X inactivation-specific transcript expression in mouse oocytes and zygotes

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Expression of the X inactivation-specific transcript (Xist) gene has previously been shown by reverse transcription–polymerase chain reaction (RT–PCR) to be present at the 4-cell stage of female mouse embryos. This early expression, which is followed by X inactivation in the extra-embryonic tissues, is maternally imprinted. By the blastocyst stage, as the embryonic lineages begin to form, the imprint is lost and expression becomes random. By applying in-situ RT–PCR, we showed that Xist is expressed even earlier in development, in unfertilized mouse oocytes as well as in pronuclei stage zygotes. Our data demonstrate Xist expression in oocytes and suggest that Xist transcripts may occur in both XX and XY zygotes. A difference in the pattern of expression (rod-like or rounded punctate signal) is found among pronuclei-stage embryos. Early expression is in agreement with findings reported in human embryos.

Key words: embryo/oocyte/X inactivation/Xist expression

Introduction

X inactivation, a mammalian dosage compensation mechanism which assures equal expression of many X-linked genes between males (XY) and females (XX), is preceded by the expression of the X gene inactivation-specific transcript (XIST/Xist), the human and mouse genes respectively (Kay et al., 1993). XIST/Xist is expressed in female-differentiated cells exclusively from the inactive X (Borsani et al., 1991; Brown et al., 1991). It encodes an untranslatable transcript (Brockdorff et al., 1992; Brown et al., 1992), which accumulates in the nucleus along the inactive chromosome (Clemson et al., 1996), and is required for the initiation of the inactivation process (Penny et al., 1996).

Initially, in undifferentiated embryonic stem cells, prior to X-inactivation, low levels of unstable Xist RNA are expressed biallelically in XX as well as in XY cells. The antisense of Xist, Tsix, possibly blocks its action on the X chromosome of undifferentiated stem cells (Lee et al., 1999). As cells differentiate, a monoallelic increase in RNA stability is observed in XX cells at the initiation of X inactivation (Brockdorff, 1998), while in XY cells it completely disappears.

During pre-implantation development low levels of Xist expression can be detected by reverse transcription–polymerase chain reaction (RT–PCR). In mice, expression was shown to be present at the 4-cell stage of female embryos (Kay et al., 1994). This early expression is maternally imprinted (transcribed only from the paternal allele), and is followed by the formation of the extra-embryonic lineages. By the blastocyst stage, as the embryonic lineages begin to form, the imprint is not seen and expression becomes random, transcribed either from the maternal or paternal allele (Monk et al., 1987; Kay et al., 1994). Recent results in early human embryos showed low levels of XIST expression in 1-cell stage embryos, with increasing amounts to the 8-cell stage (Daniels et al., 1997). Moreover, transcripts were detected in both male and female pre-implantation embryos (from the 4-cell to the blastocyst stage) (Daniels et al., 1997; Ray et al., 1997), indicating expression from the maternal allele as well.

To study the apparent differences between mouse and human, Xist expression was analysed in mouse unfertilized oocytes and in two pronuclei (PN)-stage zygotes, by indirect in-situ RT–PCR. In-situ RT–PCR during preimplantation allowed the localization of rare Xist mRNA molecules at these early stages of development, where transcripts could not be detected by other techniques, e.g. RT–PCR and fluorescent in-situ hybridization (FISH).

Materials and methods

Cell collection and slide preparation

Murine unfertilized oocytes (C57BL/6J×CBA/Ca)F1 and zygotes (C57BL/6J×CBA/Ca)F1×129/Sv) were obtained as previously described (Hogan et al., 1986). Following the removal of the zona pellucida by pronase digestion (5 mg/ml), cells (10 per slide) were mounted on Superfrost Plus glass slides (Menzel Glazer), fixed in 4% paraformaldehyde at 4°C for 24 h, air-dried, washed in phosphate-buffered saline (PBS) and dehydrated. Experiments were performed on 30 slides for zygotes and on 10 slides for oocytes.

