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Original Article

X chromosome inactivation revealed by the X-linked *lacZ* transgene activity in periimplantation mouse embryos

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ABSTRACT Using H253 mouse stock harboring X-linked *HMG-lacZ* transgene, we examined X chromosome inactivation patterns in sectioned early female embryos. X-gal staining patterns were generally consistent with the paternal X inactivation in the trophectoderm and the primitive endoderm cell lineages and random inactivation in the epiblast lineages. The occurrence of embryonic visceral endoderm cells apparently at variance with the paternal X chromosome inactivation in 7.5 dpc embryos was explained by the replacement of visceral endoderm cells with cells of epiblast origin. The frequency of cells negative for X-gal staining in 4.5-5.5 dpc X^mX^p* embryos fluctuated considerably especially in the extraembryonic ectoderm and the primitive endoderm, whereas it was less variable in the embryonic ectoderm. We could not, however, determine whether it is a normal phenomenon revealed for the first time by the use of *HMG-lacZ* transgene or an abnormality caused by the multicopy transgene.

KEY WORDS: *X chromosome inactivation, imprinting, cell lineage, X-gal staining, egg cylinder*

Introduction

One of two X chromosomes is inactivated in each somatic cells of adult mammalian females (Lyon, 1961) to compensate X-linked gene dosage difference between XX females and XY males. Cytogenetic, biochemical and developmental studies indicated that X chromosome inactivation occurs first in the trophectoderm of 3.5 days post coitum (dpc) blastocysts (Takagi, 1974; Monk and Harper, 1978; Kratzer and Gartler, 1978a; Monk, 1978), then in the primitive endoderm of implanting 4.5 dpc blastocysts (West *et al.,* 1977; Kratzer and Gartler, 1978b), and finally in the epiblast or embryonic ectoderm of 5.5 dpc egg cylinders (Gardner and Lyon, 1971; Monk and Harper, 1979; Takagi *et al.,* 1982; Rastan, 1982). Consequently, initiation of X-inactivation has been considered to be coupled with cell differentiation (Monk and Harper, 1979). Either the maternal X (X^M) or the paternal X (X^P) chromosome is randomly inactivated in cells of the epiblast lineage, but the X^P chromosome is preferentially inactivated in the extraembryonic tissues. Although these findings are considered generally correct, one cannot exclude the possibility that a minority of cells behave differently.

Tan *et al.* (1993) developed a mouse stock carrying the ubiquitously expressing X-linked *lacZ* transgene, which is subject to X-inactivation and hence has made it possible to see X chromosome inactivation in histological preparations. Detailed studies using this mouse stock disclosed several new findings that could otherwise be hardly obtained. A small number of cells, for example, may have an active X^P chromosome in the trophectoderm and primitive endoderm lineages that are characterized by the imprinted X^P-inactivation. Furthermore, random inactivation of the X^P and the X^M is completed at different times in different tissues of the epiblast lineage. Cells having two active X chromosomes are still present in such tissues as the notochord, the heart and the embryonic gut of 9.5 dpc embryos (Tan *et al.,* 1993).

In this study we employed the H253 stock to examine X chromosome inactivation in early mouse embryos with the aid of resin sectioning that increases resolution and allows better orientation of small embryos under examination than paraffin sectioning. Our main aim was to test the temporal and spatial rigidity with which X-inactivation is initiated in mouse embryogenesis previously established by various techniques, especially because Tan *et al.* (1993) found certain variability in extraembryonic tissues of

Abbreviations used in this paper: X^m, maternal X chromosome; X^p, paternal X chromosome; T16H, Searle's T(X,16)16H translocation; β-gal, β-galactosidase; X-gal, 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside; HMG, 3-hydroxy-3-methylglutaryl coenzyme A.

