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A cytogenetic and developmental study on the choice of the X chromosome
to be inactivated in the mouse

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A dissertation
submitted to the Graduate School, Hokkaido University
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for the degree
Doctor of Science

by
Takashi TADA

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Summary

For the last three decades X-chromosome inactivation has attracted much interest as a model system for the study of the genetically programmed large scale gene regulation in development. One of the theoretically most interesting aspects of the X-inactivation process would be the choice of the X chromosome to be inactivated in each embryonic cell. Available evidence shows that a single X chromosomal site, X-chromosome inactivation center (Xic), is involved in the initiation of X-inactivation both in the mouse and man, and that the presence of at least two Xic in the same nucleus is essential for the initiation of X-inactivation. Early cytogenetic studies in human subjects with sex chromosome abnormalities established that the number of the active X chromosome is always one in diploid cells and two in tetraploid cells, which suggests direct or indirect roles played by the autosomal sets in the initiation of X-inactivation. Furthermore, detailed studies in early mouse embryogenesis revealed that only the paternally derived X (X^P) chromosome is inactivated in the non-epiblast cell lineage, whereas X-inactivation is basically random in the epiblast cell lineage silencing X^P and the maternally derived X (X^M) equally frequently. Random and non-random choice occurring in the mouse embryogenesis has been attributed to the differential imprinting given to each Xic during gametogenesis and its subsequent erasure.

In order to gain insight into the nature and outcome of such imprinting, I first examined the X-inactivation pattern in $X^M X^M Y$, $X^M X^P Y$ and $X^M X^M X^P$ embryos produced by various matings involving mice hemizygous or heterozygous for the Robertsonian translocation Rb(X.2)2Ad (Adler et al., 1989). Unlike $X^M X^P Y$ embryos which grew normally, $X^M X^M X^P$ and $X^M X^M Y$ embryos attributable to nondisjunction in heterozygous or hemizygous carriers of the translocation invariably showed growth retardation or abnormal development characterized by the

deficient extraembryonic region. A cytogenetic finding worth mentioning was the frequent occurrence of $X^M X^M X^P$ cells carrying only one inactive X^P chromosome and $X^M X^M Y$ cells without any inactive X chromosome suggesting the presence of two active X^M chromosomes in those cells. Thus, it appears highly responsible for the abnormal embryonic development, and the maternal imprint on the X chromosome probably at Xic was potent enough to keep the chromosome active in occasional non-epiblast cells from $X^M X^M Y$ and $X^M X^M X^P$ embryos. Nothing abnormal was found in the inactivation pattern in epiblast cell lineage of embryos disomic for X^M . The same study suggested the paucity of $X^P O$ embryos relative to those having the complementary karyotype of $X^M X^M X^P$. Further study of embryos from matings between females heterozygous for In(X)1H and Rb2Ad males did not substantiate it. The extensive preimplantation loss of $X^P O$ embryos reported by Hunt (1991) may not be due to the paternal imprinting of the single X chromosome, but due to the maternal uterine environment and/or the deficient X chromosome dosage throughout oogenesis. The absence of any clear parental imprinting on the X chromosome other than the one at Xic further supports the failure in X-inactivation was responsible for the abnormal development of embryos disomic for X^M .

To study the behavior of the X^M chromosome in inactivation more fully, I turned, next, to ethanol-induced diploid parthenogenetic embryos because the frequency of the sex chromosomal nondisjunction in mice carrying Rb2Ad was low, and it was difficult to identify them with certainty without damaging the embryo. Two X chromosomes are always maternal in origin in parthenogenetic embryos, although difficulty arises in the evaluation of data, since every autosome is also inherited from the mother. Shortly after implantation, cell death became evident in the polar trophoctoderm of most parthenogenetic embryos. It was remarkable that parthenogenones recovered 7 to 8 days after activation closely resembled fertilized embryos disomic for X^M chromosome with the

deficient extraembryonic region. A cytogenetically identifiable inactive X chromosome occurred much less frequently in the parthenogenetic than in the fertilized blastocysts at comparable stages. The failure of X inactivation in the trophectoderm seems to mainly contribute to the early defective development of parthenogenones, although effects due to the absence of the paternal autosomes may not be neglected completely.

The possible role played by autosomes in the establishment of the X chromosome inactivation pattern was examined by identifying the inactivated X chromosome in early development of the tetraploid embryos induced by electrofusion of blastomeres at the 2-cell stage. Tetraploid embryos were characterized by severe underdevelopment of the embryonic ectoderm whereas the extraembryonic ectoderm grew much better at 7 to 8th day of gestation. The inactive X chromosome was hardly found in cells from $X^M X^M Y Y$ tetraploid embryos, whereas two X^P chromosomes were inactivated in most cells from $X^M X^M X^P X^P$ tetraploid embryos. I was interesting to find occasional cells having one or 3 inactive X chromosomes in $X^M X^M X^P X^P$ embryos. Although the data are still meager, it is now probable that the inactivation pattern in the embryonic ectoderm cells could be deviated from that predicted by the formula proposed by Harnden (1961). This seems to reflect the fact that the correct X-inactivation pattern could not be established in every tetraploid cells in the absence of the imprinting system.

Finally, I studied the nature of X-chromosome imprinting using newly isolated ES cell lines having two active, cytogenetically or biochemically distinguishable X chromosomes. These ES cell lines, when cultured in the bacteriological petri dish, formed balloon-like cystic embryoid bodies with the yolk sac-like mural region consisting of the outer visceral endoderm and the inner mesoderm layer and the more solid polar region containing the embryonic ectoderm component. Interestingly enough the inactivated X chromosome was

paternal in origin in the majority of cells from the mural region. The same was true in embryoid bodies derived from ES cells at the 30th subculture generation. Thus, the inevitable conclusion would be that the imprint responsible for the preferential X^P -inactivation was so stable that it was maintained intact during the establishment of the ES cell and subsequent subculture generations. This conclusion is at variance with the hypothesis that such imprint is lost from the X chromosome during 24 h period from 5th to 6th day of normal mouse embryogenesis. It is thus probable that the imprint is ignored when X-chromosome inactivation occurs in the epiblast of female mouse embryos.

Introduction

Female mammalian cells usually contain two X chromosomes, whereas male cells contain one X and one Y chromosome. The X chromosome bears about 150 million base pairs of DNA and a number of functional genes, but the Y chromosome carries only a few genes. Developmental abnormalities found in human and mouse individuals with an autosomal trisomy or monosomy invariably show that the genetic balance is essential for normal development not only in the case of the autosome but in the case of the Y chromosome. Genetic event that leads to inactivation on one of two X chromosome in the female cell provides the mean to accomplish it.

More than thirty years ago, Ohno (1959) found that the sex chromatin or Barr body (Barr and Bertram, 1949) was derived from a heteropycnotic X chromosome in the rat. Welshons and Russell (1959) reported that XO mice were phenotypically normal and fertile females, implying that only one active X is enough for normal female development, including sexual development. All sex-linked mutants known at that time affecting coat color caused a mottled or dappled phenotype with patches of normal and mutant color in heterozygous for an X-autosome translocation (Russell and Bangham, 1959, 1960). In 1961, Mary Lyon put forward a hypothesis that can elucidate all of these findings consistently: During the early development of female mammals, one or the other of X chromosomes becomes inactive in each somatic cell, and once established, the active state of the X chromosome is maintained through successive cell generations, resulting in cellular mosaicism consisted of the one cell type having the maternally derived X chromosome active and the other having the paternally derived X chromosome active. Thus the genetic inactivation of one of the two X chromosomes makes the dosage of the active X-linked genes equivalent in female and males (Beutler et al., 1962).

In the last three decades, considerable efforts have been devoted to study details of X chromosome inactivation especially in the human and mouse, which proved the hypothesis fundamentally correct. But our knowledge on the mechanisms of X chromosome inactivation remains unquestionably meager. It has been considered that the phenomenon of X chromosome inactivation is consisted of at least four events: (1) initiation of inactivation at a single site on X chromosome, (2) spreading or promulgation of inactivation along the entire X chromosome length, (3) stable maintenance of inactivation through DNA replication and mitosis, and (4) reactivation of the inactivated X chromosome in oocytes.

In this dissertational study, I focused my attention to one of the most important aspects of the X chromosome inactivation, the choice of the X chromosome to be inactivated during mouse embryogenesis. Before going further, I would like to outline what have been known about the control of X chromosome inactivation.

Studies in 1960s based on the number of sex chromatin bodies and asynchronously replicating X chromosomes in man showed that one and two X chromosomes remain active in diploid and tetraploid cell (Carr, 1965, 1971; reviewed by Hamerton, 1971). These cytological observations led to a hypothesis that two sets of autosomes produce factors barely enough to keep one X chromosome active or that one set of autosomes acts as a suppressor of inactivation. On this basis, Harnden (1961) proposed a formula for predicting the number of sex chromatin bodies or the inactive X chromosome in individual nuclei:

$$S=X-A/2$$

where S is the number of X chromatin bodies or inactive X chromosomes, X the number of X chromosomes, and A the number of the chromosomal sets of a cell. It remains unknown whether this relationship, though fits well with observed conditions in postnatal cases and fetuses obtained considerably long after the

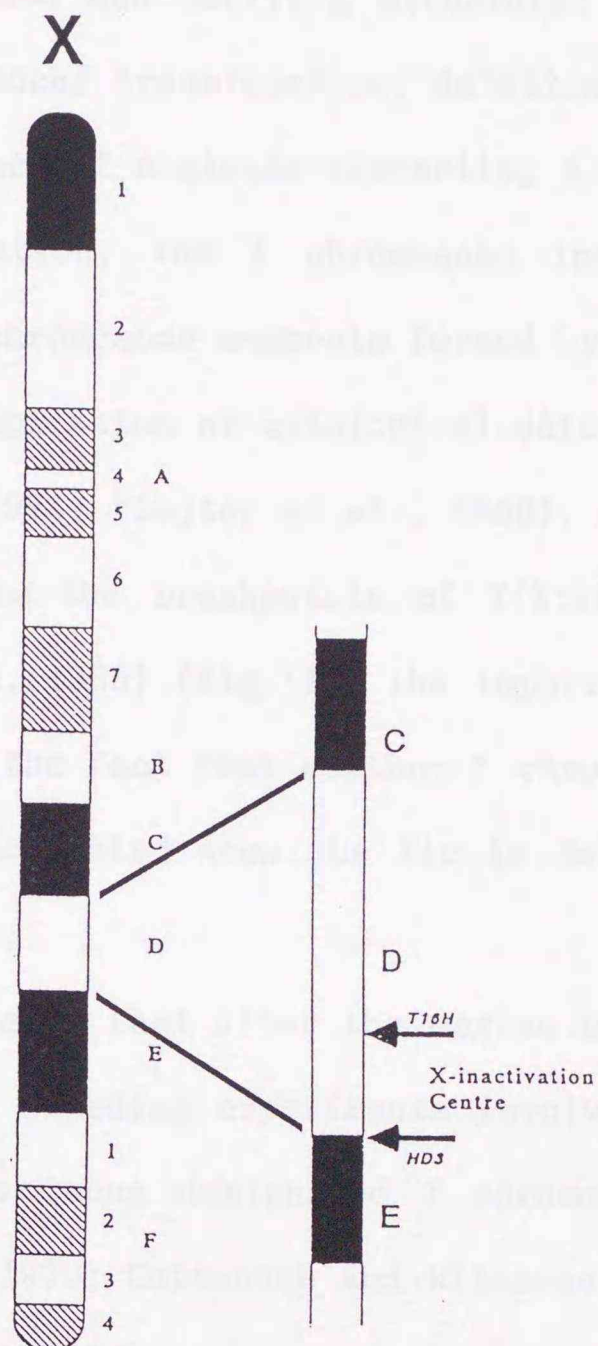


Figure 1. Localization of X-chromosome inactivation center (XIC) on the G-band map of the mouse X chromosome. Genetic studies indicate that the XIC locates to the D band, between the region distal to the translocation T16H and that proximal to the breakpoint HD3.

time of X-inactivation, had been established from the outset or resulted from subsequent cell selection. Furthermore, there is no direct evidence for the participation of the autosome in the initiation of the X-inactivation.

Evidence from mice and men carrying structural alterations of the X-chromosome such as reciprocal translocation, deletion and isochromosome formation points to the presence of a single cis-acting X chromosomal locus that is responsible for inactivation, the X chromosome inactivation center (Xic). Thus, only one of the X chromosome segments formed by reciprocal translocation could be inactivated. Compilation of cytological data localized Xic to Xq13-21 in man (Therman et al., 1974; Flejter et al., 1986), and to the D band of the mouse X-chromosome between the breakpoints of T(X;16)16H (Rastan, 1983) and HD3 (Rastan and Robertson, 1985) (Fig. 1). The importance of Xic in X-inactivation has been shown by the fact that neither X chromosomes present in a diploid XX cell can be inactivated when the Xic is deleted from one of the X chromosome (Takagi, 1980).

Various X-linked alleles that alter the degree of coat variegation were isolated in the extensive breeding experiments involving the Cattanach insertion, Is(X;inv7)1Ct. This locus designated *X chromosome controlling element* (Xce) (Cattanach et al., 1970; Cattanach and Williams, 1972) has been considered as a candidate for the Xic, because rough agreement of the map site of Xce and cytological localization of Xic. Recently, an unique X-linked gene *XIST/Xist* (X-inactivation specific transcript), which is expressed only from inactive X chromosome, was found in both human and mouse (Brown et al., 1991a; Borsani et al., 1991; Brockdorff et al., 1991). *XIST/Xist* was localized to the minimal interval of the Xic region. Although its function remains to be clarified, the combination of its map position and unique pattern of expression makes this gene a strong candidate for the Xic.

Contrary to the Lyon hypothesis, nonrandom X-inactivation was found in

marsupial species. Using interspecific hybrid kangaroos, Sharman (1971) showed that the cytologically identifiable paternal X chromosome was usually late replicating, and only the electrophoretic forms specified by the maternally derived X chromosome was expressed in somatic cells (Cooper et al., 1971, 1975). In the mouse, Takagi and Sasaki (1975) distinguished the parental origin of inactivated X chromosome in various tissues of embryos at 7.5 and 8.8 days of gestation using a morphologically distinguishable translocation X chromosome in combination with differential staining of the asynchronously replicating X chromosome. Unexpectedly, the inactivated X chromosome was paternal in origin in most cells of the extraembryonic membranes, whereas it was either paternal or maternal in origin in the fetus itself. This finding extended later to the rat was confirmed by biochemical studies using the electrophoretic variants of the X-linked enzyme, phosphoglycerate kinase 1 (PGK-1) (West et al., 1977; Harper et al., 1982) in the embryonic and extraembryonic tissues at various embryonic stages.

A cytogenetic study of Takagi (1976) and a biochemical analysis of West et al. (1977) showed that non-random X^M -expression is due to preferential X^P -inactivation and not to cell selection after random inactivation. This preferential X^P -inactivation is considered as an example of genomic imprinting, a term originally used to explain the selective elimination of the paternal X chromosome in the soma and germ lines of the fungus gnat, *Sciara coprophila* (Crouse, 1960). Brown and Chandra (1973) was the first who has incorporated the concept of imprinting to the model of X-chromosome inactivation in mammals. It has been argued that X^P -inactivation is not rigidly determined even in the trophectoderm and primitive endoderm cell lineage of the mouse, because the single X^P -chromosome of X^P0 mice is not inactivated (Frels and Chapman, 1979; Papaioannou and West, 1981) and one of two X^M -chromosomes is inactivated in diploid parthenogenetic embryos surviving beyond implantation (Kaufman et

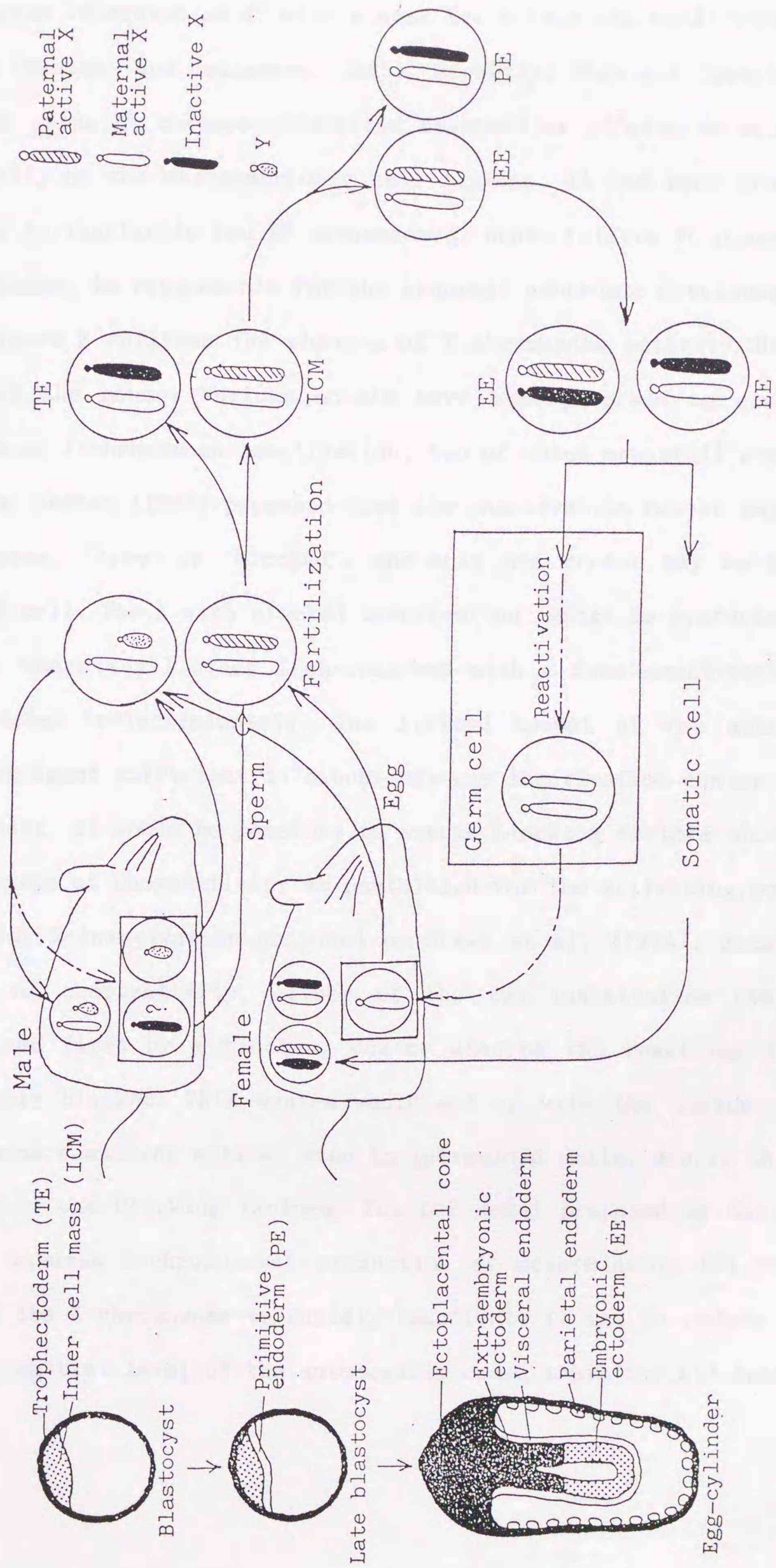


Figure 2. Changes of X chromosome activity during the life cycle of the mouse.

al., 1978; Rastan et al., 1980; Endo and Takagi, 1981). Moreover, it was reported that X^M is inactivated in a proportion of the yolk sac endoderm cells in embryos inherited an X^M with a weak Xce allele and an X^P with a strong Xce allele (Rastan and Cattanch, 1983). Recently, Shao and Takagi (1990) showed that an extra X^M chromosome exerted deleterious effects on mouse development especially on the extraembryonic cell lineage. It has been proposed that the failure to inactivate two X^M chromosomes, hence failure to compensate X-linked gene dosage, is responsible for the abnormal embryonic development.

Figure 2 outlines the changes of X chromosome activity during the life cycle of the mouse. Various models have been proposed to elucidate various aspects of X-chromosome inactivation, two of which are still worthy of consideration. Rastan (1983) proposed that the inactivation center may be in one of two states, "free" or "blocked", and only one center may be blocked in any diploid cell. The X with blocked inactivation center is protected from inactivation, whereas all other X chromosomes with a free inactivation center are inactivated indiscriminately. The limited amount of the autosomally coded blocking agent sufficient to block only one inactivation center is critical in this model. It would be possible to assume blocking factors which show a very high degree of cooperativity as postulated for the activating molecules in the model for X-inactivation proposed by Drews et al. (1974). Because of a high degree of cooperativity, either of the two inactivation centers which is recognized first by a blocking factor absorbs the remaining factors and it completely blocked. This system would end up with the choice of a single X chromosome remaining active, even in tetraploid cells, due to the high cooperativity of the blocking factors. The D+I model proposed by Gartler and Riggs (1983) assumes X-chromosomal production of determinator (D) that acts on a site of the X chromosome to rapidly inactivate it and to reduce the amount of D. The constant level of the autosomally coded inhibitor (I) that binds tight-

ly to D totally inactivating it is considered to establish the correct active-X/autosome ratio.

As mentioned above, preferential X^P -inactivation in the extraembryonic tissues of mouse embryos suggests that X^P and X^M are marked differentially during gametogenesis (Lyon and Rastan, 1984). The drastic change from X^P -inactivation to random inactivation in mouse embryogenesis in a matter of one or two days implies that the differential mark or imprint is erased before X-inactivation in the epiblast, the progenitor of the fetus proper, or it is conserved but is ignored when X inactivation occurs in the epiblast.

