Quantitative RT-PCR assays show Xist RNA levels are low in mouse female adult tissue, embryos and embryoid bodies

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SUMMARY

We have investigated expression of the Xist gene in mouse female adult kidney, embryos and embryonic stem (ES) cells undergoing in vitro differentiation as embryoid bodies. Using the quantitative RT-PCR single nucleotide primer extension (SNuPE) assay, we found that the amount of Xist RNA in adult kidney of three mouse strains was less than ~2000 transcripts per cell, with only modest differences between strains carrying different Xce alleles. Female embryos 7.5 days post coitum had the same number of Xist transcripts per cell as isogenic adult tissue. Using quantitative oligonucleotide hybridization assays after RT-PCR, transcripts per cell as isogenic adult tissue. Using quantitative RT-PCR we investigated Xist expression in ES lines heterozygous at the Pkg-I and Xist loci. We found that, while in most (XX) ES lines Xist RNA levels increased during embryoid body formation, the levels seen were less than 10% those found in adult female kidney. In addition, we found that the allelic ratio of Xist transcripts from reciprocal (XX) ES cell lines differentiating in vitro was identical to that of isogenic 10.5 to 11.5 day female embryos. These latter results suggest that there is no pattern of preferential paternal imprinting during days 1 to 9 of in vitro differentiation of ES cells. However, the influence of the Xce locus on the randomness of X-inactivation in embryos seems to operate also in ES cell lines. Our overall conclusion is that the low levels of Xist RNA in female kidney, embryos and differentiating (XX) ES cells are compatible only with models that do not require Xist RNA to cover the entire inactive X chromosome.

Key words: ES cells, X chromosome inactivation, imprinting, allele-specific assays, mouse, RNA, Xist

INTRODUCTION

X chromosome inactivation (XCI), the transcriptional silencing of one of the two X chromosomes in female mammals, occurs early in embryogenesis (Lyon, 1961). Prior to XCI, both X chromosomes are active in early female mouse embryos (Adler et al., 1977; Epstein et al., 1978; Kratzer and Gartler, 1978). Evidence from cells carrying rearranged X chromosomes shows that X-inactivation very likely spreads in cis from an X inactivation center (Xic) (Russell, 1983). Cells of the trophoderm and primitive endoderm, which do not contribute to the embryo proper, undergo X inactivation that is non-random; the paternal X chromosome is preferentially inactivated at 3.5 and 4.5 days postcoitum (dpc), respectively (Harper et al., 1982; Takagi and Sasaki, 1975). In the embryonic ectoderm, from which the embryo proper is derived, X inactivation is random and occurs at about 6.5 dpc, about 1.5 days after the time of implantation (McMahon and Monk, 1983).

The X chromosome-controlling element (Xce) affects the probability that a particular X chromosome will remain active (Cattanach and Williams, 1972; Simmler et al., 1993). In embryos heterozygous at the Xce allele, an X chromosome carrying a strong Xce allele will remain active in a higher proportion of cells than an X chromosome carrying a weak Xce allele, with the strength of the alleles varying in the order XceP > XceB > XceA. The Xce locus maps to the Xic region and has been suggested as a candidate for the X inactivation center.

The recently discovered Xist gene (XIST in human) also maps to the Xic region and is transcribed exclusively from the inactive X chromosome (Borsani et al., 1991; Brockdorff et al., 1991). The mouse Xist transcript is a 15 kb RNA with no open reading frame greater than 500 bp. Although the open reading frames are not conserved, there are regions of sequence conservation between mouse and human Xist, suggesting that the gene has a function (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1992; Hendrich et al., 1993).

Several additional lines of evidence also suggest that Xist may play a role in XCI. The Xist transcript is located almost wholly in the nucleus and is not associated with the translation components of the cytoplasm, suggesting the RNA itself may be functional, or that the Xist locus may represent a chromatin organizer region (Brockdorff et al., 1992). The onset of Xist expression in the early embryo precedes X inactivation by at least one day and the transcript is from the paternal X chromosome, reflecting the preferential paternal X inactivation in the trophoderm and primitive endoderm (Kay et al., 1993). In addition, the relationship of Xist to the Xce locus suggests a strong interaction between the two. In one study, the level of Xist RNA was found to be inversely correlated with the...
strength of the Xce allele (Brockdorff et al., 1991) and a strong Xce allele was found to modify preferential paternal Xist expression in the trophectoderm (Kay et al., 1993). However, recently it has been reported that microsatellite sequences surrounding the Xist gene are genetically separable from the Xce locus (Simmler et al., 1993). Another recent report concludes that the XIC region is not necessary for the maintenance of XCI in mouse/human somatic cell hybrids (Brown and Willard, 1994).

