Genet. Res., Camb. (1992), **59**, *pp.* 205–214 *With* 1 *text-figure* *Printed in Great Britain*

The differential staining pattern of the X chromosome in the embryonic and extraembryonic tissues of postimplantation homozygous tetraploid mouse embryos

S. WEBB, T. J. DE VRIES† AND M. H. KAUFMAN*
Department of Anatomy, University Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

(*Received 5 December 1991 and in revised form 17 January 1992*)

Summary

(C57BL × CBA)F1 hybrid female mice were mated with hemizygous Rb(X.2)2Ad males to distinguish the paternal X chromosome. Homozygous tetraploids were produced by blastomere fusion at the 2-cell stage, and 161 of these were transferred to recipients and analysed on the 10th day of gestation. 59 implants contained resorptions and 76 contained either an embryo and/or extraembryonic membranes. 38 (20, XXXX and 18, XXYY) were analysed to investigate their X-inactivation pattern. Embryonic and yolk sac endodermally- and mesodermally-derived samples were analysed by G-banding and by Kanda analysis. In the XX and XY controls, the predicted pattern of X-inactivation was observed, though 12·2% of metaphases in the XX series displayed no X-inactivation. In the XY series the Y chromosome was seen in a high proportion of metaphases.

In the XXXX tetraploids, 8 cell lineages were recognized with regard to their X-inactivation pattern, though most belonged to the following 3 categories: (XmXm)XpXp, Xm(XmXp)Xp and XmXm(XpXp). The other categories were only rarely encountered. In the embryonic and mesodermally-derived tissue the ratio of these groups was close to 1:2:1, whereas in the endodermally-derived tissue it was 1:4·11:4·88, due to preferential paternal X-inactivation. A significant but small proportion of all 3 tissues analysed displayed no evidence of X-inactivation. Indirect evidence suggests that this represents a genuine group because of the high efficiency of the Kanda staining. The presence of the Xm(XmXp)Xp category is consistent with the expectation that X-inactivation occurs randomly in 2 of the 4 X chromosomes present. The presence of small numbers of preparations with no evidence of X-inactivation and other unexpected categories suggests that these are probably selected against during development.

1. Introduction

Tetraploidy is usually a consequence of failure of cytokinesis at the first mitotic division, and is observed in about 5% of human spontaneous abortuses with a numerical chromosomal abnormality (Boué *et al.* 1975; Hassold *et al.* 1980; Lin *et al.* 1985). Rarely, tetraploidy has been reported in a live-born infant (Golbus *et al.* 1976; Pitt *et al.* 1981; Wilson *et al.* 1988, and for recent review, see Pajares *et al.* 1990). Analysis of the little literature on the placental morphology of human homozygous tetraploid conceptuses strongly suggests that they have no characteristic histopathological features (Surti, 1987). Occasionally, tetraploidy results from the trispermic fertilization of an egg, and it is believed that the placentae in these heterozygous tetraploids invariably display partial hydatidiform molar degeneration (Sheppard *et al.* 1982; Surti *et al.* 1986).

In other mammalian species, the spontaneous incidence of tetraploidy is less that 1% (reviewed in Dyban & Baranov, 1987). Consequently, in order to study the development of tetraploid mammalian embryos, various experimental techniques have been devised over the years to increase the incidence of this condition. One of the simplest ways to induce homozygous tetraploid development is by the experimental inhibition of the first or second cleavage division by cytochalasin B or D (Snow, 1973, 1975, 1976; Tarkowski *et al.* 1977). An alternative approach involves the fusion of the two blastomeres of a 2-cell stage embryo, and a variety of fusigenic stimuli have been used over the years, such as polyethylene glycol

* Corresponding author.
† Present address: Department of Pathology, University Hospital, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

(Eglitis, 1980; Eglitis & Wiley, 1981), inactivated Sendai virus (Graham, 1971; O'Neill *et al.* 1990) and electrofusion (Kubiak & Tarkowski, 1985; Kaufman & Webb, 1990). Development to term of a limited number of cytochalasin B-induced tetraploid mouse embryos was claimed by Snow (1975), but this finding has yet to be repeated by other researchers (Tarkowski *et al.* 1977; Ozil & Modliński, 1986).