The aim of present study is to gain insight into the choice of the X chromosome to be inactivated. Chapter 1 to 3 are devoted to the study of X-chromosome inactivation patterns in various early postimplantation mouse embryos and developmental effects of abnormal X-inactivation in vivo. Chapter 4 of this thesis deals with the nature of X-chromosome imprinting revealed by experiments involving newly established embryonic stem cell lines. Studies on the cystic embryoid bodies formed by the embryonic stem cells provided evidence that the imprinting that entails X^P -inactivation is not unstable one that is lost or diluted out during several rounds of cell divisions. It is likely, therefore, that random inactivation in the epiblast is resulted not from the loss of imprinting, but from the loss of sensitivity of the system to imprinting. The significance of this type of imprinting is shown by the fact that the presence of imprinting accounts for the abnormal inactivation pattern in non-epiblast cells of embryos disomic for X^M on the one hand, and functional loss of imprinting elucidates the abnormal inactivation in the epiblast cells of the tetraploid embryos on the other. The X chromosome inactivation system developed for the diploid cell may not be able to cope with tetraploid conditions without imprinting.

Chapter 1: X-chromosome inactivation and early postimplantation development in XO, XXX and XXY embryos

Materials and methods

(i) Matings of mice

The Rb(X.2)2Ad stock mice (Adler et al. 1989; Shortened to Rb2Ad hereafter) used to produce various sex chromosomal aneuploids have been maintained at the Center for Experimental Animals and Plants, Hokkaido University by the sib mating since July, 1990. To obtain X^M0 and X^MX^PY embryos due to nondisjunction at male meiosis, Rb2Ad males were mated to chromosomally normal females, females homozygous for In(X)1H (Evans & Phillips, 1975; Shortened to In1H hereafter) or those heterozygous for Is(In7;X)1Ct (Cattanach, 1961; Shortened to Is1Ct hereafter). X^M0 and X^MX^PY zygotes were expected to occur at an equal frequency.

F1 females from the cross Rb2Ad/Rb2Ad x C3H/He or C3H/He x Rb2Ad/Y were mated with males carrying In1H or Is1Ct to produce X^MX^MY , $X^MX^MX^P$ and X^P0 zygotes. The autosomal insertion into the X chromosome and the large X chromosomal inversion together with the Rb2Ad Robertsonian translocation allowed us ready identification of every X chromosome involved in these crosses. The developmental potential of the X^P0 embryo was further studied in female mice heterozygous for In1H mated with Rb2Ad/Y males.

Female mice caged with a male mouse were checked daily for the vaginal plug early in the morning, and the day when it was found was taken as day 0 of gestation. Embryos were usually recovered from decidual swellings at 9:00 to 10:00 a.m. on day 6 or 7 of gestation under the dissection microscope. These embryos were designated as 6.5 and 7.5 days post coitum (dpc) embryos, respectively.

(ii) BrdU labeling and slide preparation

Recovered embryos were photographed and incubated in Eagle's minimum

essential medium supplemented with 10% fetal bovine serum and 150 μ g/ml 5-bromo-2-deoxyuridine (BrdU) at 37°C in an atmosphere of 5% CO₂ in air. The duration of incubation was 6 and 7 h for 6.5 and 7.5 dpc embryos, respectively, including the last 1 to 2 h in the presence of Colcemid. After hypotonic treatment with 1% sodium citrate for 10 min, embryos were fixed with 3:1 methanol:acetic acid. Chromosome slides were prepared according to a modification (Takagi & Oshimura, 1973) of the air-drying method described by Wroblewska and Dyban (1969). Slides stained with freshly prepared acridine orange were examined under the fluorescence microscope for the karyotype and the X chromosomal replication pattern.

(iii) Karyotyping of oocytes and spermatocytes

Unfertilized oocytes were obtained from females heterozygous for In1H superovulated by the injection of 10 IU pregrant mare's serum gonadotropin (PMSG; Teikoku Hormone) followed 48 h later by 10 IU human chorionic gonadotropin (hCG; Teikoku Hormone). Cumulus mass recovered from swollen ampulla of superovulated females 19-20 h after hCG injection were treated with 300 IU/ml bovine testis hyaluronidase (Sigma) in M2 medium for 5 min to remove cumulus cells (Quinn et al., 1982). Immediately after hypotonic treatment and fixation, chromosome slides were prepared according to the method described above.

Testes of Rb2Ad males were prepared for light-microscopic analysis of spermatocytes at the second meiotic division according to a routine air drying method.

Table 1. Early postimplantation development of 6.5- and 7.5-day embryos

Matings	No. of				
	Mice copulated	Corpora lutea	Implants	Viable embryos	Embryos analyzed
X/X x Rb2Ad/Y					
In1H/X x	91	796	628(79)*	591(94)*	577
Is1Ct/X x					
Rb2Ad/X x X/Y	40	368	341(93)	306(90)	305
Rb2Ad/X x Is1Ct/Y	23	187	178(95)	173(97)	171
Rb2Ad/X x In1H/Y	97	777	667(86)	640(96)	637
In1H/X x Rb2Ad/Y	71	495	362(73)	329(91)	321

*Percent.

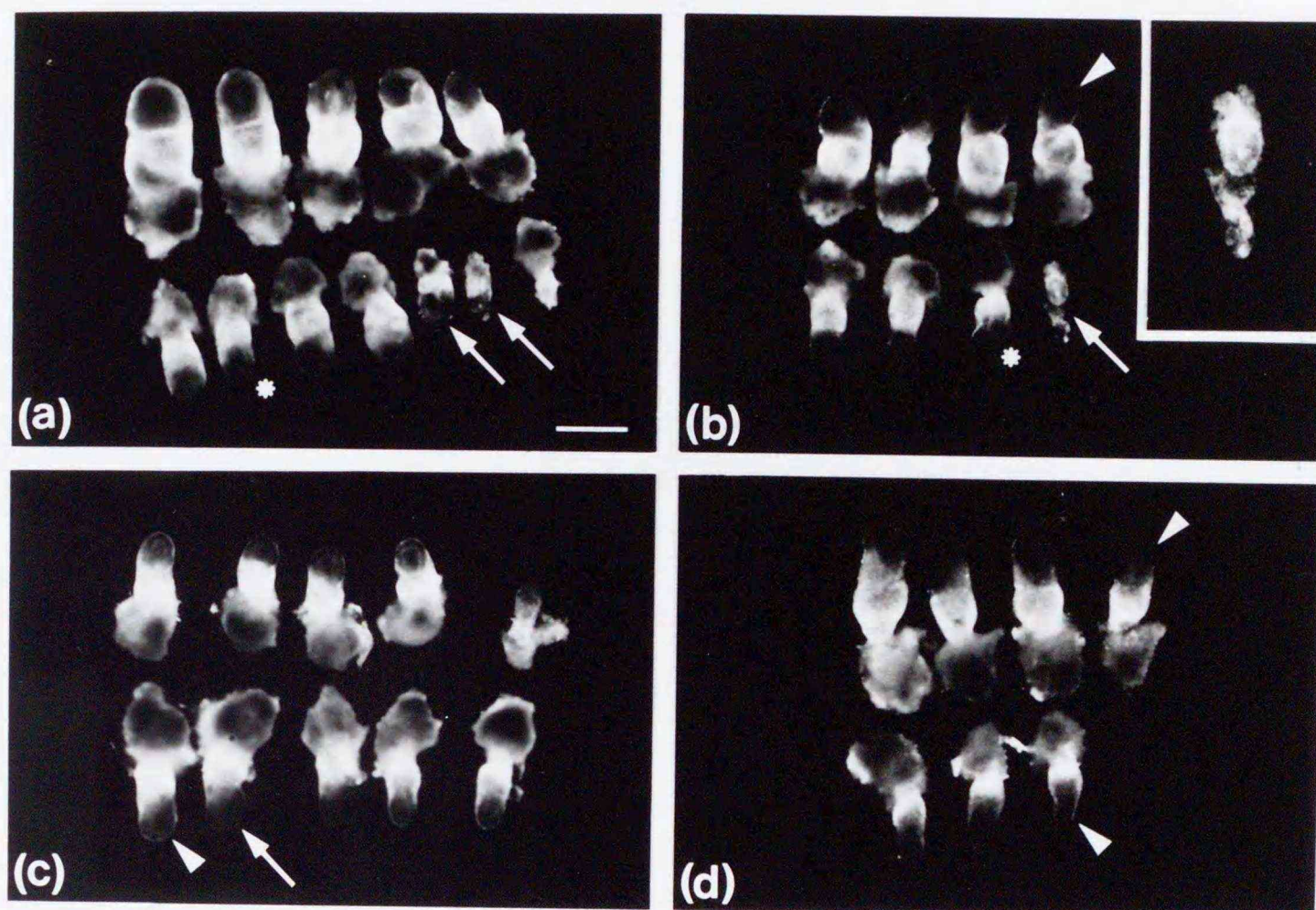


Figure 3. Gross morphology of 7.5 dpc embryos. (a and b): Littermates from Rb2Ad/X female mated with Is1Ct male. Arrows indicate the retarded $X^M X^M X^P$ and $X^M X^M Y$ embryos in (a) and the abnormal $X^M X^M X^P$ embryos in (b). A magnification of abnormal $X^M X^M X^P$ embryo is shown in the inset. Asterisks and arrowhead indicate embryos with an unidentified extra autosome and $X^P O$ embryo, respectively. (c): Littermates sired by a Rb2Ad/Y male. An arrow and arrowhead indicate $X^M X^P Y$ and $X^M O$ embryos, respectively. (d): A litter of embryos from mating involving In1H/X female. Normal and retarded $X^P O$ embryos are shown by arrowheads. Bar = 0.5 mm.

RESULTS

(i) X^M0 and X^MX^PY embryos sired by Rb2Ad/Y males

In order to obtain X^M0 and X^MX^PY embryos, Rb2Ad males were mated to females from various stocks of mice including IslCt and InlH. Data yielded by females from different stocks were essentially homogeneous, hence they were combined. The loss of ovulated oocytes before implantation estimated by the difference between the number of corpora lutea and implantation sites was extensive (Table 1), cause of which remained unknown.

Karyotype analyses of 577 embryos recovered from 91 females mated with Rb2Ad/Y males revealed that 11 and 10 were $X0$ and XXY , respectively (Table 2). The X chromosome was of maternal origin in 10 of 11 $X0$ embryos (Fig. 4e), whereas all XXY embryos were uniformly X^MX^PY (Fig. 4a). The 1:1 ratio of X^MX^PY and X^M0 embryos agreed with the expectation that sperm bearing both Rb2Ad and Y chromosomes and those bearing no sex chromosome were produced at an equal frequency by nondisjunction at the first meiotic division. Hence, it is either that these embryos with sex chromosome aneuploidies were not lost more than the background level or they were subjected to selection but the survival rate was identical in both categories.

The size and morphology of individual embryos favored the lack of selection against either X^M0 or X^MX^PY embryos at least until 7.5 dpc. All the X^MX^PY embryos recovered here, unlike X^MX^MY ones reported by Shao and Takagi (1990) and found in this study (see below), could not be distinguished from their chromosomally normal or balanced littermates by their growth (Fig. 3c). Similarly, nine X^M0 embryos grew normally (Fig. 3c), and only one showed slight growth retardation. In conclusion, available evidence shows that X^M0 and X^MX^PY embryos have been fully viable and did not suffer extensive

Table 2. Gross morphology of 6.5- and 7.5-day embryos obtained from females mated with hemizygous Rb2Ad/Y males

Gross morphology	No. of embryos								Triploid or Mosaic tetraploid
	X ^M X ^P	X ^M Y	X ^M O	X ^P O	X ^M X ^P Y	Autosomal trisomy	Autosomal monosomy		
Normal	235	236	9	0	10	0	0	0	3
Retarded	32	22	1	1	0	1	1	0	3
Severely retarded or abnormal	6	12	0	0	0	0	1	1	3
Total	273	270	10	1	10	1	2	1	9

X^P, paternal X chromosome; X^M, maternal X chromosome.

selection until 7.5 dpc. Adler et al. (1989) found that out of 115 newborn mice from the cross between X/X females and Rb2Ad/Y males 3 (2.6%) were X^M0 and 2 (1.7%) were X^MX^PY . Tease and Fisher (1991) similarly found 6 (5.0%) X^M0 and no XXY mice at birth among 120 newborns from X/X mothers mated with Rb2Ad males. The combined frequency of these newborn mice with sex chromosomal aneuploidies agreed reasonably well with the frequency obtained here soon after implantation, which suggests no selection against X^M0 and X^MX^PY zygotes throughout pregnancy.

In Rb2Ad/Y males, Adler et al. (1989) and Tease and Fisher (1991) found that the total nondisjunction frequency of the sex chromosomes was 10.8% and 9.2%, and secondary spermatocytes containing two or no sex chromosomes were equally frequent. A reasonable conclusion would be the loss of half the zygotes with sex chromosomal aneuploidies (Adler et al., 1989). Since the present data were not consistent with this explanation, we carried out a cytogenetic study of spermatocytes at the second meiotic metaphase in Rb2Ad/Y males maintained in Sapporo. Out of 600 cells from 2 males 14 (2.3%) had both Rb2Ad and Y and 15 (2.5%) had neither. Autosomal nullisomy was found in 10 cells and autosomal disomy in 3. If this were true in males used for the above crosses, it would be unnecessary to postulate extensive selection against embryos with sex chromosomal aneuploidies by 7.5 dpc. It is hard, however, to explain the low frequency of nondisjunction found by us and the higher ones reported by Adler et al. (1989) and Tease and Fisher (1991), since identification of Rb2Ad and Y chromosomes was unequivocal in most secondary spermatocytes at metaphase II.

(ii) X^P0 , X^MX^MY and $X^MX^MX^P$ embryos from Rb2Ad/X females

The dissection of Rb2Ad/X females on day 6 and 7 of gestation (Table 1)

Table 3. Gross morphology of 6.5- and 7.5-day embryos obtained from heterozygous Rb2Ad females mated with males carrying IslCt or InlH

Gross morphology	No. of embryos							
	X ^M X ^P		X ^M Y					Autosomal trisomy Triploid
	XX	Rb2X	XY	Rb2Y	X ^P O	X ^M O	X ^M X ^M X ^P	
Normal	189	171	197	169	5	3		12 6
Retarded	10	7	3	4			6	4
Severely retarded or abnormal	2	4	4	4			4	
Total	201	182	204	177	5	3	10	16 6

Rb2, Rb(X.2)2Ad chromosome; X^P, paternal X chromosome; X^M, maternal X chromosome.

showed no obvious anomaly except the increased preimplantation loss of oocytes ovulated by females mated with In1H/Y males. It was 14.2% compared to 4.8% and 7.3% in females mated with Is1Ct/Y and C3H males, respectively. Increase in the number of unfertilized oocytes or postzygotic death may explain the high preimplantation loss, but we did not pursue this point further, because it seemed to have no intimate bearing on the main issue of the present study.

Table 3 shows the results of karyotype analyses of 808 embryos obtained from 2 different crosses. As reported earlier by Adler et al. (1989) and Tease and Fisher (1991), eudiploid embryos having the Rb2Ad chromosome were significantly fewer than those lacking it. Table 3 also shows that 10 out of 808 embryos were $X^M X^M X^P$ (Fig. 2d), whereas only 4 and 5 were $X^M X^M Y$ (Fig. 2b, c) and $X^P 0$, respectively, although they are expected to occur at a comparable frequency. Three $X 0$ embryos were found to be $X^M 0$ confirming that nondisjunction of sex chromosomes occurred spontaneously in Is1Ct/Y and In1H/Y males as in chromosomally normal males (Adler et al. 1989). Embryos with the complementary karyotype of $X^M X^P Y$ could not, however, be recovered in this study. We also found two $X 0$ and six XXY embryos out of 305 progenies from the cross between Rb2Ad/X females and C3H males. Without any cytogenetic marker of the X chromosome from C3H, it was impossible to unequivocally determine whether nondisjunction occurred at male or female meiosis. If the value 3/808 obtained above is a reasonable frequency of X-Y nondisjunction in C3H males, either one $X 0$ or XXY could be ascribed to nondisjunction at male meiosis and others to nondisjunction at female meiosis. This leads us to estimate the total numbers of $X^P 0$, $X^M X^M Y$ and $X^M X^M X^P$ embryos obtained from Rb2Ad/X females mated to Is1Ct, In1H and chromosomally normal males at 6-7, 9-10, 10, respectively, being not significantly different from the theoretical ratio of 1:1:1 ($\chi^2 = 1.04$ or 0.67 , $0.5 < P < 0.7$ or $0.7 < P < 0.8$).

Adverse effects of the extra X chromosome were evident in the growth of all six XXY embryos sired by C3H males as well as every legitimate $X^M X^M Y$ and $X^M X^M X^P$ embryo sired by $In1H/Y$ and $Is1Ct/Y$ males. Since each $X^M X^P Y$ embryo found in this study grew normally, all the XXY embryos sired by C3H males should most probably be $X^M X^M Y$. The abnormality found in these embryos ranged from growth retardation by 12-24 h (Fig. 3a) to severe anomaly leading to the formation of the tiny spherical embryo (Fig. 3b) as reported by Shao and Takagi (1990). They showed that the lack or severe deficiency of the structure derived from the extraembryonic ectoderm characterized embryos having an extra copy of X^M . Severe developmental abnormalities found in some of these embryos suggest that a proportion of embryos disomic for X^M had been dead and resorbed by 7.5 dpc, although the present data does not allow us to estimate the extent of such selection. Therefore, the nearly equal frequencies of the three classes of sex chromosomally aneuploid embryos may not necessarily reflect the situation at fertilization. Failure to find any $X^M X^M Y$ and $X^M X^M X^P$ karyotype among newborn mice from $Rb2Ad/X$ females (Adler et al. 1989; Tease and Fisher 1991) implies that these hyperdiploid embryos were totally lost by parturition.

The total number of $X^P O$ embryos recovered in these matings tended to be fewer than that of $X^M X^M Y$ or $X^M X^M X^P$ embryos, but the difference was not statistically significant even if the allowance was made that $Rb2Ad$ chromosome segregated into the first polar body in 60% of meiosis I ($\chi^2 = 1.06$, $P > 0.3$). Furthermore, $X^P O$ embryos did not suffer from any specific developmental abnormality except occasional slight growth retardation.

(iii) $X^P O$ embryos from $In1H/X$ females

We studied further the developmental potential of $X^P O$ zygotes in XX mothers using conceptuses from $In1H/X$ females mated to $Rb2Ad$ males. Karyotype

Table 4. Gross morphology of 7.5-day embryos obtained from In1H/X females mated with Rb2Ad/Y males

Gross morphology	No. embryos										Autosomal trisomy
	X ^{MY}	X ^{MX} P	X ^P O	X ^P Y	X ^M O	X ^M X ^P Y	X ^M X ^M X ^P	X ^P X ^P			
Normal	136	122	20	3	5	4	1				
Retarded	1	9	8	1		2		1		1	
Severely retarded or abnormal	1	2	2	1						1	
Total	138	133	30	5	5	6	1	1		2	

X^P, paternal X chromosome; X^M, maternal X chromosome.

analyses of 321 embryos recovered from 71 pregnant In1H/X females (Table 4) identified a single rare case of $X^P X^P$, five $X^P Y$ and thirty $X^P O$ embryos (Fig. 4f) apparently derived from 0-oocytes. Other relevant sex chromosome aneuploids found were five $X^M O$ and six $X^M X^P Y$. The $X^P X^P$ embryos was moderately retarded. Although we confirmed the occurrence of a single inactive X chromosome in one metaphase cell, paucity of suitable cells precluded further study. Developmental retardation was found in 12 out of 35 embryos with $X^P O$ or $X^P X^P$ sex chromosome constitutions. In accordance with results obtained above, all the $X^M O$ and $X^M X^P Y$ grew normally except two $X^M X^P Y$ conceptuses which showed slight growth retardation.

It was reported that about 22% of oocytes produced by In1H/X females contain no sex chromosome due to a crossing over within the inverted chromosome region (Phillips & Kaufman, 1974; Mann & Lovell-Badge, 1987). Also reported were that about 6% of oocytes carry a dicentric X, and remaining 72% are chromosomally normal bearing a single X chromosome. The fate of zygotes with a dicentric X chromosome remains unknown, but most of them should be lethal before implantation. Our own chromosomal observation on unfertilized oocytes from In1H/X females gave slightly lower frequencies of chromosomally abnormal oocytes. Out of 244 oocytes examined 42 (17.2%) had no sex chromosome and 12 (4.9%) had a dicentric X chromosome. A normal X chromosome was present in 190 oocytes 181 of which appeared normal with a total of 20 chromosomes. Remaining 9 oocytes contained one or two extra autosomes.

It is likely that a half of 0-oocytes give rise to $X^P O$ and the remaining half of OY which are eliminated before implantation (Morris, 1968). Hence, the expected frequency of $X^P O$ embryos at 7.5 dpc should be, if they were not lost abundantly, about 13% based on the data reported previously, and about 10% (21/211 excluding 12 oocytes with a dicentric X and 21 OY zygotes) based on

the present data. The recovery of a total of 11.2% embryos derived from 0-oocyte at 7.5 dpc suggests that they were not lost appreciably by this stage, since the frequency was not significantly different from the one expected from our cytogenetic data on unfertilized oocytes ($\chi^2 = 0.10$, $P=0.75$).