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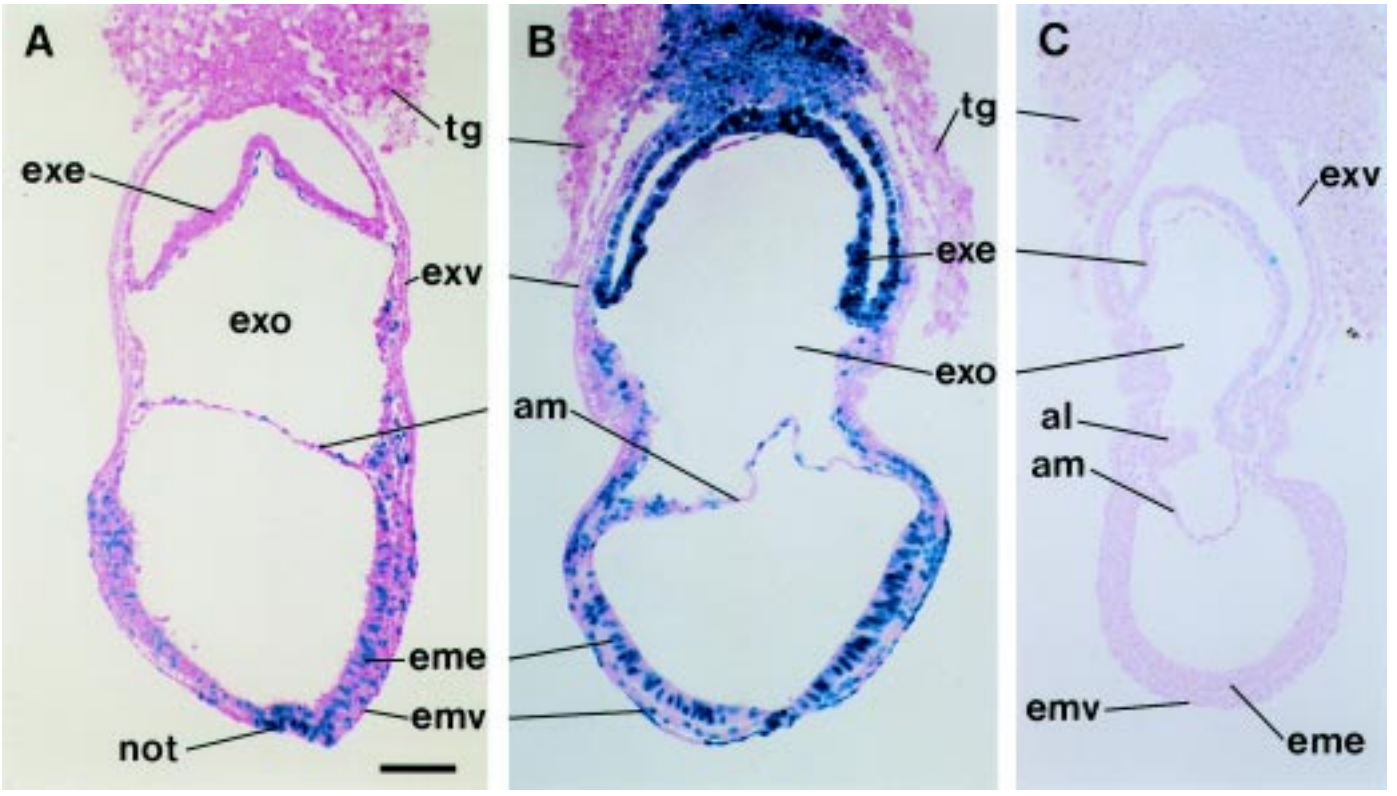

Fig. 1. Imprinted and random X-inactivation patterns revealed by X-gal histochemical staining in 7.5 dpc female mouse embryos hemizygous for X-linked *HMG-lacZ* transgene. *Embryo carrying the transgene on the paternally derived X chromosome ($X^{M}X^{P}$*) is shown in* **(A)** *and that carrying it on the maternally derived X chromosome (X^{M}*X^{P}) is shown in* **(B)**. *The transgene is carried on the paternally derived X chromosome in the embryo shown in* **(C)**, *but it also has Searle's T16H translocation. In the karyotypically normal embryos shown in (A) and (B), the paternal X chromosome is non-randomly inactivated in the extraembryonic ectoderm (exe) and extraembryonic visceral endoderm (exv), but inactivation is random in the embryonic ectoderm (eme) and mesodermal tissues. The entire T16H/+ embryo except the extraembryonic ectoderm is devoid of β-gal positive cells. Sections (A) and (B) were counterstained with eosin, but section (C) was counterstained with nuclear fast red. Bar, 100 μm. al, allantois; am, amnion; emv, embryonic visceral endoderm; exo, exocoelom; tg, trophoblast giant cell.*

5.5 dpc embryos. It was also expected to determine the temporal relation between the initiation of asynchronous X chromosome replication and the advent of β-gal negative cells in three different cell lineages in the female periimplantation embryos carrying paternally inherited *lacZ* transgene. We report here the possibility that the embryo has considerable latitude in the initiation of X-inactivation with respect to its size or the stage of development.

