and annealed to 200 ng of random hexamer for 10 min at 70°C. Annealed RNA was split in two for +RT and −RT reactions, and first-strand cDNA was synthesized in a volume of 20 μl with 200 units Moloney murine leukemia virus-RT for 1 h at 37°C followed by incubation at 80°C for 10 min. cDNA (1 μl) was used as a template for PCR under the cycling protocol reported (10) with 100 ng each of CJ11 and CJ12 primer in a 25-μl reaction. After 30 cycles, 1 μl was diluted 25-fold into a mixture of fresh buffer, dNTPs, primers, and *Taq* polymerase and extended for one additional cycle (94°C for 1 min, 55°C for 45 s, and 72°C for 2 min) to minimize possible heteroduplex products arising from annealing of *Rps12/pS12X* PCR products. Extension reactions were precipitated with glycogen, washed in 70% (vol/vol) ethanol, and dried. Pellets were digested with 4 units of *Hin*fI or *Taq*I (New England Biolabs) at 37°C or 65°C, respectively, and electrophoresed on a 2.5% agarose gel. Gels were transferred to positively charged nylon (Zetabind, Cuno) under alkaline conditions, hybridized to ^{32}P-end-labeled oligonucleotide CJ10 overnight at 50°C, and washed in 6× SSC (0.15 M sodium chloride/0.015 M sodium citrate, pH 7)/0.1% SDS at 50°C by using standard protocols (14).

Northern Blotting. Total RNA (10 μg) isolated from male and female fibroblast, male ES (J1), or female ES (EL16) cell lines was electrophoresed on a formaldehyde gel, transferred to positively charged nylon, and hybridized to a *pS12X* probe by using standard protocols (14). The *pS12X* probe was generated by PCR of genomic DNA with primers CJ9 and CJ12, followed by random priming with ^{32}P-radiolabel.

Results

P_0 Is Not Required for *Xist* Expression in Undifferentiated Cells. The dynamic regulation of *Xist* expression is recapitulated by mouse ES cells during differentiation (5, 15), and many steps of this regulation can be reenacted on mouse autosomes carrying *Xist* transgenes (16–18). To test the predictions of the promoter-switch hypothesis, we examined effects of deleting P_0 on an autosomal *Xist* transgene in ES cells. We had previously isolated a P1 clone, πJL3, which contains 80 kb of *Xic* sequence including an intact *Xist* gene, 730 bp of sequence upstream of P_1, and 60 kb of sequence downstream of *Xist* (17). Thus, the transgene lacks P_0 but contains the 400-bp P_1 promoter shown by several groups to contain the minimal *Xist* promoter for expression in somatic and ES cells (19, 20). We had shown that transgenic ES cell lines carrying a high copy number insertion of πJL3 could express *Xist* despite lacking P_0 sequence.

Because a recent study revealed that transgenic *Xist* expression depends on transgene copy number (21), we wished in this study to verify the ability of πJL3 to express *Xist* in lower copy ES cell lines. πJL3.1 and πJL3.9 carry one to two and four to five copies, respectively. By using strand-specific probes, RNA FISH revealed *Xist* expression from both the endogenous and ectopic loci in undifferentiated πJL3.1 and πJL3.9 cells (Fig. 1*A*), consistent with the prior observation that *Xist* was expressed from higher copy transgenic lines πJL3.6, πJL3.8, and πJL3.10 (17). Thus, in undifferentiated cells, P_0 sequences were dispensable for *Xist* expression on a transgene array regardless of copy number. RACE (5′) suggested three potential transcriptional start sites in transgenic cells (Fig. 1*B*): one corresponding to initiation at P_1 (360 bp band), another indicating initiation ≈200 bp upstream of P_1, and the last suggesting yet another start site ≈200 bp downstream of P_1. The observed use of additional promoters near P_1 is consistent with previous reports of multiple start sites in close proximity to the P_1 promoter (3). For purposes of discussion, we refer to this collection of start sites as "P_1." These results also indicate that the P_1 promoter is used by undifferentiated cells, contrary to the first prediction of the P_0 hypothesis.