(iv) X chromosome inactivation in XXX and XXY embryos

In three $X^M X^P Y$ embryos, like normal females, X^P replicated either precociously (Fig. 4a) or late in 230 (97%) of 236 informative extraembryonic cells, and X^P or X^M replicated asynchronously in most cells from the embryonic region (Table 5). In four $X^M X^M Y$ embryos, 26 of 460 informative metaphases apparently had no inactivated X chromosome (Fig. 4c). This frequency could be unduly low because one embryo provided 360 informative cells only 9 of which had no asynchronously replicating X chromosome. Evidence from six retarded $X^M X^M X^P$ embryos was more convincing. Thirty out of 168 informative cells had the X^P as the only X chromosome replicating asynchronously (Fig. 4d). No such cell was found among 29 cells from two grossly abnormal $X^M X^M X^P$ embryos lacking most of the extraembryonic portion. These findings suggested that those cells having a single asynchronously replicating X^P were derived mainly from the extraembryonic tissues and those cells were, in turn, responsible for the deficient extraembryonic structures. Most remaining cells had two asynchronously replicating X chromosomes indicating that random inactivation of all X chromosomes except one prevailed in the embryonic region (Fig. 4b).

Table 5. X-chromosome replication patterns in 7.5-day mouse embryos carrying an extra X chromosome

Karyo- type	No. of embryos	Gross morphology	No. of cells									
			with allocyclic					without allocyclic				
			X ^P		X ^M		X ^M X ^P		X ^M X ^M			
			P	L	P	L	P	L	L	Xs		
41, X ^M X ^M X ^P	6	Retarded	9	21				96	32	10		
	2	Abnormal						20	9	0		
41, X ^M X ^M Y	4	Retarded			1	433				26		
41, X ^M X ^P Y	3	Normal	63	377		103				5		

X^P, paternal X chromosome; X^M, maternal X chromosome; P, precociously replicating; L, late replicating.

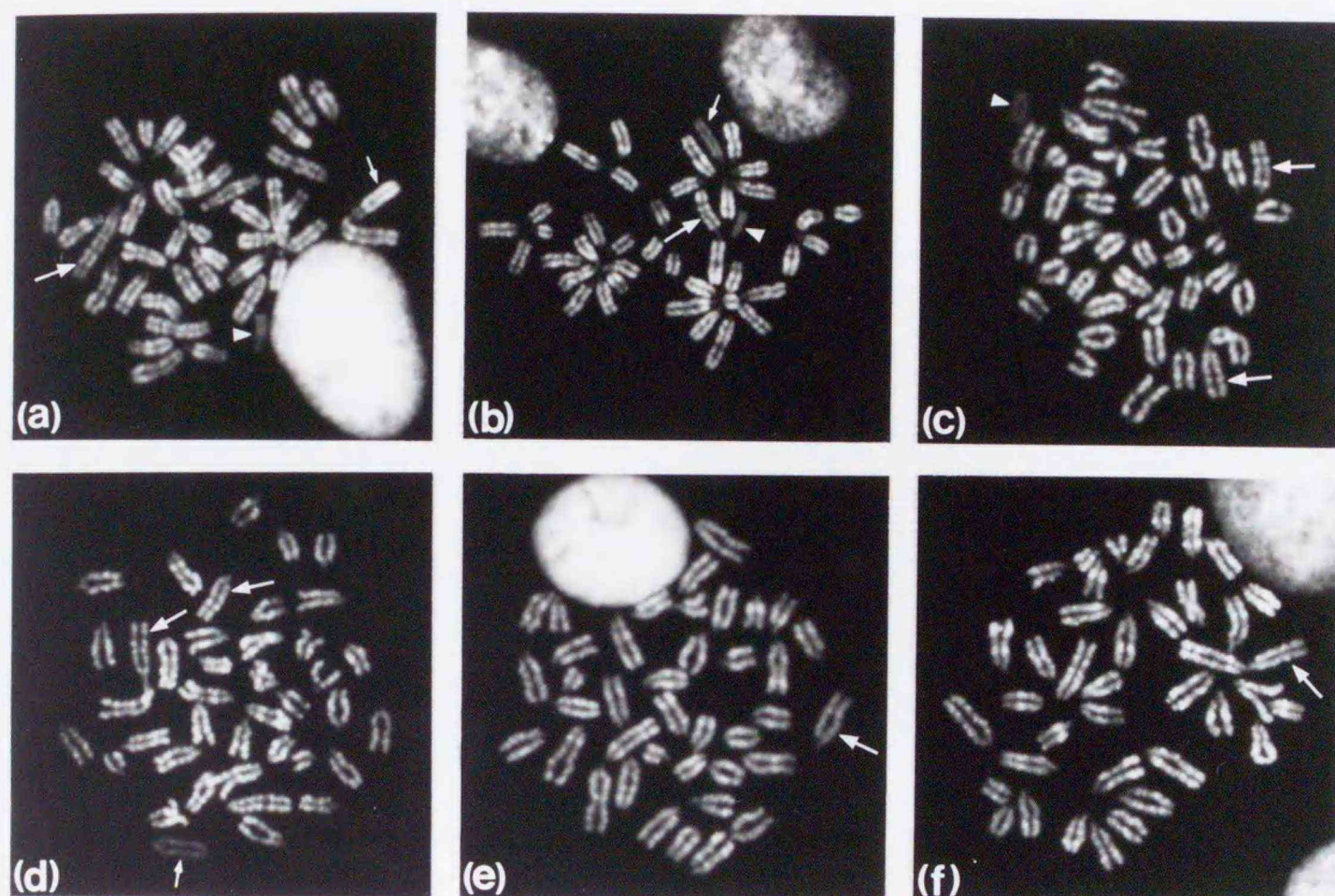


Figure 4. X chromosome replication patterns revealed by a BrdU-acridine orange procedure in sex-chromosomal aneuploids. (A): A metaphase spread of $X^M X^P Y$ embryo carrying one maternal Is1Ct and one paternal Rb2Ad. Paternal Rb2Ad replicated precociously. (B and C): Metaphases from $X^M X^M Y$ embryos carrying mateaternal Rb2Ad and normal X. One X in (B) and two Xs in (C) replicated synchronously. (D): A cell from an $X^M X^M X^P$ embryo with In1H, Rb2Ad and normal X. Only paternal In1H was late replicating. (E): A metaphase spread from an $X^M O$ embryo obtained from Is1Ct/X female mated with Rb2Ad/Y male. (F): A cell from an $X^P O$ embryo from the cross between In1H/X female and Rb2Ad/Y male. Arrowheads indicate Y chromosomes. Synchronously and asynchronously replicating X chromosomes are shown by large and small allows, respectively.

DISCUSSION

All the 16 $X^M X^P Y$ embryos recovered in this study were developmentally normal at 7.5 dpc without showing any sign of selection at earlier stages. Ten out of 11 $X^M X^M Y$ embryos, on the other hand, suffered from growth retardation or abnormal development with specifically affected extraembryonic structures as reported by Shao and Takagi (1990). Since the only notable difference between $X^M X^M Y$ and $X^M X^P Y$ sex chromosome constitution is the parental origin of the supernumerary X chromosome, it may now be positively concluded that the maternal inheritance of the two X chromosomes, hence their maternal imprinting, was responsible for the aberrant embryogenesis. The same tendency found in $X^M X^M X^P$ embryos substantiates this conclusion.

The imprinted gene(s) of X^M responsible for the developmental anomaly of $X^M X^M Y$ embryos is evidently not expressed when there is only one X^M chromosome in a cell as in the case of normal male ($X^M Y$) embryos. Hence, Shao and Takagi (1990) and Takagi (1991) hypothesized that effects of X^M imprinting are exercised through X chromosome inactivation. Furthermore, they proposed that the mechanism that induces the preferential X^P inactivation prevailed in the trophectoderm and primitive endoderm cell lineages of normal female embryos was involved in this phenomenon.

This hypothesis is supported by the occurrence of a considerable proportion of cells having no asynchronously replicating, hence genetically inactivated, X chromosome in 7.5 dpc $X^M X^M Y$ embryos. It is formally possible, however, to assume that they represented cells in which X inactivation had not yet occurred. $X^M X^M X^P$ cells having only one asynchronously replicating X chromosome can not be explained in the same way. One may still assume that X-inactivation occurs stepwise in XXX cells, one X chromosome at a time, and the

single-inactive-X cell is at the intermediate stage. But the fact that the single inactive X is always the paternally derived one, whereas inactivation is essentially random in two-inactive-X cells, i.e., inactive X chromosomes were X^M and X^M or X^M and X^P , is at variance with this assumption and suggests that both types of cells do not represent different stages of X-inactivation, but different cell lineages: one-inactive-X cells belong to the trophectoderm and primitive endoderm cell lineages, and two-inactive-X cells to the epiblast cell lineage. Shao and Takagi (1990) also postulated that X^M imprinting does not simply reduce the probability with which X^M is chosen to be inactivated, but makes it resistant to inactivation in the trophectoderm and primitive endoderm cell lineages.

X chromosome dosage compensation is essential for the survival of the mouse as proved in *Drosophila* and *Caenorhabditis elegans* (reviewed by Hodgkin, 1990). Unbalanced carriers of the T(X;16)16H translocation characterized by two complete sets of autosomes, one normal X chromosome and one X^{16} translocation chromosome (consisting of the centromeric 63% of the X chromosome and the distal half of chromosome 16) invariably die before midgestation with deficient embryonic ectoderm, severely underdeveloped ectoplacental cone and very limited mesoderm formation (Takagi & Abe, 1990). The entire X chromosome and the X chromosome part of X^{16} are functional in every cell of these embryos because X inactivation does not occur due to the loss of the inactivation center from X^{16} by the translocation.

Thus, it would not be surprising that the failure to accomplish correct X chromosome dosage compensation in the non-epiblast cell lineage entails abnormal embryonic development. The majority of diploid parthenogenetic embryos, whose chromosomes are exclusively maternal in origin, resemble fertilized embryos disomic for X^M in morphology. Tada and Takagi (1992) found that the frequency of cells having an inactive X chromosome was significantly lower in

parthenogenetic blastocysts than in normal fertilized ones, which implies that X-inactivation has occurred infrequently in the parthenogenetic trophectoderm. Furthermore, the polar trophectoderm of 5-day parthenogenones, immediately after implantation, was found degenerating. It would be reasonable that degeneration of the polar trophectoderm evokes deficient extraembryonic tissues, since both the ectoplacental cone and extraembryonic ectoderm are formed by the proliferation of the polar trophectoderm. These findings are apparently in accordance with the observation that parthenogenetic embryos with a single X chromosome grow better than those with two X chromosomes (Mann & Lovell-Badge, 1988).

Although no postpartum $X^M X^M Y$ mouse is ever found, two possible $X^M X^M X^P$ cases have been reported. Endo and Watanabe (1989) found an XXX female among phenotypically normal progenies of an X0 mouse. Although these authors favored the maternal origin of the extra X chromosome in this particular case, the lack of X chromosomal marker did not allow them to prove it. Recently, Matsuda and Chapman (1992) reported another XXX female among 200 interspecific backcross progenies between laboratory mice (C57BL/6Ros) and *Mus spretus*. A combination of in situ hybridization and Southern analyses with X-chromosomal probes favored that two of three X chromosomes are of maternal origin. However, their results do not rule out, as they pointed out, nondisjunction at the second division of male meiosis. An additional XXX case was reported by Beechey et al. (1992): The small, fertile adult female was mosaic for at least 3 cell lines and lymphomyeloid cells were practically 100% XXX. The parental origin of the additional X chromosome is unknown. The occurrence of an apparently normal 7.5 dpc $X^M X^M X^P$ embryo in a mating between In1H/X female and Rb2Ad/Y male (Table 4) may point to the possibility that certain $X^M X^M X^P$ embryos are capable of growing normally for the first one third of the pregnancy. It would be conceivable that exceptional embryos disomic for X^M survive preg-

nancy, if they manage to inactivate two X chromosomes in just enough cells to form extraembryonic tissues that are necessary to overcome the initial crisis immediately after implantation, since XX inactivation is apparently normal in the epiblast cell lineage.

Harmful effects of two X^M chromosomes may not be universal in mammals. The supernumerary X chromosome, for example, could be either paternal or maternal in origin in Klinefelter's syndrome (Jacobs et al., 1988). May et al. (1990) showed that nondisjunction at female meiosis is responsible for the origin of human XXX females in nearly 90% of cases. Furthermore, Hassold et al. (1990) reported four human cases of tetrasomy or pentasomy X whose extra X chromosomes were maternal in origin. It is possible that such difference between the man and the mouse may have arisen from a slight changes in the stability or strength of the imprint involved in the choice of the X chromosome to be inactivated.

X0 mothers are expected to produce XX, XY, X0 and OY zygotes at an equal frequency. Since OY zygotes do not develop beyond 2-cell stage (Morris, 1968; Luthardt, 1976), X0 zygotes should comprise 33% of remaining zygotes. Observations thus far made at meiosis (Kaufman, 1972; Luthardt, 1976), cleavage stage (Luthardt, 1976) and at birth (Morris, 1968; Brook, 1983) invariably showed that X0 progenies were much less frequent than expected. Our unpublished observation on embryos from X0 females also showed a severe shortage of X0 conceptuses (14/113) at 6.5-7.5 dpc. This shortage of X0 zygotes has been explained by either nonrandom segregation of the X chromosome at the first meiotic division or reduced survival of offspring. Recently, Hunt (1991) compared viability of female mice with a single X chromosome of either maternal or paternal origin using a recombinant product from the structurally abnormal Y chromosome, Y^* . She found a highly significant prenatal loss of X^P0 and X^PY^{*X} (Y^{*X} is a small and apparently inert chromosome consisted of the

centromere of the Y^* chromosome and the pseudoautosomal region) females from the cross $XY^{*X} \times XY$, but no comparable loss of $X^M Y^{*X}$ females from the cross $XX \times XY^*$. The reduced viability of $X^P O$ and $X^P Y^{*X}$ offspring advocated the effect brought about by X chromosome imprinting or uterine environment of practically XO mother (Hunt, 1991). The present observation on $X^M O$ and $X^P O$ embryos clearly supported the latter alternative, although 12 out of 35 $X^P O$ and $X^P Y$ embryos showed slight growth retardation. Most probably they are able to catch up with XX or XY littermates, because Burgoyne et al. (1983) showed that XO embryos from $In1H/X$ mothers retarded in growth at 7.25 dpc, but that they were similar to XY and XX littermates in size by 12.5 dpc. In agreement with this observation, XO and XX embryos from ICR females did not differ in development at midgestation (Omoe & Endo, 1993). It may be concluded that the effect of paternal imprinting of the X chromosome, if any, is minor, which is consistent with the finding that $X^P Y^P$ mice are fully viable (Handel & Hunt, 1991). This conclusion agrees with the fact that the single X chromosome is either paternal or maternal in origin in human XO cases. The most remarkable difference between men and mice would be that nearly 99% of human XO cases are aborted early in pregnancy.

In summary, studies of early postimplantation development of X chromosomal aneuploids revealed no parentally imprinted X-linked gene essential for the early mouse embryogenesis except for the one most probably involved in the choice of X chromosome to be inactivated. The maternally inherited X chromosome does not undergo inactivation in cell lineages other than the epiblast, although we do not know whether or not this inhibitory effect of imprinting is perfect. It was also shown that X^P imprinting did not explain preimplantation loss of $X^P O$ offspring of XO mothers.

Chapter 2: X-chromosome inactivation and early postimplantation development in parthenogenetic embryos

MATERIALS AND METHODS

Superovulation and matings of mice

Unfertilized oocytes were obtained from young female mice of 129/Sv, C3H/He, LT/Sv and hybrids between C3H/He and CBA/J. Superovulation was induced by the intraperitoneal injection of 10 IU pregnant mare's serum gonadotropin (PMSG, Teikoku hormone) followed 46-48 h later by the injection of 10 IU human chorionic gonadotropin (hCG, Teikoku hormone). Spontaneously ovulating females were mated with fertile males for control embryos. LT parthenogenones were obtained from spontaneously ovulating females mated with vasectomized males.

Parthenogenetic activation

Cumulus masses recovered from swollen ampulla of mice 15-17 h after hCG injection were treated with 300 IU/ml bovine testis hyaluronidase (Sigma) in M2 medium for 5 min to remove cumulus cells (Quinn et al., 1982). Eggs were washed with M2 medium twice, treated with 7% ethanol in M2 medium for 6 min at room temperature (Cuthbertson, 1983; Kaufman, 1983) at 10:00 or 15:00 and washed again with M16 medium twice. Extrusion of the second polar body was suppressed by the incubation with 5 μ g/ml cytochalasin B (Sigma) for 4-5 h at 37°C (Niemierko, 1975). Activated eggs were transferred to oviducts of 0.5-day pseudopregnant females anesthetized by the intraperitoneal injection of 2mg pentobarbital sodium.

Recovery of embryos

Embryos from spontaneously mated females and parthenogenetic embryos activated by ethanol at 10:00 were dissected from decidual swellings daily at 10:00-11:00 and 15:00-17:00, respectively, from day 5 to 8. Parthenogenetic

blastocysts were flashed from uteri 90 h after activation.

Histological examination

Embryos isolated from decidual swellings were fixed immediately with 2.5% glutaraldehyde in 0.1M cacodylate buffer containing 5% sucrose, postfixed with 1.0% osmium tetroxide, dehydrated with acetone, and embedded in Epon 812. Semithin sections cut at 1 μ m were stained with 1.0% toluidine blue.

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BrdU labeling and slide preparation

Blastocysts were allowed to incorporate 5-bromo-2'-deoxyuridine (BrdU: 25 μ g/ml) in Eagle's minimum essential medium supplemented with 10% fetal bovine serum for 9-10 h at 37°C. The concentration of BrdU was increased to 150 μ g/ml and incubation time was reduced to 6-7 hr in the case of postimplantation embryos. Colcemid (0.3 μ g/ml) was present in the medium for the final 2 h of incubation. Embryos were then treated with 1% sodium citrate for 10 min at room temperature, and fixed with 3:1 methanol:acetic acid fixative. Chromosome preparations were made according to a modification (Takagi & Oshimura, 1973) of the method developed by Wroblewska and Dyban (1969) which involves lactic acid treatment to dissociate individual cells at slide preparation.

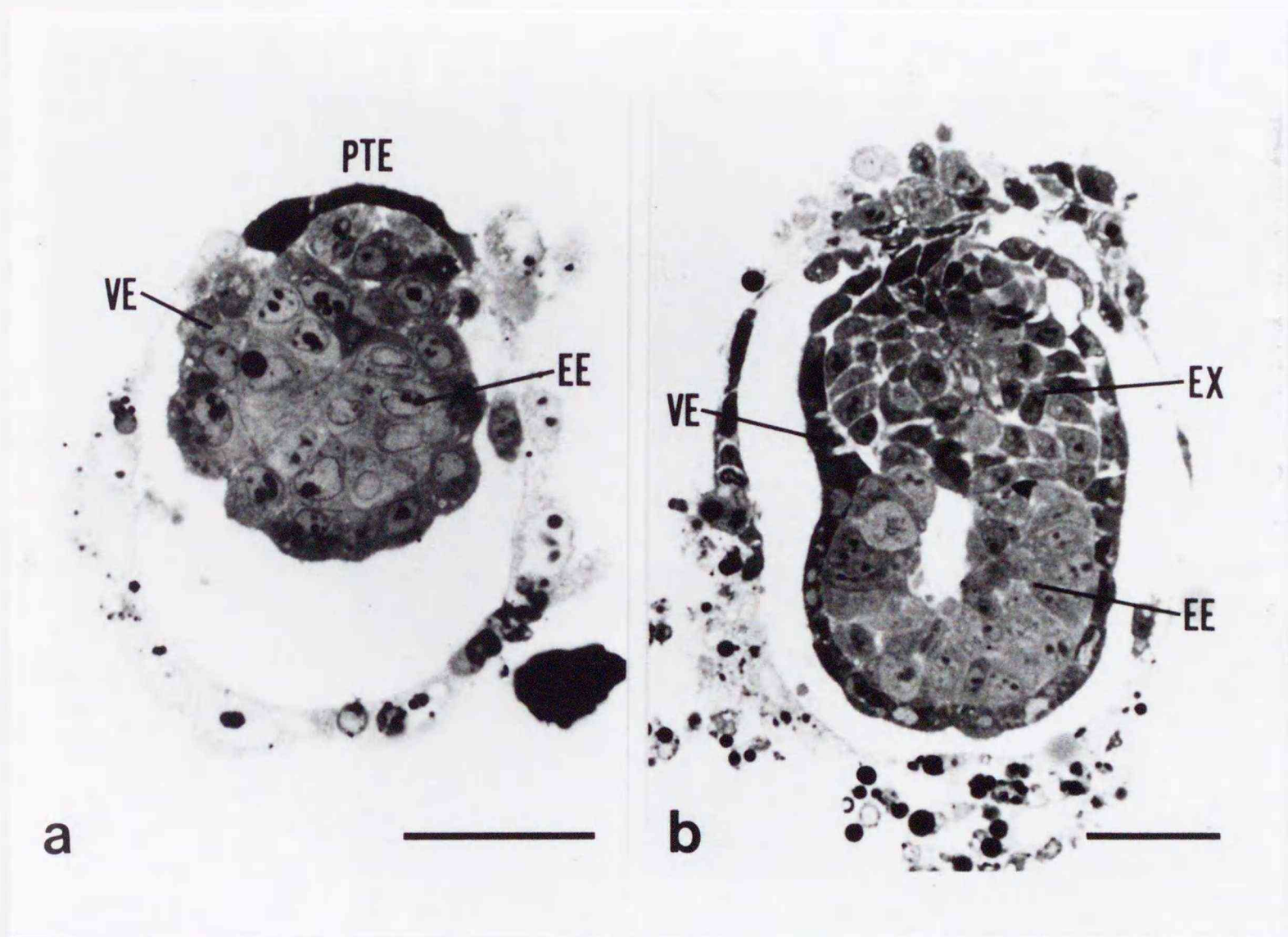


Figure 5. Longitudinal sections of 5-day embryos: (a) an ethanol-induced parthenogene, (b) a fertilized embryo. Note the complete lack of the extraembryonic ectoderm (EX) and the darkly stained polar trophectoderm (PTE) cells in the parthenogene. EE, embryonic ectoderm; VE, visceral endoderm. Bars = 50 μ m.

