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Parental imprinting on the mouse X chromosome: effects on the early development of X0, XXY and XXX embryos

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Summary

To examine the effects of X-chromosome imprinting during early mouse embryogenesis, we attempted to produce X^M0, X^P0, X^MX^MY, X^MX^PY and $X^MX^MX^P$ (where X^M and X^P stand for the maternally and the paternally derived X chromosome, respectively) making use of mouse strains bearing the translocation Rb(X.2)2Ad and the inversion In(X)1H. Unlike X^MX^PY embryos, X^MX^MY and $X^MX^MX^P$ conceptuses suffered from severe growth retardation or abnormal development characterized by deficient extra-embryonic structures at 6·5–7·5 days post coitum (dpc). A cytogenetic study suggested that two X^M chromosomes remaining active in certain non-epiblast cells were responsible for the serious developmental abnormality found in these embryos disomic for X^M. Although matings involving females heterozygous for Rb(X.2)2Ad hinted at the paucity of X^P0 embryos relative to those having the complementary karyotype of $X^MX^MX^P$, further study of embryos from matings between females heterozygous for In(X)1H and Rb2Ad males did not substantiate this observation. Thus, the extensive peri-implantation loss of X^P0 embryos shown by Hunt (1991) may be confined to X0 mothers. Taken together, this study failed to reveal a parentally imprinted X-linked gene essential for early mouse embryogenesis other than the one most probably corresponding to the X-chromosome inactivation centre.

1. Introduction

Failure to complete development of parthenogenetic embryos (Kaufman *et al.* 1977), gynogenetic or androgenetic embryos produced by pronuclear transplantation (McGrath & Solter, 1984; Surani *et al.* 1986) in mice led to the conclusion that the two parental genomes are not functionally equivalent, and both of them are necessary for normal development to occur. The concept of genomic imprinting thus revealed has prompted genetic studies for delineating affected autosomal regions, and at least 10 imprinted regions and 4 tentative ones have been identified in the mouse karyotype (Beechey & Cattanach, 1993). Mouse stocks carrying a reciprocal or a Robertsonian translocation were used for these studies because simultaneous occurrence of the identical adjacent disjunction or nondisjunction in both male and female heterozygotes results in embryos which inherit two homologous chromosomes or chromosomal segments exclusively from one of the parents but none from the other (uniparental disomy).

Imprinting on the X chromosome remains obscure, though the X chromosome, unlike autosomes, has the favourable property that monosomy is compatible with prolonged postnatal life. It has been assumed that the X chromosome bears no prominent imprints because X0 mice are phenotypically normal females capable of reproduction irrespective of the parental origin of the single X chromosome. Recently, however, Hunt (1991) reported that the patroclinous X0 (X^P0) tends to be lost during embryonic development, whereas matroclinous X0 (X^M0) does not show such tendency. The other indication of X chromosome imprinting would be the preferential inactivation of X^P in certain extra-embryonic tissues derived from the trophectoderm and the primitive endoderm cell lineage (Takagi & Sasaki, 1975; West *et al.* 1977), whereas either X^P or X^M is inactivated in embryonic tissues derived from the epiblast cell lineage (Lyon, 1961; Monk & Harper, 1979; Takagi *et al.* 1982).

Our previous study (Shao & Takagi, 1990) on

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segregants of T(X;4)37H led us to speculate that early mouse embryonic development is impaired by an extra maternally derived X chromosome, because X^MX^MY and $X^MX^MX^P$ embryos consistently showed severe growth retardation or abnormal development shortly after implantation. However, low frequency of such embryos together with technical difficulty in their identification by karyotyping and lack of observation on X^MX^PY embryos due to sterility of males bearing the T(X;4)37H translocation precluded extensive study and hence firm conclusion. We hoped that the use of Rb(X.2)2Ad (Adler *et al.* 1989) would overcome some of these difficulties and help us to compare developmental competence of X^M0 and X^P0 embryos as well for further exploration of X chromosome imprinting. Karyotypic identification of aneuploid embryos was easy and unequivocal in most cases as was expected, but the frequency of nondisjunction of sex chromosomes was unexpectedly low in female mice heterozygous for Rb(X.2)2Ad and hemizygous males, which compelled us to undertake matings involving females heterozygous for In(X)1H.

