X chromosome retains the memory of its parental origin in murine embryonic stem cells

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SUMMARY

A cytogenetic and biochemical study of balloon-like cystic embryoid bodies, formed by newly established embryonic stem (ES) cell lines having a cytogenetically or genetically marked X chromosome, revealed that the paternally derived X chromosome was inactivated in the majority of cells in the yolk sac-like mural region consisting of the visceral endoderm and mesoderm. The nonrandomness was less evident in the more solid polar region containing the ectodermal vesicle, mesoderm and visceral endoderm. Since the same was true in embryoid bodies derived from ES cells at the 30th subculture generation, it was concluded that the imprinting responsible for the preferential inactivation of the paternal X chromosome that was limited to non-epiblast cells of the female mouse embryos, was stably maintained in undifferentiated ES cells. Differentiating epiblast cells should be able to erase or avoid responding to the imprint.

Key words: mouse, embryoid body, embryonic stem cell, X chromosome, inactivation, imprinting

INTRODUCTION

In female mammals the activity of X chromosomes is under a unique developmental regulation that allows only one X to express in each cell (Lyon, 1961). This is accomplished by inactivation of one X chromosome early in embryogenesis. In individuals with supernumerary X chromosomes, all X chromosomes except one become inactivated. In spite of continuous efforts the mechanisms determining which X chromosome is to be inactivated remains totally unknown and has been the subject of extensive reviews (Gartler and Riggs, 1983; Grant and Chapman, 1988; Lyon, 1988). Studies in man and mice with structurally altered X chromosomes have indicated that inactivation is initiated at a single X chromosomal locus, the X-inactivation center (Xic), and it then spreads to the rest of the chromosome (Russell, 1964; Therman, 1974). It has been postulated that Xic is also involved in the determination of which X chromosome is to be inactivated. Genetic studies in mice revealed that alleles of X-chromosome controlling element (Xce) locus, a candidate for Xic, affect the proportion of two cell populations produced by X inactivation (Cattanach, 1975).

Cytogenetic and biochemical studies on early mouse embryos demonstrated that the two X chromosomes in female cells behave differently during development, according to their parental origin. Only Xp (paternally derived X chromosome) is inactivated in cells of the extraembryonic membranes derived from the trophectoderm and primitive endoderm, whereas either Xm (maternally derived X chromosome) or Xp is inactivated basically at random in cells of the definitive embryo derived from the embryonic ectoderm or epiblast (Takagi and Sasaki, 1975; West et al., 1977). X inactivation occurs 1 or 2 days earlier in the trophectoderm and the primitive endoderm cell lineages than in the epiblast cell lineage (Kratzer and Gartler, 1978; Monk, 1978; Monk and Harper, 1979; Sugawara et al., 1983). Hence, it has been suggested that the X chromosome is differentially modified or imprinted during gametogenesis. Persistence of an imprint would ensure inactivation of Xp in the trophectoderm and primitive endoderm and its erasure would make inactivation random in the epiblast (Gartler and Riggs, 1983; Grant and Chapman, 1988). Another possibility would be that the imprint is stably transmitted but it is ignored at inactivation in the epiblast cell lineage (Lyon, 1989).

We set out in this study to examine the nature of this parental modification using seven newly isolated mouse ES cell lines, six of which are heterozygous for X-linked markers. It was found that Xp was inactivated in the overwhelming majority of visceral endoderm cells differentiated from ES cells in vitro. Available evidence showed that Xm, inactive cells were more abundant in the mesoderm and embryonic ectoderm than in the visceral endoderm. Since these findings hold true even after 30 consecutive passages in vitro, the inevitable conclusion would be that the parental imprint, responsible for the nonrandom inactivation of Xp in mouse extraembryonic tissues, is maintained stably in ES cells. However, the present study could not determine whether the modification is erased or ignored when X inactivation occurs in the differentiating epiblast cells.
MATERIALS AND METHODS