Table 6. Early postimplantation development of ethanol-induced diploid parthenogenones

Days of development	No. of				Morphology of embryos		
	recipient females transferred	eggs	implants	viable implants	embryos sectioned	egg-cylinder	embryonic disorganized ectoderm cell mass
5	35	988	156(16) ^a	77(49) ^a	49	0	-----49-----
6	20	519	172(33)	34(20)	18	1	2
7	22	707	191(27)	30(16)	30	1	7
8	2	57	16(28)	6(38)	3	0	2

a) percent.

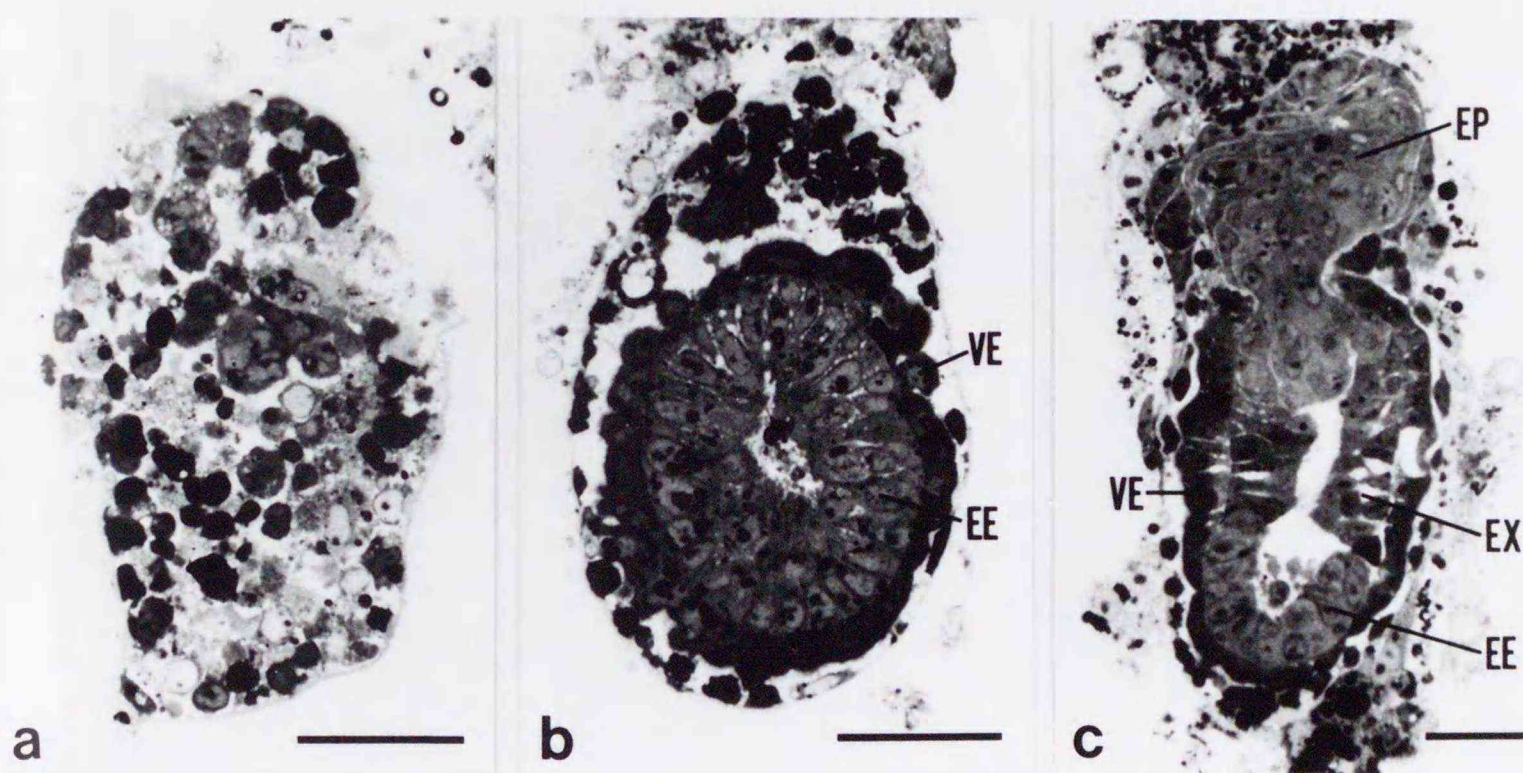


Figure 6. Longitudinal sections of ethanol-induced parthenogenetic embryos on day 7: (a) an abnormal embryo described as a disorganized cell mass in the text, (b) an abnormal spherical embryo without the extra-embryonic ectoderm (EX) and ectoplacental cone (EP), (c) a retarded egg-cylinder stage embryo. EE, embryonic ectoderm; VE, visceral endoderm. Bars = 50 μ m.

RESULTS

Parthenogenetic activation of artificially ovulated eggs and their postimplantation development

About 50-70% of unfertilized eggs treated with ethanol and incubated with cytochalasin B had two pronuclei indicative of diploid parthenogenone. Ten to 40 activated eggs were transferred to oviducts of 98 0.5-day pseudopregnant females, 79 of which carried at least one implantation site 5 to 8 days later. Live embryos were found in 59 recipients. The frequency of implantation sites with a discernible embryo was 49% on day 5, but it decreased to 20% and 16% on day 6 and 7, respectively (Table 6). Thus, most parthenogenones were incompatible with early postimplantation development.

Sizes of parthenogenones recovered on day 5 were somewhat smaller than those of chronologically comparable fertilized embryos. Parthenogenones recovered on day 6 and 7 were, however, much smaller than normally fertilized embryos. Except for 2 egg-cylinder-like embryos, postimplantation parthenogenones were tiny and rounded in shape. The implantation sites of parthenogenones were vascularized less well than those of normally fertilized embryos, and parthenogenones tended to implant less firmly than fertilized embryos, as reported in A^y/A^y embryos that are deficient in trophoblast differentiation (Eaton & Green, 1963). We assumed that parthenogenones obtained here were mostly diploid, since our cytogenetic examination failed to detect any haploid or polyploid embryos on day 5 to 7.

Histological examination of ethanol-induced parthenogenones

A total of 49 5-day parthenogenones sectioned fell into 2 classes on the basis of their histology (Table 6). The extraembryonic ectoderm was absent in

45 parthenogenones (Fig. 5), whereas it developed to a limited extent in remaining 4. Cells at the site of polar trophectoderm were homogeneously darkly stained with toluidine blue suggesting that they were dead or dying (Fig. 5A).

Developmental variability increased on day 6 and the situation was the same on day 7 (Table 6). Fifteen out of 18 embryos examined on day 6 were apparently disorganized. Partially dissociated, often pycnotic cells probably belonging to the embryonic ectoderm cell lineage occupied small presumptive yolk cavity (cf. Fig. 6A). Reichert's membrane and associated parietal endoderm cells were rarely found in these embryos. Two parthenogenones consisted of a spherical embryonic ectoderm surrounded by a thin visceral embryonic endoderm layer and a sac of Reichert's membrane (cf. Fig. 6B). We could not find any cells resembling the ectoplacental cone and extraembryonic ectoderm in these embryos. Cells in mitosis were abundant in the embryonic ectoderm layer, and the central cavity was filled with cell debris. It may be worth noting that the visceral endoderm layer was frequently discontinuous. One exceptional 6-day parthenogenone had morphology of an egg-cylinder, though much smaller than fertilized embryos at a chronologically comparable stage (cf. Fig. 6C).

On day 7, 22 out of 30 parthenogenones belonged to the disorganized type prevalent on day 6. Seven consisted of a spherical embryonic ectoderm bounded by a flat layer of the visceral endoderm and Reichert's membrane with well-developed parietal endoderm cells. Only one parthenogenone was a retarded egg-cylinder containing all primary tissues.

Spontaneous parthenogenones in LT/Sv mice

LT/Sv females were dissected 6 to 8 days after mating with vasectomized males. A total of 31 decidual swellings were found in 18 out of 83 females

Table 7. Early postimplantation development of spontaneous diploid parthenogenones from LT/Sv mice

Days of development	No. of				Morphology of embryos		
	females copulated	eggs ovulated	implants	viable embryos sectioned	egg-embryonic disorganized cylinder ectoderm	cell mass	
6	10	80	3	2	0	2	0
7	55	540	15	4	0	3	1
8	18	146	13	0	0	0	0

Table 8. Replication patterns of X chromosomes in XX embryos

Stage of development (cell no.)	No. of cells with			Total no. cells examined
	an allocyclic X		two isocyclic Xs	
	early	late		
Parthenogenetic blastocysts				
<30	1	0	6(86) ^a	7(6) ^b
30-40	0	0	20(100)	20(6)
40-50	0	0	17(100)	17(9)
50-60	0	0	10(100)	10(9)
60-70	2	0	17(89)	19(8)
>70	1	0	11(92)	12(8)
Total	4(5) ^a	0	81(95)	85(46)
Fertilized blastocysts				
<30	0	0	1(100)	1(1)
30-40	0	0	11(100)	11(7)
40-50	3(11)	0	24(89)	27(11)
50-60	12(17)	0	57(83)	69(23)
60-70	1(20)	0	4(80)	5(1)
>70	1	0	1	2(1)
Total	17(15)	0	98(85)	115(44)
5-day parthenogenetic embryos				
<100	5(11)	0	40(89)	45(19)
100-200	17(19)	0	73(81)	90(21)
200-300	4(10)	0	38(90)	42(5)
Total	26(15)	0	151(85)	177(45)
5-day fertilized embryos				
100-200	4(33)	0	8(67)	12(3)
200-300	20(39)	0	31(61)	51(6)
300-400	22(42)	0	30(58)	52(4)
400-500	8(42)	0	11(58)	19(3)
500-700	18(60)	0	12(40)	30(3)
>700	7(35)	2	11(55)	20(1)
Total	79(43)	2(1) ^a	103(56)	184(17)

a) percent; b) number of embryos examined.

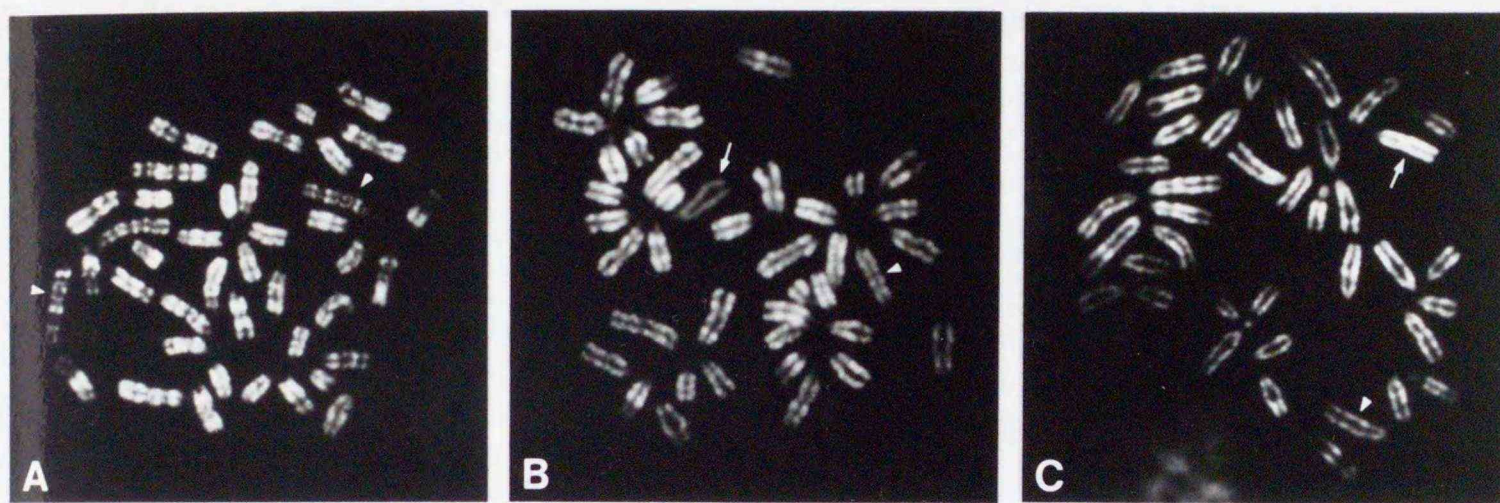


Figure 7. X-chromosome replication patterns revealed by a BrdU-acridine orange procedure in diploid parthenogenetic embryos: (A) a metaphase spread having two isocyclic X chromosomes, (B) a metaphase spread with a late replicating X chromosome, (C) a metaphase spread with a precociously (early) replicating X chromosome. Arrows and arrow heads indicate the allocyclic and isocyclic X chromosomes, respectively.

examined (Table 7). Embryos were alive in 14 implantation sites. One small and 5 larger embryos were subjected to histological examination (Table 7). The small embryo was similar to the disorganized type found in ethanol-induced parthenogenones. Larger embryos, on the other hand, consisted mainly of the embryonic ectoderm bounded by a flat layer of the visceral embryonic endoderm as seen in certain induced parthenogenones. Structures reminiscent of the ectoplacental cone or extraembryonic ectoderm never occurred in these embryos. Thus, spontaneous and induced parthenogenones resembled each other quite well.

X chromosome inactivation in parthenogenones

In early mouse embryos, the inactivated X chromosome replicates either in the earlier half of the S phase (early or precociously replicating X; Fig. 7C) or in the later half (late replicating X; Fig. 7B). X chromosome that replicates synchronously with autosomes (isocyclic X; Fig. 7A) were considered genetically active or potentially active.

X chromosome inactivation was studied in expanded blastocysts, because Sugawara et al. (1985) showed that the inactivated X chromosome was cytologically detectable first at this stage of mouse development. In this observation, metaphase spreads were considered informative if chromosome 16 were readily identified on the basis of their R-banding patterns after staining with acridine orange to assure that the cell incorporated enough BrdU in the latter part of the S phase (Takagi, 1980). Cytogenetic data summarized in Table 8 showed that X inactivation was apparently delayed or impeded in parthenogenones. Only 4 out of 85 informative cells obtained from 46 parthenogenetic blastocysts had a precociously replicating X chromosome, whereas 17 out of 115 informative spreads from 44 fertilized embryos had such element. The difference was statistically significant at 5% level ($X^2=4.26$). This compari-

son would be valid if embryos examined were at comparable stages of development and had an identical inner cell mass/trophectoderm ratio, because X-inactivation is limited to trophectoderm at this stage (Sugawara et al., 1985). Both conditions seemed to be met because all embryos studied here were expanded blastocysts with nearly identical mean cell numbers [$49 \pm 1.56(\text{SE})$ in fertilized embryos and $52 \pm 2.94(\text{SE})$ in parthenogenetic embryos] and no histological abnormality was evident in sectioned parthenogenetic blastocysts (data not shown).

The majority of 5-day parthenogenones examined consisted of 100-200 cells whereas fertilized embryos on day 5 were somewhat larger (Table 8). A precociously replicating X chromosome was found in 21 of 132 informative metaphase cells from parthenogenones comprising 100-300 cells, but a late replicating X chromosome was never detected in these embryos. In fertilized embryos with comparable total cell numbers, 24 out of 63 informative cells contained a precociously replicating X chromosome. The late replicating X was found in fertilized embryos with more than 700 cells.

Out of 101 informative cells from 13 6-day parthenogenones a precociously and a late replicating X chromosome was found in 24 and 35 cells, respectively suggesting that X-inactivation had occurred in the embryonic ectoderm (Table 9). Remaining 42 spreads had two isocyclic X chromosomes. On day 7, 91 out of 98 parthenogenetic metaphase cells examined had an asynchronously replicating X chromosome. The frequency of cells with a precociously replicating X decreased considerably as in fertilized embryos. It may be significant that there was a tendency that the frequency of cells having two isocyclic X chromosomes was higher in parthenogenones than in fertilized embryos on day 6 (Table 9).

Table 9. Replication patterns of X chromosomes in day 6 and 7 embryos

Stage of development (days)		No. of cells with			Total no. cells examined
		an allocyclic X		two isocyclic	
		early	late	Xs	
6	P	24(24) ^a	35(35) ^a	42(42) ^a	101(13) ^b
	F	185(48)	136(36)	62(16)	383(25)
7	P	12(12)	79(81)	7(7)	98(6)
	F	22(8)	235(89)	7(3)	264(9)

P, parthenogenetic embryos; F, fertilized embryos; a) percent;
b) number of embryos examined.

DISCUSSION

The frequency of postimplantation development of parthenogenones varied extensively among different experiments. The present study showed that ethanol-induced mouse parthenogenones are lost at various stages of embryonic development. Although the exact frequency is difficult to assess, a large number of parthenogenones are eliminated before implantation, and about 50% of the embryos implanted were lost by day 5. During next 24h, about half the embryos alive on day 5 are resorbed, and only 16% of initially implanted embryos are surviving on day 7. This low frequency obtained by us is comparable to those reported by Tarkowski et al. (1970), Witkowska (1973) and Kaufman and Gardner (1974) following transfer of blastocysts to the uterus. Kaufman et al. (1977) reported a frequency of 25% development to somite stage after inducing delayed implantation in blastocysts and Surani and Barton (1983) obtained a frequency up to 22% development to various postimplantation stages. It is difficult to define variables affecting the frequency and degree of development of parthenogenones at present.

Parthenogenetic embryos that managed to reach the expanded blastocyst stage were morphologically normal, but the majority of them became abnormal immediately after implantation. They consisted of loosely connected, probably embryonic ectoderm, cells enclosed in a tiny yolk cavity with poorly developed visceral or parietal endoderm. It is tempting to speculate that the absence or deficiency of the visceral endoderm is related to derangement or abortive columnar pattern formation by the embryonic ectoderm in these embryos. Fewer, but more successful parthenogenones contained a spherical embryonic ectoderm bounded by the visceral endoderm as reported in fertilized embryos with an extra copy of X^M (Shao and Takagi, 1990). Developmental abnormalities were

basically identical between spontaneous and induced parthenogenones.

In agreement with the recent finding by Surani et al. (1990), the present study showed that the most consistent and remarkable developmental abnormality in early postimplantation parthenogenones is the lack of the ectoplacental cone and the extraembryonic ectoderm. In normal embryogenesis soon after implantation, diploid polar trophoctoderm cells proliferated under an inductive influence from the inner cell mass give rise to the ectoplacental cone and extraembryonic ectoderm (Copp, 1979). Therefore, the explanation would be either that the parthenogenetic inner cell mass is defective in inducing cell proliferation in polar trophoctoderm, or that the parthenogenetic polar trophoctoderm fails to respond to an inductive signal from the inner cell mass. Data available thus far seem to favor the latter possibility. Barton et al. (1985) examined the developmental potential of the inner cell mass and trophoctoderm derived from parthenogenetic or biparental gynogenetic embryos in reconstituted blastocysts with normal trophoctoderm or inner cell mass, respectively. Observation made on day 11 or later showed that the trophoblast tissue developed very poorly in the presence of the normal inner cell mass, if the trophoctoderm tissue lacked a paternal genome. However, the inner cell mass lacking a paternal genome developed to an advanced embryonic stage if it was introduced inside a normal trophoctoderm vesicle. These findings indicate that the polar trophoctoderm is defective in most parthenogenones.

Degenerating polar trophoctoderm cells found in 5-day parthenogenones are in accordance with the lack of the ectoplacental cone and extraembryonic ectoderm in later stages. Therefore, cause(s) of cell death at the polar trophoctoderm may have a share in the early death of parthenogenones, though we have to take the complexity of developmental processes and indirectness of available data into consideration. As mentioned above, Mann and Lovell-Badge (1988) showed that parthenogenetic inviability is ultimately determined by the

possession of two sets of maternally derived autosomes, available evidence suggests that difficulties in accomplishing X inactivation may also contribute to the abnormal development of parthenogenones: (1) Fertilized, hyperdiploid $X^M X^M Y$, but not $X^M X^P Y$, male mice have never been found in postnatal populations (Russell and Chu, 1961), (2) Fertilized, $X^M X^M Y$ and $X^M X^M X^P$ embryos die early in embryogenesis (Shao and Takagi, 1990), and (3) Two X^M chromosomes tended to remain active in a proportion of cells from $X^M X^M Y$ and $X^M X^M X^P$ embryos even when X-inactivation is supposed to have taken place (Shao and Takagi, 1990). Detrimental effects of two active X chromosomes were demonstrated in unbalanced carriers of Searle's X-autosome translocation, T(X;16)16H, in which the proximal 63% of X^M and the intact X^P remain active in every cell (Takagi and Abe, 1990). Underdevelopment of the ectoplacental cone and the failure to form mesoderm characterized such embryos.