The present cytogenetic study of embryos from various matings supported an idea that an imprint on X^M which interferes with accomplishment of correct dosage compensation in non-epiblast cells explains the deficient extra-embryonic region and hence early post-implantation lethality of X^MX^MY and $X^MX^MX^P$ embryos. It was also shown that imprinting of X^P, if any, would not be directly responsible for selection against X^P0 embryos from X0 mothers.

2. 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 Centre for Experimental Animals and Plants, Hokkaido University by 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 × C3H/He or C3H/He × 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 between 09.00 and 10.00 hours 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 labelling and slide preparation*

Recovered embryos were photographed and incubated in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 150 μg ml^{-1} 5-bromo-2-deoxyuridine (BrdU) at 37 °C in an atmosphere of 5% CO_2 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 & 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 pregnant 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^{-1} 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.

3. Results

(i) *X^M0 and X^MX^MY 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 Is1Ct and In1H. Data yielded by females from different stocks were essentially homogeneous, hence they were combined. The loss of

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Table 1. *Early post-implantation development of 6·5- and 7·5-day embryos*

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

* Percent.

Table 2. *Gross morphology of 6·5- and 7·5-day embryos obtained from Is1Ct/X females mated with hemizygous Rb2Ad/Y males*

Gross morphology	No. of embryos X^MX^P	X^MY	X^M0	X^P0	X^MX^PY	Autosomal Trisomy	Monosomy	Mosaic	Triploid or tetraploid
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.

ovulated oocytes before implantation estimated by the difference between the number of corpora lutea and implantation sites was extensive (Table 1), the 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. 2*e*), whereas all XXY embryos were uniformly X^MX^PY (Fig. 2*a*). 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 chromosomes were produced at an equal frequency by nondisjunction at the first meiotic division. Hence, it is that either those 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 favoured the lack of selection against either X^M0 or X^MX^PY embryos until at least 7·5 dpc. All the X^MX^PY embryos recovered here, unlike X^MX^MY ones reported by Shao & Takagi (1990) and found in this study (see below), could not be distinguished from their chromosomally normal or balanced littermates by their growth (Fig. 1*c*). Similarly, nine X^M0 embryos grew normally (Fig. 1*c*), 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 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 & 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 & 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

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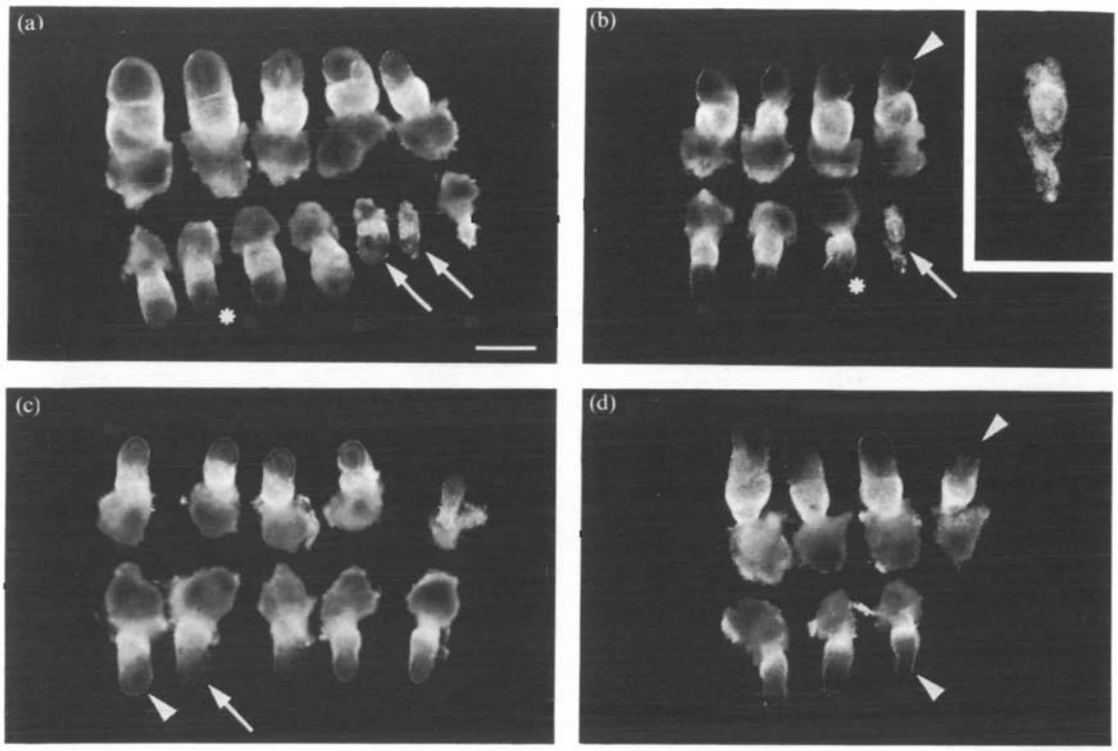