Establishment and maintenance of ES cell lines

Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10^{-4} M 2-mercaptoethanol, non-essential amino acids, and 1000 units/ml recombinant leukemia inhibitory factor (AMRAD) was used throughout the establishment and maintenance of ES cell lines. ES cell lines were established from 3.5 days post coitum (dpc) blastocysts according to the method described by Robertson (1987). 4 days after the commencement of in vitro culture of blastocysts, growing inner cell mass derived cell clumps were individually disaggregated by 0.25% trypsin/0.4% EDTA and allowed to grow in the 24-well plate for 5-8 days. After the second round of disaggregation and growth for variable periods, cells were transferred to 35 mm Petri dishes. Usually, within a few days, ES cells increased in number enough for the expansion into a 60 mm dish. The cell line was considered to be established at this stage. Subsequently, each cell line was maintained by refeeding twice a day, and subculturing every 2-3 days at the splitting ratio of 1:4.

Cell lines

Seven ES cell lines, established as described above, were used in this study. TMA-2 is heterozygous for T(X;4)37H (Searle et al., 1983) the morphologically normal X chromosome originating from the paternal C3H/He strain. Translocation chromosomes are easily recognized because the larger one (4^{X}) is distinctly the largest element in the karyotype and the smaller one (X^{E}) is smaller than the smallest autosome. Three cell lines, TMA-29, 30, and 32, were established from blastocysts obtained from the cross between female mice homozygous for Rb2Ad (Adler et al., 1989) and 129/Sv males. The maternally derived Rb2Ad chromosome is the only metacentric element in the cell. Blastocysts obtained from 129/Sv females (Pgk-1/Pgk-1) mated with C3H/HeHa males (Pgk-1/Pgk-1) gave rise to Pgk-1/Pgk-1 cell lines TMA-45 and 46, TMA-16 has the 129/Sv genetic background. The parental origin of two X chromosomes in TMA-16, 45, and 46 cannot be distinguished microscopically.

Formation of cystic embryoid bodies

Cystic embryoid bodies were produced according to the method described by Martin et al. (1977) and Robertson (1987). About 10^7 ES cells, maintained in an undifferentiated state, were seeded into a 90 mm bacteriological Petri dish to which they did not adhere. Cell aggregates appeared overnight and transformed into simple embryoid bodies, consisting of the outer endoderm layer and the inner mesodermal layer lining it, with occasional blood-filled capillaries. The amount of mesodermal cells relative to endodermal cells varied extensively in each embryoid body. The more solid, embryo-like polar region consisted of the ectodermal vesicle, mesoderm cells, Reichert’s membrane-like material and the outermost layer of the visceral endoderm. The proportion of these components also varied considerably from one embryoid body to the other.

Preferential X_P inactivation in the mural region of the embryoid body

The initial observation made on the mural region of balloon-like cystic embryoid bodies derived from TMA-2 at passage 3 revealed that X_P chromosome was inactivated in 1292 cells, whereas X_M was inactivated in only 147 cells, which was a significant departure from a ratio 1:1. This observation, suggesting preferential X_P inactivation and hence retention of the putative imprinting by the X chromosome of an established ES cell line after a series of cell divisions in vitro, prompted us to carry out more extensive cytogenetic studies in TMA-2 and other newly isolated ES cell lines with cytogenetically marked X chromosomes. A summary of chromosomal findings obtained
from separate cystic embryoid bodies formed by four ES cell lines is shown in Table 1. Data from samples yielding less than 10 informative metaphase cells were discarded. The frequency of cells having an inactivated X chromosome was relatively constant in embryoid bodies derived from TMA-2 at passages 3, 10, 20 and 30 (embryoid bodies will be designated by the passage number at which the differentiation experiment was initiated, to simplify description), although the efficiency to form the cystic embryoid body declined with in vitro passages. X inactive cells predominated mural specimens from TMA-2 cystic embryoid bodies at passages 10, 20 and 30, as
detected at passage 3. Almost all the same results were obtained in the three remaining cell lines, although experiments were limited to passages 3 and 10. Cytologically inactivated X chromosomes were mostly late replicating, but precociously replicating ones were not uncommon in these samples (Fig. 2).

