**INTRODUCTION**

During mammalian development, one of the two X chromosomes in the female embryo is randomly inactivated in the somatic cells to achieve gene dosage compensation. On the basis of the activity of X-encoded enzymes (Monk and Harper, 1978; McMahon et al., 1983) and the detection of a late replicating X chromosome (Sugawara et al., 1983), it was suggested that X-inactivation takes place shortly before the onset of gastrulation. X-inactivation is also believed to occur synchronously in all embryonic tissue lineages. Recently, we have re-examined the progression of X-inactivation in different somatic lineages using an X-linked lacZ transgene whose expression reveals the activity of the X chromosome at the single cell level. Using this approach, we have discovered that, instead of being a single event happening simultaneously in all cell types, X-inactivation proceeds with different schedules in different somatic tissues (Tan et al., 1993). Some tissues such as the notochord, the heart, the cranial mesoderm and the gut endoderm in the organogenesis-stage embryo are among the last to complete X-inactivation. These tissues also have in common that they are specified early during gastrulation (Lawson et al., 1991; Tam, 1989; Tam et al., 1993) and a substantial population of cells in these lineages is still maintaining two active X chromosomes long after the onset of X-inactivation (Tam et al., 1994; Tan et al., 1993).

Our studies so far have concerned only with the pattern of X-inactivation in the somatic cells and not the germ-line cells. The germ line in the mouse embryo is first established during gastrulation (Lawson and Hage, 1994) when some cells from the proximal epiblast of the egg cylinder differentiate to form the primordial germ cells (PGCs). PGCs are first recognised as a discrete population in the primitive streak and allantoic bud of the 7.5-day gastrulating embryo. Although inactivation of the transgene was found in some PGCs at these sites, at least 85% of the population were still expressing the lacZ gene. This suggests that, although X-inactivation has commenced during gastrulation, the majority of PGCs still possess two active X chromosomes. Transgene activity remained unchanged during the relocation of PGCs to the hindgut endoderm, but decreased abruptly when PGCs left the hindgut and migrated through the mesentery. X-inactivation was completed during the migration of PGCs, but not simultaneously for the whole population. The first wave of PGCs entering the genital ridge at 9.5 days did not immediately re-activate the silent transgene until about 24 hours later. Re-activation of the transgene took place in over 80% of PGCs entering the genital ridge at 10.5-13.5 days p.c., preceding the entry into meiosis. About 90% of the meiotic germ cells in the 14.5-15.5 day fetal ovary expressed the transgene. Similar profiles of transgene activity were observed in PGCs of embryos that have inherited the lacZ transgene from different parents, showing unequivocally that X-inactivation in the germ cell lineage is not related to parental legacy. In contrast to those germ cells in the genital ridges, a small population of PGCs that was left outside the genital ridges at 13.5-15.5 days did not re-activate the silent X. This strongly suggests that re-activation of the silent X chromosome in the female germ cells is a response to local signals in the genital ridge.

**SUMMARY**

We have determined the timing of the inactivation and re-activation of the X chromosome in the mouse primordial germ cells (PGCs) by monitoring the expression of an X-linked HMG-lacZ reporter gene. PGCs were identified by their distinct alkaline phosphatase activity and they were first localised in the primitive streak and allantoic bud of the 7.5-day gastrulating embryo. Although inactivation of the transgene was found in some PGCs at these sites, at least 85% of the population were still expressing the lacZ gene. This suggests that, although X-inactivation has commenced during gastrulation, the majority of PGCs still possess two active X chromosomes. Transgene activity remained unchanged during the relocation of PGCs to the hindgut endoderm, but decreased abruptly when PGCs left the hindgut and migrated through the mesentery. X-inactivation was completed during the migration of PGCs, but not simultaneously for the whole population. The first wave of PGCs entering the genital ridge at 9.5 days did not immediately re-activate the silent transgene until about 24 hours later. Re-activation of the transgene took place in over 80% of PGCs entering the genital ridge at 10.5-13.5 days p.c., preceding the entry into meiosis. About 90% of the meiotic germ cells in the 14.5-15.5 day fetal ovary expressed the transgene. Similar profiles of transgene activity were observed in PGCs of embryos that have inherited the lacZ transgene from different parents, showing unequivocally that X-inactivation in the germ cell lineage is not related to parental legacy. In contrast to those germ cells in the genital ridges, a small population of PGCs that was left outside the genital ridges at 13.5-15.5 days did not re-activate the silent X. This strongly suggests that re-activation of the silent X chromosome in the female germ cells is a response to local signals in the genital ridge.