Recently, viable tetraploid mouse embryos with a developmental age of about 14·5 days p.c. have been recovered (Kaufman, 1991). While Snow (1975) described a range of abnormalities involving virtually all of the body systems, we have consistently observed abnormalities involving only the craniofacial region (Kaufman & Webb, 1990). The latter are either directly or indirectly related to failure of differentiation of the forebrain and its derivatives, and include partial or complete failure of separation of the two telencephalic hemispheres. Inconstant postcranial abnormalities were also commonly observed, but were not considered to be life-threatening (Kaufman, 1991; Kaufman, 1992). However, the placentae invariably displayed characteristic histopathological features (authors, unpublished), and it appears likely that it is the inefficient functioning of this organ that leads to the premature death of these embryos.

Snow (1975) hypothesized that irregular X-chromosome inactivation might have accounted for the developmental abnormalities observed in his tetraploid embryos. While Snow (1975) did not discuss the normality or otherwise of the placentae in his tetraploids, it is possible that the abnormalities we have observed in this organ might also have resulted from irregularities of X-inactivation. The latter is a phenomenon that occurs in all female mammals in order to achieve dosage compensation for X-linked genes (Lyon, 1961, 1962), and plays a critical role in the differentiation of the extra-embryonic tissues of rodents (Monk & Harper, 1979; Monk, 1981). In the mouse, the X chromosome appear to be inactivated in different embryonic tissues at different times. X-inactivation initially occurs in the trophectoderm of 3·5-day blastocysts, then in the primitive endoderm overlying the inner cell mass at 4·5 days and in the embryonic ectoderm at 6–6·5 days post coitum (Monk, 1978; Monk & Harper, 1979; Harper *et al.* 1982). The paternally derived X chromosome is preferentially inactivated in the trophectoderm and primitive endoderm (Takagi *et al.* 1978; West *et al.* 1977; Frels *et al.* 1978; Frels & Chapman, 1980), whereas either the maternal or paternal X chromosome can be inactivated at random in the embryonic ectoderm and in the extraembryonic mesoderm (Takagi & Sasaki, 1975; West *et al.* 1977).

Because of our interest in the tetraploid placenta, we decided that it would be instructive to analyse the X-inactivation pattern in the embryonic and extra-embryonic tissues of tetraploid mouse embryos using the Kanda differential staining technique. The availability of male mice carrying a Robertsonian translocation between the X chromosome and chromosome 2, allows us to identify the parental origin of the inactive X chromosome(s) in all of the metaphase preparations analysed. This paper therefore reports on the X-inactivation pattern in post-implantation tetraploid mouse embryos, and allows us to investigate the following questions;

(i) Does the X-inactivation pattern affect the viability of tetraploid embryos?
(ii) Are the number of inactivated X chromosomes present double that observed in normal diploid embryos, as might be anticipated, or otherwise?
(iii) Is there the same pattern of preferential X-inactivation of the paternal X chromosome(s) in the endoderm-derived component of the yolk sac as occurs in normal diploid mice, or is the pattern abnormal and therefore unexpected?

2. Materials and methods

(i) *Isolation of 2-cell stage embryos*

8- to 12-week-old (C57BL × CBA)F1 hybrid female mice were injected with 5 IU PMSG and then 48 h later with 5 IU HCG to induce ovulation. After the HCG injection, the females were caged individually with fertile hemizygous Rb(X.2)2Ad male mice (Adler *et al.* 1989) kindly supplied by Dr Mary Lyon, MRC Radiobiology Unit, Harwell. The presence of a vaginal plug the next morning was taken as evidence of mating and this was considered to be the first day of gestation. Early on the second day of gestation the female mice were killed by cervical dislocation and the oviducts removed and flushed through with phosphate-buffered saline (PBS) containing 4% bovine serum albumin in order to recover the 2-cell stage embryos. The embryos were then transferred into drops of M16 tissue culture medium (Whittingham, 1971) under paraffin oil, and held in an incubator at 37 °C in an atmosphere of 5% CO_2 in air.

