SHORT PAPER

Mouse endogenous X-linked genes do not show lineage-specific delayed inactivation during development

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Summary

X chromosome inactivation (XCI) has been assumed to be complete in all cells of female mouse embryos at about 6 d post coitum (dpc). However, a recent study on β-galactosidase expression of an X-linked lacZ transgene suggests that XCI is probably not complete several days after this time in some lineages. To help resolve this issue, we analysed XCI in embryos which carry the T(X;16)16H (Searle's) translocation and are heterozygous at the X-linked Hprt and Pgk-1 genes. The quantitative RT-PCR single nucleotide primer extension (SNuPE) assay was used to measure Hprt and Pgk-1 allele-specific transcripts in embryos 9-5 dpc. No transcripts from the normal X chromosome were found in any of the tissues tested, indicating that inactivation was complete for these endogenous genes.

1. Introduction

It is generally accepted that cells of the inner cell mass of female mouse embryos begin X chromosome inactivation (XCI) by 5-5 d post coitum (dpc), and that most cells have completed XCI by 6-5 d (Grant & Chapman, 1988), as evidenced by heteropycnosis (Ohno & Lyon, 1965), asynchronous replication (Takagi, 1980) and differential Kanda staining (Rastan, 1983). Experimental difficulties in distinguishing alleles have made evidence for specific genes very limited. As measured by the activity of enzymes encoded by X-linked genes, XCI has been assumed to occur in all cells of female mouse embryos shortly before the onset of gastrulation. These studies were based upon the analysis of enzyme activity of whole embryos, which may be inadequate to detect variation in XCI among different tissue lineages. In recent studies, expression of an X-linked lacZ transgene was measured by blue histological staining for β-galactosidase activity, and the activity found in some tissues of 8-5 to 105 dpc female embryos hemizygous for the transgene suggested incomplete XCI (Tan et al. 1993; Tan & Tarn, 1993). We therefore decided to determine whether endogenous genes also show selective delay in XCI. We report here that two endogenous X-linked genes, Hprt and Pgk-1, show no apparent delay in XCI in any tissue tested.

2. Materials and methods

(i) Collection of embryos and RNA purification

Female mice carrying Searle's translocation and a normal X chromosome containing the lacZ transgene (X18Hprt1/X16 Pgk-1a Hprt1a lacZ; 16a Pgk-1a/16) were mated with males homozygous for the Pgk-1a and Hprt1a alleles (Xa Pgk-1a Hprt1a/Y). Pregnant females were killed at 9-5 dpc, and the embryos collected. For...
each embryo the caudal region, hindbrain, heart and yolk sac were dissected, then purified by the use of RNAzol B as described (Singer-Sam & Riggs, 1993). RNA was stored at —70 °C in 75% ethanol prior to use.

(ii) Analysis of Xist transcripts

For each embryo assayed, Xist RNA sequences were amplified by use of RT-PCR with the primer set 1994/2300 as described (Buzin et al. 1994). Following agarose gel electrophoresis, RT-PCR products were transferred to nylon membranes and hybridized to allele-specific Xist oligonucleotide probes. Hybridization to each 32P-labelled oligonucleotide probe was done in the presence of a 40-fold excess of non-radioactive oligonucleotide specific for the other allele (Nozari et al. 1986; Buzin et al. 1994). The probes were CATCAGACTGTAAGTACC for Xist* and GCATACAGCTAAGTACC for Xist*, and the temperature of hybridization was 46 °C.

(iii) Analysis of Pgk-1 and Hprt transcripts

Pgk-1 and Hprt sequences containing allele-specific differences were amplified from RNA of each sample by RT-PCR as previously described (Singer-Sam et al. 1992; Buzin et al. 1994), with 40 cycles of PCR following reverse transcription. Following electrophoresis on low melt agarose, amplified products of the appropriate size were excised and purified by use of gelase. Samples were analysed by the quantitative single nucleotide primer extension assay as described previously (Singer-Sam & Riggs, 1993). An example of the assay is shown in Fig. 3.