Mitotic activity was relatively low in the polar solid region in 2- to 3-week-old balloon-like cystic embryoid bodies. Hence, the number of specimens yielding at least 10 informative metaphase cells was fewer than in the mural specimens. The proportion of metaphase cells having an inactivated X chromosome in the polar region was, however, roughly equal to that in the mural tissue. Interestingly, it was found that the frequency of X\textsuperscript{P}-inactive cells was considerably low in this region: 143 X\textsubscript{M}-inactive cells, compared with more than 84 X\textsuperscript{P}-inactive cells in TMA-2 cystic embryoid bodies at passage 10, but data at passage 20 were consistent with random inactivation. The differences between the mural and the polar regions were statistically significant in TMA-2 embryoid bodies at passages 10 and 20. Shifts toward the same direction were also evident in the polar region of embryoid bodies formed by TMA-29, 30 and 32, although decrease in the frequency of X\textsuperscript{P}-inactive cells in the polar region was not as striking as in TMA-2 (Table 1). In fact, the frequency of X\textsuperscript{P}-inactive cells was significantly higher than that of X\textsubscript{M}-inactive cells in each case. However, differences between the mural and the polar regions were statistically significant, except TMA-30 embryoid bodies at passage 3 ($\chi^2=1.77, P>0.1$), and TMA-32 embryoid bodies at passage 3 ($\chi^2=0.88, P>0.25$).

Cytogenetic findings in individual polar and mural specimens are shown in Fig. 3. Although X\textsuperscript{P} inactivation was predominant in the mural regions of the majority of cystic embryoid bodies, polar specimens showing a similar
skewed X-inactivation pattern were also present. Similarly, certain mural samples were characterized by low frequencies of cells showing X<sup>p</sup> inactivation. Observation of control in vivo and cultured cells confirmed the occurrence of X<sup>p</sup> inactivation in nearly 50% of cells belonging to the epiblast or fetal cell lineage and in over 90% of cells from the extraembryonic cell lineages (Table 2) in accord with previous reports (Takagi and Sasaki, 1975). The reasons for the rather wide variation in embryoid bodies remains to be determined but a possible explanation would be variable contribution of cells belonging to different cell lineages in individual samples.

Fig. 4 is a scatter diagram showing the relationship between the proportion of X<sup>p</sup>-inactive cells in the mural region and that in the polar region of the same embryoid body. Only 23 embryoid bodies from four ES cell lines were available for comparison. The proportion of X<sup>p</sup>-inactive cells was higher in the mural region than in the polar region in 18 embryoid bodies, which was reminiscent of the situation found in the yolk sac and the fetus of the postimplantation embryos. In four embryoid bodies, the proportion was nearly equal in both regions. The remaining point represented an exceptional embryoid body showing preferential X<sup>M</sup> inactivation in nearly 50% of cells belonging to the extraembryonic cell lineages (Table 2) in accord with previous reports (Takagi and Sasaki, 1975). The reasons for the rather wide variation in embryoid bodies remains to be determined but a possible explanation would be variable contribution of cells belonging to different cell lineages in individual samples.

<table>
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<th>No. of cells with</th>
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<td></td>
<td>LRX&lt;sup&gt;M&lt;/sup&gt;</td>
<td>PRX&lt;sup&gt;p&lt;/sup&gt;</td>
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X<sup>M</sup>: maternally derived translocation X chromosome, X<sup>p</sup> in T37H/+ and Rb2 in Rb2Ad/+ cells, X<sup>p</sup>: paternally derived morphologically normal X chromosome.