Transcripts Initiated at the P_1 Promoter Are Not Intrinsically Stable and Do Not Accumulate in cis. The finding that *Xist* can be expressed from P_1 in undifferentiated cells provided the opportunity to test whether P_1 initiation led to increased *Xist* RNA half-life. The promoter-switch hypothesis predicts that P_1 transcripts would have a half-life comparable to that of *Xist* RNA in somatic female cells. We treated normal and transgenic cells with actinomycin D and analyzed *Xist* expression at different time points (Fig. 2*A*). In contrast to the prediction of the promoter-switch hypothesis, we found that the transgenic RNA was as unstable as *Xist* RNA in normal male and female ES cells. This result argued that transcription from P_1 (and from addi-

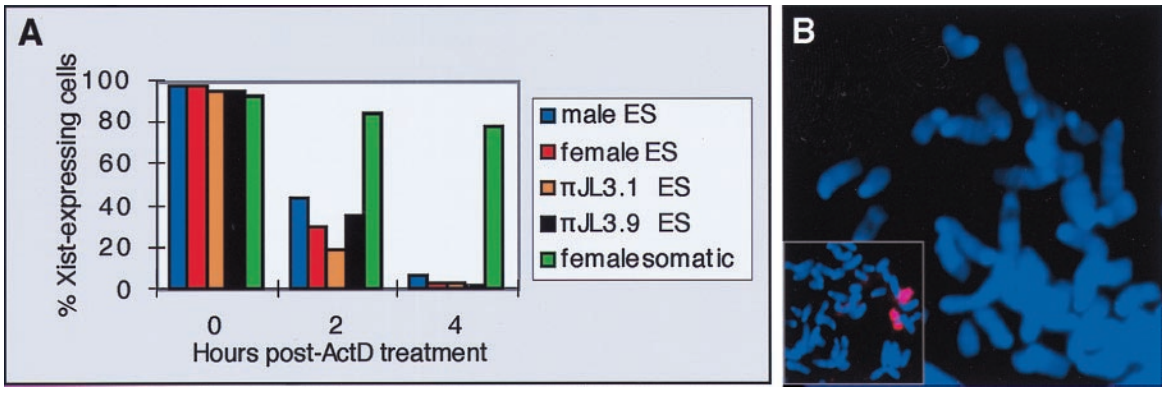

Fig. 2. Transcription from the P_1 promoter does not yield intrinsically stable RNA. (*A*) Male, female, and transgenic ES cells or fibroblasts (somatic) were treated with 5 μg/ml actinomycin D for 0, 2, and 4 h and then subjected to RNA FISH with strand-specific pooled exon 1 and exon 6 *Xist* probes. For each sample, 100 nuclei were scored for the presence of *Xist* signals at every time point. Because the female ES line is a mosaic of 40XX and 39XO cells, either one or two *Xist* signals were scored as positive. (*B*) RNA FISH on metaphase chromosomes of transgenic cells showed that *Xist* RNA did not coat the chromosome in cis either before (shown) or after differentiation (data not shown). (*Inset*) Transgenic cell line πJL2.5 (17), a control showing that *Xist* RNA could coat the autosome on cell differentiation when expressed from a transgene containing 80 kb of *Xic* sequence.

tional promoters located within 200 bp of P_1) did not yield an intrinsically stable RNA. The result argued further that neither P_0 nor any sequence upstream of −730 bp (transgene boundary) is required for production of unstable *Xist* transcripts.

A corollary of the RNA stabilization hypothesis and the promoter-switch hypothesis is that RNA stabilization leads to coating of the chromosome in cis. To test whether the P_1-initiated transcript could coat the chromosome, we performed RNA FISH on metaphase chromosomes of undifferentiated and differentiated πJL3.1 and πJL3.9 ES cells (Fig. 2*B*). We found that the P_1-initiated RNA did not coat chromosomes of either undifferentiated ES cells or their differentiating derivatives up to day 11 (embryoid bodies). Taken together, these observations showed that P_0 sequences are required neither for *Xist* expression in ES cells nor for production of unstable *Xist* transcripts.

