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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 10·5 dpc female embryos hemizygous for the transgene suggested incomplete XCI (Tan *et al.* 1993; Tan & Tam, 1993). The *HMG–lacZ* transgene, which has the mouse 3-hydroxyl-3-methylglutaryl coenzyme A reductase (*HMG CoA*) promoter driving expression of a *lacZ* gene for *E. coli* β-galactosidase, is present as a tandem 14-copy insert at a single locus on the X chromosome. In the adult and in embryonic tissues older than 11·5 dpc the *lacZ* transgene is silent in approximately half the cells, indicating normal XCI. However, at earlier stages, more than 50% of the cells in some tissues express the transgene. For example, heart tissue of 9·5 dpc embryos was seen to have 65% of cells showing blue staining for β-galactosidase. The findings strongly suggested that XCI is not complete in some lineages for several days after gastrulation (Tan *et al.* 1993; Tam *et al.* 1994*a*, *b*). 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 (X^{16}*Hprt*^{b}/X^n *Pgk-1*^{b} *Hprt*^{b} *lacZ*; 16^X*Pgk-1*^{b}/16) were mated with males homozygous for the *Pgk-1*^{a} and *Hprt*^{a} alleles (X^n *Pgk-1*^{a} *Hprt*^{a}/Y). Pregnant females were killed at 9·5 dpc, and the embryos collected. For

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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 ^{32}P-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*^{a} and GCATCAGACTAAGTACCT for *Xist*^{b}, 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 X^{16} and 16^X have the 'b' allele at both loci, whereas the normal X chromosome (X^n) has the 'a' allele. In adult Searle's females, X^n is the inactive chromosome in all cells, while chromosomes X^{16} and 16^X 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 16^X 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 X^n 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-1*^{a} and *Pgk-1*^{b} alleles, respectively; for *Hprt*, a C *v.* G polymorphism distinguished the *Hprt*^{a} and *Hprt*^{b} 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 X^n to inactivate in even a small percentage of cells of a given tissue.

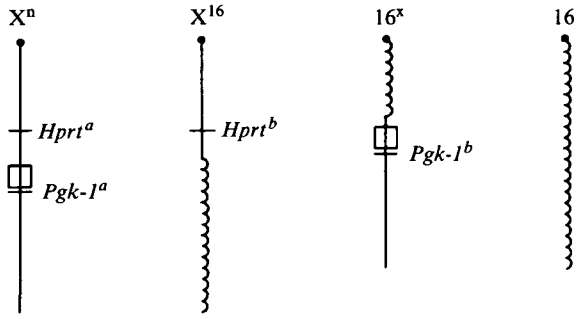

Fig. 1. Genotype of balanced Searle's heterozygote embryos (X^{16}/X^n;$16^X/16$). Straight line, X chromosome sequences; jagged line, chromosome 16 sequences. X^n, normal X chromosome. The box represents the region containing the center of X-inactivation (*Xic*).

Embryos 9·5 dpc were collected from pregnant Searle's (X^{16}*Hprt*^{b}/X^n *Pgk-1*^{b} *Hprt*^{b} *lacZ*; 16^X*Pgk-1*^{b}/16) female mice mated with X^n *Pgk-1*^{a} *Hprt*^{a}/Y male 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 spretus* (Brockdorff *et al.* 1991) is also present on the X chromosome containing the *Pgk-1*^{a} 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

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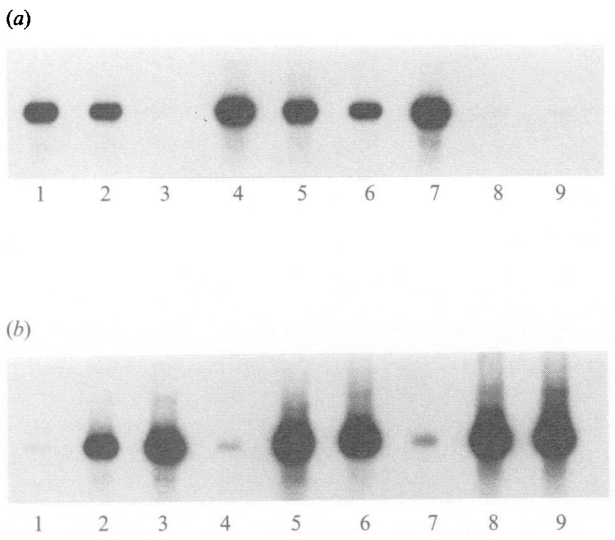

Fig. 2. Allele-specific hybridization assay for expression of *Xist*^{a} and *Xist*^{b}. RNA was isolated from individual embryos, and *Xist* RT-PCR products were hybridized to oligonucleotide probes specific for *Xist*^{a} (cis to *Pgk-1*^{a} allele) (Panel *a*) or *Xist*^{b} (cis to *Pgk-1*^{b} allele) (Panel *b*). Lanes (1), 40 ng *Xist*^{a} DNA; (2) 20 ng *Xist*^{a} + 20 ng *Xist*^{b} DNA; (3) 40 ng *Xist*^{b} DNA; (4) embryo 249-1; (5) embryo 249-2; (6) embryo 249-3; (7) embryo 247-1; (8) and (9) maternal liver (*Pgk-1*^{b}/*Pgk-1*^{b}). Embryos 249-1 and 247-1 were deduced to be of the genotype shown in Fig. 1, while embryos 249-2 and 249-3 were XX. Results of the SNuPE assay of the 9·5 dpc embryos 249-1, 249-2, and 249-3 are shown in Table 1. Embryo 247-1, dissected at 8·5 dpc, gave the same results as 249-1 (not shown).