Biochemical verification of preferential X<sup>p</sup> inactivation

We attempted to verify the above cytological findings by the expression of Pgk-1 alleles in TMA-45 and TMA-46. Pgk-1<sup>a</sup> allele closely linked to Xce was carried by X<sup>p</sup> and Pgk-1<sup>b</sup> by X<sup>M</sup> in both cell lines. A cytological observation made immediately before the initiation of differentiation confirmed that two X chromosomes were synchronously replicating in almost all cells of each cell line. The Cellogel electrophoresis allowed us to distinguish 5 classes of PGK-1 mosaic expression: exclusive PGK-1B expression, PGK-1B>PGK-1A, PGK-IB=PGK-1A, PGK-1A>PGK-1B, and exclusive PGK-1A expression. PGK-1A and PGK-IB bands stained with similar intensity in undifferentiated TMA-45 (Fig. 5) and TMA-46 substantiated that two X chromosomes in these cell lines were genetically active before differentiation. Examination of mural specimens from 51 TMA-45 cystic embryoid bodies revealed either exclusive or predominant expression of the maternal PGK-IB in 18, and equal PGK-1A and PGK-1B expression in 10 specimens (Table 3). This table also shows that the mosaic expression of PGK-1 isozymes shifted slightly in favor of PGK-1A in the polar specimens, implying increase in the proportion of cells with inactivated X<sup>M</sup> carrying the Pgk-1<sup>b</sup> allele. It is likely that the presence of considerable numbers of cells in which X inactivation had not occurred made the difference between the mural and the polar region less distinct than it in fact was. Statistical analysis was confined to 23 TMA-45 or TMA-46 embryoid bodies, two regions of which were successfully examined by electrophoresis (Fig. 6). The mural and the polar regions were ranked relative to the B form of PGK-1 for Dixon and Mood’s sign test. Results (χ<sup>2</sup>=9.39, P<0.01) seemed to verify that the proportion of PGK-1B was higher and, hence, X<sup>p</sup> was inactivated more frequently in the mural region than in the polar region.

Knowledge of mosaic expression of PGK-1 in vivo is needed for the evaluation of the above findings, since we do not know which Xce allele is on X<sup>M</sup> of 129/Sv origin. A study of 7.5 dpc embryos from the cross between 129/Sv
females and C3H/HeHa males showed that PGK-1A was slightly in excess of PGK-1B in the embryonic region, probably because the C3H X chromosome (Pgk-1\textsuperscript{a}) carried Xce\textsuperscript{c} and the 129/Sv X chromosome (Pgk-1\textsuperscript{b}) carried either Xce\textsuperscript{a} or Xce\textsuperscript{b} as expected (Fig. 5). Only PGK-1B was detected in ectoplacental cone. The situation in the extraembryonic region was inbetween that of the ectoplacental cone and the embryonic region. Hence it may be reasonable to assume that preferential X\textsuperscript{P} inactivation indeed occurred in specimens showing exclusive PGK-1B, PGK-lB>PGK-lA, or PGK-1A=PGK-lB expression.

Effects of 5-azacytidine treatment of ES cells

It is conceivable that differential DNA methylation is somehow related to the preferential inactivation of X\textsuperscript{P} in the mural region of the cystic embryoid body. Hence, we examined the effect of demethylation on the choice of the X chromosome to be inactivated in TMA-2. The undifferentiated ES cells at passage 4 were treated with 2 \( \mu \)M 5-azacytidine (5AC) for 24 and 48 hours. Cells were allowed to initiate differentiation in suspension culture 24 hours after the termination of the drug treatment. 5AC-treated cells formed balloon-like cystic embryoid bodies in exactly the same manner as untreated TMA-2. Chromosomal examination revealed that X\textsuperscript{P} was inactivated in 696 out of 860 cells obtained from mural regions of cystic embryoid bodies formed by cells exposed to 5AC for 24 hours. The result was essentially the same in cells treated for 48 hours. Thus, we could not obtain positive evidence that DNA methylation was involved in the preferential X\textsuperscript{P} inactivation in the ESC-derived cystic embryoid body under the conditions tested.