Results

Tissue specific expression pattern of HMG-lacZ in 7.5 dpc embryos

As shown in Figure 1A, $X^{M}X^{P}$* embryos recovered from wild type females mated with H253 males at 7.5 dpc typically showed nonrandom and random X chromosome inactivation in different tissues. Although the density of X-gal staining varied considerably from cell to cell, we judged cells positive for X-gal staining if their nuclei were bluer than those of trophoblast giant cells and parietal endoderm cells that were completely β-gal negative for unknown reasons (Fig. 1A and B). The extraembryonic ectoderm and extraembryonic visceral endoderm were mostly β-gal negative, but β-gal positive cells were frequently observed in the embryonic region of the visceral endoderm. The variegated X-gal staining pattern suggestive of random inactivation was evident in every tissue of the epiblast lineage, i.e. the embryonic ectoderm, mesodermal tissues including the allantois, amnion and the thin layer lining the exocoelom (Fig. 1A and B). In agreement with previous reports (Tam *et al.*, 1994a), the proportion of β-gal positive cells was high in the notochord (Fig. 1A).

Consistent with nonrandom inactivation of the X^{P}, the extraembryonic ectoderm and the extraembryonic visceral endoderm were uniformly β-gal positive, although the β-gal activity was considerably low in cells of the latter tissue in X^{M}*X^{P} embryos (Fig. 1B). The mosaic β-gal expression in tissues of the epiblast lineage closely resembled that observed in $X^{M}X^{P}$* embryos supporting the occurrence of random inactivation. In parallel with the finding mentioned above in $X^{M}X^{P}$* embryos, β-gal negative cells were not rare in the embryonic visceral endoderm of X^{M}*X^{P} embryos, whereas such β-gal negative cells were never found in X^{M}*Y embryos. By the same token, we never found β-gal-positive cells in the embryonic visceral endoderm from T16H/+ female embryos carrying the *HMG-lacZ* transgene on the paternally derived normal X chromosome (Fig. 1C). In spite of hemizygosity for the *lacZ* transgene, such embryos were mostly negative for β-gal activity heralding complete non-random inactivation of the intact X chromosome characterizing postnatal T16H/+ animals (Lyon *et al.* 1964). In the extraembryonic ectoderm, however, β-gal positive cells were often found for presently unknown reason.

X-inactivation in earlier embryos

X-gal staining pattern was further studied in X^MX^{P*} embryos at earlier developmental stages to examine the process that accomplishes the inactivation pattern observed in 7.5 dpc embryos. There was an apparent tendency that the β-gal negative cell became detectable first in the trophectoderm, second in the visceral endoderm and last in the embryonic ectoderm in agreement with findings from previous studies. In implanting 4.5 dpc X^MX^{P*} blastocysts, for example, almost all epiblast cells and primitive endoderm cells were β-gal positive, whereas a proportion of β-gal negative cells were observed in the polar trophectoderm (Fig. 2A).

The frequency of β-gal negative cells fluctuated extensively thereafter in the extraembryonic ectoderm of most 5.5 dpc X^MX^{P*} embryos. In typical cases, β-gal negative cells outnumbered β-gal positive cells in this tissue. The frequency of β-gal positive cells also varied considerably in the primitive endoderm, whereas most epiblast cells were strongly β-gal positive (Fig. 2B). β-gal negative cells in the embryonic ectoderm appeared for the first time at 6.5 dpc (Fig. 2C).

Growth of individual postimplantation embryos varies within and between litters. Probably, the size of embryos rather than their chronological age estimated from the assumption that ovulation and fertilization occurs about the midpoint of dark cycle, may better correlate with the progression of X-inactivation. Hence, we arranged the data with reference to the length of the dorsoventral axis of each embryo excluding the ectoplacental cone to deduce the course of X-inactivation. If X chromosome is inactivated simultaneously throughout the trophectoderm in fully expanded blastocysts, the frequency of the β-gal positive cell would decrease with increasing size of the embryo along a sigmoid curve. The midpoint of the descending curve should correspond to the average stage of embryo in which X-inactivation defined by the disappearance of β-gal activity finished in that tissue.

