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 XmXp* 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. Cyto genetic, 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, 1976; 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 (Xm) or the paternal X (Xp) chromosome is randomly inactivated in cells of the epiblast lineage, but the Xp 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 Xp chromosome in the trophectoderm and primitive endoderm lineages that are characterized by the imprinted Xp-inactivation. Furthermore, random inactivation of the Xm and the Xp 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: Xm, maternal X chromosome; Xp, 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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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\textsuperscript{M}X\textsuperscript{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). 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\textsuperscript{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\textsuperscript{M}\textsuperscript{X*} embryos supporting the occurrence of random inactivation. In parallel with the finding mentioned above in X\textsuperscript{M}\textsuperscript{X*} embryos, β-gal negative cells were not rare in the embryonic visceral endoderm of X\textsuperscript{M}\textsuperscript{X*} embryos, whereas such β-gal negative cells were never found in X\textsuperscript{M}\textsuperscript{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<sup>MX<sup>XP<sup>±</sup> 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<sup>MX<sup>XP<sup>±</sup> 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<sup>MX<sup>XP<sup>±</sup> 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 dorsocentral 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 litters. 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.
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.

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 Xp-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\textsuperscript{M}X\textsuperscript{P}* embryos, and β-gal negative cells in that of X\textsuperscript{M}*X\textsuperscript{P} embryos contrary to nonrandom Xp inactivation reported previously. The present study showed that, at least in X\textsuperscript{M}X\textsuperscript{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 Xp and de novo inactivation of the X\textsuperscript{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\textsuperscript{M}X\textsuperscript{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 Xp 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<sup>n</sup> 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). Interestingly 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<sup>n</sup>X<sup>P</sup>) or the maternally derived one (X<sup>M</sup>*X<sup>P</sup>) from crosses of Wild type derived one (X<sup>M</sup>*X<sup>P</sup>) carrying HMG-lacZ transgene and 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<sub>2</sub>, 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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