The Transcriptional Boundary at P_0 Results from a Ribosomal Protein S12 Pseudogene (*pS12X*) and Not an *Xist* Promoter. The existence of a promoter at P_0 was inferred from RT-PCR data indicating positive reactions downstream of primer CJ12 and negative reactions upstream (10). The promoter was reported to lie between bp −6,590 and −6,725 upstream of P_1 (see Fig. 5*A*). In the course of our analysis, we were surprised to discover that this region (bp −6,635 to −6,136) bears striking homology (94% identity over 500 bp) to mouse ribosomal protein S12 (*Rps12*) cDNA (Figs. 3*A* and 5*A*; ref. 22). This sequence has many features of a processed pseudogene (23): It contains multiple nucleotide substitutions and insertions, is punctuated by stop codons in all three frames, is flanked by direct repeats, and carries a 3′ poly(A) tract (Fig. 3*A*). We named this pseudogene *pS12X* (*Rps12-ps1* in the mouse database). As a processed pseudogene and because of data shown below, this sequence is unlikely to be transcribed. Additionally, this sequence is not conserved at the human *XIC* as determined by dot plot analysis (data not shown).

Of note, primers used to define the P_0 promoter lie within the pseudogene and are either identical (CJ10, CJ11) or nearly identical (CJ12) to the autosomally expressed *Rps12* gene (Fig. 3*A*). CJ9 lies outside of *Rps12* expressed sequence tags but yields amplification similar to that of CJ11–CJ12 primer pairs (data not shown). Because of the primers' nearly perfect identity to *Rps12* sequences, we considered the possibility that the assignment of the P_0 transcriptional boundary might be an artifact caused by cross-amplification of *Rps12* RNA. We found that RT-PCR with CJ11–CJ12 and CJ9–CJ10 primer pairs gave specific amplification in both ES cells and adult somatic tissue, including liver, brain, and fibroblasts (Fig. 3*B* and data not shown). Thus, in our hands, transcripts defined by these primers are not specific to ES cells but are ubiquitously expressed, a result that differs from the ES-specific expression profile reported previously (10). This finding suggested to us that P_0 transcription may indeed reflect *Rps12* rather than *Xist* expression.

This notion is supported by the following observations. First, Northern analysis with a *pS12X* genomic probe (PCR product of CJ9–CJ12) detected a band of 400–600 bases, consistent with the 470-base transcript reported for *Rps12* mRNA (Fig. 3*C*; GenBank accession no. X15962). This mRNA was expressed in both ES and somatic cells and in both XX and XY cells. Second, we reasoned that, if CJ11–CJ12 amplification reflected *Xist* expression, the RT-PCR product would be found only in nuclear RNA fractions and not in the cytoplasmic fraction. However, RT-PCR analysis of fractionated nuclear and cytoplasmic RNAs revealed that CJ11–CJ12 products were present in both nuclear and cytoplasmic compartments (Fig. 4*A*). Because *Xist* was exclusively nuclear (Fig. 4*A*; refs. 2 and 3), this observation argued that *Xist* and the CJ11–CJ12 product cannot be identical. Because a fraction of the CJ11–CJ12 product was also found in the nucleus, it was formally possible that CJ11–CJ12 amplified both *Xist* and *Rps12* RNA. To rule out this possibility, we performed RFLP analysis of CJ11–CJ12 RT-PCR products. In this assay, *Rps12* and the *Xic*-linked *pS12X* sequence could be distinguished by RFLPs in *Taq*I and *Hin*fI (Fig. 4*B*). Our analysis indicated that CJ11–CJ12 exclusively amplified autosomal *Rps12* RNA (Fig. 4*C*). This result held true for all lines tested and for both *Hin*fI and *Taq*I RFLPs. By mixing known amounts of *Rps12* and *pS12X* products, we determined that this assay could detect *pS12X* RNA if it were as rare as 0.1 to 0.01% of total amplified product (Fig. 4*D*). Thus, this sensitive PCR assay failed to detect the *Xist*-linked *pS12X* product (P_0 RNA). The *Rps12* origin indicated by the RFLP profiles was then confirmed by direct sequencing, revealing complete sequence identity of the CJ11–CJ12 RT-PCR product with *Rps12* cDNA (data not shown). We conclude that the P_0 transcriptional boundary was indeed an artifact of PCR cross-amplification caused by autosomally expressed *Rps12* RNA.