In-situ RT–PCR and hybridization

Intracellular cDNA amplification was performed as previously described (Nuovo, 1994). Primers were designed to span exons I–IV.
Figure 1. Schematic diagram of the X inactivation-specific transcript (Xist) cDNA region (exons I–IV) amplified by in-situ reverse transcription–polymerase chain reaction (RT–PCR). cDNA was generated from primer 9733R and amplified with primers Mx52 and Mx23bR. Exon/intron boundaries (black), primer positions (red), product size (green) and probe location (yellow) are indicated.

Figure 2. X inactivation-specific transcript (Xist) gene expression in two pronuclear (2PN)-stage zygotes. (A) Xist expression detected by in-situ reverse transcription–polymerase chain reaction (RT–PCR): rod shaped (top) and punctate (bottom) signals; (B) negative control (no reverse transcription); (C) positive control (amplified genomic DNA).

Figure 3. Different patterns of X inactivation-specific transcript (Xist) gene expression in two pronuclei (2PN)-stage zygotes, as revealed by in-situ reverse transcription–polymerase chain reaction (RT–PCR). Xist expression detected as (A) rod-like or (B) rounded punctate signals.

Table 1. X inactivation-specific transcript (Xist) gene expression as detected by in-situ reverse transcription–polymerase chain reaction (RT–PCR)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of cells analysed</th>
<th>No. of positive cells</th>
<th>Signal type (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rounded</td>
</tr>
<tr>
<td>2PN embryo</td>
<td>22</td>
<td>14</td>
<td>40</td>
</tr>
<tr>
<td>Oocyte</td>
<td>10</td>
<td>8</td>
<td>100</td>
</tr>
</tbody>
</table>

2PN = two pronuclei.

Figure 4. X inactivation-specific transcript (Xist) gene expression in unfertilized oocytes at metaphase II, as detected by in-situ reverse transcription–polymerase chain reaction (RT–PCR). (A) Xist expression; (B) negative control (no reverse transcription); (C) positive control (amplified genomic DNA).
(Figure 1), eliminating spurious results due to genomic or unspliced sequences. Cell samples were reverse transcribed at 42°C for 1 h in the presence of a downstream primer 9733R 5'-CAGCAGGCCAGC- AATTCTGG-3' (exon IV). The cDNA was amplified using primers Mx525'-GTAACCTACCCAGTGCGGG-3' and Mx23bR 5'-CTG- TATAGGCTCGGG-3' (exons I and III respectively) by 35 cycles of 1 min at 94°C, 2 min at 55°C and 30 s at 72°C, and a final extension of 10 min at 72°C. Intracellular products were visualized by hybridization with a digoxigenin (DIG)-labelled probe and detected by an alkaline phosphatase-based colorimetric system. The intronless DIG-labelled probe (Boehringer Mannheim, Mannheim, Germany) was a 111 bp PCR product generated with primers designed in the cDNA, in the junction regions of exons I/II (forward primer 9475 5'-GACTACAAGGATGAACTTTGGG-3') and of exons III/II (reverse primer 9586R 5'-GGATGTTTGCCATCTTG-3'). A 30 µl drop of hybridization solution containing 60% formamide, 2× sodium chloride/sodium citrate (SSC), 10% dextran sulphate, 200 ng/µl salmon sperm DNA and the probe was spotted on the samples. Slides were sealed with a cover-slip, followed by 5 min denaturation at 95°C and an overnight incubation at 37°C. Slide samples were washed twice in 2× SSC and once in 1× SSC at 37°C. Following post-hybridization washes, DIG-labelled molecules were visualized by an anti-DIG-conjugated alkaline phosphatase system (Boehringer Mannheim), which produces a water-insoluble purple-black dye in the presence of Nitro Blue Tetrazolium (NTB) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). In order to avoid false-positives due to non-specific and/or genomic amplifications, the RT enzyme was omitted in the negative controls. Genomic amplification of exon VI by in-situ PCR was used as a control for positive signals (primers 11483 5'-GTCCTGTCTCTCTGTG-3' and 11857R 5'-CTCGGACT- TAGCTAGGGTTTGTGTC-3'). DIG-labelled probe synthesized by PCR using primers 11664 5'-ATCTAGACAAATATACATCATC- CG-3' and 11797R 5'-TCAGCATCGTCCAGCCAGC-3') (Ayoub et al., 1997).

