Developmentally-Regulated Changes of Xist RNA Levels in Single Preimplantation Mouse Embryos, as Revealed by Quantitative Real-Time PCR

CRISTINA HARTSHORN,* JOHN E. RICE, AND LAWRENCE J. WANGH
Department of Biology, Brandeis University, Waltham, Massachusetts

ABSTRACT Xist RNA localizes to the inactive X chromosome in cells of late cleavage stage female mouse embryos (Sheardown et al., 1997: Cell 91: 99–107). Fluorescence in situ hybridization (FISH), however, does not quantify the number of Xist transcripts per nucleus. We have used real-time reverse transcription-polymerase chain reaction (RT-PCR) to measure Xist RNA levels in single preimplantation embryos and to establish developmental profiles in both female and male samples. The gender of each embryo was readily established based on Xist RNA levels, by counting Xist gene copies per cell, and by independent detection of the presence/absence of Sry, a Y chromosome-specific gene. Xist expression in males was found to be very low at all stages, as suggested by FISH. In contrast, female embryos contained measurable levels of Xist mRNA starting at the late 2-cell stage and rapidly accumulated Xist transcripts until morula stage. Xist RNA accumulation per embryo then reached a plateau, while cell division continued. We propose that during early cleavage high enough levels of Xist mRNA are transcribed to generate a pool of unbound molecules. This pool would serve to temporarily maintain X chromosome inactivation without additional transcription while the trophoderm and inner cell mass (ICM) differentiate. The ICM would then loose the paternally imprinted pattern of X inactivation originally present in all embryonic cells. Mol. Reprod. Dev. 61: 425–436, 2002.

Key Words: Xist expression; genome copy counting; mRNA copy counting; embryo sexing

INTRODUCTION

Dosage compensation of X-linked genes in the cells of female mammals is achieved by transcriptional silencing one of the two X chromosomes (Lyon, 1961). This process, known as X inactivation, is dependent on the presence of a cis-acting locus, the X inactivation center (Xic), that is required during early embryogenesis for both the initiation and spreading of inactivation along the X chromosome (Russell and Montgomery, 1965; reviewed by Rastan and Brown, 1990). The mouse Xist gene maps to the Xic region (Borsani et al., 1991; Brockdorff et al., 1991), as does its human homologue XIST (Brown et al., 1991), and it is abundantly and exclusively expressed from the inactive X in somatic cells (high level monoallelic expression) (Panning et al., 1997). The Xist transcript, a 17.4 kb molecule with no conserved open reading frame (Brockdorff et al., 1992; Hong et al., 1999), appears to be directly involved in the X chromosome inactivation, having the unique role of painting the chromosome during interphase (Moulton Clemson et al., 1996). Hypoacetylation of histone H4 and enrichment in histone macroH2A1.2 follow Xist expression and appear to play a role in the maintenance of X inactivation (Costanzi and Pehrson, 1998; Mermod et al., 1999; Gilbert et al., 2000), which does not require the presence of either the Xic or Xist DNA sequences beyond the initial phase (Brown and Willard, 1994; Csankovszki et al., 1999; Wutz and Jaenisch, 2000).

Xist transcripts in the cleavage stage mouse embryo are predominantly derived from the paternally inherited X chromosome, because of imprinting during gametogenesis (Takagi and Sasaki, 1975; Kay et al., 1993, 1994; Lee, 2000; Sado et al., 2001). These transcripts localize in cis along the X chromosome of paternal origin, eventually leading to its inactivation. Differences in DNA methylation are thought to be responsible for this early state of determination and for triggering or avoiding X inactivation (Norris et al., 1994; Panning and Jaenisch, 1996; reviewed by Goto and Monk, 1998; Lee, 2000; Sado et al., 2001). Molecular genetic experiments carried out with nested PCR demonstrate the presence of Xist transcripts in female mouse embryos as early as the 4-cell stage (Kay et al., 1994). Analysis of Xist RNA by fluorescence in situ hybridization (FISH) consistently reveals the presence of a strong signal in most interphase nuclei of female mouse embryos at the 8- to 16-cell stages, believed to colocalize with the inactive X chromosome (Sheardown et al., 1997). This pattern of accumulation of stable Xist...
transcripts from the paternally derived X chromosome persists in the trophodermal cells surrounding the blastocyst stage embryo (Takagi and Sasaki, 1975; Kay et al., 1994; Goto and Takagi, 1999), which go on to form the placenta. In contrast, the inner cell mass (ICM) within the blastocyst is pluripotent and generates the embryo and its surrounding membranes (Mintz, 1965; Tarkowsky and Wroblewska, 1967; Wilson et al., 1972; Johnson and Ziomek, 1981; Ziomek et al., 1982). By the time the epiblast is formed, the paternally imprinted pattern of X chromosome inactivation is erased within these cells and is replaced by random inactivation of either the paternally or maternally derived X chromosome. The mechanisms underlying this switch remain to be clarified, but appear to be linked to a genome-wide demethylation that takes place between the 8-cell and blastocyst stages (Monk, 1990; Monk et al., 1991; Kay et al., 1994). As one of the X chromosomes becomes recognizable inactive in each cell of the developing embryo, Xist transcription from the active X chromosome is silenced because of de novo methylation within its Xic locus (Beard et al., 1995; Panning and Jaenisch, 1996).

