LT/Sv系マウス由来2倍性単為発生胚におけるX染色体不活性化の細胞学的研究

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著者	遠藤, 澄世 高木, 信夫
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A preliminary cytogenetic study of X chromosome inactivation in diploid parthenogenetic embryos from LT/Sv mice

By Sumiyo ENDO and Nobuo TAKAGI

Chromosome Research Unit, Faculty of Science, Hokkaido University, Sapporo, Hokkaido 060

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ABSTRACT

We examined the replication behavior of the X chromosome in diploid 8.5- and 9.5-day parthenogenetic LT/Sv mouse embryos showing growth retardation and morphological anomalies. A single late replicating X chromosome was found in the extraembryonic as well as in the embryonic regions. However, the frequency of cells with a late replicating X was significantly lower in the extraembryonic region of the parthenotes than in that of normally fertilized embryos.

1. INTRODUCTION

One of two X chromosomes is genetically inactivated in somatic cells of female mammals, so that females become equivalent to males with respect to the number of the active X chromosomes (Lyon 1961). In the developing female murine embryo, X chromosome from either parent becomes inactivated at random in embryonic regions, while the paternally inherited X is predisposed to inactivation in certain extraembryonic tissues (Takagi and Sasaki 1975; Wake *et al.* 1976; West *et al.* 1977). Thus it is of special interest to study X chromosome inactivation in extraembryonic regions of diploid parthenogenetic or gynogenetic embryos in which two X chromosomes are maternal in origin.

Parthenogenesis occurs spontaneously in about 10% of ovulated eggs of inbred LT/Sv strain mice. Most of these parthenotes undergo cleavage in the oviducts, implant in the uteri, and usually die early in the postimplantation stage (Stevens 1975). This paper reports a preliminary observation on X chromosome inactivation in such parthenogenetic embryos, in terms of the allocyclic behavior as revealed by an acridine orange fluorescence technique after BrdU incorporation.

2. MATERIALS AND METHODS

Mature female LT/Sv mice were caged with vasectomized males to obtain parthenogenetic embryos. Females were examined daily for vaginal plugs. The day when the plug was found was considered as day 0 of pregnancy or

0.5 day post coitum (p.c.). Females mated with fertile males provided normally fertilized control embryos.

Copulated females were killed on day 6 to 9. Implantation sites were examined carefully under a dissection microscope and viable embryos were isolated from the decidua.

Intact embryos were exposed to 5-bromo-2 deoxyuridine (BrdU, 200 μg/ml) in Eagle's minimum essential medium supplemented with 10% fetal bovine serum for 7–9 hours in an atmosphere of 5% CO_2 in air at 37°C. Colcemid was added to the medium at the final concentration of 1 μg/ml 2 hours before harvest. At the end of incubation embryos were treated with 1% trisodium citrate for 10 min, fixed with 3 : 1 methanol/acetic acid, and stored at −20°C overnight.

Before slide preparation, each intact embryo was photographed and its length and width were measured with an aid of ocular micrometer under a dissection microscope. Chromosome preparations were made according to a modification (Takagi and Oshimura 1973) of the method developed by Wroblewska and Dyban (1969) which involves lactic acid treatment to dissociate individual cells immediately before slide preparation.

Slides were stained with acridine orange to delineate the allocyclic X chromosome. Chromosome segments which did not incorporate BrdU emit bright yellow-green fluorescence, while fluorescence becomes duller in proportion to the increasing relative amount of the incorporated base analogue. After continuous labeling with BrdU during the later part of the S phase, the allocyclic X is identified as the dullest red or the brightest green element in the cell from female mouse embryos (Takagi and Sasaki 1975). The former was considered to finish replication later, while the latter was considered to do so earlier than other chromosomes. These chromosomes will be designated as the late replicating and the early replicating X chromosome, respectively. The allocyclic X could not be recognized in certain metaphase spreads (Fig. 2b).