We found that the frequency of cells having an asynchronously replicating X chromosome is significantly low in expanded parthenogenetic blastocysts than in fertilized ones, although such tendency was not always evident in well-grown parthenogenones (Kaufman et al., 1978; Rastan et al., 1980; Endo and Takagi, 1981). Initiation of X inactivation in the mouse embryo occurs at different times in different cell lineages. X^P chromosome is inactivated in the trophectoderm and the primitive endoderm in which inactivation occurs earlier than in the embryonic ectoderm characterized by random X inactivation (Takagi and Sasaki, 1975). It is believed that the nonrandomness in these tissues is ascribed to resistance of the X^M to inactivation by imprinting conferred to it during oogenesis, and randomness in the embryonic ectoderm cell lineage is attained by the erasure of such imprint (Lyon and Rastan, 1984). This imprinting is so effective and consistent that two X^M chromosomes remained active in the majority of extraembryonic cells from mouse digynic $X^M X^M X^P$ and $X^M X^M Y$ triploids (Endo et al., 1982). Although Rastan and Cattana-

(1983) showed cytogenetically that this type of nonrandom inactivation can be overridden to a certain extent by appropriate manipulation of alleles at the Xce locus, this has not been born out by biochemical determination of the level of PGK-1 isozymes (Bucher et al., 1985). We may tentatively conclude that X-inactivation is difficult to accomplish in the trophectoderm of mouse parthenogenones since both X chromosomes carry maternal imprinting, and two copies of the active X chromosome are incompatible with survival and further differentiation of the polar trophectoderm, though why so remains unknown. It is also unknown whether failure of X chromosome dosage compensation is causally related to underdevelopment of the visceral or parietal endoderm.

The survival of some XX parthenogenones beyond midgestation could probably be due to just enough trophectoderm and primitive endoderm cells managed to inactivate an X chromosome to enable further development (Mann and Lovell-Badge, 1988). Possible implantational delay might have given embryos enough time to do so. Both X^M and X^P are apparently exempt from other imprints that seriously affect embryonic development in hemi- or homozygous conditions, since adult hemizygous X^M0 and X^P0 mice are found with reasonable frequencies (Russell, 1962). In contrast to the situation in mice, the supernumerary X chromosome could be either paternal or maternal in origin in Klinefelter's syndrome (Jacobs et al., 1988). Furthermore, nondisjunction at female meiosis is responsible for the genesis of human XXX females in nearly 90% of cases (May et al., 1990). It may be speculated that X chromosome imprinting in man is probably less powerful than in mice. Controversies on the preferential inactivation of X^P in the human placenta (Migeon et al., 1985; Harrison and Warburton, 1986) seem to reflect such situation. It would not be surprising if human parthenogenones are more well-balanced than mouse parthenogenones with both embryonic and extraembryonic structures.

Chapter 3: X-chromosome inactivation and early postimplantation development in tetraploid embryos

Materials and Methods

The female mice homozygous for the Rb(6.16)24Lub translocation received an intraperitoneal (i.p.) injection of 10 IU of pregnant mare's serum gonadotropin (PMSG; Teikoku hormone), followed 46-48 hr later by an i.p. injection of 10 IU of human chorionic gonadotropin (hCG; Teikoku hormone). Soon after hCG injection, the females were mated individually with male mice hemizygous for Rb(X.2)2Ad translocation. The presence of a vaginal plug the next morning was taken as evidence that mating had occurred. Early in the morning of the second day of gestation, the female mice were sacrificed by cervical dislocation, and the oviducts were removed and flushed with phosphate buffered saline to recover the two-cell-stage embryos.

Tetraploid embryos were produced by electrofusion of blastomeres at late 2-cell-stage as described by Kubiak and Tarkowski (1985). An electrode made of 0.1 mm thick platinum ribbon on the bottom of the fusion chamber was glued, and the second movable electrode made of a platinum wire ($D=0.2$ mm) was attached to a simple micro-manipulator (Fig. 8). The electrodes were covered by 500 μ l of 0.3 M mannitol. A single embryo was placed between them at a time and oriented so that the plane of first cleavage division was perpendicular to the electric field. The distance between the electrodes was adjusted to slightly exceed the diameter of the zona pellucida (ca 100 μ m). A single square pulse of 15 V for 100 μ sec was applied by a pulse generator (SEN-2201, Nihon Koden, Japan). Treated embryos were incubated for 2 hr in M16 medium at 37°C in an atmosphere of 5% CO₂ in air before being transferred to the oviducts of pseudopregnant recipients on the first day of pseudopregnancy. These females were autopsied on 6th to 8th day of gestation (i.e. the first day of finding a vaginal plug, when spontaneously cycling females are mated to sterile males, and subsequently termed the first day of gestation) for the histo-

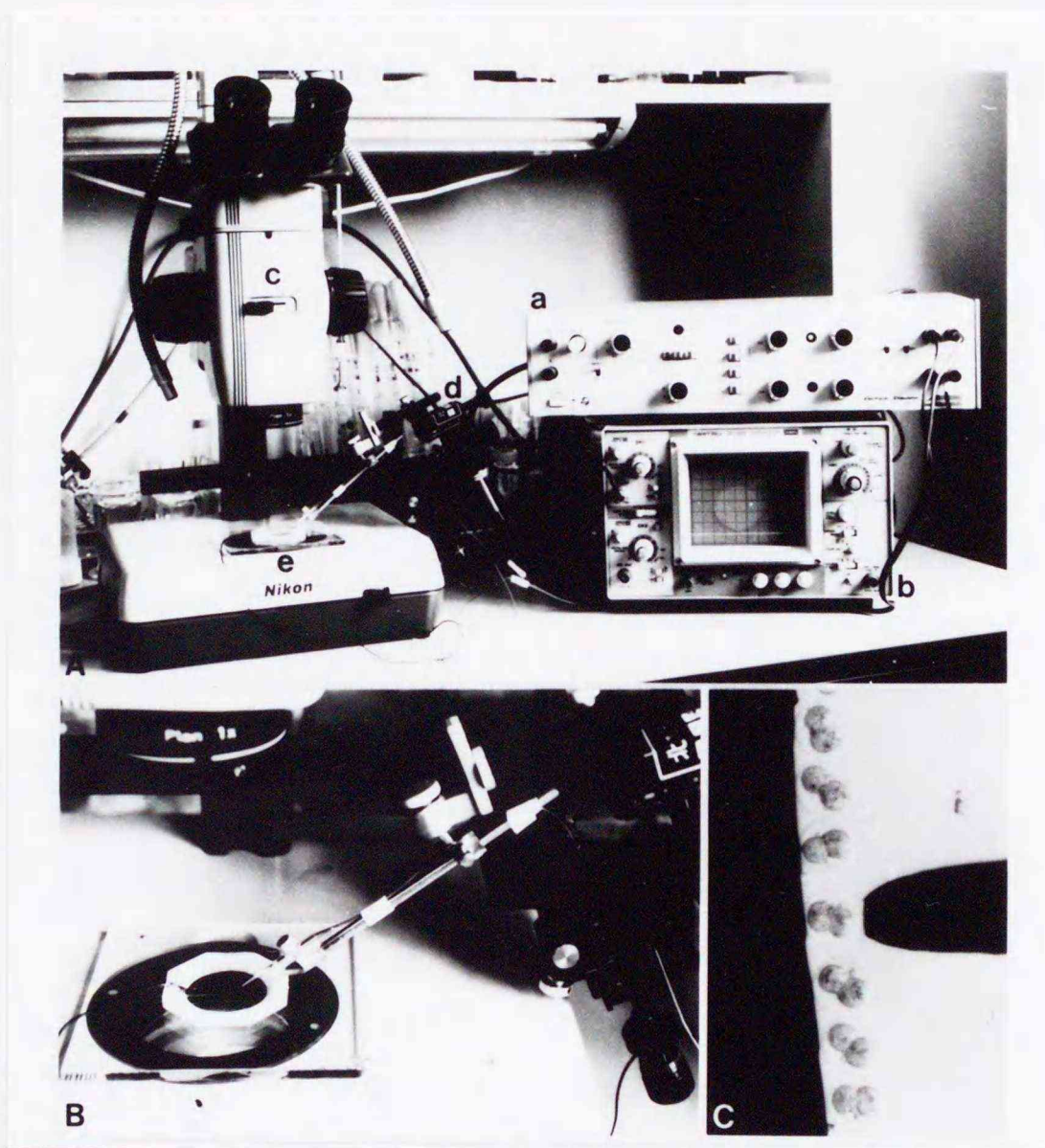


Figure 8. A set of apparatus for the electric fusion of blastomeres at 2-cell stage. A, Equipment. a, electric stimulator; b, synchroscope for monitoring; c, dissection microscope; d, simple micromanipulator; e, electric chamber. B, A chamber used for blastomere fusion. A platinum ribbon glued to the bottom of chamber as the fixed electrode and platinum wire attached to a simple micromanipulator as the movable electrode. C, Alignment of 2-cell-stage embryos between both electrodes.

logical and cytological examinations of transferred embryos.

For histological examination, embryos isolated from decidual swellings were fixed immediately with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 5% sucrose, postfixed with 1.0% osmium tetroxide, dehydrated with acetone, and embedded in Epon 812. Semithin sections cut at 1 μ m were stained with 1.0% toluidine blue.

Recovered 8th day embryos for chromosome study were incubated in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 150 μ g/ml 5-bromo-2-deoxyuridine (BrdU) at 37°C. The duration of incubation was 7 hr including the last hour in the presence of 0.2 μ g/ml Colcemid. After hypotonic treatment with 1% sodium citrate, embryos were fixed with 3:1 methanol:acetic acid. Chromosome slides were prepared according to a modification of the air-drying method described by Wroblewska and Dyban (1969). Slides were stained with freshly prepared acridine orange and examined under a fluorescence microscope.

Results

Early postimplantation development of tetraploid embryos

About 90% of embryos exposed to the electric pulse at 2-cell stage produced reconstructed one-cell tetraploid embryos. Following the transfer of 790 one-cell tetraploid embryos to the oviducts of 52 recipient mice on the first day of pseudopregnancy, 431 (55%) implantation sites were found 6-8 days later. Of these, 238 (55%) contained resorptions and 193 (45%) contained an embryo or embryonic remnant. The frequency of implantation sites was roughly equal in the 6th to 8th day of gestation, indicating that extensive resorption and embryonic loss occurred immediately after implantation was minimal, if any, during this 48 hr period of postimplantation stages (Table 10). Among 46 embryos cytologically analyzed, 27 had an XXXX and 19 had an XXYY sex chromosome constitution.

Tetraploid embryos at the 7th and 8th day of gestation were abnormal as reported previously (Snow, 1975; Tarkowski et al., 1977). Figure 9-10 illustrates morphological features of representative tetraploid conceptuses. The most remarkable abnormality was the deficient embryonic portion, whereas extraembryonic structures developed relatively well. Another point of interest was underdevelopment of the ectoplacental cone as reported in the unbalanced carrier of the T(X;16)16H translocation (Takagi and Abe, 1990). Of the seven 7-day and eight 8-day conceptuses histologically analyzed, 1 consisted mostly of trophoblastic giant cells (Fig. 9a), 5 lacked the embryonic region completely (Fig. 9b) and 8 consisted of small embryonic ectodermal tissue and well-developed extraembryonic membranes (Fig. 9c). Thus, no egg-cylinder with normal morphology was ever found in this study. Severely abnormal development of tetraploid embryos has been reported several times, but Kaufman's group

succeeded in getting much better growth. The clear disparity should be attributed to the difference present between strains of mice used for the experiment rather than techniques (Kaufman and Webb, 1990; Webb et al., 1992).

In contrast with these embryos, all of four tetraploid embryos at 6th day of gestation were morphologically similar to the day 5.5 dpc normal diploid (Fig. 10). The embryonic ectoderm remained to be small for the following two days, whereas the extraembryonic region grown well. Histological sections showed that mitotic cells were abundant in the embryonic ectoderm, but there were signs of extensive cell death (Fig. 11). Death of embryonic ectoderm cells at the developmental stage when X-chromosome inactivation is occurring seems to suggest the involvement of unbalanced X-chromosome inactivation in this phenomenon.

X-chromosome replication patterns in tetraploid embryos

Non-random inactivation of the paternally derived X chromosome occurs in all embryonic cells except the embryonic ectoderm or epiblast. Hence we attempted to examine X chromosome inactivation pattern in different embryonic regions separately. In view of the abnormal development of tetraploid embryos revealed by histological analysis, each embryo was arbitrarily cut into three regions, embryonic region, extraembryonic region and ectoplacental cone under the dissection microscope. The fraction of the ectoplacental cone might be contaminated with cells from the extraembryonic region. The extraembryonic region consisted of the outer endoderm and the inner ectoderm layer was recovered relatively free of contaminants. The embryonic region, however, comprised the embryonic ectoderm primitive endoderm and variable amount of the extraembryonic ectoderm. Asynchronous replication early or late in the S phase was employed for the recognition of the inactive X chromosome.

In the ectoplacental cone and extraembryonic regions of $X^M X^M X^P X^P$ embryos,

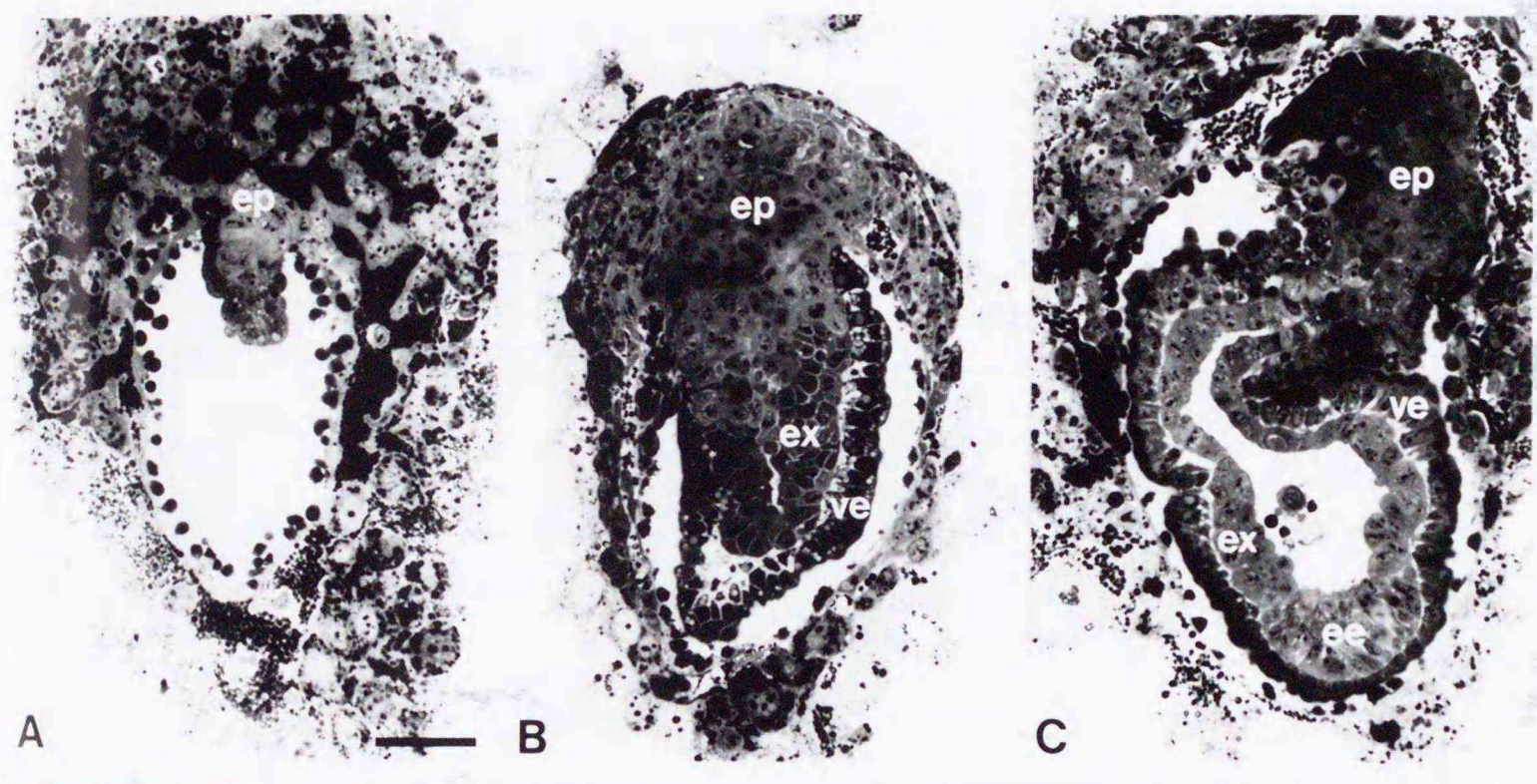


Figure 9. Longitudinal section of representative tetraploid embryos on 8th day of gestation. A: an embryo generally lacking the embryonic ectoderm (ee) and extraembryonic ectoderm (ex). B: an embryo completely lacking the embryonic ectoderm. C: an embryo with abnormal embryonic ectoderm. ve, visceral endoderm; ep, ectoplacental cone. Bars = 100 μ m.

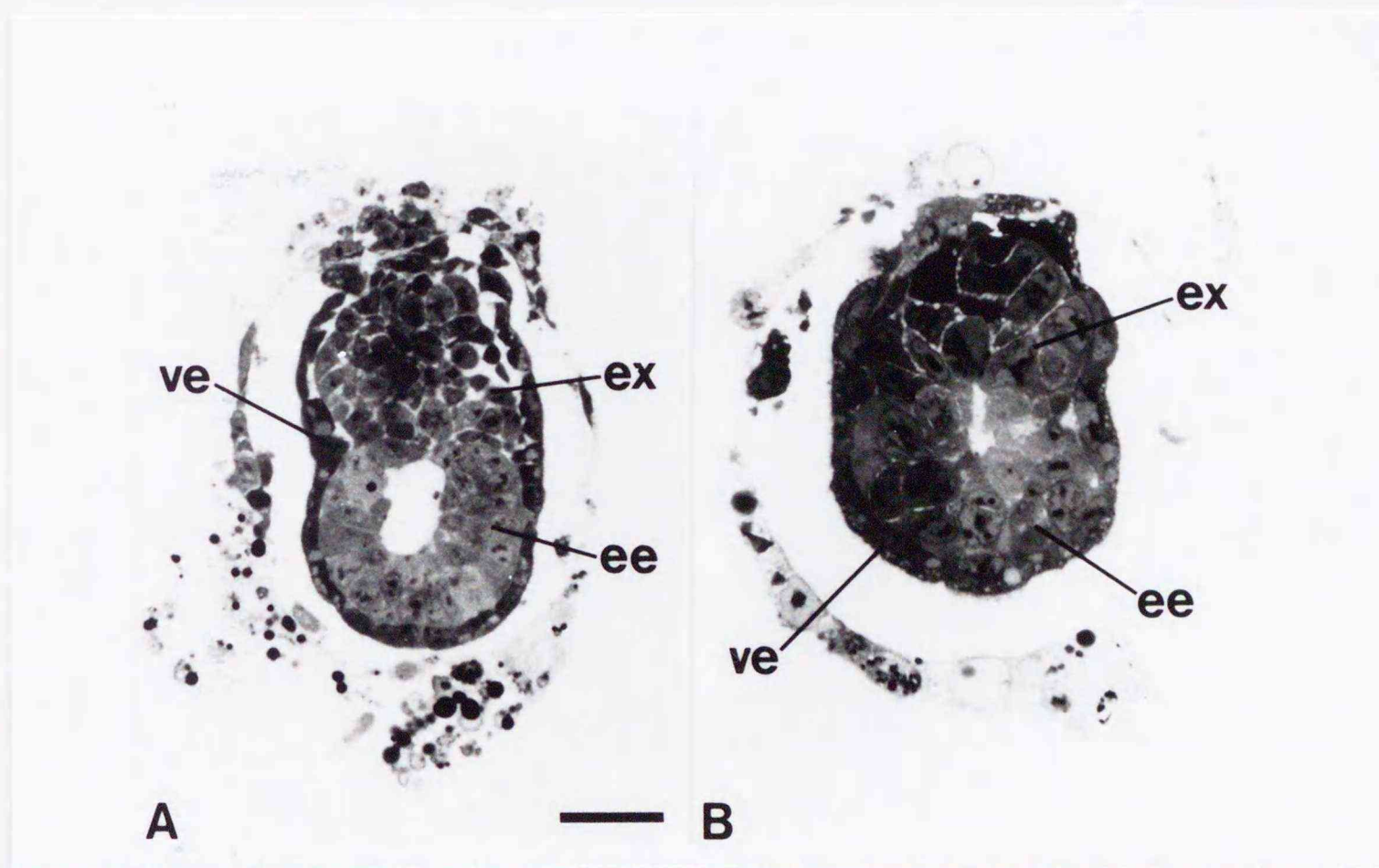


Figure 10. Longitudinal section of a 5.5-day fertilized embryo (A) and a 6th day tetraploid embryo (B). ee, embryonic ectoderm; ex, extraembryonic ectoderm; ve, visceral endoderm. Bars = 50 μ m.