Transcripts Upstream of the P_1 Promoter in Undifferentiated Cells Represent the 3′ End of *Tsix* RNA. In light of the above conclusions, we sought to address the origin of transcription in the region upstream of P_1. Given that *Tsix* antisense RNA crosses this

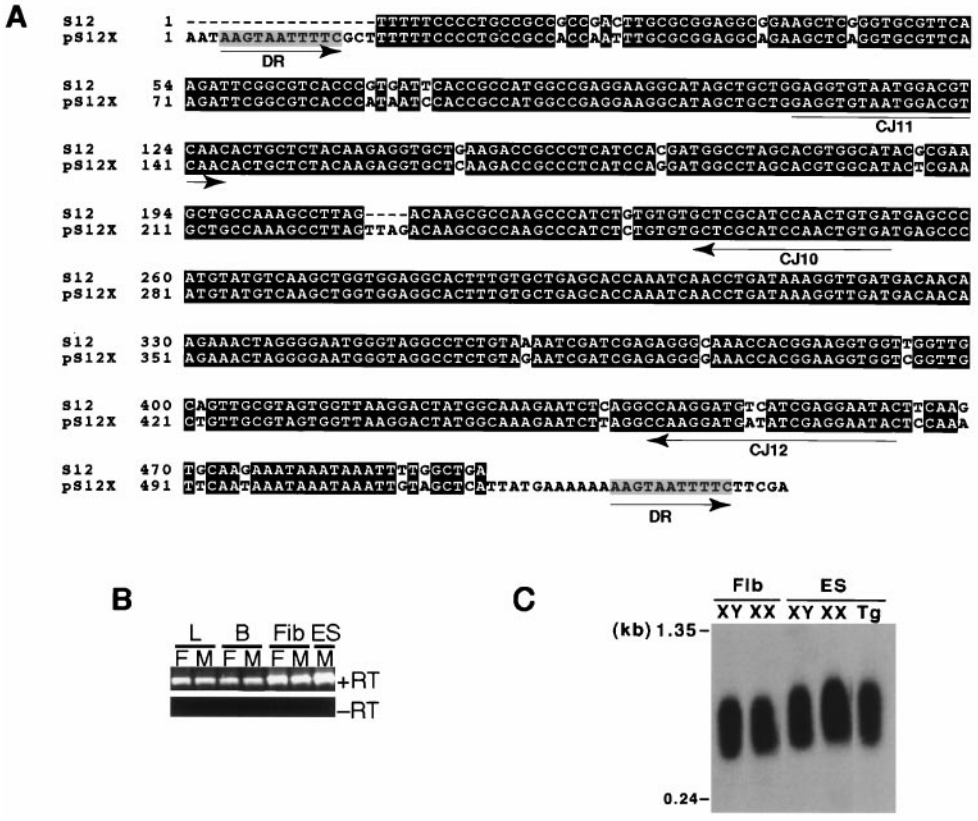

Fig. 3. Identification of a *Rps12* pseudogene, *pS12X*. (*A*) Alignment of the longest expressed sequence tag corresponding to mouse ribosomal protein S12 cDNA (GenBank accession no. AI526798) and *pS12X* (bp 3,015–2,472 of GenBank accession no. AJ010350) sequences. Direct repeats (DR) are in grey boxes. Poly(A) stretch is found immediately before the 3′ direct repeat. CJ10–CJ12 primer locations are indicated by arrows. CJ9 is located outside of known *Rps12* expressed sequence tags and cDNA sequence. (*B*) Tissue distribution of the CJ11–CJ12 RT-PCR product. Total RNA from male (M) or female (F) ES cells, adult fibroblasts (Fib), brain (B), and liver (L) was reverse transcribed by random priming and amplified with primers CJ11 and CJ12. −RT controls were processed in parallel without adding RT. (*C*) Northern blot analysis of total ES and fibroblast RNA with a pS12X probe. ES cells are male (J1, 40XY), female (EL16, a mosaic cell line: 30% 40XX and 70% 39XO), and transgenic (Tg, 116.6).