While a number of models for the role of Xist RNA in XCI have been proposed (Brockdorff et al., 1992; Brown et al., 1992), one unknown factor in devising models is the cellular concentration of Xist RNA. Quantitative information about specific RNAs is very difficult to obtain by standard techniques such as northern blotting. We have recently shown that the RT-PCR single nucleotide primer extension assay (SNuPE) can be used quantitatively (Singer-Sam et al., 1992b). Here, we have used the method to estimate the absolute level of Xist RNA in female adult tissue and in embryos isolated at 7.5 dpc, soon after the time of XCI. We also investigated Xist expression in XX embryonic stem (ES) cells, which possess two active X chromosomes and undergo XCI upon in vitro differentiation, thus providing a potential in vitro system for the study of XCI (Rastan and Robertson, 1985). We have found that the Xist RNA levels in adult tissue, embryos and differentiating ES cells are compatible with only some of the potential models for the role of Xist in XCI.

MATERIALS AND METHODS

Mouse strains

Three strains of mice were used: (1) 129/Sv – Pgk-1P XistP (129) (Jackson Lab. Stock No. JR090), (2) congenic C57BL/6J – Pgk-1P XistP (B6A) (Jackson Lab. Stock No. JR0827) and (3) standard C57BL/6J – Pgk-1P XistP (B6). The Pgk-1P allele backcrossed into B6A mice is derived from feral Mus musculus musculus mice (Chapman et al., 1989). The XistP and XistP alleles are here defined as those cis to the Pgk-1P and Pgk-1b alleles, respectively. The XistP allele contains the same GT insertion previously reported in Mus spretus DNA (J. M. LeBon and J. Singer-Sam, unpublished; Brockdorff et al., 1991).

Production of embryonic stem cell lines and embryoid bodies

ES cell lines were derived from reciprocal crosses of 129 and B6A mice essentially as described by Robertson (1987). Feeder cells used were mitomycin C inactivated STO mouse fibroblasts transfected with the neomycin and leukemia inhibitory factor (LIF) genes, kindly supplied by Frank Conlon and Elizabeth Robertson. Medium used in cell line derivation and subsequent culture was high glucose Dulbecco’s modified Eagle’s medium containing 15% (v/v) fetal bovine serum, glutamine, penicillin/streptomycin and non-essential amino acids at standard concentrations, and 10−4 M 2-mercaptoethanol. No LIF was added.

Newly derived ES cell lines were analyzed for the presence of the Y chromosome-specific Zfy gene by a PCR-based assay (Singer-Sam et al., 1990) and cell lines scoring as negative (STO fibroblasts score as negative in this assay) were then analyzed for chromosome complement. Those possessing a majority of spreads containing 40 chromosomes were considered as putative XX cell lines and heterozygosity for the Pgk-1 and Xist alleles was confirmed by an allele-specific hybridization assay (see below). The (129 × B6A)F1 ES cell lines (B6A paternal) and the reciprocal, are hereafter termed XA and AX ES cell lines, respectively. Other ES cell lines used were of the 129 genotype and are termed W9 (J. Mann, unpublished data).

ES cells at early passage numbers of 6 to 8 were differentiated as embryoid bodies in viscous methylcellulose medium, as previously described (Szabó and Mann, 1994). At intervals during differentiation, samples of embryoid bodies were added to 1 ml of RNAzol (Tel-Test, Inc., Friendswood, TX) and stored at −70°C prior to RNA purification.

RT-PCR

RNA was isolated from ES cells, embryoid bodies, embryos and adult tissues by use of the guanidinium thiocyanate/phenol-containing reagent, RNAzol B, as previously described (Singer-Sam and Riggs, 1993). Purified RNA was diluted to 100 ng/µl in 75% ethanol and stored at −70°C.

RT-PCR was carried out as previously described (Singer-Sam et al., 1992b) with modifications. Prior to RT-PCR, RNA samples were incubated (under mineral oil) in 18 µl of a partial RT mix containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 1 mM dNTPs, 1 U/µl RNasin and 0.05 U/µl RNase-free DNAse RQ1 (Promega) at 37°C for 20 minutes, then at 99°C for 5 minutes to inactivate the DNase. Tubes were put on ice and 2 µl of a mix was added to give final concentrations of 2 U/µl RNasin, 1 µM downstream primer and 2.5 µM Moloney murine leukemia virus reverse transcriptase (BRL). Reverse transcription was carried out at 42°C for 15 minutes, then 99°C for 5 minutes, followed by equilibration at 50°C. 80 µl PCR mix was added to the tubes, giving a final reaction mixture of 100 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 µM dNTPs, 0.2 µM upstream and downstream primers and 2.5 units/reaction of AmpliTaq DNA polymerase (Perkin Elmer-Cetus).