(ii) *Electrofusion and transfer of fused tetraploid embryos to pseudopregnant recipients*

The 2-cell stage embryos that were to be fused were transferred into microdrops of a non-electrolyte solution of 0·3 M mannitol (Kubiak & Tarkowski, 1985). The same solution was also present in the fusion chamber which consisted of a plastic tissue culture dish which contained two platinum wires of 250 μm diameter fixed parallel to each other on the bottom of the dish with a space of about 600 μm between them. The platinum wires were connected to a Digitmer pulse stimulator set at 200 V, with a pulse duration of 50 μs. The 2-cell stage embryos, in batches of 10, were then placed between the two platinum wires in the chamber and the pulse stimulator was

triggered. The embryos were removed immediately and washed through 4 drops of tissue culture medium and then returned to the incubator. Within 15–30 min the two blastomeres had fused in a high proportion of cases to form a single blastomere. These 1-cell (tetraploid) embryos were then transferred unilaterally to the oviducts of recipients on the first day of pseudopregnancy (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). The recipients were anaesthetized with tribromoethanol (Avertin, Winthrop; dose 0·02 ml/g body weight of a freshly prepared 1·2% solution of Avertin in 0·9% saline).

(iii) *Isolation of tetraploid embryos on the 10th day of gestation*

The recipients were autopsied on the 10th day of gestation and the number of implantation sites, resorptions, and embryos isolated was recorded. The decidual swellings were isolated from the uterine horns into PBS. The developmental stage of the embryos recovered, whether normal or abnormal, and their crown–rump length (if 'turned') were noted at this time. The embryos, where size permitted, were separated from their extraembryonic membranes and divided into two halves and each was then transferred into tissue culture medium containing colcemid (1 ml of a 0·1% solution of colcemid in 100 ml of TC199), and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 2–3 h.

(iv) *Enzyme separation of the yolk sac*

After an initial incubation period of 2–3 h, the yolk sac was separated into its two component parts, namely an inner mesodermally-derived layer and an outer endodermally-derived layer, using a modification of the enzyme separation technique described by Levak-Svajger *et al.* (1969). For this procedure, the yolk sac samples were placed in 1 ml of an enzyme solution which contained 2·5 g of pancreatin and 0·5 g of trypsin per 100 ml of PBS, and were retained in this solution for 2–3 h at 4 °C. The yolk sac samples were then transferred into individual solid watch-glasses containing 2–3 ml of M2 medium (Quinn *et al.* 1982), and the separation of the two layers was completed mechanically using watchmakers' forceps. The mesodermally-derived and endodermally-derived components were in all cases readily distinguished, principally because blood vessels were only associated with the mesodermally-derived samples.

(v) *The Kanda staining technique and analysis*

Where possible, four samples were obtained from each conceptus, namely two portions of embryonic tissue, the yolk sac endodermally-derived sample and the yolk sac mesodermally-derived sample. One of the embryonic tissue samples was processed for G-banding analysis. The remaining three samples were processed separately using a modification of the Kanda technique (Kanda, 1973) which differentially stains the inactive X chromosome. For the latter procedure, the samples were retained in a 0·5% hypotonic solution of KCl at room temperature for 7 min, then transferred into a hot solution of hypotonic KCl at 50 °C and maintained at this temperature for 12–13 min. All samples were fixed in 3:1 methanol: glacial acetic acid at 4 °C for a minimum of 20 min. Slides were prepared using a modification of the cell spreading technique outlined by Evans *et al.* (1972) and subsequently stained for 5 min in a 3% Giemsa solution.

Analysis of the X-inactivation status of these preparations was made on a sample of chromosome spreads where all of the individual chromosomes present could be distinguished and counted. Metaphase spreads in which the Rb(X.2) translocation, though present, was not readily distinguished (except that is in the case of XXYY tetraploids) were automatically excluded from the analysis. Since the possibility exists that a low frequency of aneuploid embryos could be generated when normal mice are mated to hemizygous mice bearing the Rb(X.2) translocation (Adler *et al.* 1989), this factor could have accounted for some of these embryos. In the following text, the parental origin of the X chromosome is designated as Xm or Xp, where the X chromosome is of maternal or paternal origin, respectively, while the inactive chromosome(s) are enclosed in parenthesis e.g. (XmXm)XpXp.

(vi) *Production of control diploid embryos*

In a parallel control study, 2-cell stage embryos that had not been exposed to electrofusion were transferred to recipients and isolated on the 10th day of gestation. The embryos and their extraembryonic membranes were treated in the same way as the tetraploid tissues detailed above.