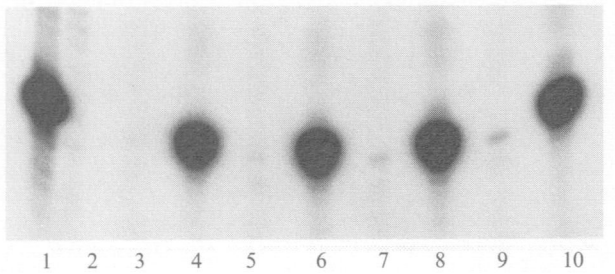

Fig. 3. Example of SNuPE assay. RNA was purified from the caudal region (lanes 3–4), hindbrain (lanes 5–6), heart (lanes 7–8) and yolk sac (lanes 9–10) of a Searle's embryo 9·5 dpc (see Fig. 1 for genotype). After RT-PCR, gel-purified amplification products served as a template for the SNuPE assay as previously described (see text). Controls included amplified templates containing only the *Pgk-1*^{a} (lane 1) or *Pgk-1*^{b} (lane 2) polymorphism. Each template was incubated with Taq polymerase and the same oligonucleotide primer, and either ^{32}P-dCTP, specific for *Pgk-1*^{a} (lanes 1, 2, 3, 5, 7, 9) or ^{32}P-dATP, specific for *Pgk-1*^{b} (lanes 4, 6, 8, 10). After denaturing polyacrylamide gel electrophoresis, a Molecular Dynamics PhosphorImager was used for volume integration of each spot. Total accumulated counts X 10^{-3}: (lane 1) 44054; (lane 2) not determined; (lane 3) 10·8; (lane 4) 20517; (lane 5) 20·6; (lane 6) 24470; (lane 7) 40·2; (lane 8) 32830; (lane 9) 60·0, (lane 10) 28125.

normal X, but not 16^X. Typical results obtained by this assay are shown in Fig. 2. Two of the embryos, shown in lanes 4 and 7, respectively, express *Xist*^{a} RNA almost entirely, and were deduced to be of the desired genotype. The low *Xist*^{b} signal is comparable to background levels seen with this method.

Table 1.* *Ratio of* Pgk-1^a/Pgk-1^b *and* Hprt^a/Hprt^b *transcripts in 9·5 dpc embryos*

Sample	Genotype	*Pgk-1a/b*	*Hprta/b*
249-1	$X^{16}/X^n;16^X/16$		
Caudal		0·001	0·002
Hindbrain		0·001	0·002
Heart		0·002	0·002
Yolk sac		0·003	0·006
295-1	$X^{16}/X^n;16^X/16$		
Caudal		< 0·001	0·003
Hindbrain		0·003	< 0·001
Heart		0·010	0·006
Yolk sac		0·009	0·012
295-4	$X^{16}/X^n;16^X/16$		
Caudal		< 0·001	< 0·001
Hindbrain		0·002	< 0·001
Heart		0·002	0·003
Yolk sac		0·004	0·003
295-5	$X^{16}X^n;16^X/16$		
Caudal		< 0·001	< 0·001
Hindbrain		< 0·001	< 0·001
Heart		< 0·001	< 0·001
Yolk sac		< 0·001	< 0·001
249-2	XX		
Caudal		1·16	0·90
Hindbrain		0·93	0·81
Heart		0·56	0·53
Yolk sac		0·14	0·35
249-3	XX		
Caudal		2·04	1·15
Heart		1·47	0·58
Yolk sac		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).

Embryos were dissected into four fragments comprising (*a*) the hindbrain and associated craniofacial tissues, (*b*) the lower third portion of the trunk (caudal), (*c*) the heart, and (*d*) the yolk sac which includes both the mesoderm and the endoderm, and analysed by the RT-PCR SNuPE assay as shown in Fig. 3. These tissues were chosen because previous studies (Tan *et al.* 1993) had indicated that at 9·5 dpc, the hindbrain should have only one active X chromosome, while the caudal third (incorporating the hindgut) and the heart should have a proportion of cells harbouring two active X chromosomes. Table 1 summarizes the results obtained from XX, XY, and Searle's (X^{16}/X^n; $16^X/16$) embryos at 9·5 dpc. The normal XX embryos clearly show expression of both alleles at roughly similar levels, although expression

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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*^{b} maternally derived X chromosome illustrates the sensitivity and limits of the SNuPE 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^n 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 β-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.* 1994*a*, *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 β-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^n;16^X/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.* 1994*a*, *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.* 1994*a*).

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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