For RT-PCR of Pgk-1 transcripts, the primers PGK445 (AGCT-GAGGCGGCAAAATTGAT) and PGK750 (GFAAGGCG-CATTCCACCAACA) were used under conditions previously described (Singer-Sam et al., 1992b). For Xist transcripts, two primer sets were used. The first, set 1994/2300 (TAAAGGACTACTTTAAGGGCT, upstream primer; TACTCAGACATTCCCTGGCA, downstream primer) was used to amplify a 307 bp RT-PCR product corresponding to positions 12,691 to 12,997 of Xist cDNA. In some experiments, formamide was added to the reaction mixture after the DNase step, giving a concentration of 10% in the reverse transcriptase step and 2% in the PCR step. In later experiments without formamide, primer 2300 was added to the reaction mixture at 50°C after the DNase step. The mixture was then heated to 95°C for 5 minutes and equilibrated to 50°C prior to addition of reverse transcriptase and additional RNasin. For amplification, a cascade reaction was used consisting of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute for the first two cycles, followed by 2-cycle sets in which the annealing temperature was sequentially 50°C, 48°C, 45°C and 43°C. In the remaining 30 cycles, the annealing temperature was 41°C. The elongation step of the final cycle was at 72°C for 5 minutes. MgCl2 concentrations were 3.75 mM for the RT step and 2 mM for the PCR reaction.

The second Xist primer set, Top1/Top2 (Borsani et al., 1991) (CAGGATCTCTTCACTTCTCAGGAGA, upstream primer; CAGGATGCCTTGTAACTTCTTATGGG, downstream primer) gave a 251 bp RT-PCR product including positions 12,239 to 12,475 of Xist cDNA. In later experiments T7-Top2 (TAATAGCAGCTC-TATAGGTTGTCATTC TTCACTTG, containing the promoter for T7 polymerase 5’ to the Top2 sequence) was used as the downstream primer, as it was found to increase the signal after amplification. Conditions of amplification for Top1/Top2 or Top1/TT-Top2 primer sets were 94°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute (34 cycles).

For absolute quantitation, an Xist internal standard differing by a single base from the endogenous sequence was added just prior to the PCR step. The internal standard DNA was prepared from total RNA.
by RT-PCR with primers Top2 and Top1Mut (TTCACATCGTCT-CACCTGAGACCGAATCTACTG+TCCAGCCAGGT), so that it contained a C residue in place of A at position 12,271 of the Xist cDNA sequence. The internal standard was gel purified prior to use.

Quantitative assays

The relative location of the various primers used for RT-PCR and the base differences used to assay for allele-specific transcripts or for absolute quantitation are shown in Fig. 1. Three assays were used to quantify RT-PCR products: the SNuPE assay and direct hybridization or allele-specific hybridization to oligonucleotide probes after Southern blotting. The quantitative SNuPE assay was carried out as previously described (Singer-Sam et al., 1990, 1992b; Singer-Sam and Riggs, 1993). In brief, gel-purified RT-PCR products (5 ng) and a SNuPE primer just 5’ to the variant base were incubated with Taq polymerase and either [32P]dATP or [32P]dCTP. SNuPE primers were PGK471 (TCCGAGCCCTCAGTCTCA), for the detection of a C/A allelic difference at position 489 of the Pgk-1 cDNA sequence (Boer et al., 1990), and MXT 12,253 (CACTTGAGACGGAACTAAC*TCCAGCCAGTG), so that it contained 1 pmole/ml 32P-labeled probe specific for one allele and 40 pmole/ml of each template component. SNuPE primers were PGK471 (TCCGAGCCCTCAGTCTCA), for the detection of a C/A allelic difference at position 489 of the Pgk-1 cDNA sequence (Boer et al., 1990), and MXT 12,253 (CACTTGAGACGGAACTAAC*TCCAGCCAGTG), so that it contained 1 pmole/ml 32P-labeled probe specific for one allele and 40 pmole/ml of the unlabeled oligonucleotide specific for the opposite allele. Temperatures for hybridization were: Pgk-1/C, 47°C; Xist/G, 46°C. After hybridization, the membranes were washed three times for 5 minutes each in 0.1 SSC at the hybridization temperature and exposed to Kodak XAR-5 film or analyzed quantitatively. Ratios of the two alleles were determined by comparing the signal ratios of the experimental samples to a standard curve determined in the same experiment. The probes used were: Pgk-1/C, PGK496G. TCCTAGTG*TGACACGTA (detects ‘C’ at position 489); Pgk-1/G, PGK496T. TCCTAGTTGACACGTA (detects ‘A’ at position 489); Xist/G, XIST CAS. CATCAGACTAAGTACC (detects GT insert after position 12,853); Xist/C, XIST DOM. GCATCAGACTAAGTACC (detects allele with no insert).