3. Results

We used mouse embryos carrying the T(X;16)16H translocation (Searle’s translocation) (Lyon et al. 1964), which also had distinguishable alleles at the X-linked Pgk-1 and Hprt loci (see Fig. 1). The translocated chromosomes X16 and 16X have the ‘b’ allele at both loci, whereas the normal X chromosome (Xn) has the ‘a’ allele. In adult Searle’s females, Xn is the inactive chromosome in all cells, while chromosomes X16 and 16X are active, even though the latter contains the region of the X chromosome coding for the center of XCI (Xic). XCI is thought to be random initially, but shortly after inactivation there is a rapid selection against those cells which have inactivated the 16X chromosome (McMahon & Monk, 1983). Therefore, in the background of the Searle’s translocation, any RNA expressed from the Hprt and Pgk-1 alleles found on Xn would be evidence for a delay in XCI. To detect allele-specific expression, we used the single nucleotide primer extension (SNUPE) assay following amplification by RT-PCR (Singer-Sam et al. 1992; Singer-Sam & Riggs, 1993). In brief, an oligonucleotide that binds adjacent to a single nucleotide difference in the cDNA sequence of two alleles is used to prime extension for one base only, using a single, radioactively labelled nucleotide. If the sequence of the template is not complementary immediately 3′ to the primer, a base will not be added. In the case of Pgk-1, a C v. A polymorphism was used to distinguish the Pgk-1a and Pgk-1b alleles, respectively; for Hprt, a C v. G polymorphism distinguished the Hprt* and Hprt alleles (Boer et al. 1987; Johnson et al. 1988; Singer-Sam et al. 1992). The assay seemed ideally suited for this study since prior work established that less than 1% expression of an allele differing by only one nucleotide can be detected (Singer-Sam et al. 1992). This sensitive detection of transcripts from both X chromosomes allowed us to measure the failure of Xn to inactivate in even a small percentage of cells of a given tissue.

Embryos 9.5 dpc were collected from pregnant Searle’s XnHprt*/Xn Pgk-1a Hprt*/lacZ; 16XPGk-1a/16 male mice mated with Xn Pgk-1b Hprt*Y female mice. The female embryos of interest will be the balanced heterozygotes carrying Searle’s translocation shown in Fig. 1. The cross will produce, in addition, two other classes of embryos; XY and XX. XY embryos were identified by an RT-PCR assay for the presence of the Y-linked Zfy gene (Singer-Sam et al. 1990). Some embryos were analysed as well by histological staining for the presence of the lacZ transgene; female embryos expressing β-galactosidase were presumed to have two intact X chromosomes. In addition, an RT-PCR assay for Xist was performed, making use of our observation that the same polymorphism previously found for Xist cDNA from Mus sprattus (Brockdorff et al. 1991) is also present on the X chromosome containing the Pgk-1a allele. Using primers that flank this allelic difference, and then an allele-specific hybridization assay (Buzin et al. 1994), we were able to determine whether Xist RNA was present (diagnostic for the presence of two X chromosomes, one active and one inactive), as well as which allele was being expressed. Embryos of the desired genotype should express Xist RNA from the

Fig. 1. Genotype of balanced Searle’s heterozygote embryos (Xn/Xn; 16X/16X). Straight line, X chromosome sequences; jagged line, chromosome 16 sequences. Xn, normal X chromosome. The box represents the region containing the center of X-inactivation (Xic).
Table 1. * Ratio of Pgk-1°/Pgk-1° and Hprt°/Hprt° transcripts in 9.5 dpc embryos

| Sample | Genotype   | Pgk-1a/b | Hprt a/b |
|--------|------------|----------|----------|
| 249-1  | X°/X°;16°/16 | 0.001    | 0.002 |
|        | Caudal     | 0.001    | 0.002 |
|        | Hindbrain  | 0.002    | 0.002 |
|        | Heart      | 0.003    | 0.006 |
|        | Yolk sac   | 0.002    | 0.003 |
| 295-1  | X°/X°;16°/16 | <0.001   | <0.001 |
|        | Caudal     | <0.001   | <0.001 |
|        | Hindbrain  | <0.003   | <0.001 |
|        | Heart      | <0.010   | 0.006 |
|        | Yolk sac   | <0.009   | 0.012 |
| 295-4  | X°/X°;16°/16 | <0.001   | <0.001 |
|        | Caudal     | <0.002   | <0.001 |
|        | Hindbrain  | <0.002   | <0.001 |
|        | Heart      | <0.002   | <0.001 |
|        | Yolk sac   | 0.004    | 0.003 |
| 295-5  | X°X°;16°/16 | <0.001   | <0.001 |
|        | Caudal     | <0.001   | <0.001 |
|        | Hindbrain  | <0.001   | <0.001 |
|        | Heart      | <0.001   | <0.001 |
|        | Yolk sac   | <0.001   | <0.001 |
| 249-2  | XX         | 1:16     | 0:90 |
|        | Caudal     | 0.93     | 0.81 |
|        | Hindbrain  | 0.56     | 0.53 |
|        | Heart      | 0.14     | 0.35 |
|        | Yolk sac   | 2:04     | 1:15 |
|        |            | 1:47     | 0:58 |
|        |            | 0:81     | 0:51 |
| 249-5  | XY         |          |        |
|        | Caudal     | 0.002    | 0.003 |
|        | Hindbrain  | <0.001   | 0.007 |
|        | Heart      | 0.001    | 0.004 |
|        | Yolk sac   | <0.001   | 0.006 |