In spite of the extensive literature focusing on these remarkable patterns of Xist gene expression, a quantitative developmental profile of Xist RNA levels in single embryos of each sex has not yet been reported. Techniques such as RT-FISH and in situ RT-PCR are only roughly quantitative, and solely visualize molecules that are localized to recognizable loci within the nucleus. Conventional methods of PCR and nested PCR amplification (Kay et al., 1994) rely on non-quantitative methods of end product analysis, such as gel electrophoresis, and have largely been carried out using groups of male and female embryos. Single nucleotide primer extension analysis allows RNA quantification, but its complexity restricts its application to a small number of samples (Buzin et al., 1994). Even more recent methods involving radioactive dot blotting to measure PCR products from single embryos (Latham et al., 2000) still show very high variability. In order to circumvent these limitations, we have optimized real-time PCR (Heid et al., 1996; Pierce et al., 2000) to measure Xist levels in single mouse embryos. Real-time PCR and RT-PCR are recently developed techniques for the quantification of genomic and RNA templates (reviewed by Orlando et al., 1998; Freeman et al., 1999; Bustin, 2000). The real-time RT-PCR method allowed us to measure not only the levels of total Xist mRNA present in embryos at different developmental stages, but also to directly count the number of genomes within each sample, and thus, to calculate RNA levels on a per genome basis. Moreover, it enabled the parallel analysis of the presence/absence of the Scy gene, thus providing an independent criterion for embryo sexing. Xist RNA levels differed substantially in male and female samples from the 4-cell stage onwards, and can, therefore, be used to sex individual embryos very early during development. We anticipate that real-time PCR of individual blastomeres will make it possible in the future to distinguish mRNA differences in single cells of morula stage embryos that presage cellular differentiation in developing blastocysts.

MATERIALS AND METHODS

Embryo Culture and Nucleic Acid Isolation

Frozen late 2-cell stage mouse embryos (43.5 hr post hCG) were obtained from Embryotech Laboratories, Inc. (Wilmington, MA). Embryos were thawed for 2 min at room temperature followed by 1 min at 37°C, and were washed twice in Modified HTF Medium Hepes + 5% Synthetic Serum Substitute (both products from Irvine Scientific, Santa Ana, CA) at 37°C. After 5 min, embryos were transferred to 35-μl droplets of GEM-PS medium (Duncan Holly Biomedical, Bedford, MA) + 5% Synthetic Serum Substitute, overlaid with a very thin layer of Mineral Oil (embryo tested) previously washed in Water for Embryo Transfer (both products from Sigma Chemical Company, St. Louis, MO) and equilibrated with 7.3% CO2 at 37°C. After two washes, embryos were transferred to new droplets in groups of 3 or 4, and cultured in the presence of 7.3% CO2 in order to maintain a pH of 7.3. Samples were moved to fresh medium when cultured for more than 48 hr. Embryos harvested at the 2-cell stage were cultured for 1 hr. One-cell embryos were used for the study of the pronuclear stage and cultured for 45 min. At each developmental stage, about 20 individual embryos were denatured using a Micro RNA Isolation Kit (Stratagene, La Jolla, CA) and the resulting nucleic acids (DNA and RNA) were purified using the phenol:-chloroform:isoamyl alcohol procedure recommended by the manufacturer. For most experiments, each sample was divided into three aliquots. Two aliquots were used for separate analysis of Xist and Scry genomic DNAs, the third aliquot was reverse transcribed, and the resulting cDNA was used as a measure of Xist RNA.

Preparation of Xist “RT” and “No RT” Samples

Nucleic acids extracted from each embryo (see above) were used to prepare “RT” and “No RT” samples for Xist PCR. “RT” samples contained cDNA plus genomic DNA. “No RT” samples had only genomic DNA. Reverse transcription was carried out with ThermoScript™ (GIBCO-BRL, Life Technologies, Rockville, MD) using random hexamer primers according to the suggested protocol but in the absence of DTT, in a total volume of 10 μl. Extreme care was taken to avoid RNase contamination and all equipment of a dedicated area was treated with RNase ERASE (ICN Biomedicals, Costa Mesa, CA) before each experiment. RNase-free test tubes and pipette tips were purchased from USA Scientific (Ocala, FL). “No RT” samples were obtained by omitting the reverse transcriptase in the assay mix, but were otherwise treated as the other samples. In order to preserve genomic DNA in all the samples, enzymatic digestion with DNase was not included in our protocol.
Real-Time PCR