3. Results

(i) *Control series*

The control series consisted of 26 diploid conceptuses isolated on day 10 of gestation. Of these, 14 were XX and 12 were XY. The incidence of differential staining in the metaphase spreads isolated from the various tissues of XmXp diploid embryos showed that in the 623 metaphase spreads analysed from the embryonic tissues, 282 (45·3%) displayed a single inactive Xm chromosome, 265 (42·5%) displayed a single inactive Xp chromosome and 76 (12·2%) showed no darkly staining X chromosome(s). In the mesodermally-derived tissue, out of 197 metaphase spreads analysed,

https://doi.org/10.1017/S0016672300030494 Published online by Cambridge University Press

Table 1. *The differential staining pattern of the X chromosome in metaphase spreads isolated from XmXp diploid control tissues*

Tissue Analysed	No. of metaphases with no darkly staining X chromosome (%)	No. of metaphases with one darkly staining X chromosome (%)		Total no. analysed
		(Xm)	(Xp)	
Embryonic	76 (12·2)	282 (45·3)	265 (42·5)	623
Mesodermally-derived	31 (15·7)	76 (38·6)	90 (45·7)	197
Endodermally-derived	48 (33·8)	9 (6·3)	85 (59·9)	142

76 (38·6%) displayed evidence of a single inactive Xm chromosome, 90 (45·7%) displayed a single inactive Xp chromosome and 31 (15·7%) showed no darkly-staining X chromosome(s). By contrast, in the endodermally-derived tissue, out of 142 metaphase spreads analysed, 9 (6·3%) showed evidence of an inactive Xm chromosome, 85 (59·9%) showed evidence of an inactive Xp chromosome and 48 (33·8%) showed no darkly-staining X chromosome. In the embryonic and mesodermally-derived tissue, X-inactivation is random with regard to the parental origin of the X chromosome (embryonic tissue $\chi^2 = 0{\cdot}528$, $P > 0{\cdot}5$; mesodermally-derived tissue $\chi^2 = 1{\cdot}181$, $P > 0{\cdot}3$). The X-inactivation pattern of the endodermally-derived tissue was found to be non-random ($\chi^2 = 61{\cdot}446$, $P > 0{\cdot}001$; see Table 1).

The X-inactivation status of the single X chromosome present in the metaphase spreads isolated from the various tissues of XY diploid control embryos was also studied. The findings demonstrated that in 229 metaphase spreads obtained from embryonic tissue, 171 metaphases from mesodermally-derived tissue and 150 metaphases from endodermally-derived tissue no darkly-staining X chromosomes were observed. The Y chromosome, however, was invariably recognised as a very small darkly-staining chromosome in most of these preparations, and confirmed the very high efficiency of the Kanda staining technique in relation to this material.

(ii) *Tetraploid series*

Following the transfer of 161 1-cell 'fused' tetraploid embryos to the oviducts of 14 pseudopregnant recipients, 135 (83·9%) implantation sites were observed on the 10th day of gestation. Of these, 59 (43·7%) contained resorptions and 76 (56·3%) contained either an embryo and/or extraembryonic membranes, and 38 (50%) of these were analysed using the Kanda staining technique. Twenty had an XXXX and 18 had an XXYY sex chromosome constitution.

(*a*) *XmXmXpXp tetraploid conceptuses.* Of the 20 conceptuses analysed in this series, 7 consisted of extraembryonic membranes only, 10 were represented by an embryonic vesicle associated with extra-embryonic membranes, and 3 were somite-stage embryos with associated extraembryonic membranes (see Table 2). The three developmentally most advanced embryos recovered consisted of an 'unturned' embryo with about 8 pairs of somites, an advanced 'unturned' embryo with about 15–20 pairs of somites, and a 'turned' embryo with about 25–30 pairs of somites with a crown–rump length of 2·46 mm.

Eight distinct cell lineages could be identified in the various tissues of this group of tetraploid embryos.

1. No inactive X chromosomes present, i.e. XmXmXpXp.
2. A single inactive X chromosome of maternal origin, i.e. (Xm)XmXpXp.
3. A single inactive X chromosome of paternal origin, i.e. XmXm(Xp)Xp.
4. Two inactive X chromosomes of maternal origin, i.e. (XmXm)XpXp (see Fig. 1*a*).
5. Two inactive X chromosomes of paternal origin, i.e. XmXm(XpXp) (see Fig. 1*b*).
6. Two inactive X chromosomes, one of maternal origin and the second of paternal origin i.e. Xm(XmXp)Xp (see Fig. 1*c*).
7. Three inactive X chromosomes, two of maternal origin and one of paternal origin, i.e. (XmXmXp)Xp.
8. Three inactive X chromosomes, one of maternal origin and two of paternal origin, i.e. Xm(XmXpXp).