RESULTS

Xist transcripts in female adults and embryos

The level of Xist expression in female adult mouse kidney and 7.5 dpc embryo RNA was determined by RT-PCR SNuPE assay. An internal standard DNA, differing by one nucleotide from the endogenous Xist sequence, was co-amplified with the reverse transcribed Xist cDNA. To determine the optimal amount of the internal standard to use, we first carried out the SNuPE assay using a constant amount of RNA and variable amounts of added internal standard (Fig. 2A). When the internal standard/endogenous RNA signal is plotted vs. the concentration of the standard, a hyperbolic curve is seen; the most accurate measurement is taken in the linear portion of the curve, where the signal ratios are most similar (Gilliland et al., 1990). Choosing internal standard amounts in this range, the absolute number of molecules of Xist cDNA/ng of total RNA in adult tissue was determined in several experiments. The results are shown in Table 1. We find 1654±228 molecules of Xist cDNA in 1 ng of total RNA from B6 female adult kidney, and 633±61 molecules and 431±164 molecules/ng RNA in strains 129 and B6A, respectively. Analysis by the Wilcoxon rank-sum test showed that, while the values for strains 129 and B6A are not significantly different from each other (P=0.3434), strain B6 has significantly more Xist RNA than either 129 or B6A (P=0.0001 and P=0.0009, respectively).

One important factor for absolute measurement is the efficiency of reverse transcription. We therefore did a control experiment, shown in Fig. 2B, using a constant amount of endogenous RNA and various amounts of added RNA internal standard instead of DNA. A comparison of Fig. 2A and B shows that to obtain comparable signal ratios requires considerably more of the RNA than the DNA internal standard. Calculations based on measurement in the linear range of the assay led to the conclusion that 5% as much DNA as RNA standard was required to obtain comparable ratios, providing a measurement of the efficiency of the reverse transcription step.

A second factor in calculating Xist RNA concentrations is the amount of total RNA per cell. Extrapolating from published values of total RNA in 8- to 16-cell and early blastocyst stages (~40 pg/cell) (Pikó and Clegg, 1982), regenerating rat liver (~60 pg/10^6 cell) (Reiners and Busch, 1980) and our own values for ES cells based on RNA yield (~30 pg/cell), we estimate that the cells we used contain between 30 and 60 pg of RNA/cell. Thus, B6 female adult kidney is estimated to contain 1-2×10^5 Xist RNA molecules per cell (~1700 cDNA molecules/ng RNA ÷ 0.05 efficiency of transcription × 0.03 or
To measure Xist RNA levels in individual 7.5 dpc individual embryos, where the amount of material is limiting, co-amplification was done at only one concentration of internal standard DNA, 5×10^4 molecules/reaction mix. The results of the assay of three female (XX) and one male (XY) embryo are shown in Fig. 3. Lanes (a) show the signal from embryo RNA, while lanes (b) show the signal due to the internal standard. The male (XY) embryo RNA shows no signal after subtraction of background (~1%, not shown). The female (XX) embryos all show a signal comparable to the internal standard. Table 1 shows the result of calculation based on the internal standard/template ratio; 1690±222 molecules of Xist cDNA/ng RNA, corresponding to 1-2×10^3 molecules RNA per cell, the same results as those seen in isogenic adult female kidney.