Two sets of primers were designed from mouse Xist and Sry sequences retrieved from GenBank (accession no. L04961 and X67204, respectively). One set of primers, 6271/6566 (5'-TTGTCGAGGCATCTTATGAT-3', upstream primer; 5'-AAACCCACATTTCGAATG-3', downstream primer) amplified both the cDNA and genomic DNA within Xist exon 1. A second set of primers, 8531/8693 (5'-TCATCGAGGGCTAAAGTG-3', upstream primer; 5'-CAACAGGCTGCAATAAAAAG-3', downstream primer) amplified a mouse specific sequence within the Sry gene. Real-time PCR of both Xist and Sry was carried out in an ABI Prism 7700 Sequence Detector using SYBR® Green (FMC BioProducts, Rockland, ME) as the detector. The “threshold cycle” (C_T) at which each fluorescent signal was first detected above background was used as a measure of the number of template copies present at the start of the reaction by comparison to a standard scale prepared from male mouse genomic DNA (Sigma). DNA concentration of the standard was converted into genome number using a 6 pg/genome size (Vendrely and Vendrely, 1949). Serial dilutions were freshly prepared for each experiment from a stock solution of at least 100 genomes/μl, and run in triplicates. DNase- and RNase-free, sterile 18 megohm H_2 O (Sigma) was used for all dilutions. A difference of one cycle between two C_T determinations represented a two-fold difference in the number of templates amplified, a lower C_T value indicating an earlier detection of the fluorescent signal and, therefore, more templates present at the start of the reaction (Heid et al., 1996). Purity and specificity of both Xist and Sry amplicons were confirmed by analysis of both their size and melting temperature. Products size was established by electrophoresis of the samples through a 3% agarose gel. Melting profiles for each sample were generated at the end of PCR from the accured PCR products, as the negative derivative of the products' fluorescent signal measured at increasing temperatures (Bernard et al., 1997, see below). A single melting peak is characteristic of each PCR product, and occurs at a temperature determined by its sequence.

One-sixth (blastocysts) to all (1- or 2-cell embryos) of the material derived from a single embryo was used for each PCR determination, depending on the developmental stage of the embryo. Accordingly, the PCR assay volume was adjusted to either 50 or 100 μl, such that no more than 10% of the total volume was accounted for by the RT-derived samples (5 or 10 μl, respectively) in order to minimize the effects of the RT buffer components on the PCR assays. The conditions for Xist PCR were as follows: 50 mM Tris, pH 8.3, 2.5 mM MgCl_2, 0.3 μM each primer, 0.25 mM each dNTP, 1: 62,500 SYBR Green (from a “10,000× concentrate in DMSO” purchased from FMC), and 1 unit of Taq polymerase (Promega, Madison, WI). The polymerase was incubated with TaqStart antibody (Clontech, Palo Alto, CA) for 5 min before addition to the reaction mixture (hotstart PCR). The cycling profile included a denaturation step, 3 min at 95°C, followed by 45 four-step cycles. The temperature profile of these cycles was: 95°C (10 sec), 53°C (15 sec), 72°C (15 sec), 78°C (10 sec). Fluorescence readings were acquired at 78°C, in order to exclude fluorescent signals because of the possible formation of primers dimers late in the reaction. At the end of the PCR assays, melting profiles were recorded in the 55–95°C range at 0.2°C intervals. The conditions for Sry PCR were as follows: 100 mM Tris, pH 8.3, 4 mM MgCl2, 20 mM KCl, 0.3 μM each primer, 0.25 mM each dNTP, 1:62,500 SYBR Green (see above), and 1 unit of Taq polymerase. The polymerase was incubated with TaqStart antibody, as described for Xist PCR. The cycling profile started with a denaturation step, 3 min at 95°C, followed by 45 four-step cycles. The temperature profile of these cycles was: 95°C (10 sec), 63°C (30 sec), 72°C (25 sec), 82°C (15 sec). Fluorescence readings during PCR were acquired at 82°C and melting profiles of the PCR products were also recorded, as detailed for Xist.

Microscopy

The developmental stage of embryos was visually identified using an Olympus IX70 inverted microscope equipped with Hoffman modulation contrast optics and a Hitachi VK-C370 video camera (Hitachi, Ltd., Japan). Images were collected with Snappy 2.0 software (Play Inc., Rancho Cordova, CA).

RESULTS

Xist RNA Levels Measured in Single Preimplantation Embryos Allow Gender Identification

Real-time PCR is a technique that allows the amount of DNA present in a sample to be measured as a function of how quickly a fluorescent signal is first observed above a threshold (C_T value) during the process of sequence amplification (see Materials and Methods). We have used this approach to count the number of Xist gene copies and Xist RNA molecules recovered from single male and female preimplantation mouse embryos. Nucleic acids isolated from each samples comprised both DNA and RNA, and were subdivided into aliquots processed in parallel. One aliquot was subjected to PCR without prior reverse transcription to give us a measure of the number of genomic DNA copies, while a second aliquot was treated with reverse transcriptase to give us a measure of total Xist DNA, genomic DNA plus cDNA. By comparing the C_T values of the two aliquots, we were able to deduce the number of Xist RNA molecules originally present in the embryo.