3. RESULTS

Table 1 summarises the frequency of viable parthenogenetic embryos on day 6 to 9 of gestation. A total of 16 parthenotes were obtained so far, all of which were severely retarded in development. Four 7.5-day parthenotes were minute rounded masses being about 150 μ in major axis. They seemed to correspond to the 5.5–6.0-day normal embryo in size. Three out of six 8.5-day parthenotes closely resembled to 7.5-day parthenotes. Remaining three embryos grown slightly better had an appearance of a stunted egg cylinder with poorly developed extraembryonic region (Fig. 1a). The ectoplacental cone appeared normal.

Three out of six 9.5-day parthenotes grew exceptionally well being at the

Table 1. *Postimplantation development of parthenogenetic embryos from LT/Sv mice mated with vasectomized males*

Day of development	No. of copulated females	No. of females with impl. (%)	No. of impl. (mean)	No. of living embryos (%)
6.5	2	0	0	0
7.5	15	6 (40.0)	7 (1.2)	4 (57.1)
8.5	14	5 (35.7)	11 (2.2)	6 (54.5)
9.5	19	6 (31.6)	13 (2.2)	6 (46.2)
Total	50	17 (34.0)	31 (1.8)	16 (51.6)

impl.: implants.

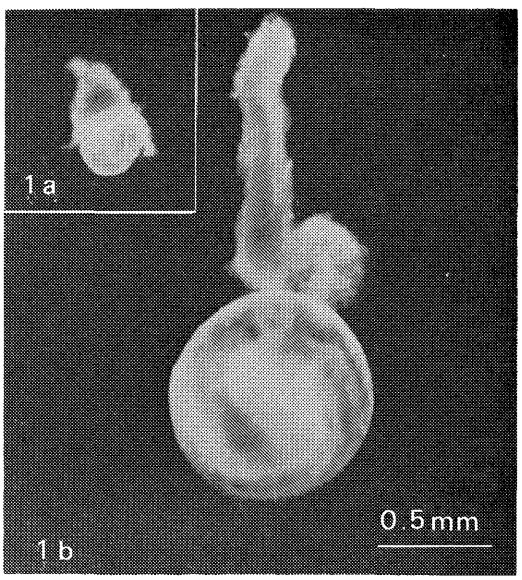

Fig. 1. Fixed LT/Sv parthenotes. (a) A severely retarded 8.5-day conceptus without clear differentiation between embryonic and extraembryonic regions. (b) A 9.5-day parthenote at the head fold stage, showing hypoplastic chorionic ectoderm.

head fold stage with the well developed yolk sac, amnion, and allantois. Foetuses were comparable to those of normal 8.5-day conceptuses. Gross morphological abnormalities included the absence or underdevelopment of the chorion, failure of the dorsal end of the allantois to fuse with the chorion, and an unidentified elongated structure on the dorsal end of the embryo instead of the normal ectoplacental cone (Fig. 1b). Three remaining 9.5-day embryos consisted of two small rounded tissue masses apparently indistinguishable from

Table 2. *Frequency of cells with an allocyclic X chromosome in female LT/Sv mouse embryos 6.5–8.5 days after fertilization*

Area examined	Whole body	Embryo proper	Allantois	Yolk sac	Chorion
Age(d)	6.5	8.5	8.5	8.5	8.5
No. of embryos examined	5	6	3	7	6
No. of cells examined	421	303	75	405	211
% cells with allocyclic X (range)	85.7 (70.2-87.5)	100	100	95.8 (83.7-100)	96.2 (75.0-100)
% late X (range)	80.8 (40.6-85.7)	100	100	93.6 (83.7-100)	69.7 (32.0- 98.7)
% early X (range)	5.0 (1.5-46.9)			2.2 (0 - 8.0)	26.5 (1.3- 68.0)

Table 3. *Frequency of cells with late replicating X chromosome in diploid parthenogenetic LT/Sv mouse embryos*

Embryo no.	Age (d)	Region examined	No. of cells examined	No. of cells with late replicating X chromosome (%)
1	8.5	W	57	54 (95)
2	8.5	W	83	37 (45)
3	9.5	E	111	101 (91)
		EX	26	6 (23)
4	9.5	E	34	33 (97)
		EX	11	11 (100)
5	9.5	E	94	94 (100)
		EX	36	30 (83)
6	9.5	E	41	41 (100)
		EX	110	85 (77)

W: Whole embryo; E: Embryonic region; EX: Extraembryonic region.