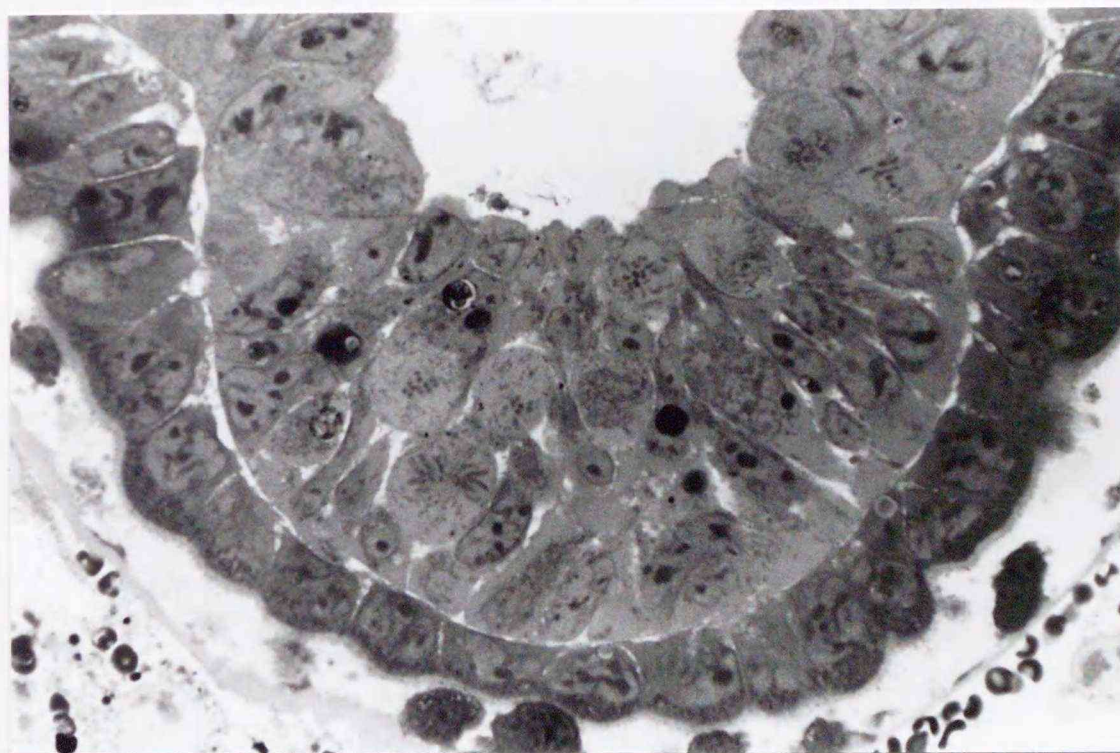


Figure 11. A section of the embryonic ectoderm (ee) of 8th day tetraploid embryo. Mitosing cells are abundant, whereas many cells stain darkly, the sign of cell death.

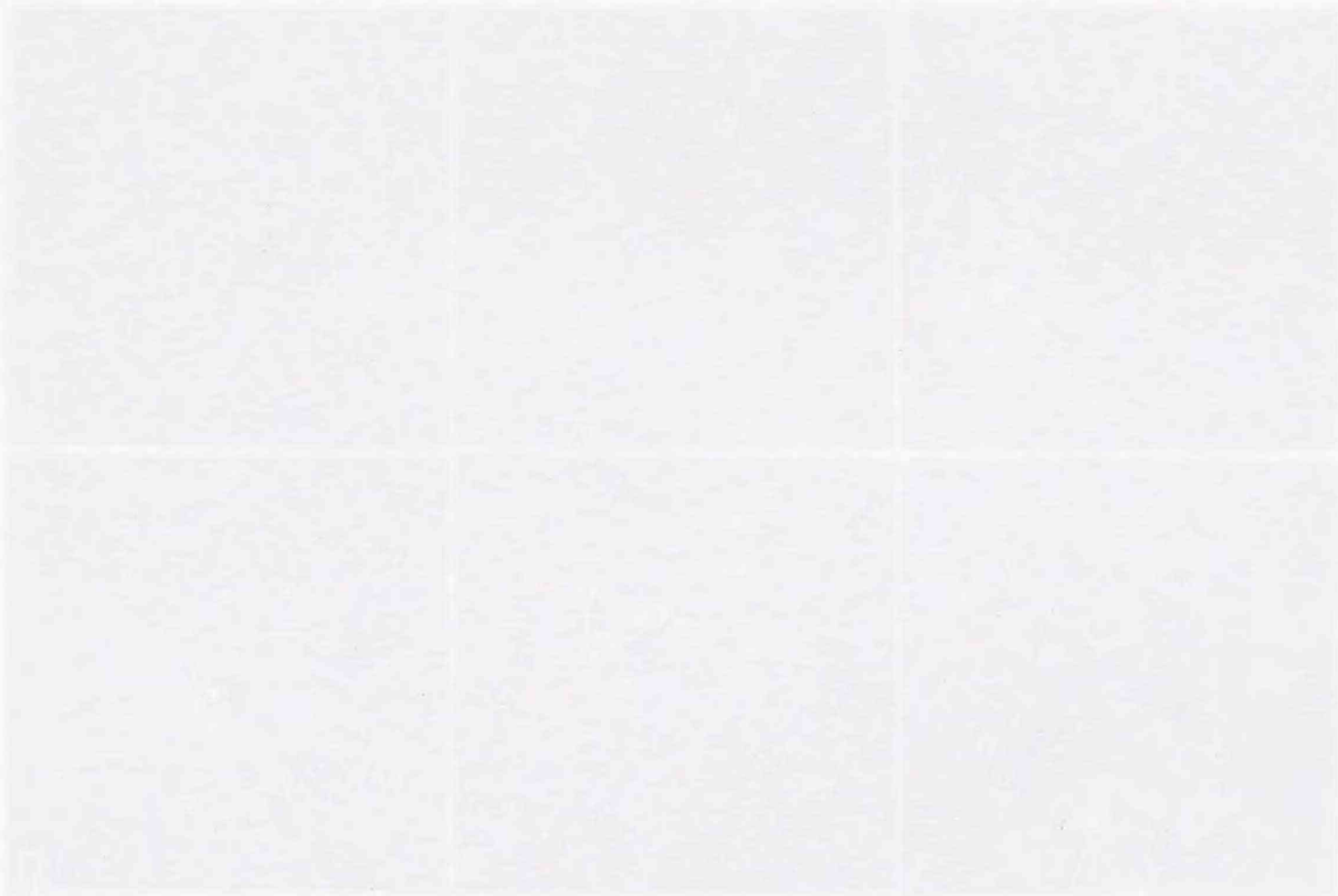
Table 10. Early postimplantation development of tetraploid embryos produced by electrofusion

Days of development	No. of			
	Recipient females	Embryos transferred	Implants	Viable implants
6th	2	33	18(55)	10(56)
7th	12	181	104(58)	37(36)
8th	38	576	309(54)	146(47)

(), percent.

the vast majority of metaphase spreads had two asynchronously replicating X-chromosomes (Table 11). Out of 80 and 35 metaphases from the extraembryonic region and ectoplacental cone, 57 (84%) and 32 (91%) carried two allocyclic X-chromosomes (Fig. 12B), respectively. All inactivated X chromosome of these cells but one were paternally derived and about half of them replicates precociously. It was interesting to find six cells having 3 apparently inactive X chromosomes (Fig. 12C) and three cells having only one such chromosome (Fig. 12A) among cells from the extraembryonic region. Only two cells from the ectoplacental cone had three inactive X chromosomes. Extreme underdevelopment of the embryonic region allowed us to appraise only 16 metaphase cells: 13 had two asynchronously replicating X chromosomes, one cell each had one and three such chromosomes, and one had four synchronously replicating X chromosomes (Fig. 12D). No allocyclic X-chromosome in 109 out of 110 cells from the extraembryonic ectoderm and ectoplacental cone regions of $X^M X^M Y Y$ embryos. The high frequency of $X^M X^M X^P X^P$ cells with inactivated X^P -chromosomes in the ectoplacental cone and extraembryonic region indicated the occurrence on non-random X^P -inactivation (Table 11) in the trophectoderm and primitive endoderm as in diploid female embryos probably due to parental imprinting. The significance of cells with one or three inactivated X chromosomes is difficult to evaluate. They might show either that unbalanced X-inactivation indeed occur in XXXX tetraploid embryos or that they are artifacts due to insufficiency of cytogenetic techniques employed here. By means of Kanda's hot hypotonic techniques, Webb et al. (1992) also recognized the occurrence of cells showing such unbalanced X-inactivation. Hence, we feel that they are not artifacts although further study is essential. If this is the case, the frequency of unbalanced X-inactivation could be high in the embryonic ectoderm, because the proportion of non-epiblast contaminants is excessively high in our samples of

the embryonic region.



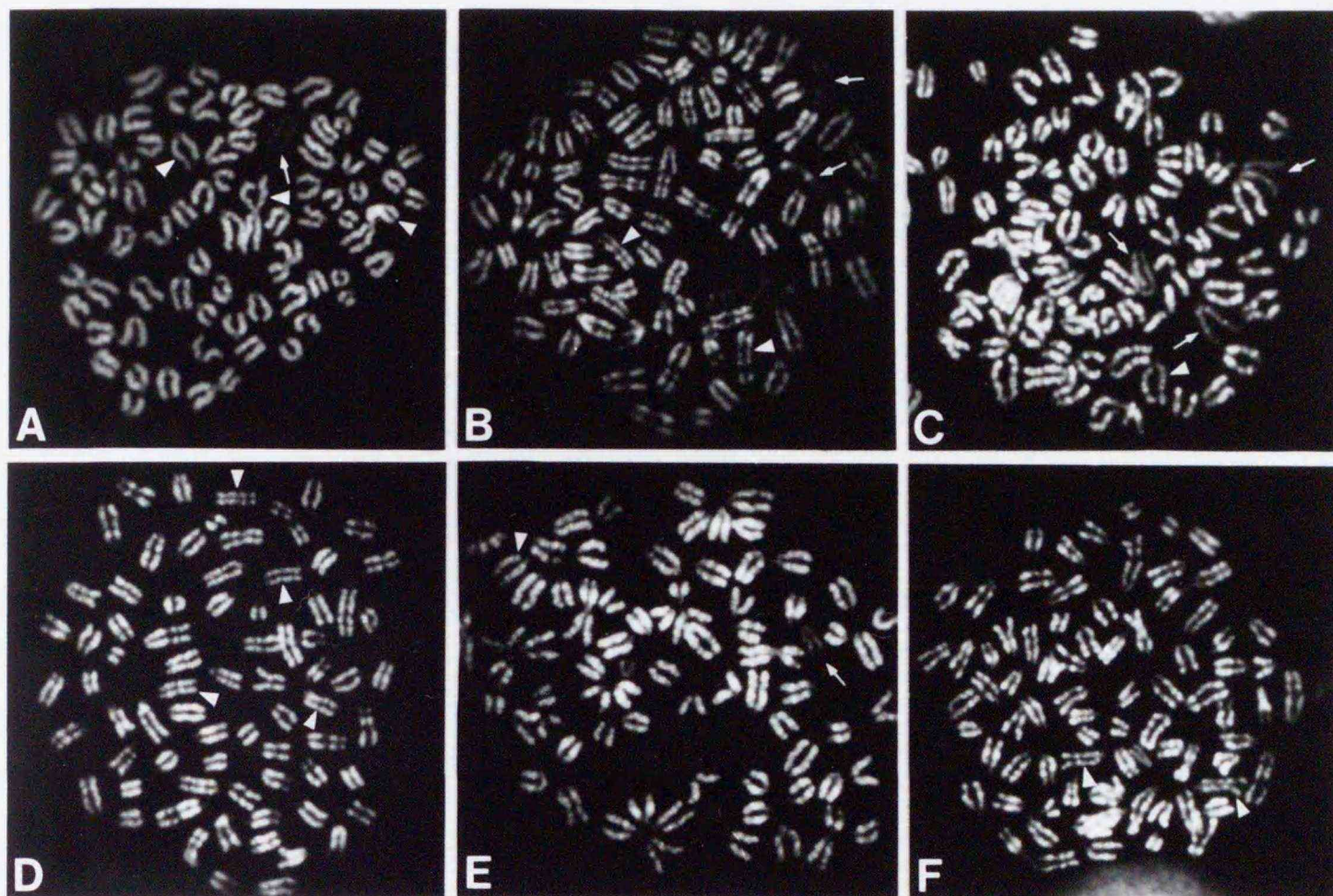


Figure 12. X-chromosome replication patterns by using a BrdU-acridine orange procedure in tetraploid embryos. A: A XXXX metaphase spread having one late replicating X^M and three synchronously replicating X chromosomes. B: A XXXX cell with two late replicating X^P chromosomes. C: A XXXX metaphase spread carrying three late replicating X chromosomes. D: A XXXX metaphase spread having four synchronously replicating X chromosomes. E: A XXYY metaphase spread with one late replicating X chromosome. F: A XXYY cell with two synchronously replicating X chromosomes. Arrows and arrowheads indicate the allocyclic and isocyclic X chromosomes, respectively.

Table 11. X chromosome replication patterns in $X^M X^M X^P X^P$ tetraploid embryos on the 8th day of gestation

Inactivated X chromosomes		Embryonic region	Extraembryonic region	Ectoplacental cone	Whole embryo
None		1(6)	4(5)	1(3)	2(3)
X^M	P	0	0	0	0
	L	0	0	0	1(2)
X^P	P	1(6)	1(1)	0	0
	L	0	2(3)	0	1(2)
$X^M X^P$	P	0	0	0	1(2)
	L	1(6)	1(1)	0	0
$X^P X^P$	P	3(19)	34(43)	18(51)	18(30)
	P/L	0	3(4)	0	2(3)
	L	9(56)	29(36)	14(40)	34(56)
$X^P X^P X^M$	P	0	2(3)	0	1(2)
	L	1(6)	4(5)	2(6)	1(2)
Total		16	80	35	61

P, precociously replicating; L, late replicating; (), percent.

Discussion

Spontaneous tetraploid embryos relatively rare in mammals. In the human, tetraploid embryos can implant but they are usually aborted in early pregnancy, the exception being some liveborn infants described in the literature (Golbus et al. 1976; Pitt et al. 1981; Scarborough et al. 1984; Shiono et al. 1988). Most tetraploid abortuses consist of fetal membranes only and do not contain any recognizable embryonic structures (Carr, 1971; Hamerton, 1971). Experimentally produced mouse tetraploid embryos can implant at high frequency, but rarely form embryonic structures (Snow, 1975, 1976; Tarkowski et al. 1977). Occasional embryos proceed through organogenesis and reach term (Snow, 1973), but these embryos could have been diploid/tetraploid mosaics (Tarkowski et al. 1977). When diploid $\leftrightarrow$ tetraploid chimeras are made, the tetraploid cells are selected against quickly during fetal development in most cases (Lu and Markert, 1980; Nagy et al. 1990). Thus, the tetraploid cells seem to be unable to participate fully in normal embryonic development. The abnormal development of the tetraploid embryos may be ascribed to altered nuclear/cytoplasmic ratio (Epstein, 1986), trophoblastic alteration (Jacobs et al., 1982), delayed cell division (Tarkowski et al., 1977), or abnormal active X-chromosome/autosome ratio. Snow (1975) had predicted that the developmental problems encountered by tetraploids are physiological and numerological in nature. The present histological analysis of tetraploid embryos at early postimplantation stage revealed that the underdevelopment of the embryonic ectoderm was not due to low mitotic activity but to extensive cell death after implantation. It may not be a mere coincidence that the death of embryonic ectoderm cells occurred almost simultaneously with the initiation of X-chromosome inactivation in the embryonic ectoderm. An intriguing possibility would

be that imbalance of active X-chromosome/autosome ratio resulted from irregular X-chromosome inactivation is responsible for the cell death occurring in embryonic ectoderm.

Two paternally derived X-chromosomes were inactivated in the overwhelming majority of the extraembryonic cells of XXXX tetraploid embryos. It may be reasonable to assume that the mechanism causing non-random X^P -inactivation in the non-epiblast cells of diploid XX embryo is also acting in tetraploid embryos. Webb et al. (1992) demonstrated by the Kanda's differential staining technique in two darkly-staining, inactive X chromosomes, in 78% of endodermally-derived cells of XXXX tetraploid embryos on the 10th day of gestation. However, two paternal X-chromosomes were inactivated in only 49% of two-inactive X cells. Webb et al. (1992) also found that one or three X chromosomes were inactivated in 1 and 5% of cells in which X-inactivation had occurred. They speculated that the frequency of these cell was higher in younger embryos and selection made it considerably low on the 10th day of gestation. The paucity of cells from the embryonic region observed in this study precluded any conclusion on the frequency of cells with aberrant X chromosome inactivation in XXXX embryos on the 8th day of gestation.

XXXX and XXY tetraploid embryos are phenotypically identical. The assumption that aberrant X-inactivation is mainly responsible for the abnormal development of XXXX tetraploid embryos predicts that X-inactivation also occurs in XXY tetraploid cells. Recently, Takagi found variable patterns of X-chromosome inactivation in differentiating XXXX, XXX and XXY near-tetraploid hybrid cell clones produced by fusing XX and XO embryonal carcinoma (EC) cells with lymphocytes carrying a morphologically distinguishable X chromosome(s) in vitro. Unexpectedly, cells carrying a single inactive X chromosome predominated in cells from well developed yolk sac-like structure of the balloon-like cystic embryoid bodies differentiated from EC hybrid cells with four or three

X chromosomes. The X chromosome reactivated by cell fusion was not inactivated more frequently than other X chromosomes. Although no inactivation was detected in any differentiating XXY EC hybrids, previous study in XY rat lymphocyte x XO PSA1 EC hybrids undoubtedly showed that XXY sex chromosome constitution in tetraploid cells is compatible with X-inactivation. Further careful analysis of X-inactivation in epiblast cells would help elucidate relationship between the active X-chromosome dosage and the developmental potential of tetraploid embryos, throw light on the determination of X-chromosome to be inactivated in mice.

Chapter 4: Stable X-chromosome imprinting in embryonic stem (ES) 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 in origin. Translocation chromosomes are easily recognized because the larger one (4^X) is distinctly the largest element in the karyotype and the smaller one (X^4) 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 Rb(X.2)2Ad (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^b/Pgk-1^b*) mated with C3H/HeHa males (*Pgk-1^a/Y*) gave rise to *Pgk-1^a/Pgk-1^b* 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 can not 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 core, within a few days. They grew larger and became cystic by eccentric cavitation. By day 10 in suspension culture, some of cystic embryoid bodies had expanded greatly, resulting in balloon-like cysts. In one experiment, TMA-2 at the 4th passage was treated with 2 μ M 5-azacytidine for 24 or 48 h before embryoid body formation.

Cytogenetic Examination

Chromosomes of undifferentiated ES cells and balloon-like cystic embryoid bodies were examined by a replication banding method involving 5-bromo-2'-deoxyuridine (BrdU; 150 μ g/ml) incorporation and acridine orange staining (Dutrillaux et al., 1973). The duration of BrdU incorporation was 7-8 h in the case of undifferentiated ES cells and 8-9 h in the case of cystic embryoid bodies. Slides of chromosomes were prepared from undifferentiated ES cells by a routine air-drying method. A modified form of the air-drying method, originally developed for postimplantation mouse embryos by Wroblewska and Dyban (1969), was adopted for the chromosome study of the cystic embryoid bodies. After BrdU incorporation and Colcemid treatment, cystic embryoid

bodies were placed in 1% sodium citrate for 10 min and fixed in 3:1 methanol:acetic acid. At slide preparation, each embryoid body was separated into the mural and the polar region with the aid of sharp tungsten needles, under a binocular microscope and air dried on separate slides. Chromosomes from primary cultures of the tail and lung tissues of newborn mice heterozygous for T37H, and postimplantation embryos heterozygous for Rb2Ad to act as control were examined by the same replication banding method.

Electrophoretic Analysis of PGK-1 Allozymes

Balloon-like cystic embryoid bodies were separated into mural and polar regions in PBS. 7.5 dpc embryos were cut into three regions, roughly approximating to the embryonic, extraembryonic and ectoplacental cone regions. Extracts of isolated tissues were prepared by three rounds of freezing and thawing in 1-25 μ l PBS followed by centrifugation. Samples thus obtained were electrophoresed on Cellogel strips (Chemetron) according to the method by Bucher et al. (1980).

Histological Examination

Balloon-like cystic embryoid bodies were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 5% sucrose, postfixed with 1.0% osmium tetroxide, dehydrated with acetone and embedded in Epon 812. Semithin sections cut at 1 μ m were stained with 1.0% toluidine blue.