### Xist RNA levels in differentiating ES cells

Since ES cells have widely been considered as a possible in vitro system for the study of XCI, we measured relative Xist levels in these cells after the start of in vitro differentiation. The cells were observed to undergo the normal pattern of differentiation, forming a visible outer endoderm layer by days 3 to 4, and multilayered and cystic embryoid bodies by day 9. RNA was prepared from samples taken at various times during the differentiation of six F1 (XX) ES cell lines, one F1 (XY) line and two (XX) 129 lines. Fig. 4 shows their Xist RNA levels relative to that of an adult female kidney control (strain B6). Most XX lines expressed low levels of Xist RNA prior to differentiation and showed an increase to less than 3% of the

### Table 1. Xist RNA levels in female adults and embryos

<table>
<thead>
<tr>
<th>Strain</th>
<th>Xist cDNA molecules/ng RNA</th>
<th>Xist transcripts/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 (Pgk-1^b Xist^b) adult kidney</td>
<td>1654±228 (11)</td>
<td>1-2×10^3</td>
</tr>
<tr>
<td>7.5 dpc embryos</td>
<td>1690±222 (3)</td>
<td>1-2×10^3</td>
</tr>
<tr>
<td>129 (Pgk-1^b Xist^b) adult kidney</td>
<td>633±61 (7)</td>
<td>0.4-0.8×10^3</td>
</tr>
<tr>
<td>B6A (Pgk-1^b Xist^b) adult kidney</td>
<td>431±164 (5)</td>
<td>0.3-0.6×10^3</td>
</tr>
</tbody>
</table>

Calculations were based on the SNuPE assay after RT-PCR of 100 ng total RNA + DNA internal standard (see text). The relative signal from the endogenous RNA was multiplied by a factor of two to take into account the fact that the DNA internal standard, but not the cDNA, is double-stranded. For each set of SNuPE assays, control lanes contained amplified products corresponding to endogenous cDNA or internal standard sequences alone. The signals obtained with these purified standards provided a background for mismatch incorporation (~1% of correct incorporation), which was subtracted from the value for each corresponding sample. The corrected ratios are proportional to the ratio of cDNA to internal standard added. Values were expressed as molecules of Xist cDNA/ng RNA; from these values, Xist transcripts/cell were estimated as described in the text. Amounts given are the mean ± s.e.m. The numbers in parentheses show the number of separate determinations for each sample, except for embryos, where the number of individual embryos assayed is given.

RNA, corresponding to 1-2×10^3 molecules RNA per cell, the same results as those seen in isogenic adult female kidney.

**Fig. 2. Quantitative SNuPE assay for Xist RNA with (A) DNA internal standard and (B) RNA internal standard. (A) RNA (100 ng) isolated from adult female B6 kidney was assayed for Xist transcripts by the quantitative SNuPE assay following amplification by RT-PCR. The amount of internal standard DNA added to samples 1 to 5 prior to PCR was 5×10^6, 1×10^6, 5×10^5, 1×10^5 and 5×10^4 molecules, respectively. For each amplified sample, the signal due to the endogenous sequence (lanes a) and the internal standard (lanes b) is shown. For each lane, only the signal from the major band, representing the SNuPE product of the expected size, was used in calculation. The minor bands of incorrect size are most likely due to misincorporation. RT-PCR primers, Top1/T7-Top2; SNuPE primer, MXT 12,253. (B) Conditions were the same as described in A except that an RNA internal standard was used. The amount of internal standard added to samples 1 to 4 prior to RT-PCR was 1×10^7, 5×10^6, 2×10^6 and 1×10^6 molecules, respectively. The internal standard was synthesized by amplification of the DNA standard with primers T7Top1 (TAATACGACTCACTATAGGGTTCACATCTGCTCC-ACITTGAGA) and Top2, followed by in vitro transcription of the PCR product with T7 polymerase (BRL protocol). After digestion with RNase-free DNase, phenol extraction and ethanol precipitation, the resuspended RNA was quantitated by comparison with RNA of known concentration on ethidium-bromide stained agarose gels. RNA was then diluted in TE + 10 mM NaCl, 0.8 U/μl RNasin, 1 mM DTT and 1 μg/ml glycerogen. Lanes a, endogenous sequence; lanes b, internal standard.

**Fig. 3.** Quantitative SNuPE assay for Xist RNA in 7.5 dpc embryos. RNA (100 ng) isolated from single 7.5 day B6 male or female embryos was assayed as described in Fig. 2. The amount of the internal standard DNA added was 5×10^4 molecules/reaction. Lanes (a), endogenous cDNA; lanes (b), internal standard. The sex of each embryo was determined by a PCR assay for a Y-specific gene (Zfy), using DNA isolated from the trophoderm of that embryo.
level of adult kidney by day 4 of embryoid body formation (AX.1, AX.4, XA.7, W9.12). In some ES lines, this increase was transient, followed by a return to day 0-1 levels (AX.1, AX.4), while in others Xist RNA levels remained at ~2-3% of adult levels by day 6 (XA.7, W9.12). As expected, the XY line, XA.6, did not express detectable Xist RNA. However, some XX ES lines also showed either no or very little increase during differentiation (AX.5, XA.8, W9.8).