The situation in which only one X chromosome was inactivated was only rarely encountered. In those instances in which this was the case, the maternal X chromosome was found to be inactivated more often than the paternal X chromosome. Metaphase spreads in which three inactive X chromosomes were present were also only rarely seen, and no preparations were observed in which all four X chromosomes present were inactivated.

The vast majority of XmXmXpXp metaphase spreads showed two darkly staining X chromosomes. Out of 107 metaphase spreads from embryonic tissue, 97 (90·6%) showed two darkly-staining X chromosomes. Out of 207 metaphase spreads from meso-

https://doi.org/10.1017/S0016672300030494 Published online by Cambridge University Press

Table 2. *Developmental stage and sex chromosome constitution of tetraploid conceptuses isolated on the 10th day of gestation*

	Stage recovered	Sex chromosome constitution	
		XXXX	XXYY
1.	Extraembryonic membranes only	7	5
2.	Embryonic vesicle + extraembryonic membranes	10	10
3.	Somite stage embryo + extraembryonic membranes	3	3
	Total	20	18

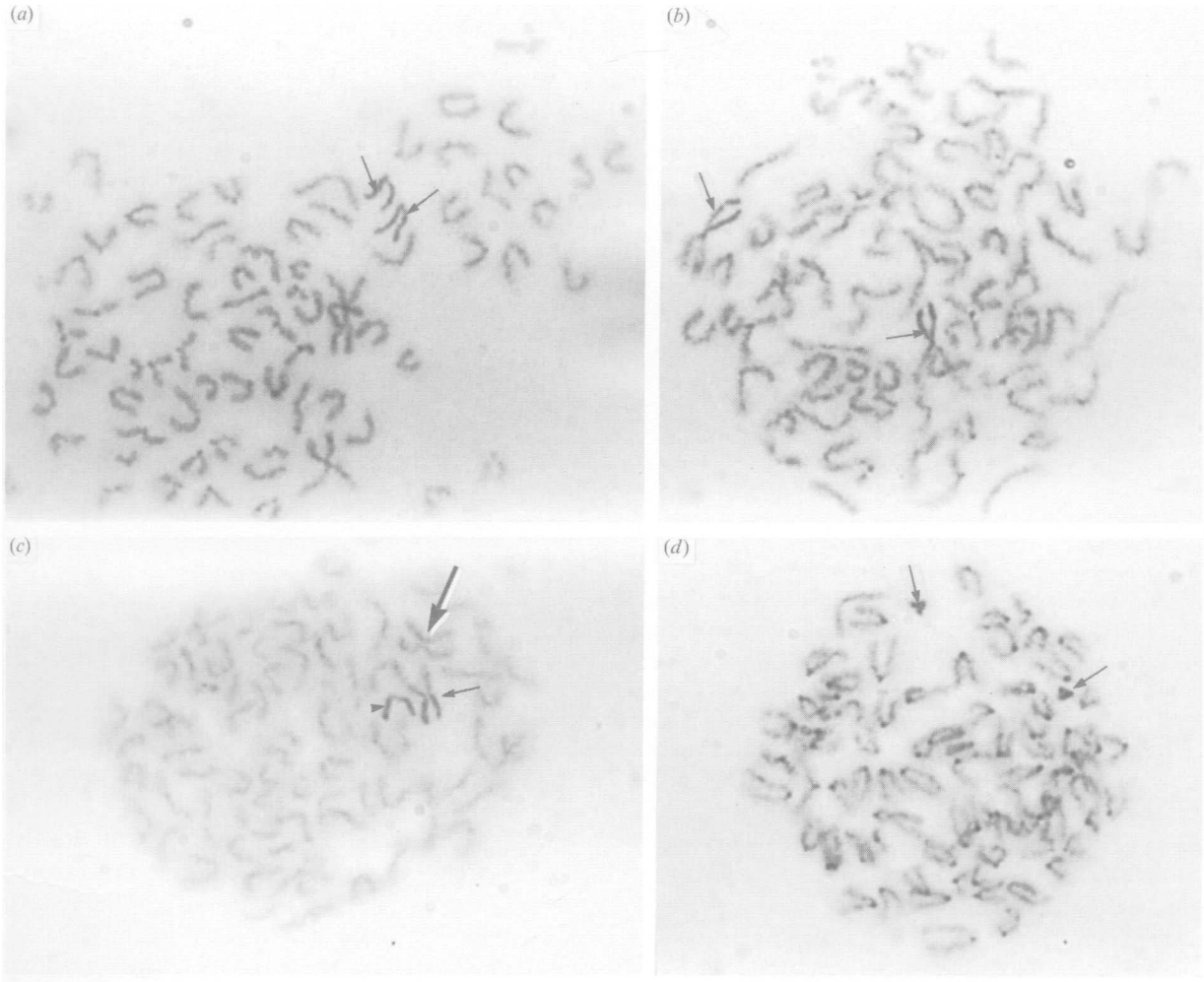

Fig. 1. (*a*)–(*d*) are representative metaphase spreads from homozygous tetraploid embryonic and extraembryonic tissues stained by the Kanda technique to investigate the pattern of X chromosome inactivation in these tissues. In (*a*)–(*c*), metaphase spreads from XXXX tetraploid embryos in which the three commonest X-inactivation patterns observed, in each of which two of the four X chromosomes present are inactivated, are illustrated. (*a*) Metaphase spread in which the two maternally-derived X chromosomes (arrows) are inactivated. This spread therefore has an (XmXm)XpXp inactivation pattern. (*b*) Metaphase spread in which the two paternally-derived X chromosomes (arrows) are inactivated. In this preparation, one half of the paternally-derived 'marker' chromosomes are darkly-staining. This spread therefore has an XmXm(XpXp) inactivation pattern. (*c*) Metaphase spread in which one maternally-derived (arrow head) and one paternally-derived (arrow) X chromosome is inactivated. As in (*b*), one half of this paternally-derived 'marker' chromosome is darkly-staining. This spread therefore has an Xm(XmXp)Xp inactivation pattern. Note that the second paternally-derived 'marker' chromosome (large arrow) has no darkly-staining region. (*d*) Metaphase spread from an XmXmYY embryo in which no evidence of X-inactivation is observed. The two Y chromosomes present are darkly-staining (arrows).