**Key words:** X-inactivation, chromosomal re-activation, primordial germ cells, X-linked lacZ transgene, mouse embryo
and proliferation of PGCs take place during the next 2-3 days resulting in the colonisation of the genital ridges by about 25000 germ cells (Tam and Snow, 1981; Eddy et al., 1981; Gomperts et al., 1994). In female embryos, germ cells cease mitosis and enter meiosis by 13.4 to 14.5 days p.c. to form primary oocytes (McLaren, 1983a).

Previous studies assaying the activities of X-linked enzymes have shown that premeiotic germ cells in the genital ridges have only one active X chromosome (Gartler et al., 1980; McMahon et al., 1981; Kratzer and Chapman, 1981; Johnston, 1981), suggesting that PGCs are not a privileged population that is spared of X-inactivation (Monk, 1981). Re-activation of the silent X chromosome occurs later as germ cells enter meiosis presumably to ensure that an active X chromosome is present in every differentiating oocyte (Monk and McLaren, 1981). This raises the question of when X-inactivation occurs before the germ cells reach the genital ridge. Analysis of X-inactivation coat colour mosaicism has suggested that inactivation of the germ-line precursors can occur as early as 3.5-4.5 days p.c. in the inner cell mass of blastocysts (Gardner et al., 1985) and the process is likely to be completed during gastrulation (McMahon et al., 1983). Direct assay of X-chromosome activity by biochemical measurements of enzyme activity is only applicable for a pure cell population such as those localised in the fetal gonad. These assays are difficult to perform on early stages when a small population of 50-5000 PGCs is migrating and interspersing with other somatic tissues (Eddy et al., 1981; McLaren, 1983a, 1993). The precise schedule of X-inactivation in the early PGCs is therefore still unknown. In the present study, we have assayed X-linked lacZ transgene expression in individual PGCs (Tan et al., 1993) to examine the X-chromosome activity of PGCs at all stages of their migration and differentiation. Specifically, we aim to pinpoint the timing of the inactivation and the re-activation of the X chromosome and to find out if X-inactivation is indeed random for the germ-line lineage.

MATERIALS AND METHODS

Animals and collection of embryos

The activity of the X chromosome was monitored by the expression of an X-linked transgene containing the promoter of a mouse housekeeping gene, 3-hydroxy-3-methylglutaric acid reductase (HMG CoA), linked to a SV 40 T antigen nucleus localisation signal sequence and the E.coli lacZ gene. Mice carrying this transgene are females and have only one active X chromosome (McLaren, 1983a). Re-activation of these Xp *X 16m embryos any PGCs that have not initiated X-inactivation or have re-activated the silent X chromosome.

Embryos and fetuses were collected from the pregnant mice from the mating of H253 and (C57BL/6xDBA/2)F1 strains at 7.5 to 15.5 days p.c. Female (Xp *X 16m) embryos produced by the T16H and H253 matings were collected at 9.5-10.5 days p.c. The extraembryonic tissues of 7.5-day embryos and fragments of the head or tail were screened for lacZ expression by staining in X-gal solutions (Tam and Tan, 1992) to identify the female transgenic embryos. The embryonic portion of the 7.5-day and 8.5-day embryos, the trunk of the 9.5-11.5 days embryos, and the genital ridges and associated mesonephros of 13.5-15.5 day fetuses were fixed for 5-15 minutes in 0.5% glutaraldehyde in 0.1 M pH 7.2 phosphate-buffered saline (CytoSystem).