We used B6 as a control because we originally believed that strain differences in Xist expression would not be significant. However, we subsequently found (Table 1) that Xist RNA levels in the two parent strains from which the ES lines were derived, B6A and 129, are 25% and 40% of the B6 levels, respectively. Correcting for these strain differences, we calculate the maximum Xist RNA level in embryoid bodies to be 2.5% ÷ (0.25 to 0.4) = 6% to 10% that of isogenic female adult. If we also assume that ~40% of the cells of 4-day embryoid bodies have undergone XCI (Rastan and Robertson, 1985), then we estimate the level of Xist RNA in those ES cells undergoing XCI to be 2.5% ÷ 0.4 ÷ (0.25 to 0.4) = 16% to 25% that of isogenic adult cells.

**Allele-specific Xist expression in ES cells**

Because the X chromosomes in our (XX) ES cell lines were genetically marked with different Xist alleles, we were able to determine whether allele-specific expression of Xist RNA in early differentiating ES cell lines reflected preferential paternal or random XCI. To measure allele-specific expression, we used either the SNuPE assay or allele-specific hybridization (see Materials and Methods). While the former has been shown to be very accurate, quantitative measurements have not previously been reported for the latter method. As shown in Fig. 5, the ratios of two alleles can be quantitatively determined by allele-specific hybridization, although it is not as accurate as the RT-PCR SNuPE assay.

We measured allele-specific Xist transcripts in the reciprocal ES lines, AX.1 and AX.7, on days 1-9 of in vitro differentiation. The results were the same for all samples, even when, in one experiment, embryoid bodies were separated by Ficoll density gradient centrifugation into inner core and endoderm-like cells (data not shown). Therefore, the ratios shown in Table 2 were calculated using pooled data from all experiments. It can be seen that Xist\(^b\) represents 71.5% and 64.2% of the total Xist transcripts in lines, AX.1 and AX.7, respectively, results not significantly different from each other (P=0.391). Thus, Xist expression appears to be unaffected by parental origin of the X chromosome.

For comparison, we also measured allele-specific expression from Pgk-1 and Xist, in female (129 × B6A)\(^F_1\) embryos at 10.5 to 11.5 dpc. Table 2 shows that 68.2% of the total Pgk-1 transcripts are from the Pgk-1\(^a\) allele, while 68.3% of Xist transcripts are from the Xist\(^b\) allele. These results are consistent with what is known of the relative strength of the Xce allele in B6A and 129 strains (B6A contains the strong Xce allele (Johnston and Cattanach, 1981), while strain 129 most likely carries the Xce\(^a\) allele, which is more likely to become inactivated (Simmler et al., 1993)). While our assays measure steady state transcript levels, which may result from many factors in addition to the effect of Xce locus, our results are consistent

**Fig. 4.** Relative Xist RNA levels in differentiating ES cells. To determine levels of Xist RNA from ES cells taken at various times during differentiation, the region between primer set Top1/Top2 was amplified by RT-PCR. Following electrophoresis and transfer, the membrane was hybridized with the \(^{32}\)P-labeled oligonucleotide probe MXT 12,253. Levels of Xist RNA from ES cell lines are expressed as a percentage of that in the female adult kidney control (strain B6). For each experiment, a dilution series was made of the control adult kidney RNA and values in ES cells were determined from the linear portion of the standard curve. In the two \(^F_1\) hybrid series AX and XA, the Pgk-1\(^a\) and Xist\(^b\) alleles were of maternal and paternal origin, respectively. All lines displayed an XX phenotype, except for XA.6, which is XY.

**Fig. 5.** Standard curve for allele-specific hybridization assay. Mixtures of known concentrations of Pgk-1\(^a\) and Pgk-1\(^b\) PCR products were run on 2% agarose gels, transferred to nylon membranes and hybridized to the oligonucleotides \(^{32}\)P-PGK 496G or \(^{32}\)P-PGK 496T (see Materials and Methods). Percentages of counts specific for each allele were determined for each point and plotted versus the percentage of that allele in the mixture. Points from five separate experiments are included.
with the expectation that the \( Pgk-1^a \) allele on the B6A X chromosome and the \( Xist^b \) allele on the 129 X chromosome, are active in more than 50% of the cells in the embryo. Also, since allelic expression of \( Xist \) transcripts is so similar in the 10.5-11.5 dpc embryos and differentiating ES cells, we conclude that, while there is no parental imprinting in day 1-9 embryoid bodies, the effect of the \( Xce \) allele is probably still intact.