Fourteen to twenty embryos at each developmental stage, from the fertilized egg (pronuclei) to the late blastocyst stage, were initially analyzed for total Xist DNA. Figure 1 shows representative results obtained from 4-cell stage and early blastocyst stage embryos. It is immediately apparent from these plots that, in either case, the samples can be grouped into two
non-overlapping sets: those in which Xist amplicons accumulated above the threshold early in the reaction (smaller Ct value), and those in which Xist amplification reached the same level several cycles later (larger Ct value). This observation was extended to all groups of embryos between the 4-cell and blastocyst stages. Because the timing of signal amplification in real-time PCR reflects the number of templates present at the start of the reaction, we postulated that the samples with early Xist signals were probably female because they contained both genomic DNA and substantial levels of cDNA, while those with delayed Xist signals were probably male and only contained Xist genomic DNA. Comparison of PCR signals generated with and without reverse transcription proved that early signals were due to the presence of Xist RNA in the samples. For instance, in Figure 2A,B (purported female embryos) omission of reverse transcription caused the PCR signals to shift to the right substantially (red and green signals are not overlapping). In contrast, the shift was negligible in the purported male embryos at the same stage, Figure 2C,D (black and green signals overlap).

This Xist mRNA-based approach was used to divide embryos at each stage into two groups. Between the 4-cell stage and the late blastocyst stage, 52% of the 88 embryos investigated displayed early Ct values, and were presumed to be female (the percentages ranged from 42 to 58% at each individual stage, see Table 1). We then analyzed the samples’ genomic DNA-only signals, by calculating and averaging the corresponding Ct values for each group of embryos. A comparison of the mean Ct values of the genomic Xist signals charted in Figure 2A–D (green lines only) is presented in Figure 3. At the 4-cell stage, the difference between the mean female Ct and the mean male Ct was 0.7 cycles (ΔCt = −0.7), and at the blastocyst stage the same calculation yielded ΔCt = −0.8 cycles. Similar delta values, of about one PCR cycle, were observed at each of the other embryonic stages (data not shown) and in each case are in accord with the fact that female embryos should generate “No RT” PCR signals one cycle earlier than male embryos with equal numbers of cells, because female cells contain two X chromosomes while male cells contain only one.

The validity of the above analyses depends on the accuracy of the real-time PCR method. The quantitative reliability of our reaction conditions was established by measuring Ct values generated from spectroscopically quantified amounts of mouse genomic DNA. The resulting values were used to generate a standard curve correlating genomic Xist template copy numbers vs. Ct values (Fig. 3). As described in Materials and Methods, the Ct values obtained for the purported female and male embryos were evaluated using this standard curve.

The validity of the above analyses also depends on the specificity of the amplicons produced by the real-time PCR method. The issue of amplicon specificity becomes particularly acute when the amount of the template present at the beginning of the reaction is small (Pierce et al., 2000; Hartshorn, unpublished communication). The oligonucleotide primers used in these experiments were carefully selected for their high degree of specificity for the Xist transcript as well as for the Xist gene, considering that amplification in whole genome samples is more likely to generate non-specific products. Closed-tube real-time PCR in the presence of SYBR Green allows the product(s) of each reaction to be individually evaluated at the end of the assay by analysis of the DNA melting profile (see Materials and Methods). The melting profiles in the insets to Figure 3 demonstrate the presence of a single PCR product at the expected melting temperature in all the genomic
Fig. 2. Xist real-time PCR in female or male 4-cell embryos and early blastocysts, with or without reverse transcription. Panels A and B. Xist expression was measured in single embryos expected to be female from the previous analysis (see Fig. 1). In each case, reverse transcription was performed on part of the sample (red, cDNA + genomic DNA), while it was omitted on an equivalent aliquot processed in parallel (green, genomic DNA only). Panel A: 4-cell embryos, 1/2-embryo equivalent per assay. Panel B: Early blastocysts, 1/6-embryo equivalent per assay. In both cases, "RT" samples generated a signal much earlier than the "No RT" samples. Panels C and D: An analysis similar to the previous one was carried out on embryos purported to be male. Experimental conditions were the same used for the female embryos. Black, "RT" samples, cDNA + genomic DNA. Green, "No RT" samples, genomic DNA only. Panel C: 4-cell embryos; the two groups cannot be distinguished. Panel D: Early blastocysts; the two groups are overlapping, although "RT" samples have a mean C_{T} slightly smaller than the "No RT" samples.

<table>
<thead>
<tr>
<th>TABLE 1. Genome Counting in Single Embryos by Xist Real-Time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developmental stage assessed by microscopy</td>
</tr>
<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>1-Cell</td>
</tr>
<tr>
<td>2-Cell</td>
</tr>
<tr>
<td>4-Cell</td>
</tr>
<tr>
<td>8-Cell</td>
</tr>
<tr>
<td>Morula</td>
</tr>
<tr>
<td>Early blastocyst</td>
</tr>
<tr>
<td>Late blastocyst</td>
</tr>
</tbody>
</table>

<sup>a</sup>Assuming synchronous cell division.
<sup>b</sup>Normalized for embryos sex (one Xist DNA copy per male genome, two copies per female genome).
<sup>c</sup>Cell number confirmed by modulation contrast microscopy.
<sup>d</sup>Cell number could not be confirmed by microscopy and was based on published estimates (Johnson et al., 1986; Monk et al., 1991).
levels of Xist cDNA (in the inset) contained no Sry DNA, while all Sry-positive embryos contained minimal amounts of Xist cDNA. Indeed, from the compacted morula stage onward, all purported male embryos (a total of 19 embryos) contained Sry DNA (Table 2). Prior to the compacted morula stage, 8 of 12 embryos deemed to be male by Xist analysis tested positive for Sry. In contrast, none of the purported female embryos at any stage tested positive for Sry DNA (total samples = 38).