Contrary to the expectation, we found it difficult to draw a simple sigmoid curve because the frequency of β-gal positive cells fluctuated too much in the extraembryonic ectoderm and in the visceral endoderm, as shown in Figure 3A and B. We suspected that technical factors account for this unexpected finding. However, further scrutiny of our data suggested that the contribution of such factors is minimal, if any. For example, four embryos represented by filled triangles were from the same litter which were fixed, stained and embedded together minimizing possible occurrence of technical variation among littermates. Although their sizes were almost the same, the proportion of the β-gal negative cells differed noticeably among them. Embryos represented by $^+$▲ and *▲ in Figure 3A-C correspond to those shown in Figure 2B and D, respectively. It is worth while noting that the strength of color produced by X-gal reaction was identical in embryonic ectoderm cells irrespective of the frequency of β-gal negative cells in the extraembryonic ectoderm.

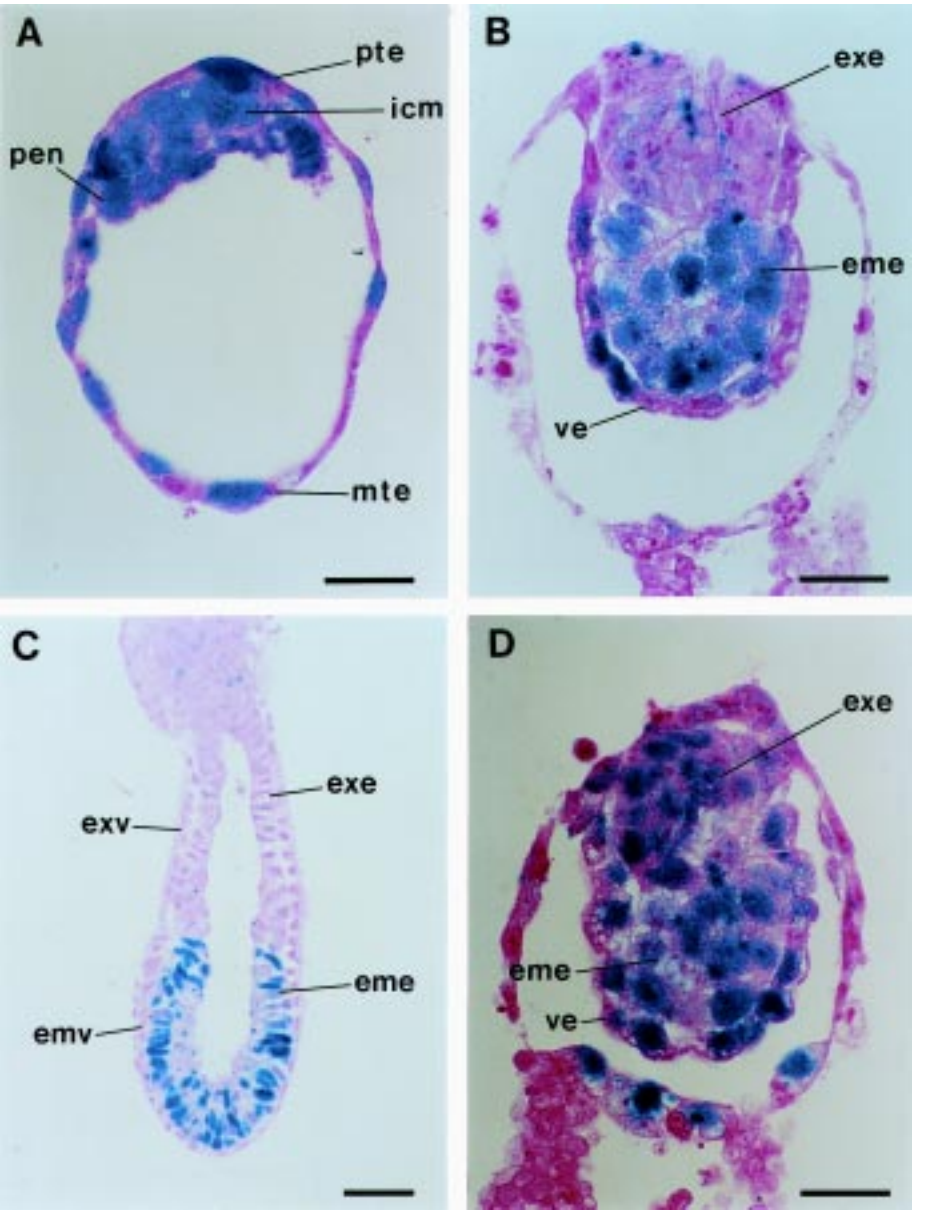

Fig. 2. *HMG-lacZ* expression pattern in $X^{P*}X^M$ embryos during early development. *All cells are β-gal positive in a 4.5 dpc blastocyst differentiating the primitive endoderm (pen)* **(A)**. *In an embryo collected at 5.0-5.5 dpc* **(B)**, *all embryonic ectoderm cells (eme) are β-gal positive, but a number of β-gal negative cells are present in the extraembryonic ectoderm (exe) and visceral endoderm (ve). Extraembryonic ectoderm and visceral endoderm cells are completely negative for β-gal in 6.5 dpc embryos* **(C)**. *β-gal negative cells appear in the embryonic ectoderm for the first time at this stage.* **(D)** *A littermate of the embryo shown in (B). The proportion of β-gal positive cell considerably differs between these littermates especially in the extraembryonic ectoderm and visceral endoderm. emv, embryonic visceral endoderm; exv, extraembryonic visceral endoderm; icm, inner cell mass; mte, mural trophectoderm; pte, polar trophectoderm. Bar, 25 μm.*

Thus, it is difficult to attribute the apparent difference in the frequency of β-gal positive cells to variables associated with various steps from fixation to embedding of embryos.

The situation was almost the same in the visceral endoderm (Fig. 3B). Note that all visceral endoderm cells were β-gal positive in certain embryos 100 μm in length, but the same tissue was totally unstained in embryos of comparable sizes (Fig. 3B). In this tissue again, it was impossible to draw a simple curve delineating the relation between the decrease of β-gal positive cells and the size of embryos. Thus, it was difficult to estimate the accurate time lag between chromosomal inactivation defined by replication asynchrony or heterochromatinization of the X chromosome and disappearance of β-gal activity in these tissues. The embryonic ectoderm showed less variability with clear transition at embryos 200 μm in size (Fig. 3C). Thus the time lag between chromosomal inactivation and loss of β-gal activity should be less than 24 h.