The fixed specimens were dehydrated in a graded series of ethanol, alcohols, and propylene oxide before being put into an embedding box and the entire box was embedded in an epoxy resin (LKB 7007). The sections were cut on a Jung microtome 15 μm thick. Dewaxed sections were treated with 3% (v/v) hydrogen peroxide for 30 minutes to block endogenous peroxidase activity.

Various controls for the immunostaining and histochemical procedure were performed by omitting the primary or secondary antibody and the enzyme substrate. Tissues of transgenic Xp *Y embryos were used as positive controls for the staining reaction. Tissues of non-transgenic XX and XY embryos were used as negative controls for assaying the intensity of background staining for scoring β-gal staining.

Analysis of lacZ expression

X-chromosome activity in PGCs at different sites of the embryo was assayed by scoring the number of ALP-expressing (ALP-positive) cells that contained β-gal in the nucleus. Nuclei were scored positive only when they were darkly stained with brown DAB product (Fig. 1A,B). The percentage therefore tends to be a conservative estimate of the proportion of PGCs that expresses the lacZ transgene. To achieve representative sampling of the PGC population, 30-700 ALP-positive cells were scored in every second or fifth sections of each specimen depending on the age of the embryo. The score was then used to compute the percentage of PGCs expressing the lacZ transgene. A score of 100% indicates that both X chromosomes are active in the entire PGC population sampled. Conversely, a score of 50%, irrespective of the parental origin of the transgene indicates
complete random X-inactivation. Values intermediate between 50% and 100% suggest that an inactive X chromosome is found in some cells of the population. The course of X-inactivation in PGCs can therefore be monitored when the score decreases from 100% to 50% and re-activation by the subsequent return to 100%. The changes in score were examined for PGCs localised in different positions along the migratory pathway at different embryonic ages. Fetal germ cells in fetuses older than 15.5 days were not studied because of the loss of the alkaline phosphatase activity (Cooke et al., 1993) and the lack of nuclear localisation of β-gal in the meiotic germ cells (Tam and Zhou, unpublished observation).

RESULTS

The specificity of immunostaining for β-gal has been tested with control tissues. Whole 10.5- to 13.5-day X<sup>m</sup>*Y embryos and 15.5-day X<sup>m</sup>*Y genital ridges displayed uniform staining of the nuclei but non-transgenic embryo only showed weak background staining. Exclusion of either the primary or the secondary antibody also resulted in negative staining. Trophoblasts that express high levels of alkaline phosphatase activity were obtained from X<sup>P</sup>X<sup>m</sup> transgenic embryo (which displays preferential X<sup>P</sup> inactivation in extraembryonic tissues). Immunostaining for β-gal followed by histochemical detection of ALP revealed distinct nuclear and cell membrane localisation respectively of these two enzymes in the same cell (Fig. 1A). When the same staining protocol was applied to 13.5-day X<sup>P</sup>X<sup>m</sup> genital ridges, ALP-positive PGCs showing positive or negative nuclear β-gal immunostaining could be distinguished unequivocally (Fig. 1B). Trophoblasts typically show stronger alkaline phosphatase activity than PGCs but the intensity of nuclear immunostaining is similar between the positive PGCs and the ectoplacental cone trophoblast (compare Fig. 1A with Figs 1B, 3D,G,H). Besides the alkaline phosphatase activity on the cell membrane, PGCs can also be identified by the strong enzyme activity in the perinuclear Golgi complex (e.g. Figs 1C-E, 3D; Ginsburg et al., 1990). It is therefore feasible to assay directly the activity of the X-linked lacZ transgene and ALP expression at single PGC level. The intensity of immunostaining may vary between specimens but the identification of the positively stained nuclei was always ascertained by taking into account of the background staining of negative controls (non-transgenic tissues) included in each run. Only intensely brown DAB-stained nuclei were scored as positive, thus the quantitation given in Tables 1 and 2 is likely to represent the minimal proportion of PGCs expressing the lacZ transgene in every case.