**Real-Time PCR Provides a Way of Counting the Number of Genomes Per Embryo**

Real-time PCR measurements of Xist genomic DNA also provided a means of counting the total number of genomes present in single embryos. The amount of Xist genomic DNA in each embryo was measured and correlated to the embryonic stage assessed by microscopy. The resulting data, combined with the knowledge of which embryos were male and which were female, permitted us to estimate the number of genomes per embryo based on the assumptions that all cells were diploid and each female cell contained twice as much Xist DNA as each male cell (Table 1). The results show that at the early stages of development the mean number of genomes per embryo measured by PCR closely corresponds to the mean number of cells per embryo determined by visual inspection. The numbers of genomes detected at the early and late blastocyst stages are higher than the presumed cell numbers per embryo, as anticipated by the presence of endoreduplication.

Using the same approach, we next compared genomic DNA levels in individual embryos that were co-cultured to the early blastocyst stage, in order to determine whether they achieved equal numbers of genomes even when they had developed at different rates. The fastest growing blastocysts had 3–5-fold more genomes per embryo than those that developed slowly (Table 3), showing a trend similar for both male and female embryos. These data provided the basis for calculating Xist RNA levels in this same group of female embryos, as described below.

**Xist Expression Levels in Developing Female and Male Embryos**

The combination of technologies described above allowed us to measure average Xist RNA levels in female and male embryos at the pronuclear, 2-cell, 4-cell, 8-cell, morula, early blastocyst, and late blastocyst stages (Fig. 5A), and to calculate the number of transcripts per genome over the course of development (Fig. 5B). The numbers of individual embryos analyzed at each stage are reported in Table 1.

**Xist gene expression can first be detected as early as the 2-cell stage in female embryos.** None of the 20 embryos tested at the pronuclear 2PN stage generated a Xist signal consistent with expression, although reverse transcription had been carried out on all samples. This result indicates either the absence of stored maternal Xist RNA in the unfertilized mouse...
Fig. 4. Sry real-time PCR in single early blastocysts. The presence of the Sry gene was investigated in a group of early blastocysts previously sexed based on Xist expression levels. One-third embryo equivalents were used for Sry PCR. Inset: Xist RT-PCR of the same embryos (from Fig. 1B). Line coding of the Sry signals was determined by the experiment in the inset, revealing a perfect accordance between the two PCR tests for the purpose of embryo sexing (broken line, male; solid line, female).

**TABLE 2. Concordance Between Xist Expression Levels and the Presence of Sry in Identifying Embryos' Sex**

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Sry-positive/Total embryos</th>
<th>Male&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Female&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Cell</td>
<td></td>
<td>5/9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/9</td>
</tr>
<tr>
<td>Morula (before compaction)</td>
<td></td>
<td>3/4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/8</td>
</tr>
<tr>
<td>Morula (compacted)</td>
<td></td>
<td>4/4</td>
<td>0/3</td>
</tr>
<tr>
<td>Early blastocyst</td>
<td></td>
<td>9/9</td>
<td>0/10</td>
</tr>
<tr>
<td>Late blastocyst</td>
<td></td>
<td>6/6</td>
<td>0/8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>27/31</td>
<td>0/38</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sex of embryos was determined based on the levels of Xist expression.

<sup>b</sup>Sry amplification failure in some of the samples containing fewer cells could be likely because of a skewed distribution of the genomic material among tubes, as nucleic acids extracted from each embryo had to be divided in three aliquots (see Materials and Methods).

**TABLE 3. Genome Number and Culture Time are Inversely Correlated in Blastocoe1-Forming Embryos**

<table>
<thead>
<tr>
<th>Early blastocyst stage embryos&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Female&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Male&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomes per embryo&lt;sup&gt;c&lt;/sup&gt; in culture</td>
<td>Hours</td>
<td>Genomes per embryo&lt;sup&gt;c&lt;/sup&gt; in culture</td>
</tr>
<tr>
<td>18</td>
<td>47</td>
<td>18</td>
</tr>
<tr>
<td>18</td>
<td>47</td>
<td>18</td>
</tr>
<tr>
<td>24</td>
<td>44.5</td>
<td>24</td>
</tr>
<tr>
<td>45</td>
<td>44.5</td>
<td>36</td>
</tr>
<tr>
<td>48</td>
<td>44.5</td>
<td>84</td>
</tr>
<tr>
<td>96</td>
<td>43.5</td>
<td>84</td>
</tr>
</tbody>
</table>

<sup>a</sup>Embryos placed in culture at the 2-cell stage were harvested as soon as they presented an identifiable blastocoe1.

<sup>b</sup>Embryos' sex was established by measuring the levels of Xist mRNA and determining the presence/absence of the Sry gene in each sample.

<sup>c</sup>The number of genomes in each embryo was determined by genomic Xist copy counting, assuming diploidy in all cells and the presence of two X chromosomes in all female cells and one X chromosome in all male cells.