Fig. 1. Gross morphology of 7·5 dpc embryos. (*a*, *b*) Littermates from Rb2Ad/X female mated with Is1Ct male. Arrows indicate the retarded $X^MX^MX^P$ and X^MX^MY embryos in (*a*) and the abnormal $X^MX^MX^P$ embryos in (*b*). A magnification of abnormal $X^MX^MX^P$ embryo is shown in the inset. Asterisks and arrowhead indicate embryos with an unidentified extra autosome and X^P0 embryo, respectively. (*c*) Littermates sired by a Rb2Ad/Y male. An arrow and arrowhead indicate X^MX^PY and X^M0 embryos, respectively. (*d*) A litter of embryos from mating involving In1H/X female. Normal and retarded X^P0 embryos are shown by arrowheads. bar, 0·5 mm.

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 & 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 days 6 and 7 of gestation (Table 1) showed no obvious anomaly except the increased pre-implantation loss of oocytes ovulated by females mated with In1H/Y males. This 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 pre-implantation 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 two different crosses. As reported earlier by Adler *et al.* (1989) and Tease & 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 $X^MX^MX^P$ (Fig. 2*d*), whereas only 4 and 5 were X^MX^MY (Fig. 2*b*, *c*) and X^P0, respectively, although they are expected to occur at a comparable frequency. Three X0 embryos were found to be X^M0, 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^MX^PY could not, however, be recovered in this study. We also found two X0 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 determine unequivocally 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 X0 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^P0,

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Table 3. *Gross morphology of 6·5- and 7·5-day embryos obtained from heterozygous Rb2Ad females mated with males carrying Is1Ct or In1H*

Gross morphology	No. of embryos									
	X^MX^P		X^MY							
	XX	Rb2X	XY	Rb2Y	X^P0	X^M0	$X^MX^MX^P$	X^MX^MY	Autosomal trisomy	Triploid
Normal	189	171	197	169	5	3			12	6
Retarded	10	7	3	4			6	4	4	
Severely retarded or abnormal	2	4	4	4			4			
Total	201	182	204	177	5	3	10	4	16	6

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

Table 4. *Gross morphology of 7·5-day embryos obtained from In1H/X females mated with Rb2Ad/Y males*

Gross morphology	No. embryos								
	X^MY	X^MX^P	X^P0	X^PY	X^M0	X^MX^PY	$X^MX^MX^P$	X^PX^P	Autosomal trisomy
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.

X^MX^MY and $X^MX^MX^P$ embryos obtained from Rb2Ad/X females mated to Is1Ct, In1H and chromosomally normal males as 6–7, 9–10, 10, respectively, which are not significantly different from the theoretical ratio of 1:1:1 ($\chi^2 = 1{\cdot}04$ or 0·67, $0{\cdot}5 < P < 0{\cdot}07$ or $0{\cdot}7 < P < 0{\cdot}8$).