RESULTS

Cystic Embryoid Body Formation in vitro

Like PSA1 embryonal carcinoma cell line studied in detail by Martin et al. (1977), ES cell lines used in this study formed balloon-like cystic embryoid bodies by 10 to 11 days in suspension culture and kept growing for some time thereafter. These cystic embryoid bodies comprised the expanded wall (mural region) and the more solid polar region. Histological examination (Fig. 13) revealed that the wall, most probably corresponding to the yolk sac of the postimplantation embryo, was made of the outer visceral 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 the 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

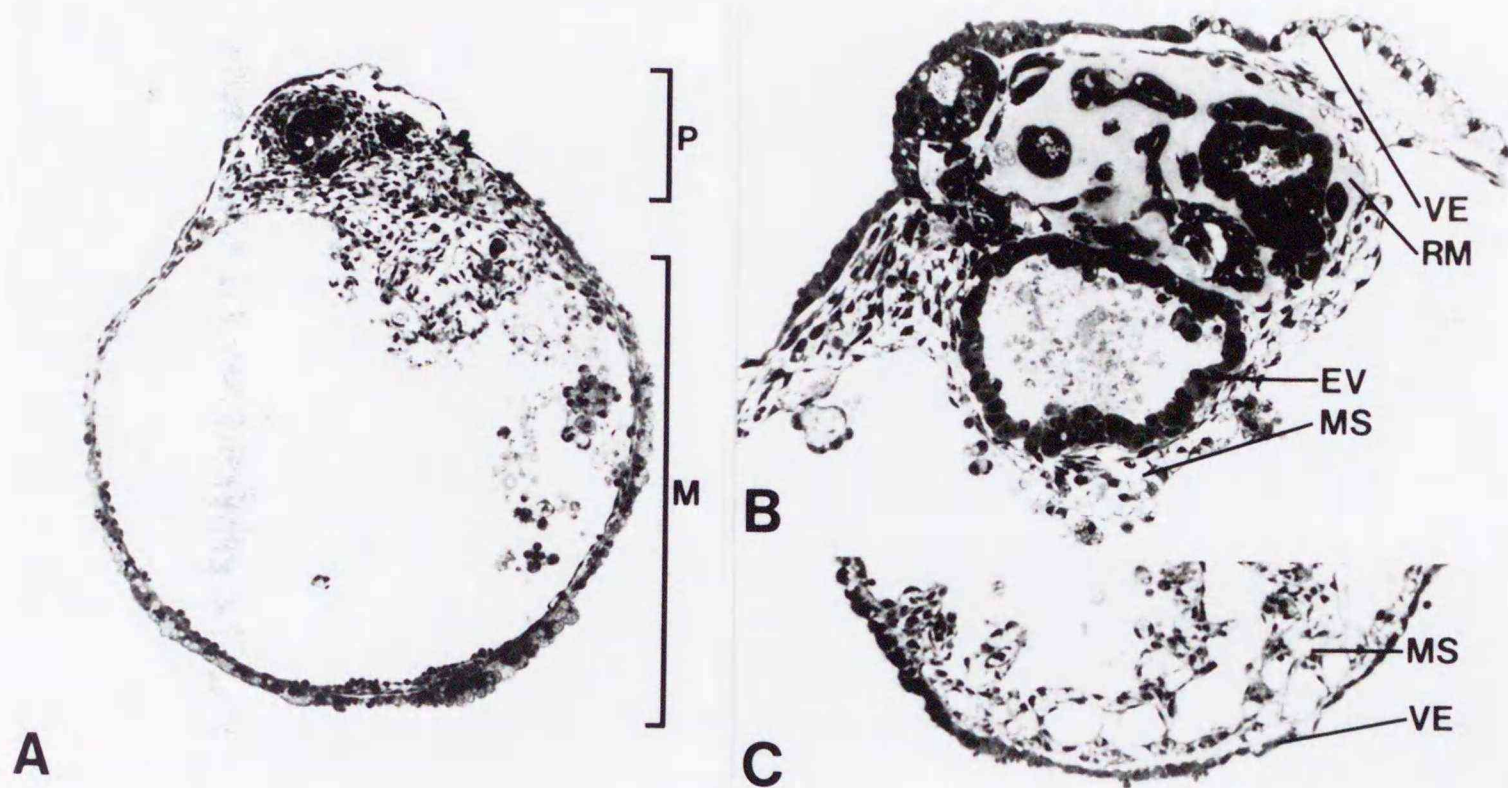


Figure 13. Histological sections of representative balloon-like cystic embryoid bodies formed in vitro. (A) A longitudinal section (P, polar region; m, mural region). (B) The polar region of another embryoid body comprising visceral endoderm (VE), ectodermal vesicle (EV), Reichert's membrane-like material (RM) and mesoderm (MS). (C) The mural region of an embryoid body consisting of the visceral endoderm layer (VE) and ample mesodermal cells (MS). Bar=0.4 mm (A), 0.1 mm (B,C).

Table 12. X chromosome replication patterns in cystic embryoid bodies from four ES cell lines

Cell line	Passage*	No.of samples examined	No. of cells with				% cells with an inactive X ^P	No. of cells with two SRXs	Total cells examined	% cells with an inactive X
			LRX ^P	PRX ^P	LRX ^M	PRX ^M				
Mural tissue										
TMA-2	3	10	1254	38	147	0	89.8	518	1957	73.5
	10	5	78	137	35	1	85.7	180	431	58.2
	20	4	81	10	4	0	95.8	52	147	64.6
	30	1	14	0	1	1	87.5	4	20	80.0
TMA-29	3	17	600	32	161	0	79.7	138	932	85.2
	10	4	77	20	1	0	99.0	46	144	68.1
TMA-30	3	13	256	12	126	27	63.7	189	610	69.0
	10	5	109	9	5	0	95.9	62	185	66.5
TMA-32	3	9	155	11	50	8	74.1	57	281	79.7
	10	2	28	3	1	0	96.9	15	47	68.1
Polar tissue										
TMA-2	10	4	82	2	107	36	37.0	66	293	77.5
	20	2	38	0	22	0	63.3	41	86	52.3
TMA-29	3	6	206	4	128	0	62.1	70	408	82.8
	10	5	104	7	66	7	60.3	112	296	62.2
TMA-30	3	6	218	7	118	1	65.4	75	419	82.1
	10	5	60	1	39	3	59.2	112	215	47.9
TMA-32	3	3	71	9	38	2	66.7	53	173	69.4
	10	3	35	0	10	33	44.9	45	123	63.4

* , The passage at which suspension culture was initiated; LRX^P, Late replicating paternal X; PRX^P, Precociously replicating paternal X; LRX^M, Late replicating maternal X; PRX^M, Precociously replicating maternal X; SRXs, Synchronously replicating X-chromosomes.

Table 13. X chromosome replication patterns in 7.5 dpc XX embryos from Rb2Ad/Rb2Ad x 129/Sv matings, and primary cultures of T37H/+ newborns from T37H/+ x C3H/He matings

Specimen	No. of cells with		
	LRX ^M	PRX ^P	LRX ^P
Rb2/+ embryo			
Embryonic			
1	90	1	125
2	81	3	91
3	122	1	92
4	49		55
Total	342	5	363
Extraembryonic			
1	4	16	70
2	1	7	50
3	2	21	92
4	6	16	72
Total	13	60	284
T37H/+ fibroblast			
1	174		183
2	37		45
3	52		59
4	119		129
Total	382		416

X^M; Maternally derived translocation X chromosome, 4^X in T37H/+ and Rb2 in Rb2Ad/+ cells, X^P; Paternally derived morphologically normal X chromosome.

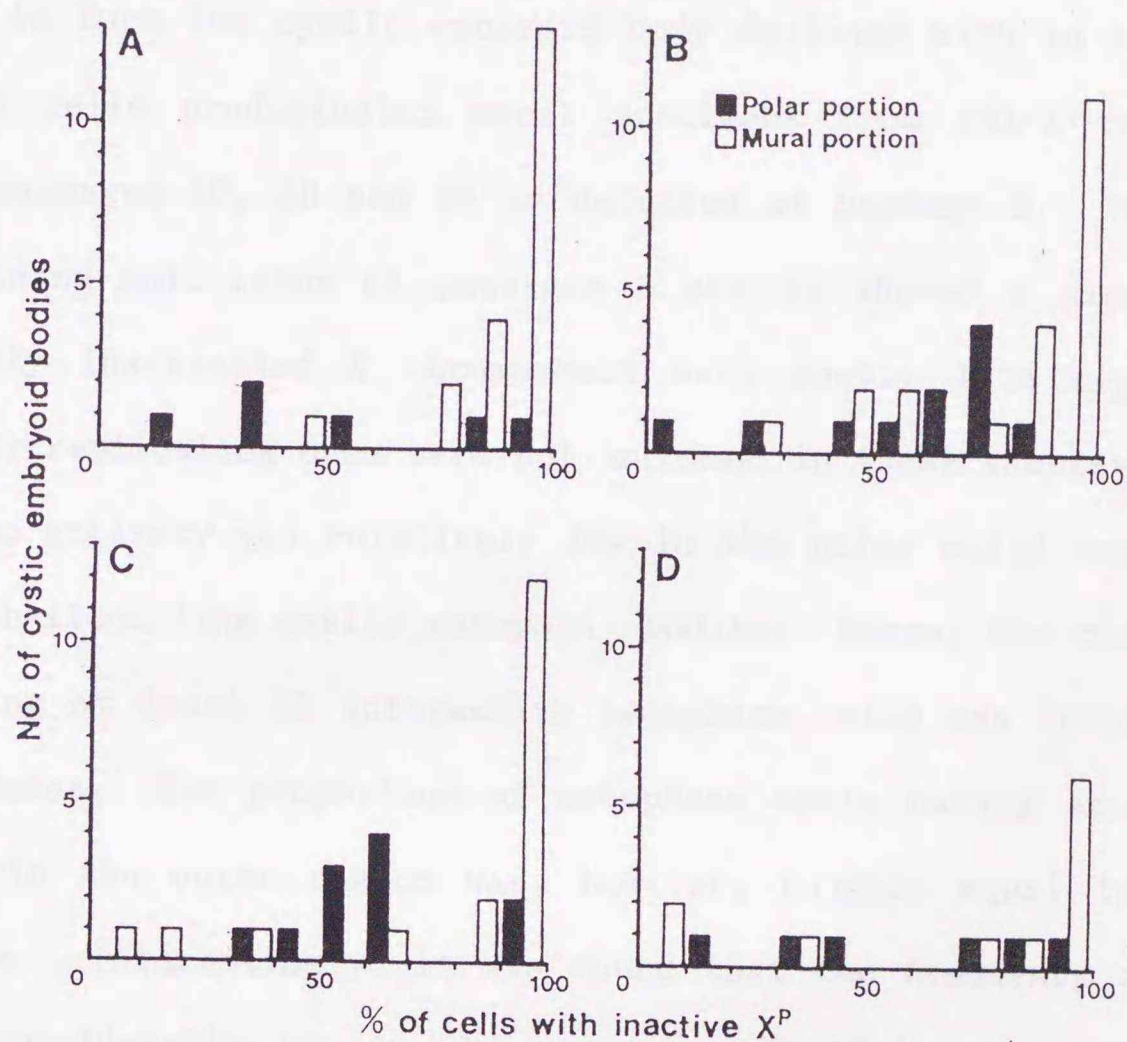


Figure 14. X-chromosome inactivation in the mural and polar regions of cystic embryoid bodies differentiated from four ES cell lines, TMA-2 (A), TMA-29 (B), TMA-30 (C) and TMA-32 (D). In each ES cell line examined, the mural region is characterized by preferential X^P-inactivation and polar portion by more random inactivation.

embryoid bodies formed by four ES cell lines is shown in Table 12. 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^P -inactive cells predominated mural specimens from TMA-2 cystic embryoid bodies at passages 10, 20 and 30 as detected at passage 3. Findings in the three remaining cell lines at passages 3 and 10 showed a similar tendency. Cytologically inactivated X chromosomes were mostly late replicating, but precociously replicating ones were not uncommon in these samples (Fig. 14).

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^P -inactive cells was considerably low in this region: 143 X^M -inactive cells, compared with more than 84 X^P -inactive cells in TMA-2 cystic embryoid bodies at passage 10, but data at passage 30 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, 20 and 30. 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^P -inactive cells in the polar region was not as striking as in TMA-2 (Table 12). In fact, the frequency of X^P -inactive cells was significantly higher than that of X^M -inac-

tive 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. 15. Although X^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^P inactivation. Observation of control in vivo and cultured cells confirmed the occurrence of X^P 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 13) 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. 16 is a scatter diagram to showing the relationship between the proportion of X^P -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^P -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 predominant X^M inactivation in the mural and the polar region. The t -test showed that the proportion of X^P -inactive cells between the mural and the polar region of the same embryoid body was significantly different ($t=3.09$, $P<0.005$). However, the correlation coefficient was margin

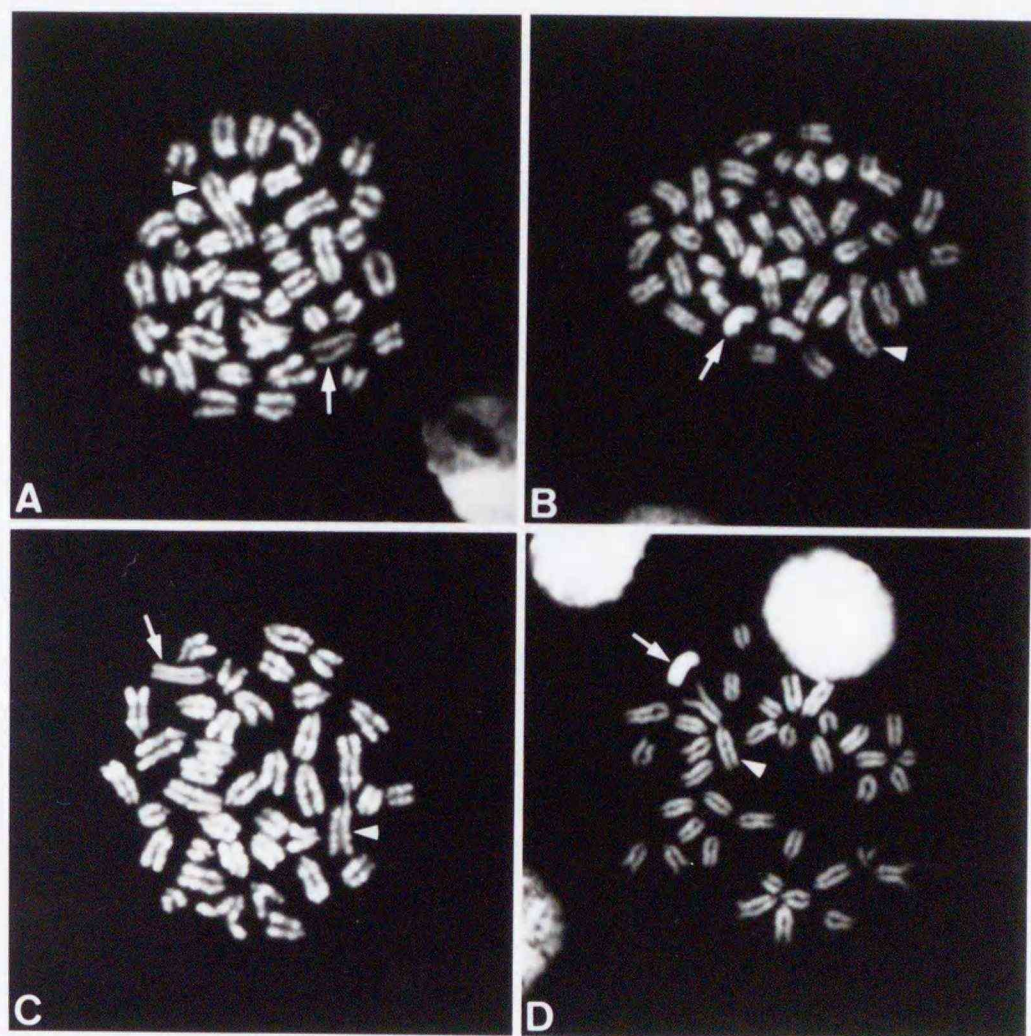


Figure 15. X-chromosome replication patterns revealed by a BrdU-acridine orange procedure in cystic embryoid bodies. Metaphase spreads heterozygous for T37H translocation (A and B) and those heterozygous for Rb2Ad translocation (C and D). The late replicating normal X (X^P) fluoresced faintly in A and C, whereas the precociously replicating normal X (X^P) fluoresced brightly in B and D. Arrows and arrow heads indicate the allocyclic and isocyclic X chromosomes, respectively.

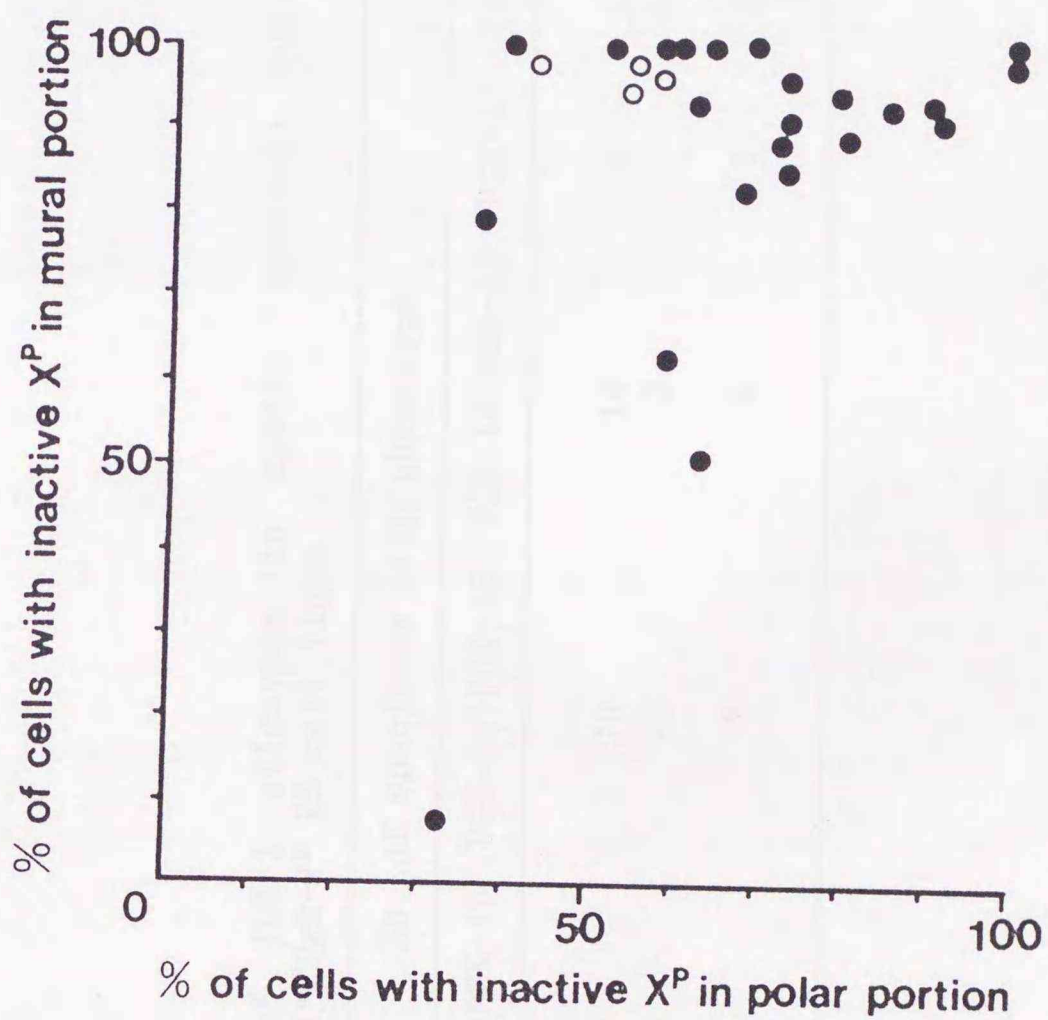


Figure 16. A scatter diagram showing the frequency of X^P -inactivation in the polar and mural region of the same cystic embryoid bodies. Preferential X^P -inactivation occurs in the mural region, but not in the polar region.

Table 14. Expression of PGK-1 allozymes in cystic embryoid bodies differentiated from *Pgk-1^a/Pgk-1^b* ES cell lines

ES cell lines	No. of specimens with phenotype				
	PGK-1A	PGK-1A>PGK-1B	PGK-1A=PGK-1B	PGK-1A<PGK-1B	PGK-1B Total
TMA-45					
Mural	3	20	10	14	4 51
Polar	2	14	13	3	32
TMA-46					
Mural	1	7	2	5	1 16
Polar	3	5			8

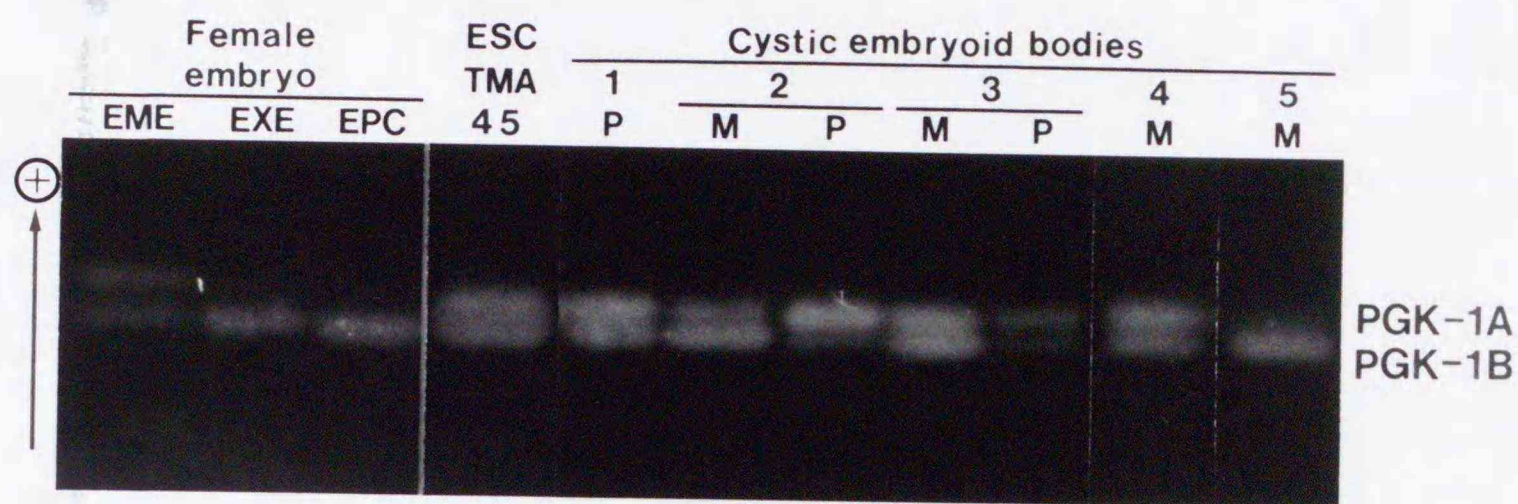


Figure 17. PGK-1 allozyme expression in cystic embryoid bodies from TMA-45 (*Pgk-1^a/Pgk-1^b*) ES cell line. In a control 7.5 dpc heterozygous female embryo, the embryonic ectoderm (EME) was characterized by PGK-1A band slightly stronger than PGK-1B band most probably due to *Xce^c* allele tightly linked to *PGK-1^a*. PGK-1B band was predominant in the extraembryonic ectoderm (EXE) and only PGK-1B band was detected in the ectoplacental cone (EPC). PGK-1A and PGK-1B bands were almost equal in undifferentiated TMA-45. In cystic embryoid bodies 2 and 3, complementary PGK-1 phenotype was found in the mural (M) and the polar (P) samples indicating preferential inactivation of *X^P* carrying *Pgk-1^a* in the mural region. Both *Pgk-1* alleles were equally expressed in the polar sample 1 and the mural sample 4. *Pgk-1^a* allele was completely suppressed in the mural sample 5.

ally significant, showing certain interaction between these two regions. It may be worth mentioning that significant correlation disappeared if the exceptional embryoid body with preferential X^M inactivation was excluded from analysis.