RNA was consistently present in about half of the embryos at the 4-cell stage (263 ± 141 copies/total embryo) onward, leading to their identification as female as detailed above, while some 2-cell stage
Fig. 5. Developmental profile of Xist expression in single embryos. A: Xist mRNA copies measured per total embryo at different developmental stages. The C_T values derived from PCR determinations were converted into Xist copy number by comparison with the standard curve of Figure 3. Average genome numbers per embryo were calculated based on genomic Xist copy counts as shown in Table 1. Messenger RNA copy numbers were deduced as the difference between genomic DNA + cDNA copy number ("RT" sample) and genomic DNA only copy number ("No RT" sample) for each embryo. Xist mRNA copy numbers obtained for female (■) or male (×) embryos at each stage were averaged, and are shown on the graph ± SD. (SD bars are not visible for lower values because they are extremely small.) Results relative to pronuclear stage embryos are shown on the ordinate. B: Xist mRNA levels per genome in developmentally advancing embryos. The number of Xist mRNA copies measured per total embryo was divided by the average number of genomes present in embryos of the corresponding stage, and the resulting mean values of Xist expression per genome were graphed as a function of development, ± SD. Genome numbers per embryo were calculated assuming diplody in all cells. Female embryos, ■; male embryos, ×. After the morula (approximately 16 cells) stage, Xist mRNA levels per total female embryo did not increase proportionally with the rate of cell division, which led to a drop in the Xist mRNA levels per genome.

embryos (4 out of 18) contained approximately 60 copies of Xist RNA per embryo (30 copies/cell), and were, therefore, presumed to be slightly more advanced female embryos.

Xist RNA levels measured in female embryos show a biphasic distribution. Xist RNA levels increased continuously and dramatically during the first four cell cycles in female embryos. Morula stage embryos were found to contain about 5,500 copies (5,465 ± 1,184) of Xist RNA (Fig. 5A), or an average of 390 molecules (+/− 85) per genome (Fig. 5B). After the morula stage, total Xist RNA levels per female embryo remained steady and then rose to about 6,800 copies (6,797 ± 2,894) in late blastocysts. However, because embryos continued to grow, Xist RNA levels per genome decreased to 129 copies (+/− 26) in early blastocysts and again to 41 copies (+/− 17) per genome in late blastocysts. Xist RNA levels in late blastocysts varied more widely than in cleavage stage embryos, perhaps reflecting increasing differences in the numbers of genomes per embryo, because of endoreduplication and divergent rates of development. This possibility is in accord with the fact that the embryo with the single highest level of Xist expression measured in our experiments (about 11,000 total copies of Xist RNA) was rated by microscopy as having considerably progressed through zona hatching, hence being the most developmentally advanced among all embryos utilized for this study (data not shown).

Decline of Xist mRNA copy numbers per genome in early blastocysts is linked to the developmental stage and is independent of time in culture. Closer analysis of some of the data presented in Figure 5B provided further information about the factors affecting Xist RNA levels at specific stages of development. Figure 6 analyzes Xist RNA levels in individual embryos, rather than presenting an average value of Xist expression at a certain embryonic stage. Nine female embryos were cultured together and harvested over a 6.5 hr period, as soon as a blastocoele could be observed under the microscope. Based on this criterion, six of these embryos were rated as early blastocysts, while the three slowest embryos did not present a visible cavity and were collected as compacted morulas. In accord with Table 3, embryos that reached the early blastocyst stage first possessed more genomes and were likely composed of more cells than the slower embryos. Analysis of the data in Figure 6 shows that Xist RNA levels per genome steadily decrease as the number of genomes per embryo increases, but embryos with equivalent numbers of genomes have very similar amounts of Xist RNA.

DISCUSSION

Using real-time PCR, we have constructed a convenient and reliable quantitative assay with which to measure Xist RNA and DNA levels in very small numbers of cells, and have thus been able to investigate the dynamics of Xist gene expression in preimplantation mouse embryos. The resulting data, collected from 126 single early embryos and normalized to internal genome copy numbers, provide the first complete quantitative profile of Xist RNA levels in developing male and female embryos. Furthermore, analysis of these data validates the accuracy of our methodology on the basis of several complementary criteria. First, genomic Xist copy numbers measured by real-time
Fig. 6. Decreasing levels of Xist expression in coel-forming embryos are a function of genome number rather than culture time. Xist mRNA levels measured in nine female embryos at the compacted morula to early blastocyst stage were tabulated as single data points. The embryos were cultured together and collected sequentially over a 6.5 hr period, as soon as a blastocoele could be visually detected in each sample. A cavity could not be seen in three of the embryos, which were collected at the end of the experiments and rated as compacted morulas. Genome numbers individually calculated for each of the blastocyst are shown above in Table 3, genome numbers for the compacted morulas were established with the same procedure. Embryos presenting more genomes were the faster growing in the group (the 96-genome blastocyst was collected after 43.5 hr in culture), while embryos with fewer genomes were the slower developing (the 12-genome compacted morula was collected after 50 hr in culture). Total Xist mRNA levels measured for the individual samples were divided by the number of genomes detected in each embryo. Results were thus plotted on a per genome basis, as in Figure 5B. Xist expression was found to decrease in a developmental stage-dependent manner rather than with longer culture times. A more pronounced decrease in Xist mRNA molecules per genome was in fact apparent in the faster growing rather than in the slower embryos.