Table 1. The expression of HMG-lacZ transgene in the primordial germ cells of mouse embryos at 7.5 to 15.5 days

<table>
<thead>
<tr>
<th>Age (days p.c.)</th>
<th>Location of germ cells</th>
<th>% lacZ expressing germ cells in X&lt;sup&gt;P&lt;/sup&gt;*X&lt;sup&gt;m&lt;/sup&gt; embryos (n)</th>
<th>% lacZ expressing germ cells in X&lt;sup&gt;P&lt;/sup&gt;X&lt;sup&gt;m&lt;/sup&gt; embryos (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 days</td>
<td>PS/AL</td>
<td>81.0±3.5(3)</td>
<td>86.5±2.7(8)</td>
</tr>
<tr>
<td>8.5 days</td>
<td>Gut</td>
<td>80.0±3.8(17)</td>
<td>85.1±2.7(9)</td>
</tr>
<tr>
<td>9.5 days</td>
<td>Mesentery</td>
<td>81.6±5.0(4)</td>
<td>87.8±6.1(11)</td>
</tr>
<tr>
<td>10.5 days</td>
<td>Mesentery</td>
<td>54.6±2.9(4)</td>
<td>59.4±2.5(10)</td>
</tr>
<tr>
<td>11.5 days</td>
<td>Genital ridge</td>
<td>72.4±6.0(3)</td>
<td>76.4±3.8(6)</td>
</tr>
<tr>
<td>12.5 days</td>
<td>Extragonadal</td>
<td>42.1(1)</td>
<td>55.7±3.8(6)</td>
</tr>
<tr>
<td>13.5 days</td>
<td>Genital ridge</td>
<td>58.6±5.1(8)</td>
<td>59.5±2.5(5)</td>
</tr>
<tr>
<td>14.5 days</td>
<td>Genital ridge</td>
<td>67.0±3.9(9)</td>
<td>73.3±6.7(5)</td>
</tr>
<tr>
<td>15.5 days</td>
<td>Extragonadal</td>
<td>47.6±7.6(6)</td>
<td>51.7±3.4(9)</td>
</tr>
<tr>
<td>15.5 days</td>
<td>Genital ridge</td>
<td>69.9±5.9(7)</td>
<td>72.5±1.6(9)</td>
</tr>
<tr>
<td>16.5 days</td>
<td>Extragonadal</td>
<td>57.1(1)</td>
<td>56.0±5.9(6)</td>
</tr>
<tr>
<td>17.5 days</td>
<td>Genital ridge</td>
<td>80.3±3.5(2)</td>
<td>81.7±1.9(6)</td>
</tr>
<tr>
<td>18.5 days</td>
<td>Extragonadal</td>
<td>61.2±7.4(7)</td>
<td>58.7±3.9(9)</td>
</tr>
<tr>
<td>19.5 days</td>
<td>Genital ridge</td>
<td>79.8±2.3(9)</td>
<td>87.3±1.8(9)</td>
</tr>
<tr>
<td>20.5 days</td>
<td>Extragonadal</td>
<td>74.2±13(3)</td>
<td>52.9±4.5(2)</td>
</tr>
<tr>
<td>21.5 days</td>
<td>Ovary</td>
<td>87.7±0.2(3)</td>
<td>90.7±1.4(4)</td>
</tr>
<tr>
<td>22.5 days</td>
<td>Extragonadal</td>
<td>63.5±0.2(3)</td>
<td>50.6±0.6(2)</td>
</tr>
<tr>
<td>23.5 days</td>
<td>Ovary</td>
<td>90.0±1.9(5)</td>
<td>90.5±1.6(5)</td>
</tr>
</tbody>
</table>

The percentage of expressing cells in the PGC population found at different sites is compared between embryos carrying the transgene on different X chromosomes.