Results

Using in-situ RT-PCR, we were able to show Xist expression in 2PN-stage zygotes as well as in meiotic metaphase II (MII) unfertilized oocytes. Figure 2A illustrates Xist RNA amplification signals in 2PN stage zygotes. In cells where the reverse transcriptase enzyme was omitted, no signal was detected (Figure 2B). As a positive control for product accumulation and detection, a genomic sequence of Xist was amplified (Figure 2C).

Two kinds of signals were observed in the zygotes: the purple-black stain appeared either as a rod-like (Figure 2A top; Figure 3A) or a smaller rounded (Figures 2A bottom and 3B) signal. These two different patterns of product accumulation were evenly distributed among the cells analysed and thus may represent a genuine difference in the level of Xist expression between embryos.

Slides that show amplification had RNA transcripts in the majority of the cells (Table I). Assuming that some cells did not stain for technical reasons and that there was an equal representation of both male and female embryos, our data suggest that Xist expression may occur in XX as well as in XY zygotes.

Figure 4A shows the presence of Xist RNA transcripts, in two unfertilized mouse oocytes; Figure 4B and C represent negative and positive controls respectively. In oocytes, we could detect signals of the rod-like kind only. The poor quality of these cells is presumably due to the harsh treatment involved in the sample preparation, that renders them morphologically irregular.

Discussion

Using in-situ RT–PCR, we showed Xist expression in 2PN zygotes as well as in MII unfertilized oocytes. These are the earliest stages of Xist expression reported to date in mice and demonstrate transcription and splicing of Xist RNA in female gametocytes. Furthermore, although we were unable to distinguish between XX and XY embryos due to technical limitations, our data shows that Xist transcripts may occur in both male and female zygotes. This observation is in close agreement with the previously reported findings in human embryos (Daniels et al., 1997; Ray et al., 1997). We assume that Xist is expressed from both maternal and paternal X chromosomes, as in undifferentiated XX and XY murine embryonic stem cells (Panning and Jaenisch, 1996; Johnston et al., 1998).

Nevertheless, it should be emphasized that biallelic expression should not necessarily implicate the absence of imprinting: imprinting may not only be an all-or-none phenomenon. The differential behaviour of the parental alleles could also be explained by a difference in the level of transcription or in processing, stabilization or localization of the RNA transcript, which may have not been detected in our system. The meaning of the different pattern of product accumulation, visualized as rod-shaped or rounded punctate signals, has not been established; it could be due to a difference in the level of Xist expression among embryos.

It should be emphasized that these signals detected in 2PN zygotes and unfertilized oocytes by in-situ RT–PCR cannot be compared with the signals described in embryonic stem cells by Johnston et al. who used FISH (Johnston et al., 1998). It is still unclear why only one signal was detected in all zygotes, when two signals would be expected in the XX fertilized oocytes. As a whole, in-situ RT–PCR has the advantage of higher sensitivity. Although it is not a quantitative technique, it can detect major differences in expression; however, it may be difficult to determine fine localization.

The significance of Xist expression at the 2PN stage zygote, much earlier than the onset of X inactivation, is not well understood. It may have no role in development, but it is possible that the early and imprinted expression prior to embryo compaction (8-cell stage), is crucial for non-random X inactivation, leading to the differentiation of the extra-embryonic tissues, as suggested by experimental data (Marahrens et al., 1997). To address this question a refinement of our experimental approach is required, where transcripts can be quantified and analysed for their parental origin, for example by simultaneous sexing by FISH. Methods should be further developed to allow the topological localization of signals in whole mount blastocyst-stage embryos.

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