region (11), we asked how much of upstream transcription in undifferentiated cells could be attributed to *Tsix*. Relevant to this point, much of the prior work (10) used double-stranded *Xist* probes and randomly primed PCR and therefore did not distinguish between sense and antisense transcription. In this study, we carried out strand-specific RT-PCR in undifferentiated cells at various intervals within a 12-kb region surrounding P_1 (Fig. 5*A*). We found that transcription upstream of P_1 was exclusively in the antisense orientation (Fig. 5*B*), consistent with previous analysis (11). By including more PCR cycles, weak bands in the sense orientation were detectable at position 6 (Fig. 5*B*). The idea of minor transcriptional start sites in the immediate vicinity of P_1 agrees with 5′ RACE analysis of πJL3 transgenic lines (Fig. 1*B*) and with previous analysis (3). Sense transcription was never observed upstream of position 6. We conclude that transcription upstream of P_1 can be accounted for by the 3′ end of *Tsix*. These results argue further against a functionally distinct *Xist* isoform in undifferentiated ES cells.

Discussion

To test the promoter-switch hypothesis, we have examined the effects of deleting P_0 sequences on a *Xist* transgene and found that deleting P_0 did not abolish *Xist* expression in cis in undifferentiated transgenic cells. *Xist* expression could be initiated from the P_1 promoter and other minor promoters within 200 bp of P_1 in undifferentiated cells. This finding is consistent with previous reports that the minimal promoter for *Xist* expression in ES and somatic cells is contained with a 400-bp region immediately upstream of the P_1 promoter (19, 20, 24). These results indicate that the P_0 region is not required for *Xist* transcription on a transgene array in undifferentiated ES cells. We also found that these P_1-initiated *Xist* transcripts were not intrinsically more stable, contrary to the predictions of the promoter-switch hypothesis. Therefore, we believe that P_0 sequences are required neither for expression nor for rapid turnover of *Xist* RNA in ES cells.

Further testing revealed that many observations relating to P_0 can be accounted for by *Tsix* and *Rps12* expression. The data presented herein did not substantiate the existence of an ES-specific promoter at P_0. Although amplification with the P_0 primer pairs, CJ9–CJ10 and CJ11–CJ12, was previously reported to be ES-specific, we have found that RT-PCR products were present in both ES and adult cells. We believe that the transcriptional boundary at P_0 is an artifact caused by cross-amplification of a coincidental pseudogene for the highly expressed autosomal *Rps12* gene, a conclusion supported by the following findings. First, Northern analysis with the *pS12X* pseudogene as a probe indicated robust expression in all cell types tested. Secondly, CJ9–CJ10 and CJ11–CJ12 primers gave amplification in both ES and somatic cells. Third, CJ11–CJ12 products were found in both cytoplasmic and nuclear compartments, whereas *Xist* RNA was found only within the nucleus. Fourth, a sensitive RFLP analysis of CJ11–CJ12 amplified products failed to detect any *Xic*-derived transcription. Because *pS12X* is not conserved at the human *XIC* (data not shown), the