### DISCUSSION

**The RT-PCR SNuPE assay for quantitation of RNA levels**

We have used the quantitative RT-PCR SNuPE assay to measure levels of \( Xist \) RNA in female embryos and adult kidney. The method should be generally applicable for quantitative measurement of specific RNAs and should be particularly useful where quantitation is impossible by other means, such as the study of low abundance RNAs or where analysis of the amount of starting material is low. Use of an internal standard amplified by the same set of primers as the template RNA, as previously described (Gilliland et al., 1990), allows for great accuracy. In addition, use of the SNuPE assay to detect internal standards differing by only a single base from the template avoids problems associated with other detection methods, for example, potential incomplete digestion when restriction enzymes are used to distinguish template from internal standard PCR products. To correct for the efficiency of transcription, a control experiment was performed using an \( Xist \) RNA segment synthesized in vitro as the internal standard instead of DNA; for RNAs shorter than the 15 kb \( Xist \) transcript, it would be practical to consider using full-length in vitro transcribed RNA standards.

**Relative \( Xist \) levels and strength of the \( Xce \) allele**

We found the \( Xist \) RNA levels of the three strains tested to be very similar, with at most a 4-fold difference in level (see Table 1), although in previous work on hybrids between \( Mus spretus \) and C57BL/10, a large inverse correlation was found between \( Xce \) strength and \( Xist \) RNA levels (Brockdorff et al., 1991). We do see a modest correlation in the case of B6 (relatively weak \( Xce \) allele, higher \( Xist \) RNA levels) vs. B6A (relatively strong \( Xce \) allele, lower RNA levels). We also see a small (albeit statistically insignificant) difference between strains B6A and 129, consistent with the predominant expression of \( Xist^b \) and \( Pgk-1^a \) RNA seen in (129 × B6A)F1 hybrid embryos (Table 2). However, a recent report (Simmler et al., 1993) showed that three microsatellite DNA markers associated with the very weak \( Xce \) allele are found on the 129 X chromosome. If strain 129 carries the \( Xce \) allele, then its \( Xist \) RNA levels should be greater than that of strain B6, carrying the moderate \( Xce \) allele, yet Table 1 shows no such correlation. While our results so far are only suggestive, they imply that the correlation previously seen between strength of \( Xce \) locus and low levels of \( Xist \) RNA is not absolute.

### \( Xist \) expression in embryoid bodies

We found low levels of \( Xist \) RNA in most XX ES lines prior to differentiation and an increase to less than 10% of the adult level as the cells formed embryoid bodies. The low levels in ES cells in culture are most likely due to the small amount of differentiation that takes place when ES cells are grown on a feeder layer, although we cannot rule out the possibility of a low level of \( Xist \) expression in ES cells prior to differentiation and XCI. We do not understand what factors prevented some lines from expressing \( Xist \) RNA, or why in other lines the increase was transient. Further study of these lines may help in analysis of \( Xist \) regulation.

Since ES cells most closely resemble early blastocyst inner cell mass (ICM) cells (Beddington and Robertson, 1989) and since the first step in ES cell differentiation in suspension as embryoid bodies is the formation of an outer endoderm layer similar to the primitive endoderm formed from the ICM, we expected to find some evidence for the preferential paternal XCI that accompanies primitive endoderm formation. Our results, however, seen in Table 2, show no evidence for preferential paternal XCI. In two reciprocal differentiating ES hybrid lines, AX.1 and AX.7, there was no preferential expression of the paternal \( Xist \) gene, even in the first days of differentiation, suggesting that the imprint on the paternal X chromosome is lost or masked. Our results are similar to the report of random XCI in day 1 to 15 embryoid bodies, based on qualitative RT-PCR assays of \( Xist \) RNA and measurement of PGK1 isozyme activity (Norris et al., 1994). In that study, hybrids were derived from a backcross between mice of an unspecified strain carrying the \( Pgk-1^a \) allele and strain 129/Ola/Hsd. The level of PGK1 isozyme from the two alleles was equal, although RT-PCR data showed more \( Xist \) RNA to be produced from the \( Pgk-1^a \)-carrying chromosome. The ratios are different from ours, possibly because of genetic background differences. In a recent report, Tada et al. (1993) observed preferential paternal XCI in the mural region of cystic embryoid bodies starting at day 10 of in vitro differentiation. It is possible that a small subset of endoderm cells, which we would have missed in our assays, undergo preferential paternal XCI during days 1 to 9 of embryoid body formation. However, it is interesting to note that paternal \( Pgk-1 \) transcripts have been detected in early embryos prior to the time of preferential paternal XCI (Singer-Sam et al., 1992a) and that autosomal imprinted genes have also been found to show imprinted expression at embryonic stages subsequent to those at which imprinting is not seen (Latham et al., 1994; Szabó and Mann, 1994).