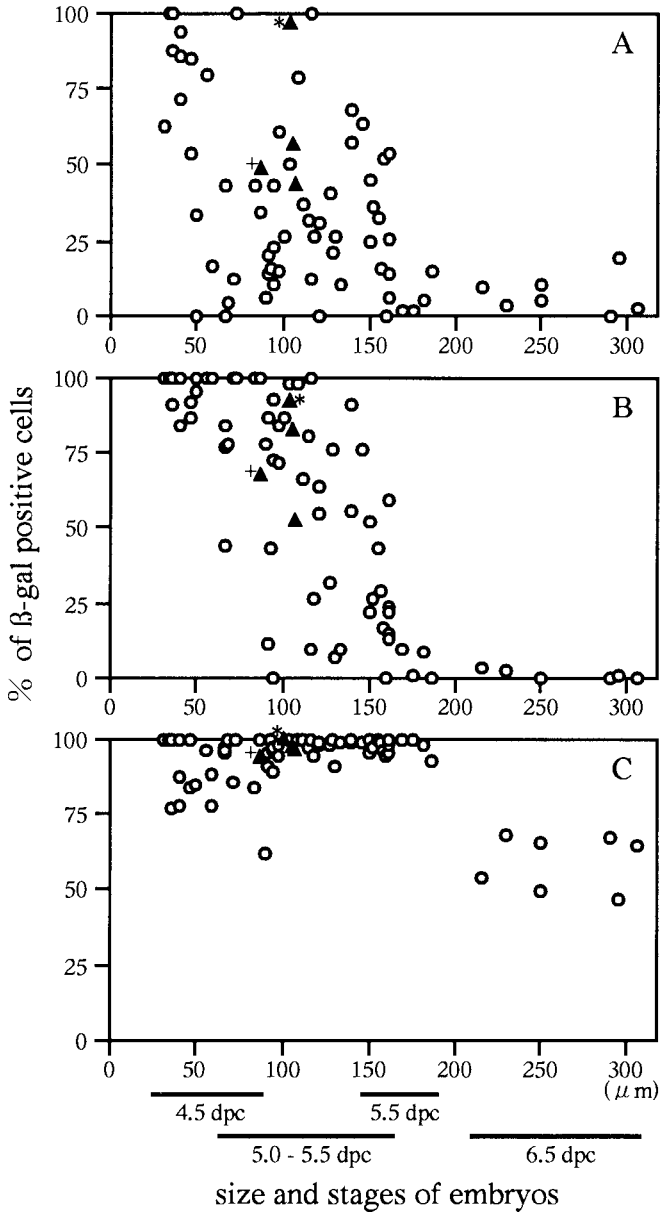

Fig. 3. Initiation of X-inactivation in the extraembryonic ectoderm (A), visceral endoderm (B), and embryonic ectoderm (C) of $X^M X^{P*}$ embryos disclosed by the loss of β-gal activity during periimplantation development. *The vertical axis shows the percentage of β-gal positive cells and the horizontal axis represents the dorsoventral length of embryos. Gestation days are also indicated. Embryos shown by solid triangles were derived from the same litter. +▲ and *▲ correspond to the embryo shown in Figure 1B and D, respectively.*

Discussion

Expression of *HMG-lacZ* transgene is by no means a perfect chronometer of X inactivation as previous studies suggested (Tan *et al.*, 1993; Tam *et al.*, 1994a). However, the H253 mice has considerably improved the level of resolution in the study of X chromosome inactivation, and provided a number of novel findings because under certain conditions all cells are potentially informative as to the activity state of the X chromosome in mice (Tan *et al.*, 1993; Tam *et al.*, 1994a,b). This is the invaluable merit because techniques used thus far tended to rely on a minority of cells composing the entire embryo, e.g. metaphase cells incorporated 5-bromo-2-deoxyuridine in the S phase, or ignoring a minority of cells, e.g. extract from the whole embryo or tissue disregarding individual cells.

Present findings were generally compatible with preferential X^P-inactivation in the extraembryonic ectoderm and extraembryonic visceral endoderm, and random inactivation in the embryonic ectoderm as shown by Tam *et al.* (1994a). An interesting exception is that there were a substantial number of β-gal positive cells in embryonic visceral endoderm of $X^M X^{P*}$ embryos, and β-gal negative cells in that of $X^{M*}X^P$ embryos contrary to nonrandom X^P inactivation reported previously. The present study showed that, at least in $X^M X^{P*}$ embryos, the paternally derived *HMG-lacZ* transgene has been inactivated both in the embryonic and extraembryonic region of the visceral endoderm by 6.5 dpc. Does activation of the inactive X^P and *de novo* inactivation of the X^M has occurred in this tissue or is it elucidated in a different way? Previous studies indicated that three germ layers of the fetus are exclusively of epiblast origin (Gardner and Papaioannou, 1975). Furthermore, clonal analysis of epiblast fate by injecting horseradish peroxidase into single cells (Lawson *et al.*, 1991) provided evidence for insertion of epiblast-derived cells into the endoderm layer by midstreak to late streak stages. Since the inactivated X chromosome is extremely stable, cellular replacement would be a more likely explanation. This view is supported by the observation that the embryonic visceral endoderm cells were consistently β-gal negative in 7.5 dpc T16H/+ embryos in which the normal X chromosome bearing *lacZ* transgene is inactivated in all somatic cells.

This study showed that the timing of X-inactivation judged by the loss of β-gal activity in $X^M X^{P*}$ embryos may vary among different embryos. It is possible that this observation has disclosed hitherto unknown heterogeneity in the progression of X-inactivation in the trophectoderm and primitive endoderm cell lineages of mouse embryos. A possible factor that contributed to the apparently asynchronous X-inactivation would be delayed activation of the X^P together with the *HMG-lacZ* transgene after fertilization (Tam *et al.*, 1994a). The occurrence of a considerable number of β-gal negative cells in certain 4.5 dpc embryos is consistent with delayed activation. An alternative explanation for the observed variability would be a peculiarity of multicopy transgenes. Expression from