https://doi.org/10.1017/S0016672300030494 Published online by Cambridge University Press

Table 3. *The differential staining pattern of the X chromosome in metaphase spreads isolated from XmXmXpXp tetraploid tissues*

Parental origin of inactivated X chromosomes	Embryonic tissue (%)	Mesodermally-derived tissue (%)	Endodermally-derived tissue (%)
No X chromosomes inactivated	7 (6·5)	20 (9·7)	22 (18·9)
(Xm)	3 (2·8)	9 (4·3)	1 (0·9)
(Xp)	0	2 (0·9)	1 (0·9)
(XmXm)	26 (24·3)	38 (18·4)	9 (7·8)
(XmXp)	49 (45·8)	95 (45·9)	37 (31·9)
(XpXp)	22 (20·6)	41 (19·8)	44 (37·9)
(XmXmXp)	0	1 (0·5)	0
(XmXpXp)	0	1 (0·5)	2 (1·7)
Total no. of metaphases analysed	107	207	116

Table 4. *The differential staining pattern in various tissues of XmXmXpXp conceptuses in which two inactivated X chromosomes were present analysed according to the developmental stage achieved*

Developmental stage	Embryonic tissue			Mesodermally-derived tissue			Endodermally-derived tissue		
	(XmXm)	(XmXp)	(XpXp)	(XmXm)	(XmXp)	(XpXp)	(XmXm)	(XmXp)	(XpXp)
1. Extraembryonic membrane only	—	—	—	22	43	24[d]	4	13	19[h]
2. Embryonic vesicle + extraembryonic membranes	13	24	14[a]	10	37	12[e]	4	21	23[i]
3. Somite stage embryo + extraembryonic membranes	13	25	8[b]	6	15	5[f]	1	3	2[j]
Total	26	49	22[c]	38	95	41[g]	9	37	44[k]

a, b, c, d, e, f, g are not significantly different from the expected value, $P > 0{\cdot}05$.
h, i, j, k are significantly different from the expected value, $P < 0{\cdot}05$.

dermally-derived tissue, 174 (84·1%) showed two darkly-staining X chromosomes, and out of 116 metaphase spreads from endodermally-derived tissue, 90 (77·6%) showed two darkly-staining X chromosomes (see Table 3). The (XmXm):(XmXp):(XpXp) ratios in the embryonic and mesodermally-derived tissues were 1:1·9:0·85 and 1:2·5:1·08, respectively. By contrast, the endodermally-derived tissue had a ratio of 1:4·11:4·88, and clearly indicated that non-random X-inactivation had occurred in this tissue, with clear evidence of preferential paternal X chromosome inactivation.