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^MX^MY and $X^MX^MX^P$ embryo sired by In1H/Y and Is1Ct/Y males. Since each X^MX^PY embryo found in this study grew normally, all the XXY embryos sired by C3H males should most probably be X^MX^MY. The abnormality found in these embryos ranged from growth retardation by 12–24 h (Fig. 1*a*) to severe anomaly leading to the formation of the tiny spherical embryo (Fig. 1*b*) as reported by Shao & Takagi (1990). They showed that the lack or severe deficiency of the structure derived from the extra-embryonic 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 do not allow us to estimate the extent of such selection. Therefore, the nearly equal frequencies of the three classes of sex chromosomally aneuploid embryos do not necessarily reflect the situation at fertilization. Failure to find any X^MX^MY and $X^MX^MX^P$ karyotype among newborn mice from Rb2Ad/X females (Adler *et al.* 1989; Tease & Fisher, 1991) implies that these hyperdiploid embryos were totally lost by parturition.

The total number of X^P0 embryos recovered in these matings tended to be fewer than that of X^MX^MY or $X^MX^MX^P$ embryos, but the difference was not statistically significant even if allowance was made that the Rb2Ad chromosome segregated into the first polar body in 60% of meiosis I ($\chi^2 = 1{\cdot}06$, $P > 0{\cdot}3$). Furthermore, X^P0 embryos did not suffer from any specific developmental abnormality except occasional slight growth retardation.

(iii) *X^P0 embryos from In1H/X females*

We studied further the developmental potential of X^P0 zygotes in XX mothers using conceptuses from In1H/X females mated to Rb2Ad males. Karyotype analyses of 321 embryos recovered from 71 pregnant In1H/X females (Table 4) identified a single rare case of X^PX^P, 5 X^PY and 30 X^P0 embryos apparently derived from 0-oocytes. Other relevant sex chromosome aneuploids found were 5 X^M0 and 6 X^MX^PY.

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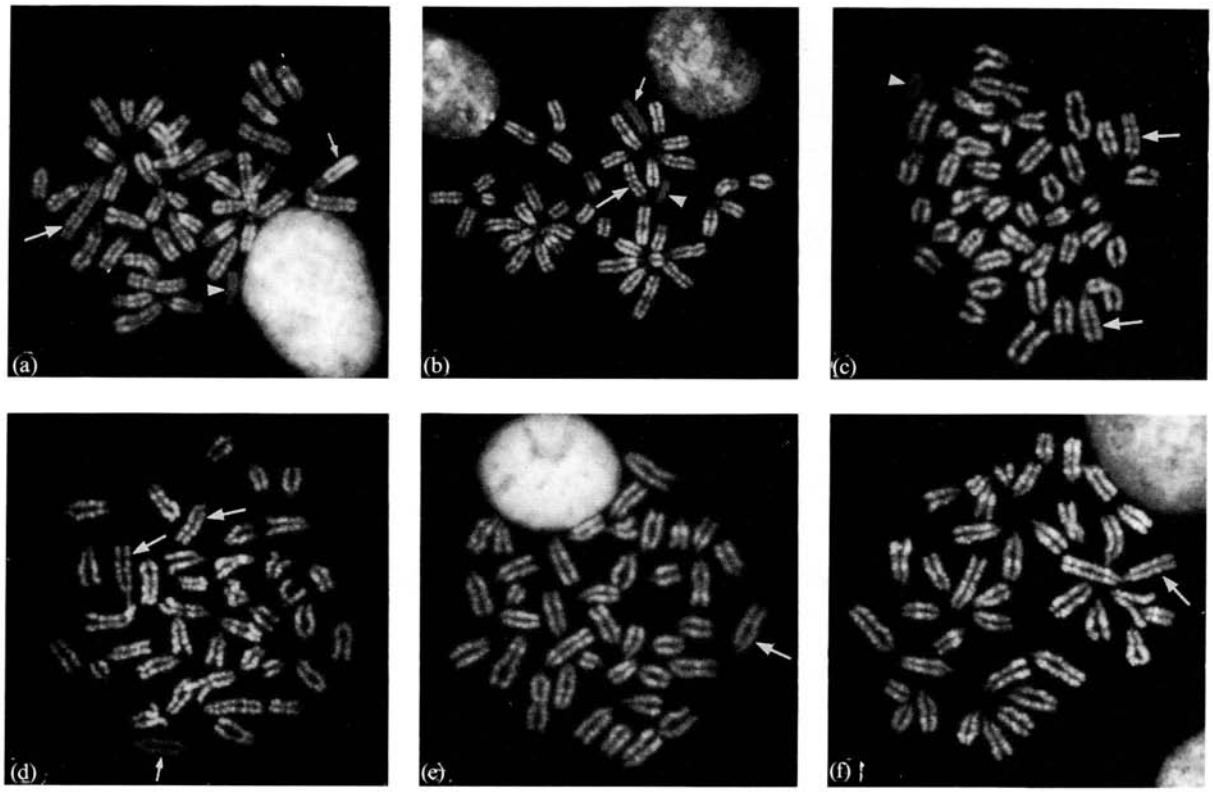