PS, Primitive streak; AL, Allantoic bud; n, number of samples.

Table 2. Expression of the HMG-lacZ transgene by primordial germ cells located at different migratory milestones in 7.5- to 15.5-days X<sup>P</sup>X<sup>m</sup> and X<sup>P</sup>*X<sup>m</sup> embryos

<table>
<thead>
<tr>
<th>Location of germ cells (n)</th>
<th>Age (days p.c.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS/AL</td>
<td>85.1±2.2(11)</td>
</tr>
<tr>
<td>Gut</td>
<td>86.2±4.6(15)</td>
</tr>
<tr>
<td>Mesentery/Ectoplacental</td>
<td>75.1±3.1(9)</td>
</tr>
<tr>
<td>Extragonadal</td>
<td>82.9±2.3(26)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>58.1±1.9(14)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>58.9±3.2(13)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>58.4±3.5(15)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>50.4±3.5(15)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>50.4±3.5(15)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>56.2±5.8(7)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>59.8±3.8(16)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>65.7±9.4(3)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>58.3±3.5(3)</td>
</tr>
<tr>
<td>Genital ridges</td>
<td>90.2±1.2(10)</td>
</tr>
</tbody>
</table>

Data are pooled for both types of hemizygous transgenic embryos in Table 1.

PS, Primitive streak; AL, Allantoic bud; n, number of samples.

more PGCs in the genital ridges expressing the transgene (Fig. 1F) with advancing age. Most (>90%) of the meiotic germ cells in the fetal ovary expressed the lacZ transgene (Table 1; Fig. 1G), but this is so in only about 50-65% of the ALP-positive cells found outside the gonad (Fig. 1H).

Fig. 2 and Table 2 show the progression of inactivation and re-activation of the lacZ transgene in PGCs at different embryonic ages. 81-86% of PGCs was actively expressing the lacZ transgene at 7.5 and 8.5 days, when they are still in the primitive streak, the allantoic bud or the gut endoderm. This percentage dropped rapidly to below 60% for PGCs in the mesentery, suggesting that X-inactivation begins as the PGCs start their migration and is completed during migration. The first wave of PGCs entering the genital ridge at 9.5 days did...
not immediately re-activate the silent transgene until about 24 hours later. From 10.5 days onwards, transgene activity increased in the PGCs that had colonised the genital ridge until over 90% of them were expressing the transgene at 14.4–15.5 days (Fig. 2). Re-activation of the silent transgene therefore occurs when PGCs enter the genital ridge and is taking place ahead of the onset of meiosis (Table 2). The timing of the inactivation and re-activation of the $\beta$-galactosidase is staggered for different waves of migrating PGCs. In contrast to those PGCs in the genital ridges, only 50–74% of PGCs which were migrating in the mesentery (at 10.5–13.5 days) or those that failed to enter the genital ridges (at 14.5–15.5 days) expressed the transgene. These strongly suggest that re-activation of the X chromosome does not occur in migrating PGCs or in germ cells that are lodged in extragondadal sites after 12.5 days.

Transgene activity in PGCs of 9.5 and 10.5-day $\text{X}^{p*}\text{X}^{16m}$ embryo (Fig. 3A) was examined to find out if the delay in X-inactivation in premigratory PGCs and the enhanced transgene expression in postmigratory PGCs in the genital ridges are due to the presence of cells that contain two active X chromosomes. The X-gal staining pattern of cells in the mid-gut region of the $\text{X}^{p*}\text{X}^{m}$ (Fig. 3B) shows a concentration of X-gal stained cells in the gut endoderm and the genital ridges. Many of these cells are likely to be germ cells which contain two active X chromosomes as revealed by the location of the X-gal stained cells in the gut endoderm and the genital ridges. Most of these cells are expressing the $\beta$-galactosidase is influenced by the rate of degradation of the protein following the cessation of transcription. Some indication of the longevity of the enzyme may be borne in mind when interpreting the data.