PCR were, on average, close to those expected for early embryos with recognizable numbers of cells. Second, individual female embryos with equivalent numbers of genomes contained very similar levels of Xist transcripts, even when they were grown in separate experiments. Such a result suggests that our methods for recovering RNA and constructing cDNA are reliable and reproducible. A higher degree of variation among samples would likely be observed if the amount of extracted RNAs only represented a small fraction of the total per embryo. Third, levels of Xist RNA at each embryonic stage were highly reproducible, once they were analyzed in terms of the rate of embryo development.

In addition to quantifying gene expression, measuring Xist mRNA levels has allowed us to identify the sex of embryos from the 4-cell stage onwards, although it was not possible for technical reasons to determine the presence of the Sry gene in all embryos purported to be male at this early stage. (In order to circumvent this problem, we are presently constructing a multiplexed RT-PCR system for the simultaneous detection of Xist RNA and the Xist and Sry genes; Hartshorn et al., unpublished communication.) Other pieces of evidence derived from our data support the assumption that quantification of Xist transcripts in early embryos can be used for gender identification. First, groups of embryos at each developmental stage appeared to be composed of male and female individuals at a roughly 1:1 ratio, as would be predicted by random sampling. Second, samples identified as female contained an average number of Xist genomic copies higher (approximately double) than the males, indicative of the number of X chromosomes.

The sensitivity of the cDNA assay presented in this study is several-fold greater than that of methods based on the detection of single-copy genes (Mulder et al., 1993), and we anticipate that it will be possible to analyze Xist RNA levels in single blastomeres in the future. The presence of multiple cDNA molecules per female cell, like the use of a repeated genomic DNA sequence (Pierce et al., 2000), makes the possibility of false negatives extremely unlikely. None of the pronucleus stage embryos had detectable Xist RNA, but some of the 2-cell embryos did, indicating that Xist transcription begins late in the second cell cycle. These measures of Xist gene expression are earlier than have been reported using either nested RT-PCR of pooled embryos (Xist cDNA was detected at the 4-cell stage; Kay et al., 1994) or FISH analysis (a single bright spot was visible within each nucleus of 8-cell female embryos; Sheardown et al., 1997). Our detection of Xist transcripts in single 2-cell stage embryos extends the findings of Latham and Rambhatla (1995), who reached the same conclusion using pooled samples, and is similar to the situation described in bovine embryos (De La Fuente et al., 1999). Expression of the human homologous XIST prior to second cleavage has also been reported (Daniels et al., 1997). Our results do not support a recent report of Xist RNA present in pronuclear stage mouse zygotes presumed to be of both sexes (Avner et al., 2000). However, that study was carried out using in situ RT-PCR without confirming the specificity of the amplified product in male and female embryos at later stages of development.

Xist RNA is not the only transcript of the Xist gene sequence. Blastocyst-stage embryos and embryonic stem cells are known to contain low levels of Tsix RNA, an antisense transcript of the same genomic region (Lee et al., 1999; Lee, 2000). Tsix expression exclusively occurs on the maternally derived X chromosome, opposite to Xist RNA production, and does not start until blastocyst formation (Sado et al., 2001). Although this antisense transcript has not yet been rigorously quantified at any stage of development, Tsix RNA molecules are most likely measured in all studies, such as ours, in which Xist cDNA is prepared without use of strand-specific primers. Our findings clearly demonstrate, however, that Tsix RNA levels must be very low in male blastocyst-stage embryos that only contain an active X chromosome, because total Xist + Tsix RNA copy numbers are very low in these samples. The same must be true for female embryos, because Tsix transcription is absent from the inactive,
Xist-expressing X chromosome (reviewed by Mlynarczyk and Panning, 2000) and the accumulation of antisense RNA molecules produced by the active X is known to be very low. This conclusion is in accord with relative measurements of Xist and Tsix RNA levels (Lee et al., 1999) and with in situ hybridization analysis of Tsix-specific probes, which reveals “pinpoint” fluorescent spots on the active X chromosome of both male and female embryonic cells (Lee et al., 1999; reviewed by Mlynarczyk and Panning, 2000).

Cell cleavage in mammals is not synchronous and cell cycle duration is known to vary with in vitro culture conditions and genetic strains. Moreover, as mouse embryos approach the blastocyst stage, either in vivo or in vitro, at least some cells of the differentiating trophectoderm undergo endoreduplication (Gardner and Davies, 1993) and contain DNA in amounts greater than 4C (Barlow and Sherman, 1972; Barlow et al., 1972). In order to account for these factors, we concluded that it would be more accurate to express our measurements of Xist RNA accumulation in terms of genomes per embryo rather than cells per embryo. The numbers of genomes counted via the real-time PCR method were in concordance with visually assessed cell numbers in early embryos, giving us confidence in the accuracy of the method. Therefore, the genome numbers higher than the expected cell numbers found in more developed samples were regarded as indicative of DNA duplication and factored into our calculations.