Multicellular origin of the embryoid bodies

It is formally possible that the prevalence of X\textsuperscript{P} inactivation in the mural region of the cystic embryoid body resulted from clonal proliferation of an X\textsuperscript{P}-inactive progenitor cell rather than preferential inactivation of X\textsuperscript{P}. To test this possibility, we produced chimeric embryoid bodies by mixing two (TMA-16 and TMA-30) or three (TMA-2, TMA-16 and TMA-30) chromosomally distinguishable ES cell lines. Cytogenetic examination showed that all the nine balloon-like cystic embryoid bodies studied were chimeric, not only in the mural tissue but in the polar region (data not shown). Although the number of embryoid bodies examined was small, it is likely that preferential X\textsuperscript{P} inactivation was indeed responsible for the predominance of X\textsuperscript{P}-inactive cells in the mural tissue of the cystic embryoid body.
DISCUSSION

Findings presented above clearly show that $X^P$ was preferentially inactivated in the yolk sac-like mural region of the cystic embryoid bodies formed by the ES cell lines even after they were allowed to proliferate for a long time in tissue culture, and that the proportion of cells having an inactive $X^M$ was significantly higher in the polar region than that in the mural region. As shown by histological examination, the mural region consists of the outer visceral endoderm layer and variable amounts of the inner mesodermal layer, whereas the polar region comprises the ectodermal vesicle, mesoderm and the outer visceral endoderm. Although the evidence obtained in this study itself is far from conclusive, what has been found in vivo favors $X^P$ inactivation in the visceral endodermal layer and random inactivation in the mesodermal layer and the embryonic ectodermal cells of the cystic embryoid body. Rather wide variation in the inactivation mosaicism in both the polar and mural regions may reflect variable amounts of contaminating visceral endoderm cells in the former, and mesodermal cells in the latter.

Paterno and McBurney (1985) reported that $X$ chromosome inactivation is random in the extraembryonic endoderm-like cells from P10 embryonal carcinoma (EC) cell line differentiated in the presence of retinoic acid. They considered their results to be consistent with the mitotic dilution model, because a large number of mitoses have taken place since the $X$ chromosomes of P10 cells had been imprinted during gametogenesis. The mitotic dilution hypothesis postulates that random $X$ inactivation in the embryonic ectoderm cells is due to loss of, or falling below, a threshold of the imprint during the mitotic events after $X$ inactivation in the primitive endoderm. The present findings, however, showed that the ‘memory’ of parental origin carried by the $X$ chromosome is not easily lost in ES cell lines during repeated mitoses as is autosomal imprinting. It is difficult to elucidate this discrepancy, although such imprint may not last indefinitely.

A preferential $X^P$-inactivation system in the mouse has been the subject of continual controversy. On the one hand, it has been considered separate from and ancillary to the system that achieves $X$ inactivation (Gartler and Riggs, 1983), since the inactivated $X$ is found in the $X^MX^M$ mouse parthenogenetic embryos (Kauffman et al., 1978), and $X^MX^M$ ovarian teratomas (Linder and Power, 1970) and $X^PX^P$ hydatidiform moles in man (Tsukahara and Kajii, 1985). Furthermore, Rastan and Robertson (1985) succeeded in inducing $X$ inactivation in ES cell lines isolated from parthenogenetic blastocysts. Rastan and Cattanach (1983) claimed that $X^P$ inactivation can be overridden to a certain extent by an appropriate combination of alleles at the $Xc$ locus. They showed that $X^M$ is inactivated in a considerable proportion of the yolk sac endoderm cells of female mouse embryos carrying the $Xc^e$ allele on $X^M$ and the $Xc^e$ allele on $X^P$. However, Bücher et al. (1985) failed to confirm this by experiments determining the expression of PGK-1 allozymes. The issue is not settled yet because results of a recent study of $Xist$ gene expression by RT-PCR (Kay et al., 1993) were in favor of the finding by Rastan and Cattanach (1983) at the blastocyst stage, but consistent with the report made by Bücher et al. (1985) at 14.5 dpc. Reviewing various systems of chromosome inactivation, Lyon and Rastan (1984) postulated that the imprinting simply diminishes the probability with which the $X^M$ chromosome is selected to be inactivated.