* The genotype of the embryos was determined as described in the text. The embryonic tissues indicated were assayed by quantitative RT-PCR SNUPE assay. It should be noted that the assay is a ratio of two alleles, and the small numbers are informative (see raw data in legend to Fig. 3).
of the ‘a’ alleles in yolk sac is low, as expected in extraembryonic tissue partly derived from cells known to undergo preferential inactivation of the paternal X chromosome. For tissues of the embryo proper, some variation from a 1:0 ratio is expected because of the random nature of XCI and the small number of progenitor cells at the time of XCI. Whether the lower ratio in heart is significant cannot be determined with present data. The XY embryo, which has only a \(Pgk-1^p\) maternally derived X chromosome illustrates the sensitivity and limits of the SNaPSE assay. The background incorporation is 0.1-0.2% for \(Pgk-1\), which has a C/A mismatch and 0.4-0.7% for \(Hprt\), which has a C/G mismatch. In all cases the Searle’s female embryos showed essentially no \(Pgk-1\) or \(Hprt\) transcripts from the inactive X chromosome; the very low signal obtained in some cases is comparable to that seen for the XY embryo control.

4. Discussion

Our results show that the endogenous X-linked \(Pgk-1\) and \(Hprt\) genes are not significantly expressed from \(X^+\) at 9.5 dpc in the four tissues tested, suggesting that there is no delay in XCI at the level of transcription. This is an unexpected finding in view of previous work showing the persistence of \(\beta\)-galactosidase staining in more than 50% of the cells of some tissues of embryos carrying an X-linked \(lacZ\) transgene.

Among the possible explanations for the discrepancy between these and the previous results might be the difference in genetic background in the two experiments. However, this possibility has been ruled out by a recent study (Tam et al. 1994a, b), in which histological staining was done on 9.5 dpc Searle’s embryos carrying the \(lacZ\) transgene on the normal X chromosome. After mating of transgenic male mice (\(X\)lacZ/Y) with Searle’s females, \(F_1\) 9-5-d embryos were stained for \(\beta\)-galactosidase activity, and three classes of embryos were observed; unstained, strongly stained and sparsely stained. The unstained embryos were presumed to be XY or Searle’s females. Histological examination of the strongly stained embryos showed mosaic \(lacZ\) expression, as expected for \(X/X\)lacZ embryos. The sparsely stained embryos, thought to be of the genotype \(X^{16}/X^a;16^{5}/16\), showed the same tissues expressing \(lacZ\) from the presumed inactive X chromosome as was previously described for \(X\)lacZ/X embryos (Tan et al. 1993; Tam et al. 1994a, b), thus confirming the delay in XCI of the \(lacZ\) transgene. As was previously recognized, this result could be caused by tissue-specific differences in stability of \(lacZ\) mRNA or protein. A more likely and interesting possibility is that the transgene is inactivated more slowly than endogenous X-linked genes, even though it is known that the transgene does become inactivated in some tissues by 8-5 dpc, and remains stably inactivated in all other somatic tissues after 11-5 dpc (Tam et al. 1994a).

Why would this be? The delay could result from the special repetitive structure of the transgene, which is a 14-unit tandem array extending for 123 kb. There is evidence for the slow spread of methylation and transcriptional silencing in at least one case of tandem, autosomally integrated adenoviruses (Orend et al. 1991). The \(HMG\ CoA\) reductase promoter is an autosomal CpG island and thus could be resistant to methylation silencing, although methylation is now thought likely to be secondary to the primary silencing event. The higher-order domain structure of the transgene array could be suboptimal for XCI, and it is also formally possible that the integration of a 123 kb segment of foreign DNA disrupts a sequence or structure necessary for the establishment of XCI. Additional studies will be needed to distinguish between these possibilities, but they all imply that endogenous X-linked mouse genes have evolved to be susceptible to efficient inactivation by XCI. Relevant to this, it is known that several endogenous human genes are not subject to XCI (Gartler & Riggs, 1983). Thus an intriguing possibility is that there are elements in these genes that give immunity against inactivation, and they might be present in perhaps rudimentary form in the \(HMG-lacZ\) transgene.

5. Conclusion

Using a sensitive assay system in which very low levels of expression from the inactive X chromosome would have been detectable, we found no evidence for expression of the X-linked \(Pgk-1\) and \(Hprt\) genes in embryos 9-5 dpc. Our results thus offer no support for the idea of a delay of XCI of endogenous genes.

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