Of the less commonly encountered groups, that in which no darkly-staining X chromosome was present was encountered in 6·5, 9·7 and 18·9% of the metaphase spreads analysed in the embryonic, yolk sac mesodermally-derived and yolk sac endodermally-derived tissues, respectively (see Table 3). The metaphase spreads in which either only a single or occasionally three darkly-staining X chromosomes were present, were only very rarely encountered (see Table 3).

The differential staining pattern of the two inactivated X chromosomes in each of the three tissues analysed, and the developmental stage achieved by these embryos revealed that in each of the developmental stages studied the embryonic and mesodermally-derived tissues displayed random X-inactivation, whereas in the endodermally-derived tissues, preferential paternal X-inactivation had occurred (see Table 4). This observation would appear to indicate that the X-inactivation status of a tetraploid embryo has no obvious influence on its development potential.

(*b*) *XmXmYY tetraploid conceptuses.* Of the 18 conceptuses in this group, 5 consisted of extra-embryonic membranes only, 10 consisted of an embryonic vesicle with associated extraembryonic membranes, and 3 consisted of somite-stage embryos

https://doi.org/10.1017/S0016672300030494 Published online by Cambridge University Press

Table 5. *The differential staining pattern of the X chromosome in metaphase spreads from XmXmYY tetraploid tissues*

Tissue analysed	No. of metaphases with no darkly staining X chromosome (%)	No. of metaphases with one X chromosome darkly staining (Xm) (%)	No. of metaphases with two X chromosomes darkly staining (XmXm) (%)	Total no. of metaphases analysed
Embryonic	110 (98·2)	2 (1·8)	0	112
Mesodermally-derived	102 (98·1)	2 (1·9)	0	104
Endodermally-derived	99 (99·0)	1 (1·0)	0	100

with associated extraembryonic membranes (see Table 2). The three developmentally most advanced stages recovered in this series consisted of an 'unturned' embryo with about 4 pairs of somites, an 'unturned' embryo with about 15–20 pairs of somites, and an advanced 'unturned' embryo with about 20 pairs of somites. Only two distinct cell lineages were observed in this tetraploid series, and the pattern of X-inactivation in all three tissues analysed was similar. Out of 316 metaphase spreads analysed, only 5 (1·6%) displayed one darkly-staining X chromosome. The remaining 311 metaphase spreads (98·4%) showed no darkly-staining X chromosome (see Table 5). The Y chromosomes were recognizable as two small darkly-staining chromosomes in virtually all of the preparations analysed (see Fig. 1*d*).

It is of critical importance in relation to the analysis of this material to note that in any particular embryo, a similar range of X-inactivation patterns was observed in each of the tissues analysed. This allowed the findings from comparable tissues from various embryos to be amalgamated together.

4. Discussion

The results obtained from this study show that by use of the Kanda differential staining technique, the X-inactivation pattern of diploid control embryos and tetraploid conceptuses could be established. Furthermore, the use of male mice bearing the Rb(X.2) translocation enabled the parental origin of the inactivated X chromosome(s) present to be determined. As expected, in the XmXp diploid control embryonic and mesodermally-derived tissues, X-inactivation was found to be random with regard to the parental origin of the inactive X chromosome. By contrast, the X chromosome in the endodermally-derived tissue showed a non-random pattern, with clear evidence of preferential paternal X-inactivation. A very high proportion of the metaphase spreads in the XY diploid control embryos displayed no darkly-staining X chromosome, and the Y chromosome was always seen as a very small darkly-staining chromosome, being observed in the majority of preparations analysed.