Cytogenetic studies in mouse embryos, on the other hand, suggested that the imprinting is more potent than that postulated by Lyon and Rastan (1984), and that its potency may not be exactly the same in mouse and man. Shao and Takagi (1990) demonstrated that an extra $X^M$ is harmful to early development, using mice carrying $T(X;4)37H X/autosome$ translocation. They found that $X^MX^MY$ and $X^MX^MPX^P$ embryos were either severely retarded or grossly abnormal as early as 6.5 dpc. Our recent observation (Tada et al., 1993) in embryos obtained from matings involving an $Rb(X;2)2Ad$ translocation invariably showed that $X^MX^PY$ but not $X^MX^MY$ embryos grow normally. Histological studies indicated that the abnormality in mouse embryos having an extra $X^M$ is mainly due to poor development of the extraembryonic tissues. Cytogenetic data suggested that failure to achieve dosage compensation of $X$-linked genes in the trophectoderm is responsible for the deficient extraembryonic tissues. Recently, Tada and Takagi (1992) showed the presence of trophectodermal cells in which $X$ inactivation failed to occur in abnormally growing mouse parthenogenetic embryos. Thus, it is likely that the maternal imprinting does prevent $X^M$ from inactivation in the trophectoderm and probably also in the primitive endoderm. It remains unknown, however, whether this imprinting is effective and hinders the establishment of a single-active-$X$ condition in every precursor cell of the extraembryonic tissue of $X^MX^MY$ and $X^MX^MPX^P$ embryos. These findings in mice are at variance with what is known to occur in man. The supernumerary $X$ chromosome can be either paternal or maternal in origin in Klinefelter’s syndrome (Jacobs et al., 1988), and nondisjunction at female meiosis is responsible for XXX females in nearly 90% of the cases (May et al., 1990). Apparently the extra $X^M$ itself does not seriously affect early human embryonic development. It is tempting to postulate, therefore, that preferential $X^P$ inactivation may not prevail in the extraembryonic tissues of human conceptuses.

The paternal origin per se does not automatically leads the $X$ chromosome to inactivation in the extraembryonic tissues of the mouse embryo. Our previous study on unbalanced carriers of $T(X;16)16H$ [[X,X,16,16], where normal X is paternal in origin, and maternally derived $X^{16}$ contains 63% of the centromeric segment of the X without Xic] did not provide any positive evidence for the occurrence of $X$ inactivation in either the embryonic or, extraembryonic tissues (Takagi, 1980). This observation is obviously consistent with the widely accepted notion that the $X$-inactivation mechanism is turned on only when not less than two inactivation centers are present in the same nucleus. The behavior of the $X$ chromosome in the extraembryonic region of $X^PX^P$ androgenones is of special interest for the elucidation of the nature of the paternal imprinting to the $X$ chromosome.

The biochemical nature of imprinting remains totally unknown. It could involve such reversible traits as chromatin structure and DNA methylation. There are at least two inde-
pendent regulatory mechanisms of gene expression during the course of cell differentiation. The one operating in undifferentiated cells blocks expression of genes regardless of DNA methylation (Niwa et al., 1983) and the other, operating in differentiated cells represses expression of genes in which DNA is methylated (Niwa, 1985). An analogous situation may be envisaged for Xic to achieve both preferential inactivation and random inactivation in the presence of the same imprinting, although it is formally possible that the imprint is erased in differentiating cells of epiblast lineage. A recent study in murine Igf2, an endogenous imprinted gene, failed to detect parent-specific differences in either DNA methylation or sensitivity to nuclease at the promoter region, but revealed parental methylation differences in a region several kilobases upstream of the first exon (Sasaki et al., 1992). Detailed analysis of candidates for Xic, including Xist (Brown et al., 1991), may help clarify the mechanism of imprinting, and its relevance to X-chromosome inactivation. Various ES cell lines would be invaluable for this purpose, since they faithfully maintained the imprint that causes preferential Xp inactivation in the extraembryonic cell lineages.

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REFERENCES


condensation on the proximal part of the Human Xq: A hypothesis. 


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