The present study demonstrates a biphasic pattern of Xist RNA accumulation in developing female embryos. Xist RNA copy numbers measured in whole embryos increase sharply during early cleavage and plateau at the morula stage. The number of Xist molecules per genome, therefore, declines dramatically between morula and late blastocyst stages, as embryos continue to grow. Because X chromosome-associated Xist transcripts are unusually stable over extended periods of time in cells in culture (Moulton Clemson et al., 1996), it is very unlikely that this change in Xist levels is because of RNA degradation. Moreover, our data demonstrate that the decrease is independent of culture time, since it is more evident in the faster rather than the slower growing blastocysts.

It is far more likely that these quantitative changes reflect alterations in Xist gene expression accompanying cell lineage differentiation, which begins in compacted morulas. Two main lineages are formed in blastocysts as a result of this process: the trophectoderm and the ICM. Trophectodermal cells give rise to the placenta whose cells continue to exhibit the paternally inherited pattern of X chromosome inactivation. In contrast, cells of the ICM generate the embryonic membranes and the embryo proper. By the time the epiblast has formed, the imprinted pattern of X chromosome inactivation is replaced in these cells by random inactivation of either the paternal or the maternal X chromosome (Kay et al., 1993, 1994; Sheardown et al., 1997). However, even though Xist RNA levels are known to be very low in cells of the ICM, the three-fold decrease in Xist RNA copies per genome that takes place between morula and early blastocyst stage cannot be accounted for by emergence of the ICM, because the ICM only comprises about 30% of total cells in the 32-cell stage embryos (Barlow et al., 1972; Kesslering et al., 1991).

The finding that Xist RNA levels present in embryos between morula and blastocyst stage remain almost constant is also not explained either by a decrease in the rate of the mitotic cycle or by endoreduplication. It is known that the length of the cell cycle in mouse embryos increases to about 18 hr by the sixth cleavage (64-cell stage) (Kiesling et al., 1991). Assuming that a steady number of Xist transcripts is needed to maintain X chromosome silencing, this delay in cell division should slow, but not prevent, further Xist RNA accumulation in the total embryo. In addition, during the same period of time some of the cells within the differentiating trophectoderm undergo endoreduplication. Cells that become polyploid replicate their entire genomes, but do not divide, hence they maintain equal numbers of active and inactive X chromosomes (Webb et al., 1992; Takagi, 1993; Panning and Jaenisch, 1998). Alternatively, cells that become aneuploid by selectively duplicating an X chromosome inactivate all of its copies except one (Brown et al., 1992; reviewed by Migeon, 1994; Brockdorff, 1998). Our data clearly demonstrate that genomic replication continues throughout preimplantation development, regardless of whether it is always followed by cell division. In either case, total Xist RNA levels should keep pace with, or exceed, the increase in genome copies per embryo recorded at each stage, and the number of Xist transcripts per gene copy should remain steady or increase throughout development. This is, however, not what we have observed.

We suggest that the plateau in Xist RNA accumulation that starts at morula stage is linked to cellular differentiation and reprogramming of the inactive X chromosomes. This hypothesis is in accord with that of Kay et al. (1994) who suggested that Xist transcription in cleavage stage embryos depends on an oocyte-derived factor that is depleted by the time morulas compact. Alternatively, Xist RNA molecules synthesized prior to morula stage may be mostly stable, while a temporary switch to expression of primarily unstable transcripts (reviewed by Kuroda and Meller, 1997; Panning et al., 1997; Sheardown et al., 1997) may take place at the time of blastocyst formation. We further speculate that the amount of stable Xist RNA that accumulates in cleavage stage cells exceeds that needed to inactivate the paternally marked X chromosome within these cells. If this is the case, the extra Xist molecules may not be bound to the inactive X chromosome and may, in fact, be located elsewhere in the nucleus or even in the cytoplasm. Moreover, if Xist accumulation pauses at morula stage, it is tempting to speculate that an asymmetric distribution of excess Xist molecules among dividing cells of the morula could influence the subsequent fate of those cells. Cells
receiving more Xist would maintain the paternally imprinted pattern of X chromosome inactivation and would go on to form the trophectoderm. These cells would resume Xist synthesis at a modest rate in the late blastocyst. In contrast, dividing morula stage cells that received little or none of the extra Xist RNA would escape the paternal pattern of silencing and would eventually switch to the random mode of X chromosome inactivation characteristic of embryonic cells. It should be possible in the future to test these ideas by using real-time PCR to quantify the amount of Xist RNA in individual blastomeres. Differences between the morula’s outer and inner cells (Johnson and Ziomek, 1981, 1982) will be of particular interest, as these cells represent the first step toward the formation of trophectoderm and ICM and are believed to become two distinct cell populations by differential inheritance of soluble factors.

ACKNOWLEDGMENTS

We thank Dr. Ken Pierce and Ariel Levine for sharing their embryo culture expertise, and Dr. J. Aquiles Sanchez for many helpful comments. Hamilton-Thorne Biosciences, Inc. is gratefully acknowledged for a grant supporting this research.

REFERENCES