transgenes on the inactivated X chromosome is not rare. So far as we are aware, three of five X-linked transgenes studied were shown to escape X inactivation totally or partially (Krumlauf *et al.*, 1986; Goldman *et al.*, 1987; Wu *et al.*, 1992). Tan *et al.* (1993) showed that a proportion of fetal cells were positive for X-gal staining in 9.5 dpc female embryos carrying T(X;16)16H and *HMG-lacZ* transgene in which the X^{P}* is inactivated selectively. Results of a sensitive SNuPE assay (Lebon *et al.,* 1995) showed that *HMG-lacZ* transgenes could remain active on the inactivated X chromosome in certain cells under certain conditions. In the absence of further knowledge about the regulation of *HMG-lacZ* expression particularly in early embryonic stages, it is difficult to judge which alternative is more likely.

In spite of close correlation between X-inactivation and DNA methylation in somatic cells (Pfeifer *et al.*, 1990; Norris *et al.*, 1991), such correlation is not present in certain extraembryonic tissues (Kratzer *et al.*, 1983). It has also been shown that X-inactivation is less stable in cells of chorionic villi than in somatic cells of epiblast origin (Migeon *et al.*, 1986). Interesting in this context is the finding that the chicken transferrin transgene is subject to X-inactivation in the fetus but escapes it in the yolk sac (Goldman *et al.*, 1987) where DNA methylation is not involved in X-inactivation. The present study showed that the timing of X-inactivation examined by X-gal histochemistry is much more variable in two extraembryonic tissues than in the embryonic ectoderm in which X-inactivation involves DNA methylation. Therefore, it is tempting to speculate that the absence of DNA methylation is correlated with the extensive variability in the frequency of β-gal negative cells in the derivatives of the polar trophectoderm and the primitive endoderm. It is also likely that the *lacZ* gene of *E. coli* origin is methylated upon integration into mouse genome. The variable degree of methylation among different tissues and cells may be responsible for the irregular expression of β-gal in these tissues. Future investigation will possibly show critical roles of DNA methylation played for the variability stability and completeness of X-inactivation.

Materials and Methods

H253 transgenic mouse stock generated by Tan *et al.* (1993) carries 14 tandem copies of an 8.9 kb DNA fragment containing the promoter of a mouse housekeeping gene, 3-hydroxy-3-methylglutaryl coenzyme A reductase, an SV40 T antigen nucleus localization signal sequence and *E. coli lacZ* gene *(HMG-lacZ)*. Under the control of the ubiquitously active promoter, the *lacZ* gene encodes nucleus-localized β-galactosidase (β-gal) which is readily detectable by X-gal histochemistry in individual nuclei. These transgenes integrated in the A6 region of the X chromosome (Tam *et al.*, 1994a) are subject to X-inactivation (Tan *et al.*, 1993).

To estimate the inactivation time based on the disappearance of β-gal activity in the trophectoderm and the primitive endoderm cell lineage, we produced hemizygous female embryos carrying the *HMG-lacZ* transgene either on the paternally derived X chromosome (X^{M} X^{P}*) or the maternally derived one (X^{M}*X^{P}) from crosses of Wild type ♀X H253 ♂ and ♀XH253 Wild type ♂, respectively. The H253 stock has C57BL/6 and DBA/2 genetic background, whereas wild type females used in this study were from the inbred X0 stock of C57BL/6 and CBA/J genetic background maintained at our laboratory. These XX females showed typical tabby stripes indicating that X-inactivation was not skewed significantly. Female mice carrying Searle's T(X;16)16H translocation (abbreviated hereafter to T16H) were mated with H253 males to obtain female embryos heterozygous for the translocation (T16H/+) carrying *HMG-lacZ* transgene on the normal X chromosome.

Embryos were recovered from females at 4.5-7.5 days post coitum (dpc). Recovered embryos were fixed in 4% paraformaldehyde in PBS for 0.5-1.0 h at 4°C. Embryos were washed in rinse buffer (1/15M Sorensen's phosphate buffer, pH 7.0 containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40), and stained in the rinse buffer containing 1mg/ml 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide overnight at 37°C. Embryos postfixed overnight in 10% formalin were dehydrated through the graded ethanol series and embedded in JB-4 resin (Electron Microscopy Sciences). The specimens were sectioned at 2-4 μm in thickness with glass knives, and sections were counterstained with eosin or nuclear fast red. The proportions of the β-gal positive and β-gal negative cells were determined under a phase contrast microscope.

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