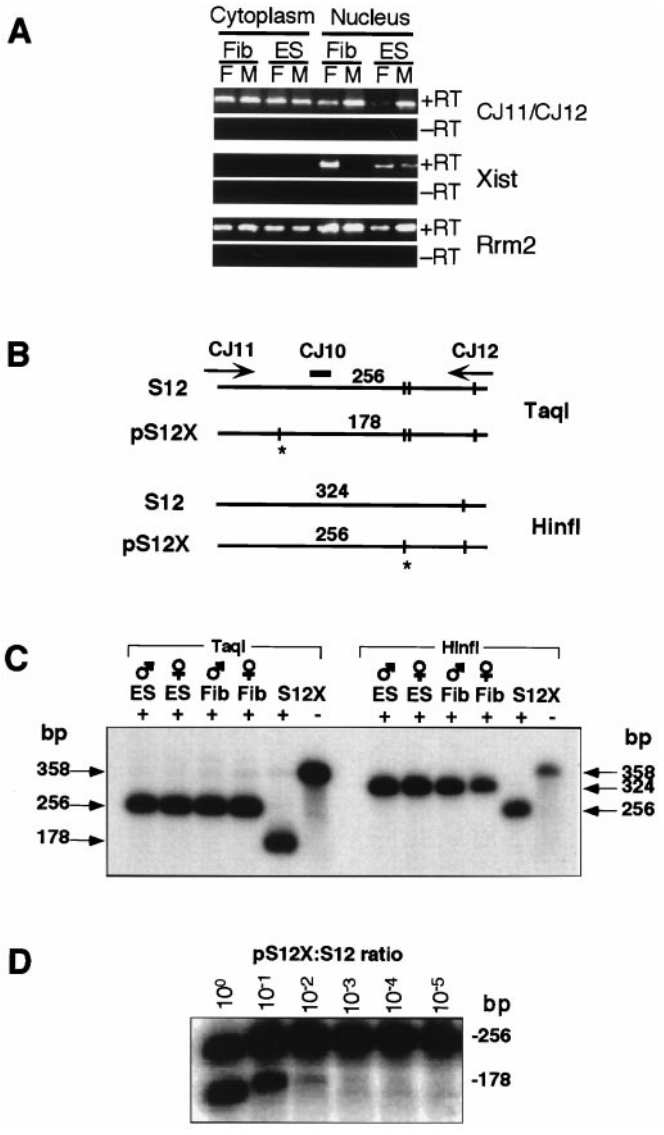

Fig. 4. The RNA detected by CJ11–CJ12 is found in the cytoplasm and is exclusively derived from autosomal *Rps12* expression. (*A*) Nuclear and cytoplasmic distribution of the ''P_0 RNA'' isolated from male (M) and female (F) fibroblasts (Fib) and ES cells, RNA was reverse transcribed and amplified with CJ11 and CJ12. *Xist* was amplified with primers Mix20–Mx23b (12), which spans exons 3 to 6 of *Xist*. (*B*) *Taq*I and *Hin*fI restriction maps for *pS12X* and *Rps12* fragments bounded by CJ11 and CJ12. Sizes are shown for polymorphic fragments. Asterisks indicate RFLP positions. (*C*) RFLP analysis of CJ11–CJ12 RT-PCR products. PCR products were diluted and extended one cycle to minimize heteroduplex formation and then digested with *Taq*I or *Hin*fI. Polymorphic restriction fragments were detected by hybridization to radiolabeled nested oligonucleotide CJ10. + and − indicate the presence or absence, respectively, of restriction enzyme during incubation. (*D*) Sensitivity of the RFLP assay of CJ11–CJ12 amplification. A constant amount of *Rps12* RT-PCR product was mixed with 10-fold dilutions of *pS12X* PCR product, digested with *Taq*I, and visualized by hybridization to CJ10 oligonucleotide. *pS12X* fragments were visible at 10^{-3} dilution (shown) and at 10^{-4} dilution on the original autoradiogram (data not shown).

pseudogene sequence itself seems unlikely to play a role in *Xist* regulation.

An additional complication for the promoter-switch hypothesis arises from the presence of antisense (*Tsix*) transcription upstream of P_1. Our data suggest that transcription upstream of P_1 is only of antisense origin. Relevant to this finding, the original