Fig. 2. X chromosome replication patterns revealed by a BrdU–acridine orange procedure in sex-chromosomal aneuploids. (*a*) A metaphase spread of X^MX^PY embryo carrying one maternal Is1Ct and one paternal Rb2Ad. Paternal Rb2Ad replicated precociously. (*b*, *c*) Metaphases from X^MX^MY embryos carrying maternal Rb2Ad and normal X. One X in (*b*) and two Xs in (*c*) replicated synchronously. (*d*) A cell from an $X^MX^MX^P$ embryo with In1H, Rb2Ad and normal X. Only paternal In1H was late replicating. (*e*) A metaphase spread from an X^M0 embryo obtained from Is1Ct/X female mated with Rb2Ad/Y male. (*f*) A cell from an X^P0 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 arrows, respectively.

The X^PX^P embryo 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^P0 or X^PX^P sex chromosome constitutions. In accordance with results obtained above, all the X^M0 and X^MX^PY grew normally except two X^MX^PY conceptuses which showed slight growth retardation.

It was reported that about 22% of oocytes produced by In1H/X females contained no sex chromosome due to a crossing over within the inverted chromosome region (Phillips & Kaufman, 1974; Mann & Lovell-Badge, 1987). Also, about 6% of oocytes carried a dicentric X, and the remaining 72% were 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 observations 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. The remaining 9 oocytes contained one or two extra autosomes.

It is likely that half the 0-oocytes give rise to X^P0 and the remaining half to 0Y which are eliminated before implantation (Morris, 1968). Hence, the expected frequency of X^P0 embryos at 7·5 dpc should be, if they were not lost at high frequency, about 13% based on the data reported previously, and about 10% (21/211 excluding 12 oocytes with a dicentric X and 21 0Y zygotes) based on the present data. The recovery of a total of 11·2% embryos derived from 0-oocytes 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 ($\chi^2 = 0{\cdot}10$, $P = 0{\cdot}75$).

(iv) *X chromosome inactivation in XXX and XXY embryos*

In three X^MX^PY embryos, like normal females, X^P replicated either precociously (Fig. 2*a*) or late in 230 (97%) of 236 informative extra-embryonic cells, and X^P or X^M replicated asynchronously in most cells from

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Table 5. *X-chromosome replication patterns in 7·5-day mouse embryos carrying an extra X chromosome*

Karyotype	No. of embryos	Gross morphology	No. of cells with allocyclic X^P P	X^P L	X^M P	X^M L	X^MX^P L	X^MX^M L	Without allocyclic Xs
$41,X^MX^MX^P$	6	Retarded	9	21			96	32	10
	2	Abnormal					20	9	0
$41,X^MX^MY$	4	Retarded			1	433			26
$41,X^MX^PY$	3	Normal	63	377		103			5