**DISCUSSION**

Similar to other studies of X-chromosome activity, results of our study are based on the expression of the protein product and not the transcription of the X-linked gene. Clearly, the determination of the timing of X-inactivation basing on the disappearance of $\beta$-galactosidase is influenced by the rate of degradation of the protein following the cessation of transcription. Some indication of the longevity of the enzyme may be

---

**Fig. 1.** (A) Trophoblastic tissues of a 7.5-day $\text{X}^{p}\text{X}^{16m}$ embryo showing strong alkaline phosphatase (ALP) activity on the cell membrane (dark purple staining) and the localisation of $\beta$-galactosidase ($\beta$-gal) in the nucleus (brown staining). The presence of multiple nuclei in some cells (arrows) is due to the formation of syncytiotrophoblast. (B) ALP-positive premigratory germ cells (PGCs) in the genital ridge of a 13.5-day $\text{X}^{p}\text{X}^{16m}$ embryo showing examples of different immunostaining reactions for $\beta$-gal (arrowheads): cells expressing $\beta$-galactosidase; arrows: cells not expressing the transgene. (C) PGCs (arrowheads) in the primitive streak (ps) and the allantois (al) of a 7.5-day $\text{X}^{p}\text{X}^{16m}$ embryo showing expression of the lacZ transgene. rm, Reichert’s membrane; en, visceral endoderm. (D) PGCs in the gut endoderm (gn) of a 9.5-day embryo showing lacZ expression. Some PGCs in the mesenchyme outside the gut (arrowheads) show weak to negative immunostaining of the nuclei. (E) Migrating PGCs in the mesentery (ms) of a 10.5 day embryo showing mosaic lacZ expression. Arrowheads point at PGCs which display little transgene expression. (F) Expression of the lacZ transgene in PGCs in the genital ridge (gr) at 10.5 days p.c. (G) lacZ expression in the germ cells of a 14.5-day fetal ovary. Arrowheads point at PGCs which display no transgene expression. (H) Examples of germ cells outside the genital ridge (arrowheads) of a 13.5 day embryo showing weak to negative expression of the lacZ transgene. By contrast, most germ cells in the genital ridge (gr) strongly express the reporter gene. $\beta$-gal in the nuclei was detected by immunostaining using diaminobenzidine which gives a brown coloration. ALP activity was revealed by histochemical detection using NBT/BCIP reagents which produces a purplish blue deposit on the cell membrane. Bar, 50 $\mu$m (A,B,C,F,G); bar, 20 $\mu$m (D,E,H).