7.5-day parthenotes in size and shape and an egg cylinder with the atrophic embryonic region measuring 410 μ in length.

Replication behavior of the X chromosome was studied in six diploid parthenogenetic embryos, two 8.5-day parthenotes comparable in general appearance to the one shown in Fig. 1a, three 9.5-day parthenotes at the head fold stage (Fig. 1b) and one 9.5-day parthenote at the egg cylinder stage. The embryonic and the extraembryonic regions were examined separately in the 9.5-day parthenotes. As a control, five 6.5- and seven 8.5-day normal LT/Sv embryos were examined. Their sizes or developmental stages seemed to correspond to those of the parthenotes examined here. Each 6.5-day embryo was processed as a whole, while 8.5-day embryos were dissected into embryo proper, yolk sac, allantois and chorion, which were examined separately. The results are summarised in Tables 2 and 3. Those cells in which most autosomes failed to show

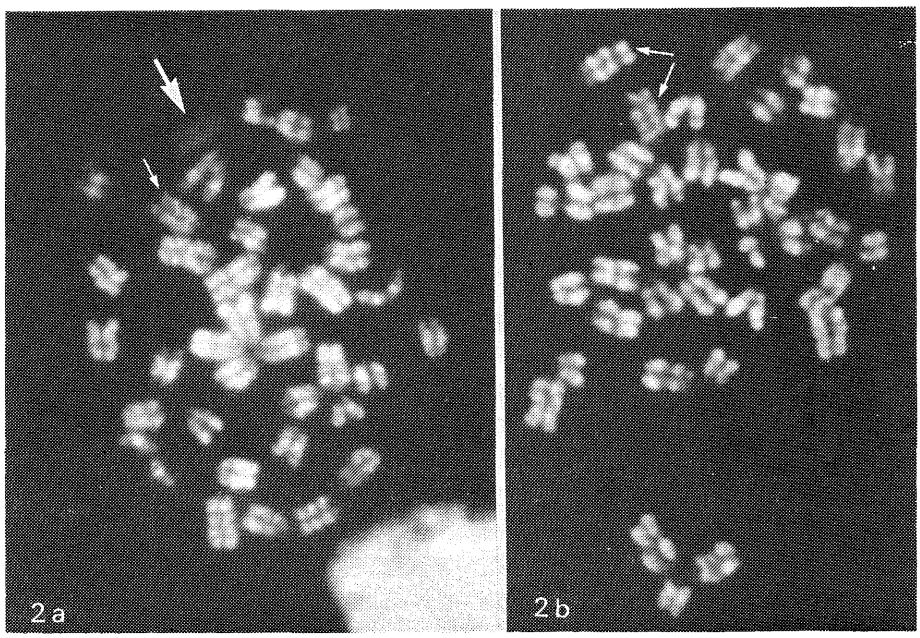

Fig. 2. Acridine orange stained metaphase spreads from LT/Sv parthenotes. (a) A cell with a late replicating X chromosome (large arrow) and an isocyclic X (small arrow). (b) A cell with two isocyclic X chromosomes (small arrows).

clear R-bands (Dutrillaux *et al.* 1973) were excluded from the count.

A single late or early replicating X chromosome was found in 361 out of 421 cells from normal 6.5-day embryos. Remaining 60 spreads showed no asynchronously replicating X chromosome. The proportion of cells having an asynchronously replicating X chromosome became considerably high in 8.5-day embryos. Every cell from the embryo proper and allantois and 591 out of 616 cells from the yolk sac or chorion had an asynchronously replicating X chromosome.

In 8.5-day parthenotes, on the other hand, a single late replicating X was found (Fig. 2a) only in 91 out of 140 cells. The frequency was significantly lower than that in normal 6.5-day embryos ($\chi^2=28.8960$, $p<0.005$). The same tendency was evident between extraembryonic regions of 9.5-day parthenotes and those of 8.5-day normal conceptuses ($\chi^2=92.9911$, $p<0.005$). Chi-square test also suggested that there were more spreads without an allocyclic X chromosome in the embryo proper from 9.5-day parthenotes than in that from normal 8.5-day conceptuses ($\chi^2=12.0577$, $p<0.01$). However, the fact that 10 of 11 cells having no allocyclic X chromosome were recovered from one of four embryos examined throws doubt on the significance of this finding. Further noticeable was our failure to find an early replicating X chromosome in all parthenotes studied here, while it occurred in normal embryos with variable

frequencies.