### Table 2. Allele-specific expression of \( Xist \) transcripts in differentiating ES cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sample</th>
<th>% ( Pgk-1^a ) RNA (n)</th>
<th>% ( Xist^b ) RNA (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AX.1 ( Pgk-1^a ) Xist(^b)/( Pgk-1^b ) Xist(^b)</td>
<td>n.d.</td>
<td>71.5±1.5 (11)</td>
<td></td>
</tr>
<tr>
<td>AX.7 ( Pgk-1^b ) Xist(^b)/( Pgk-1^b ) Xist(^b)</td>
<td>n.d.</td>
<td>64.2±3.8 (15)</td>
<td></td>
</tr>
<tr>
<td>Embryos ( Pgk-1^b ) Xist(^b)/( Pgk-1^a ) Xist(^a)</td>
<td>68.2±2.3 (8)</td>
<td>68.3±2.5 (8)</td>
<td></td>
</tr>
</tbody>
</table>

RNA was isolated from ES hybrid cell lines (AX.1 and AX.7) at various times during differentiation and from female F1 (129 × B6A) 10.5 to 11.5 dpc embryos. Allelic ratios were determined for \( Xist \) by the allele-specific hybridization assay; for \( Pgk-1 \), the SNuPE assay was used (see Materials and Methods). Percentages are the mean ± s.e.m.; n.d., not determined; n, the number of individual embryos assayed or separate determinations of ES cell samples. The primer set 1994/2300 was used for \( Xist \); primer set 445/750 was used for \( Pgk-1 \) (see Fig. 1).
Significance of Xist RNA levels

Our results show that there are less than 2000 copies of Xist RNA per cell in female adult kidney and 7.5 dpc embryos. For those ES lines expressing Xist RNA upon differentiation, the estimated levels are even lower. It is interesting that spermatogenic cells, which undergo XCI and which contain Xist transcripts (MacCary and Dilworth, 1992; Richler et al., 1992, Salidto et al., 1992), have levels comparable to embryoid bodies (our unpublished data) and that Mus spretus levels are much lower than those of B6 (Brodkoff et al., 1991, and our unpublished data). These results are consistent with the idea that XCI can occur in cells with even lower levels of Xist RNA than those reported in Table 1.

Despite circumstantial evidence, reviewed in the Introduction, that Xist may play a role in XCI, the function of Xist RNA is still elusive. The fact that Xist transcripts are found only in the nucleus is suggestive of a nuclear function, but does not rule out the possibility that transcription of Xist is a consequence of the initiation of X chromosome inactivation rather than a cause, or that the act of transcription rather than the RNA product is important in XCI, as has been suggested (Brodkoff et al., 1992; Brown et al., 1992). Although in F1 hybrids of Mus spretus x Mus musculus domesticus, the Xist RNA concentration was inversely correlated with the strength of the Xc allele, that does not appear to be always the case in our experiments. Other evidence that the Xist and Xce loci may not be identical is the recent report of Simmler et al. (1993) that microsatellite sequences surrounding the Xist gene are genetically separable from the Xce locus. Furthermore, recent data of Brown and Willard (1994) show that deletion of the XIC region including human XIST does not affect the maintenance of XCI in somatic cell hybrids.

If Xist does play a role in XCI, can any models be ruled out by our data? One simple model is that Xist RNA forms a structural component of the Barr body, binding to some major component of inactive X chromatin, making it inaccessible to transcription factors, for example. If Xist RNA were to bind everywhere along the inactive X chromosome, estimated to be 1.5x10^8 bp, at least 7,000 molecules of the 15,000 base transcript would be required. There do not appear to be enough Xist RNA molecules for it to play such a role. If matrix attachment sites occur at a frequency of one in 10^9 base pairs, there would be ~10^3 such sites on the inactive X chromosome. Although in some cells there might be enough Xist transcripts to bind at each such site, the laws of mass action make such binding seem questionable. Thus it seems more likely that Xist RNA is acting in the vicinity of the X-inactivation center, or, alternatively, that it may be transcribed as a result of altered conformation of the inactive X chromosome prior to visible XCI at 6.5 dpc, as a consequence rather than a cause of the initial inactivation event. In any case, study of the factors leading to activation of transcription of Xist RNA should further our understanding of the initiation of XCI.

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REFERENCES


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