---

**Fig. 2.** The progression of inactivation and re-activation of the $\text{HMG-\text{lacZ}}$ transgene in the primordial germ cells and meiotic germ cells of hemizygous transgenic female embryos produced by mating H253 mice to (C57BL/6 $\times$ DBA/2)$F_1$ mice. Data are drawn from Table 2. The dashed line at 50% indicates the completion of X-inactivation. The decrease in the percentage of lacZ-expressing PGCs during 8.5 to 10.5 days in extragondadal sites except the gut indicates progressive X-inactivation in the migrating germ cells. The increase in the percentage of PGCs in the genital ridges expressing lacZ transgene from 10.5 days onwards indicates progressive re-activation of the X-linked transgene. By contrast, PGCs that are migrating to the genital ridge and those that remain outside the genital ridges after 12.5 days do not seem to re-activate the transgene.
provided by the pattern of \( \beta \)-galactosidase expression in preimplantation embryos (Tam et al., 1994). At the first two cleavages, \( \beta \)-galactosidase activity in the blastomeres is encoded by the maternal transcript inherited by way of the oocyte cytoplasm. Extinction of enzyme activity is first seen at the 2-cell stage and is completely gone after the 4-cell stage. If the maternal mRNA remains active only until the 2-cell stage (Knowland and Graham, 1972; Braude, 1979; Clegg and Piko, 1982; Flach et al., 1982), then the loss of enzymatic activity by the 4-cell stage might imply that the enzyme is only detectable for 12-18 hours or for one cell division after it is synthesised. Whether a similar longevity of \( \beta \)-galactosidase is found in different somatic and germ cell lineages of the postimplantation embryo is not known. Our studies on the expression of the \( \text{lacZ} \) reporter gene in preimplantation embryos and in somatic tissues of postimplantation embryos and adult mice (Tan et al., 1993;
Tan and Breen, 1993; Tan et al., 1994) have shown that the transgene activity reflects faithfully the pattern of X-chromosome activity during development. Work is in progress to test if the regulation of transgene activity coordinates with other X-linked genes such as Hprt and Pgk-1 located at other loci on the same chromosome. More recently, we have shown that this transgene, if located on the normal X chromosome in female embryos heterozygous for the Searle’s translocation, will be non-randomly inactivated in a manner similar to that displayed by other X-linked genes (Tam, Singer-Sam, Riggs and Tan, unpublished). Furthermore, in the mesodermal and neural tissues that are known to complete X-inactivation at 8.5 days, β-galactosidase activity is not longer detected at 9.5 days in the X<sup>16</sup>X embryos (Tam et al., 1994), suggesting that at least in these tissues, the enzyme activity is not likely to last for more than 24 hours after gene inactivation. The activity of the HMG-<i>lacZ</i> transgene is therefore capable of revealing the trend if not every detail of the progression of X-inactivation and re-activation in the mouse primordial germ cells.

Results of this study provide the most direct evidence for the inactivation and re-activation of an X-linked transgene during the differentiation of the germ-line lineage in the mouse. By following the expression of the transgene in the PGC populations, we have discovered that X-inactivation has occurred in about 25-30% of the PGCs that could be identified by ALP activity during gastrulation. At least 60-70% of the PGCs still possess two active X chromosomes at 8.5 days in the early-embryonic stage embryo. This finding is particularly significant in view of the recent report that both X chromosomes remain active (Stewart et al., 1994) in female embryonic stem cells that are derived from PGCs of 8.5-day embryos (EG cells, Resnick et al., 1992; Matsui et al., 1992). X-inactivation as indicated by the expression of the <i>Xist</i> gene only occurs in XX EG cells upon differentiation into embryoid bodies (Stewart et al., 1994). These EG cells are therefore most likely to be derived from PGCs that have not initiated X-inactivation, raising the possibility that the active status of both X chromosomes may be a necessary prerequisite for maintaining pluripotency of any female stem cell lines (Tada et al., 1993; Monk, 1981; Robertson et al., 1983).

Contrary to previous conclusions that are based on the analysis of the relative level of expression of isozyme (McMahon et al., 1981, 1983) and the inheritance of X-linked coat colour chimaerism (Gardner et al., 1985), X-inactivation is not complete in the PGC population during gastrulation. Embryonic fragments containing PGCs and other somatic cells have been isolated from the primitive streak and allantoic bud of 7.5-day gastrulating mouse embryos. The methylation status of two X-linked genes (<i>Pgk-1</i> and <i>G6pd</i>) has been examined and the CpG islands of both genes are found to be unmethylated (Grant et al., 1992). On the basis that X-inactivation is complete in PGCs at this developmental stage, it has been inferred that the lack of methylation of the X-linked genes may facilitate the subsequent re-activation of these genes in the female germ cells (Monk et al., 1987). Our results, which show the expression of the X-linked transgene in PGCs at 7.5-8.5 days, raise another possibility that the lack of methylation might be due to the fact that X-inactivation has not occurred in most PGCs at 7.5 days when methylation was studied. If this is true, then the methylation of X-linked genes would be detectable in the migrating PGCs at the completion of X-inactivation. This would be followed by a de-methylation step when re-activation of the X-linked genes takes place in intraglandular meiotic germ cells. A proper test of this hypothesis will require the examination of the methylation status of X-linked genes in a pure population of migrating PGCs isolated from the mesentery (Cooke et al., 1993) or on single PGCs in situ.