Biochemical Verification of Preferential X^P Inactivation

We attempted to verify the above cytological findings by the expression of *Pgk-1* alleles in TMA-45 and TMA-46. *Pgk-1^a* allele closely linked to *Xce^c* was carried by X^P and *Pgk-1^b* by X^M 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-1B=PGK-1A, PGK-1A>PGK-1B, and exclusive PGK-1A expression. PGK-1A and PGK-1B bands stained with similar intensity in undifferentiated TMA-45 (Fig. 17) 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-1B in 18, and equal PGK-1A and PGK-1B expression in 10 specimens (Table 14). Table 14 also shows that the mosaic expression of PGK-1 isozymes apparently shifted in favor of PGK-1A in the polar specimens, implying increase in the proportion of cells with inactivated X^M carrying the *Pgk-1^b* 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. The mural and the polar regions were

ranked relative to the B form of PGK-1 for Dixon and Mood's sign test. Results ($\chi^2=9.39$, $P<0.01$) seemed to verify that the proportion of PGK-1B was higher and, hence, X^P 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 the Xce allele is on X^M 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 ($Pgk-1^a$) carried Xce^c and the 129/Sv X chromosome ($Pgk-1^b$) carried either Xce^a or Xce^b as expected (Fig. 17). Only PGK-1B was detected in ectoplacental cone. The situation in the extraembryonic region was in between that of the ectoplacental cone and the embryonic region. Hence it may be reasonable to assume that preferential X^P -inactivation indeed occurred in specimens showing exclusive PGK-1B, $PGK-1B>PGK-1A$, or $PGK-1A=PGK-1B$ 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^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 μ 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^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^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^P inactivation in the mural region of the cystic embryoid body resulted from clonal proliferation of an X^P -inactive progenitor cell rather than preferential inactivation of X^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^P inactivation was indeed responsible for the predominance of X^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

mal 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 $X^M X^M$ mouse parthenogenetic embryos (Kaufman et al., 1978), and $X^M X^M$ ovarian teratomas (Linder and Power, 1970) and $X^P X^P$ 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 Cattanch (1983) claimed that X^P -inactivation can be overridden to a certain extent by an appropriate combination of alleles at the *Xce* locus. They showed that X^M is inactivated in a considerable proportion of the yolk sac endoderm cells of female mouse embryos carrying the *Xce^a* allele on X^M and the *Xce^c* allele on X^P . However, Bucher 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 Cattanch (1983) at the blastocyst stage, but consistent with the report made by Bucher 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 the 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 $X^M X^M Y$ and $X^M X^M X^P$ embryos were either severely retarded or grossly abnormal as

early as 6.5 dpc. Our recent observation (T.Tada, N.Takagi and I.D.Adler in press) in embryos obtained from matings involving an Rb(X.2)2Ad translocation invariably showed that $X^M X^P Y$ but not $X^M X^M Y$ 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 $X^M X^M Y$ and $X^M X^M X^P$ 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,X¹⁶,16,16), where normal X is paternal in origin, and maternally derived X¹⁶ 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 ex-

traembryonic 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 $X^P X^P$ 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 independent 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 nucleases at the promoter region, but revealed parental methylation differences in a region several kilobases upstream of the first exon (Sasaki and Jones, 1992). Detailed analysis of candidates for Xic, including *Xist* (Brown et al., 1991), may help clarify the mechanism of imprinting and X inactivation as well. Various ES cell lines would be invaluable for this purpose, since they faithfully maintained the imprint that causes preferential X^P inactivation in the extraembryonic cell lineages.

Concluding remarks

Imprinting in Mammals

Since the rediscovery of Mendel's laws of inheritance, it has been a basic principle of genetics that nuclear genes inherited from each parent function equally in the offspring. Nevertheless, over the past 50 years, evidence has been accumulating that Mendel's laws are not always correct. The maternal and paternal copies are differentially modified or marked in the parental germ lines, with as the consequence that they become functionally inequivalent in the embryo. This concept is called imprinting or genomic imprinting.

A first indication that maternal and paternal genomes are required for mammalian embryonic development comes from the finding that successful parthenogenesis does not occur. In the mouse, parthenogenones can complete preimplantation development and initiate implantation, but their development usually terminates at midgestation (Kaufman, 1983). The lack of some cytoplasmic factors contributed by the sperm was excluded as the cause of parthenogene failure because it was found that uniparental gynogenetic embryos, produced by removal of the male pronucleus following suppression of the second polar body extrusion, failed to survive (Surani and Barton, 1983). Homozygosity for lethal genes produced by transferring female pronuclei into haploid fertilized eggs, showed the same deficiencies as found in parthenogenetic embryos (McGrath and Solter, 1984). Studies of Surani et al. (1984) and Barton et al. (1984) demonstrated that gynogenetic embryos showed poor postimplantation development, whereas the transfer of a male pronucleus into a haploid parthenogenetic eggs allowed development to term and also that gynogenetic embryo with two female pronuclei failed in trophoblast development, whereas androgenetic embryos with two male pronuclei showed an overgrowth of the

trophoblast. This opposite nature of gynogenetic and androgenetic embryo development suggested that the maternal genome may be more important for the development of the embryo proper, whereas the paternal genome may be important for the development of the trophoblast. The differential modification of the maternal and paternal genomes is essential for successful development of the conceptus (McGrath and Solter, 1984; Surani et al. 1984).

Mouse genetic studies using Robertsonian and reciprocal translocations have shown that certain autosomal regions are subject to the imprinting. The genetic techniques used to detect imprinting effect is one of intercrossing heterozygotes for translocations and, with the use of marker genes, ascertaining from the progeny whether gametes with reciprocal types of chromosomal imbalance can complement each other and give rise to normal, viable zygotes. Duplication of maternal or paternal copies with corresponding paternal/maternal deficiencies in chromosomally balanced zygotes leads to lethality or produce opposite phenotypes depending on the particular disomy/nullisomy involved (Cattanach and Kirk, 1985; Cattanach, 1986; Beechey and Cattanach, 1993). These results are summarized in imprinting map (Beechey and Cattanach, 1993). Imprinting regions and imprinting effects has been identified on five chromosomes (chromosome 2, 6, 7, 11, 17). Possible differential recoveries of maternal and paternal disomies of chromosome 1, 5, 9 and 14 may represent examples of imprinting (Cattanach and Beechey, 1990). The cytogenetic evidence clearly demonstrates that it is not the whole genome, but only regions of a few chromosomes that are subject to imprinting. It is also likely that only a minority of mouse genes within these regions are responsible for the phenomena found. No convincing evidence of genetic background effects has been found for any of the imprinting phenomena distinguished in the mouse genetic experiments.

Previously, certain transgenic insertions show differing methylation of

CpG dinucleotides and expression levels according to parental route of transmission (Reik et al., 1987; Sapienza et al., 1987; Swain et al., 1987; Hadchouel et al., 1987). DNA methylation is one of the most important modifications which is heritable, reversible and implicated in the control of gene expression, although the imprinting may not be solely explained by it.

The molecular basis of imprinting is still elusive. So far, four mouse endogenous genes are known subject to parental imprinting. *Insulin-like growth factor II (Igf2)* (Dechiara et al., 1991) and *Snrpn* (Cattanach et al., 1992) on chromosome 7 are active on the paternally derived chromosome, and *insulin-like growth factor II receptor (Igf2r)* (Barlow et al., 1991) on chromosome 17 and *H19* (Bartolomei et al., 1991) on chromosome 7 are active on the maternal homologue. It is interesting to know that two closely linked genes, *Igf2* and *H19* genes are imprinted in opposite manner on the same chromosome. Molecular analysis of 5' region of the *Igf2* allele by Sasaki et al., (1992) demonstrated that CpG island that comprises one of the two strong promoters is unmethylated at both alleles and DNase I hypersensitive sites are present in the strong promoter regions irrespective of their parental origin. They have found, however, parental methylation differences in a region several kilobases upstream of the first exon. Detailed comparison of imprinted genes will help clarify the molecular mechanism of epigenetic modification. If a consensus sequence controlling imprinting is revealed, it would help to isolate the X chromosome inactivation center.

Developmental potential of the cells with two active X-chromosomes

Two X chromosomes are shown to be active in oocytes (Epstein, 1969) and female cells at early preimplantation stages (Epstein, 1972). During early embryogenesis, X-chromosome inactivation occurs in cells that undergo differentiation but does not occur in cells which remain pluripotent. Biochemical

and cytogenetical studies of LT-1 embryonal carcinoma (EC) cells derived from a spontaneous ovarian teratoma has suggested that both X-chromosomes are genetically active when the cell line is maintained in the undifferentiated state, and that one of the X chromosomes becomes inactivated when the cells are differentiated in vitro (Martin et al., 1978; Takagi and Martin, 1984). A similar result was obtained by McBurney and Strutt (1980) in clonal populations from P10 EC cell line obtained from female embryos heterozygous for electrophoretic variants of the X-linked gene coding for PGK-1. Furthermore, it was reported that one X chromosome is totally lost from undifferentiated P10 EC cells (McBurney and Strutt, 1980; Paterno and McBurney, 1985) and parthenogenetic ES cells (Rastan and Robertson, 1985). These findings imply that two active X-chromosomes are not desirable not only in differentiated cells but also in undifferentiated cells.

It has been shown that two active X^M -chromosomes due to failure of X-inactivation exert deleterious effects on development of the extraembryonic tissues of mouse embryos (Shao and Takagi, 1990; Tada and Takagi, 1992; These studies). This seems to indicate that the dosage compensation of X-chromosome genes is essential for normal development of the extraembryonic ectoderm. Development of the embryonic ectoderm (epiblast) cells carrying two active X-chromosomes was observed in female embryos with two complete sets of autosomes, one maternal X chromosome and one X^{16} translocation chromosome consisting of the proximal segment of the X chromosome and distal half of chromosome 16 (X^nX^{16} embryos) (Takagi and Abe, 1990). X^nX^{16} embryos are functionally disomic for the proximal 63% of the X chromosome and trisomic for the distal segment of chromosome 16, because X-inactivation does not occur in cells of these embryos, probably due to the lack of the inactivation center on X^{16} . These chromosomally unbalanced embryos show general underdevelopment of the ectoplacental cone, embryonic ectoderm cells and embryonic and extraembryonic

mesoderm. The underdevelopment of embryonic tissues may be explained by inadequate nutrition due to the poor developed ectoplacental cone or alternatively by abnormal dosage compensation of X-chromosome genes. Detailed studies in chimeras involving X^nX^{16} ES cells would be invaluable for understanding the relationship between X inactivation and cell differentiation.

Genes escaping X-chromosome inactivation

The stable inactivation of one of two X chromosomes in females can be spread over 150 Mb of DNA, turning off several thousands of genes. However, genes in the pseudoautosomal pairing segment (PAR, pseudoautosomal region) of X and Y chromosomes are expressed from both active and inactive X chromosomes. In human PAR, the *MIC2* cell surface antigen gene and X-linked steroid sulfatase (STS)-encoding gene were shown to escape X-inactivation. In addition to these two genes, at least six other genes are expressed from both human X chromosomes (Brown and Willard, 1989; Schneider-Gadicke et al., 1989; Fisher et al., 1990; Franco et al., 1991; Yen et al., 1992). The *STS*, Kallman syndrome gene *KALIG1* and *GS1*, which is a cDNA of unknown function, are tightly clustered in Xp22.3, although *ZFX*, zinc-finger protein gene, *A1S9T/UBE1*, ubiquitin-activating enzyme E1, *DXS423*, anonymous gene of unknown function and *RPS4X*, ribosomal protein S4 are widely scattered over the proximal long arm and the distal and proximal short arm region of the X chromosome. Three of these genes escaping X-inactivation on the human X chromosome have been evaluated in the mouse. Surprisingly, all three of *Zfx*, *Rps4* and *A4s9* have been subject to inactivation in the mouse (Adler et al., 1991; Ashworth et al., 1991; Kay et al., 1991; Zinn et al., 1991). It is not known whether these X-linked human genes receive the X-inactivation signal or they are unresponsive to such a signal.

A study on STS has led to the serendipitous identification of a gene,

XIST, which is transcribed only from inactive X chromosomes (Brown et al., 1991a). Southern hybridization analyses of structurally abnormal X-chromosomes using probes from the Xq13 region showed that the *XIST* gene locates in the region of X-chromosome inactivation center on the human X chromosome. This implicates *XIST* in some aspect of X-inactivation (Brown et al., 1991b). *Xist*, murine homolog of the *XIST* was isolated by screening cDNA libraries using the human probe (Borsani et al., 1991; Brockdorff et al., 1991). The detailed mapping of *Xist* using *Mus musculus domesticus*-*M. spretus* interspecific backcross panels indicated that the map location of *Xist* was completely consistent with the location of the human *XIST*. Exclusive expression of murine *Xist* from the inactive X-chromosome was confirmed both by PCR analyses of reverse transcribed (RT) RNA and by northern blot analyses. It was also expressed in males from the single X-chromosome that was inactivated during spermatogenesis (Richler et al., 1992; Salido et al., 1992) but not expressed in the female germ line (Grant et al., 1992). Recently, Brown et al. (1992) and Brockdorff et al. (1992) sequenced 17 kb of human *XIST* cDNA and 15 kb murine *Xist* gene, respectively. Although both genes included several tandem repeats in common, they conserved no open reading frames (ORFs) and thus they do not appear to encode a protein. Available evidence shows that *XIST/Xist* transcripts appeared to be maintained in the nucleus. Indeed, fluorescence in situ hybridization using a *XIST* genomic probe invariably showed that they were localized to a site corresponding precisely to the position of the heterochromatic Barr body at the periphery of the nucleus. These findings support a role for *XIST/Xist* in X-inactivation, possibly as a functional RNA or as a chromatin organizer region. It is also possible that *XIST/Xist* may be involved in the choice of the X chromosome to be inactivated.

The absolute level of murine *Xist* gene expression appears to be inversely correlated with the strength of *Xce* allele (Brockdorff et al., 1991), and only

Xist allele on the paternally derived X-chromosome is expressed in cells of the extraembryonic lineages (Takagi and Sasaki, 1975; West et al., 1977; Harper et al., 1982). These findings support an intimate role played by *Xist* in X-inactivation. But formal proof must await the outcome of such powerful tests as destroying *Xist* function by homologous recombination in either cultured cells or in embryonic stem cells.

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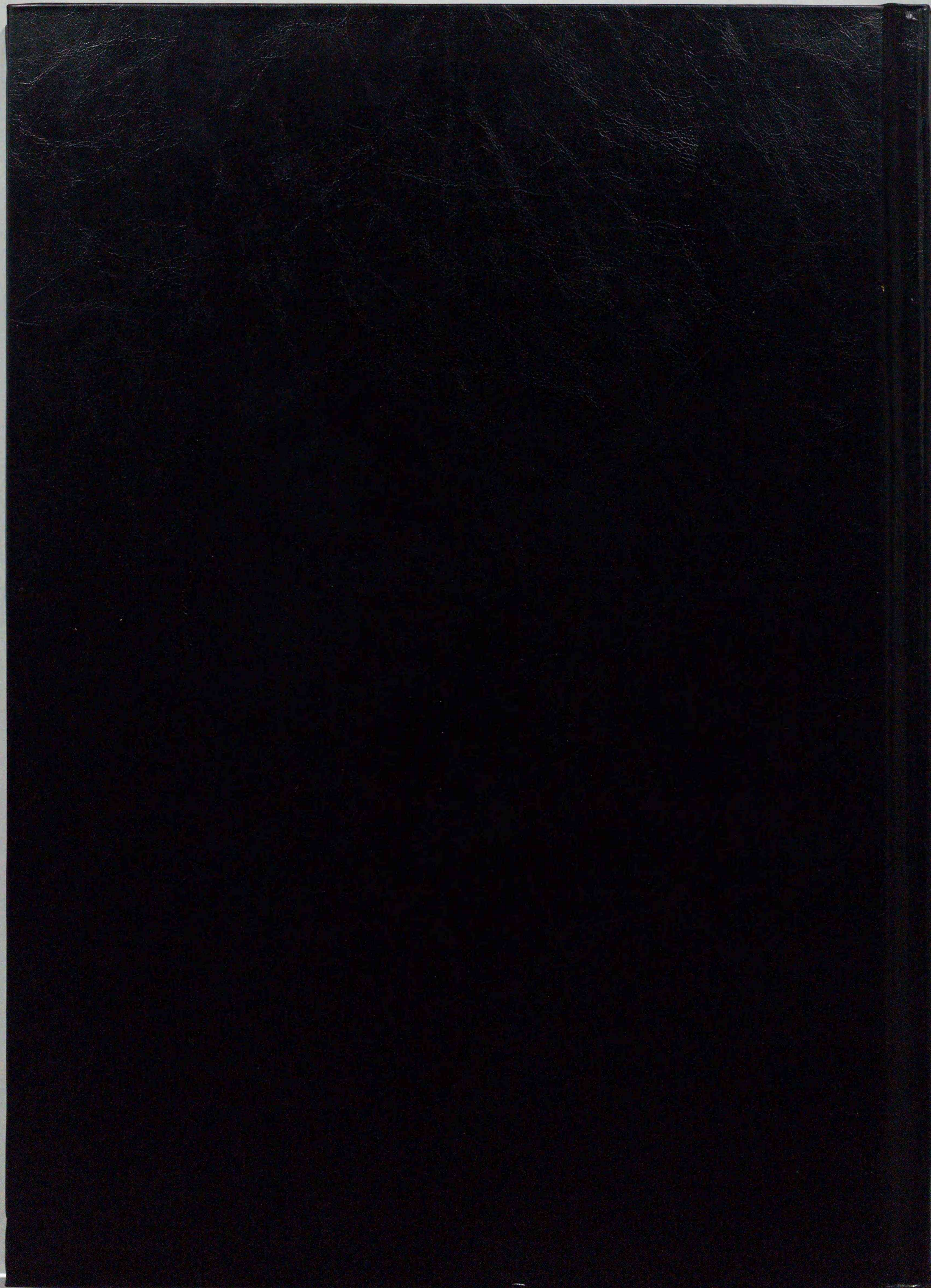
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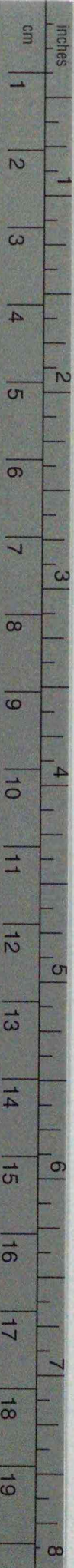
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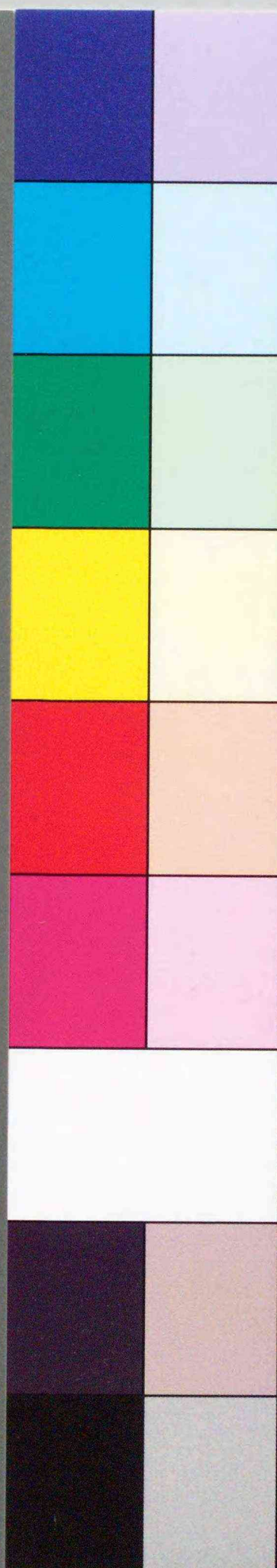




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