4. DISCUSSION

LT/Sv parthenotes obtained were characterized by poor or abnormal development of the extraembryonic region, most probably derived from the extraembryonic ectoderm. Such abnormalities were observed consistently in the present study, although they had not been reported in both ovarian and uterine parthenotes of LT/Sv mice (Stevens 1975). The anomaly may not be common to parthenogenetic conceptuses in general. Artificially activated eggs transferred to pseudopregnant females may develop normally for some time in spite of retardation by about one day (Reviewed by Graham 1974). Kaufman *et al.* (1977) obtained two apparently normal forelimb-bud stage embryos with a beating heart and yolk sac circulation.

The X chromosome seems to be inactivated in different embryonic tissues at different times at least in the mouse. Recent studies indicated that X inactivation occurs in the order of trophectoderm of 3.5-day blastocysts, primitive endoderm differentiated on the blastocoelic surface of the inner cell mass at 4.5 days p.c., and embryonic ectoderm or epiblast at 6.0–6.5 days p.c. (Monk 1978; Monk and Harper 1979). The paternally derived X chromosome is preferentially inactivated in the trophectoderm (Takagi *et al.* 1978; Frels *et al.* 1979; Frels and Chapman 1980) and primitive endoderm (West *et al.* 1977), whereas either X can be inactivated at random in the embryonic ectoderm (Takagi and Sasaki 1975; West *et al.* 1977).

Recently, a late replicating or heterochromatic, hence most probably inactive X chromosome was demonstrated not only in the embryonic region (Kaufman *et al.* 1978) but in the yolk sac endoderm and mesoderm (Rastan *et al.* 1980) from artificially formed parthenogenetic mouse embryos. Thus, X chromosome inactivation can take place without paternal genome even in tissues such as yolk sac endoderm derived from the primitive endoderm (Gardner and Papaioannou 1975), in which non-random paternal X inactivation occurs in normal development. Although data presented by Rastan *et al.* (1980) do not suggest any anomaly in X inactivation in their parthenogenetic yolk sac, the incidence of extraembryonic cells with a late replicating X chromosome was significantly lower in our LT/Sv parthenotes than in normal embryos. This observation suggests that X chromosome differentiation was disturbed to some extent in LT/Sv parthenotes. One may postulate that the maternally inherited X chromosome is somehow more resistant to inactivation than the paternally inherited one only early in embryogenesis, and inactivation failed in some cells of LT/Sv parthenotes in the absence of paternal X. Difference between the two X chromosomes should have disappeared by 6.0–6.5 days of pregnancy. An alternative possibility would be effects of two maternally derived auto-

somal sets on inactivation. Two equally active autosomal sets would have produced more regulatory molecules than required for selecting only one X chromosome to remain active (Ohno 1973), resulting in a proportion of cells with two active X chromosomes. Further studies are necessary to distinguish between these two possibilities.

There has scarecely been compelling evidence for the necessity of X chromosome inactivation in female mammals. Studying early embryonic development and inactivation patterns in balanced and unbalanced carriers of a murine X-autosome translocation T(X; 16) 16H, Searle's translocation, we obtained evidence indicating that two active X chromosomes in diploid embryonic cells may cause severe impairment of early embryogenesis (Takagi 1980). If our inability to identify an asynchronously replicating X indeed reflects failure of inactivation in most cases, the prevalence of such cells might have hindered embryonic development. Nearly complete absence or severe underdevelopment of trophectoderm derivatives such as chorion and ectoplacental cone in LT/Sv parthenotes is in accord with this expectation. Failure to find any cell with an early replicating X, which is usually restricted to derivatives of trophectoderm and primitive endoderm of normal embryos (Takagi 1978), in our parthenotes may imply defective inactivation or secondary depletion of progenitor cells of such tissues. Further studies in younger parthenotes will give us information pertaining to many facets of X chromosome inactivation.

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