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

the embryonic region (Table 5). In four X^MX^MY embryos, 26 of 460 informative metaphases apparently had no inactivated X chromosome (Fig. 2*c*). 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 $X^MX^MX^P$ embryos was more convincing. Thirty out of 168 informative cells had the X^P as the only X chromosome replicating asynchronously (Fig. 2*d*). No such cell was found among 29 cells from two grossly abnormal $X^MX^MX^P$ embryos lacking most of the extra-embryonic portion. These findings suggested that those cells having a single asynchronously replicating X^P were derived mainly from the extra-embryonic tissues and those cells were, in turn, responsible for the deficient extra-embryonic 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. 2*b*).

4. Discussion

All the 16 X^MX^PY 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^MX^MY embryos, on the other hand, suffered from growth retardation or abnormal development with specifically affected extra-embryonic structure as reported by Shao & Takagi (1990). Since the only notable difference between X^MX^MY and X^MX^PY sex chromosome constitution is the parental origin of the supernumary 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 $X^MX^MX^P$ embryos substantiates this conclusion.

The imprinted gene(s) of X^M responsible for the developmental anomaly of X^MX^MY embryos is evidently not expressed when there is only one X^M chromosome in a cell as in the case of normal male (X^MY) embryos. Hence, Shao & Takagi (1990) and Takagi (1991) hypothesized that the effects of X^M imprinting are exercised through X chromosome inactivation. Furthermore, they proposed that the mechanism that induces the preferential X^P inactivation 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^MX^MY embryos. It is formally possible, however, to assume that they represented cells in which X inactivation had not yet occurred. $X^MX^MX^P$ 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 & 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 carrier 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 under-

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developed 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 centre 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 environment. The majority of diploid parthenogenetic embryos, whose chromosomes are exclusively maternal in origin, resemble fertilized embryos disomic for X^M in morphology. Tada & 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 extra-embryonic tissues, since both the ectoplacental cone and extra-embryonic 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^MX^MY mouse is ever found, two possible $X^MX^MX^P$ cases have been reported. Endo & Watanabe (1989) found an XXX female among phenotypically normal progenies of an X0 mouse. Although these authors favoured 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 & 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 suggested that two out of three X chromosomes were of maternal origin. However, as they pointed out, their results do not rule 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 three 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 $X^MX^MX^P$ embryo in a mating between In1H/X female and Rb2Ad/Y male (Table 4) may point to the possibility that certain $X^MX^MX^P$ embryos are capable of growing normally for the first one third of the pregnancy. It is conceivable that exceptional embryos disomic for X^M survive pregnancy if they manage to inactivate two X chromosomes in just enough cells to form extra-embryonic 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 supernumary 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 a difference between man and the mouse may have arisen from a slight change 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 0Y zygotes at equal frequencies. Since 0Y zygotes do not develop beyond the 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 birth (Morris, 1968; Brook, 1983) invariably showed that X0 progenies were much less frequent than expected. Our unpublished observations 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 non-random 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 consisting 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^MY^{*X} females from the cross $XX \times XY^*$. The reduced viability of X^P0 and X^PY^{*X} offspring could be caused by X chromosome imprinting or uterine environment of the practically X0 mother (Hunt, 1991). The present observation on X^M0 and X^P0 embryos clearly supports the latter alternative, although 12 out of 35 X^P0 and X^PY 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 X0 embryos from In1H/X mothers were retarded in growth at 7·25 dpc, but that they were similar in size to XY and XX littermates by 12·5 dpc. In agreement with this observation, X0 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^PY^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 X0 cases. The most remarkable difference between men and mice would be that nearly 99% of human X0 cases are aborted early in pregnancy.

In summary, studies of early post-implantation development of X-chromosomal aneuploids revealed no parentally imprinted X-linked gene essential for 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 this inhibitory effect of imprinting is perfect. It was also shown that X^P imprinting did not explain pre-implantation loss of X^P0 offspring of X0 mothers.

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