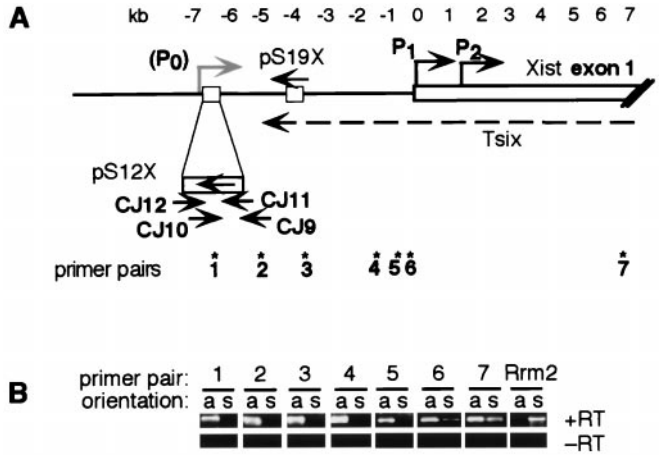

Fig. 5. Map of the region upstream of the P_1 promoter and positions of primers used. (*A*) A map of the 7-kb region upstream of Xist. The positions of the previously described pseudogene *pS19X* (27), the *Tsix* and *Xist* genes, and the newly identified pseudogene, *pS12X*, are shown. The precise 3′ end of *Tsix* has not been defined (denoted by dotted line). Each asterisk designates a sense and antisense primer pair (see *Material and Methods*). (*B*) Strand-specific RT-PCR at positions 1–7 and at *Rrm2*. Identical results were obtained from male, female, and transgenic ES cells. s, sense (*Xist* strand); as, antisense (*Tsix* strand).

mapping of P_0 was carried out exclusively by RT-PCR and largely without regard to transcript orientation (10). Strand-specific RNA FISH was performed in one experiment and was reported to show expression only in the sense (*Xist*) orientation at positions −3 kb and −1 kb upstream of P_1. We cannot explain this apparent discrepancy. However, our results are consistent with the original mapping of *Tsix* relative to *Xist* (11) and with a subsequent study showing exclusive antisense transcription in the region upstream of P_1 (25). Furthermore, a recent promoter knockout of *Tsix* showed that, in the absence of antisense RNA upstream of P_0, no transcription was detectable in the P_0–P_1 region with either sense-specific or double-stranded RNA FISH probes (26). Finally, substantiation of a 5′ end at P_0 would require nuclease protection, primer extension, or 5′ RACE. We have been unable to recover the 5′ end of any potential *Xist* isoform in the vicinity of P_0 by using 5′ RACE or primer extension (data not shown).

If P_0 sequences are not required for *Xist* regulation, do other upstream sequences play a role in X inactivation? A prior study did suggest the presence of at least one element in the 30-kb region immediately upstream of P_1 (17). ES cells carrying the πJL3 transgene could express *Xist* while they were in the undifferentiated state, but differentiation led to *Xist* repression in five independently derived cell lines. This result contrasts with ES cell lines carrying *Xist* transgenes containing an additional 30 kb of sequence upstream of P_1, all of which enabled *Xist* up-regulation and RNA accumulation in cis upon cell differentiation. The data therefore point to the existence of a positive regulatory element upstream that potentiates high-level *Xist* expression. Importantly, this element cannot be an ES cell-specific element such as the proposed P_0 promoter, because prior data argued that this element acts as a potentiator in somatic and not ES cells (17).

We began this study by testing the predictions of the promoter-switch hypothesis and have found no evidence for functionally distinct promoters upstream of P_1. Our conclusions have technical and mechanistic implications for the study of X inactivation. First, this study underscores the importance of using strand-specific probes for *Xic* analysis. Indeed, because *Xist* expression has historically been examined with double-stranded

probes, this study urges reconsideration of some conventionally held wisdom regarding *Xist*. Other properties previously ascribed to *Xist* may actually belong to *Tsix*. Second, although we remain open to the idea that a promoter other than P_0 regulates *Xist* RNA stability, no data presented here or anywhere else support such a mechanism. Although alternative *Xist* promoters might indeed exist, the available genetic and biochemical evidence makes other mechanisms equally plausible. Because knockout analysis shows that *Tsix* regulates *Xist* expression in cis, one possibility is that the antisense RNA alters *Xist* RNA half-life, perhaps by RNA duplex formation or by recruitment of other regulatory factors. It is also possible that control of *Xist* up-regulation resides in elements not yet defined. We hope that the point of view presented herein will instigate a search for additional elements and stimulate new ideas on regulatory mechanisms.

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