X-inactivation commences in the PGCs when they are relocated from the epiblast to the primitive streak and the allantois during gastrulation. Further progression to complete X-inactivation seems to be associated with the initiation of migration from the gut endoderm. Results obtained from studying <i>lacZ</i> expression in X<sup>16</sup>X female embryos suggests that about 80% of the PGCs in the gut are positive for β-galactosidase immunostaining. A possible interpretation is that these PGCs have not yet initiated X-inactivation and thus possess two active X chromosomes. In this respect, the PGC population is similar to other somatic tissues such as the heart, the notochord and the hindgut which contain many cells with two active X chromosomes and consequently display a late schedule of X-inactivation (Tan et al., 1993; Tam et al., 1994). X-inactivation is completed asynchronously for the whole PGC population and it happens during the migration of PGCs in the mesentery. At 10.5 days, about 30% of the migrating PGCs in the mesentery has not yet completed inactivation and they were mostly found at the root of the mesentery (Fig. 3D). Re-activation of the silent X chromosome is also not synchronous among the PGCs. The first waves of PGCs reaching the genital ridge at 9.5 days have not re-activated the X chromosome until a day later. A similar lag may also happen when later waves of PGCs arrive at the genital ridge. Re-activation of the X chromosome therefore takes place progressively in the PGC population and first begins about 2-3 days ahead of the onset of meiosis. This sequence of events is similar to that revealed by the changes in the activity of the X-encoded HPRT of germ cells at 11.5 to 17.5 days p.c. (Monk and McLaren, 1981). Ultimately, about 80-90% of the PGC have re-activated the X-linked <i>lacZ</i> transgene as they enter meiosis from 13.5 days onwards. A comparison of the expression of the <i>lacZ</i> transgene on X chromosomes of different parental origin further shows that not only the inactivation process (this study and that of McMahon et al., 1981 basing on PGK-1 isozyme analysis) but the re-activation event also occurs independently of the parental origin of the two X chromosomes of the female PGCs.

Migrating germ cells that are left outside the genital ridge do not seem to re-activate the X chromosome even at 15.5 days. Entry into a genital ridge environment may therefore be a critical trigger to initiate re-activation of the X chromosome. Such a trigger is likely to act locally within the developing ovary. Furthermore, this signal may be relayed through direct cell contact among the PGCs since a recent study has shown that extensive intercellular contact is maintained during the early migration of the PGC and among PGCs in the genital ridge (Gomperts et al., 1994). Ectopic germ cells have been found in the mesonephros and adrenal gland (Francavilla and Zamboni, 1985). In the female fetus, these ectopic germ cells will proceed to meiosis at about 16.5-17.5 days. They will not form functional germ cells and degenerate postnatally (McLaren, 1983b; Upadhyay and Zamboni, 1982). It would be interesting to find out if re-activation of the X chromosome may occur in the ectopic germ cells or not after 15.5 days and if every germ cells in the fetal ovary will eventually have two active X chromosomes. Attempting to enter meiosis without the proper re-activation of the X chromo-
some may underlie the failure to accomplish oogenesis and leads to the massive loss of germ cells in the fetal ovary during normal development (Baker, 1972; McLaren, 1993) and in teratogen-treated fetuses (Tam and Liu, 1985).

This work was supported by project grants from the National Health and Medical Research Council of Australia to PPLT and SST and by a donation from Mr James Fairfax to CMRI. We thank Dr Ian van Driel (Department of Immunology and Pathology, Monash University) for the gift of rabbit anti-β-galactosidase antibody, Dr Ron Weinberger and Ms Jenny Meaney for the advice on immunostaining, and Professor Peter Rowe and Dr Robyn Jamieson for reading the manuscript.

REFERENCES


(Accepted 11 July 1994)