Snow (1976) had earlier predicted that the X-inactivation pattern in tetraploid mice might well be similar to, but double the pattern found in diploid mice, with the presence of two inactive X chromosomes in female tetraploids, and no inactive X chromosomes in male tetraploids. However, our findings indicate that the X-inactivation pattern in tetraploid female embryos is in fact not only random with regard to parental origin in the embryonic and mesodermally-derived tissues, but it is also random with regard to which combination of two out of the four X chromosomes are destined to become inactivated. The observed ratio of (XmXm):(XmXp):(XpXp) was not significantly different from 1:2:1. This is different from the predicted ratio of 1:4:1 if it is assumed that any two of the four X chromosomes present inactivate at random. However, the significance of this difference between the observed and expected ratio may be explained on the grounds that there might be an element of selection against individuals with an (XmXp) inactivation pattern. It is of interest that in the yolk sac endodermally-derived tissue, the ratio of these three X-inactivation patterns was 1:4·11:4·88, and displayed clear evidence of preferential paternal X-inactivation. However, even in this tissue the presence of a small but significant number of metaphases in which the two maternally-derived X chromosomes were inactivated clearly indicates that although paternal X-inactivation predominates, it is not exclusive. A similar situation is also seen in diploid parthenogenetic mouse embryos where a single X chromosome inactivates in a high proportion of the yolk sac endodermally-derived tissue, despite the absence of a paternally-derived X chromosome, and in the absence of imprinting (Rastan *et al.* 1980).

In the XmXmYY embryos, the majority of metaphases (98·4%) displayed no evidence of X chromosome inactivation. The situation observed in this group of tetraploids would seem therefore to be consistent with the situation observed in normal diploid XY embryos.

The other category that justifies brief consideration is that in which no cytological evidence of X-inactivation is observed in the XX diploid and XXXX tetraploid groups. The incidence of this category

appears to vary between the tissues analysed, so that in the embryonic tissues the incidence of this group was 12·2 and 6·5% (in the diploids and tetraploids, respectively); in the extraembryonic mesodermally-derived tissue the comparable figures were 15·7 and 9·7%, while in the extraembryonic endodermally-derived tissue the comparable figures were 33·8 and 18·9%, respectively. While there is clearly no direct evidence to indicate whether this category results from a failure of the Kanda technique to stain inactivated X chromosome(s) in these preparations, or represents a genuine group in which all the X chromosomes present are active, the indirect evidence suggests that the latter is more likely to be the case. Evidence from the XY diploids and XXYY tetraploids suggests that the Kanda technique is in fact extremely efficient in that the Y chromosome(s) is unequivocally demonstrated as a small darkly-staining chromosome in the vast majority of preparations from these two groups. A similar finding was reported in a previous study (Speirs *et al.* 1990) where it was also observed that the incidence of this category was substantially higher in the extraembryonic endodermally-derived preparations compared to the situation observed in the embryonic tissue and in the extraembryonic mesodermally-derived tissue. The existence of such a cell lineage has also been postulated by Endo *et al.* (1982), using the Brdu technique to identify the inactive X chromosome, in their analysis of the visceral yolk sac of LT-derived XXY digynic triploid embryos.

One other curious group that has yet to be accounted for is represented by the few preparations (1·8%) in the XXYY tetraploids which display one darkly-staining X chromosome, though it should be noted that no preparations were observed in the XY diploid series in which the single X chromosome that was present was inactivated. Similarly, in the XXXX tetraploids, 3·7% out of a total of 430 metaphase preparations analysed displayed only a single inactive X chromosome, while only 0·9% displayed cytological evidence of 3 inactive X chromosomes. As has been noted in the Results section, no preparations were observed in which all four X chromosomes present were inactivated. However, the very small number of preparations involved precluded the establishment of any relationship between the incidence of these various patterns of X-inactivation and their tissue of origin.

One explanation for the presence of only relatively small numbers of these unexpected classes, and possibly also the Xm(XmXp)Xp class, is that they are selected against during development. It is possible therefore that the incidence of these classes might be proportionately higher if similar embryos were analysed at earlier stages of development, shortly after the X-inactivation event occurs.

In this study certain technical difficulties were encountered. The developmental potential of the tetraploid conceptuses produced from the mating of F1 hybrid females to Rb(X.2)2Ad males was much poorer than had previously been encountered when male mice carrying the Robertsonian translocation Rb(1·3)1Bnr were used (Kaufman & Webb, 1990). In particular, the number of viable somite-stage embryos recovered in this series was about half of that previously encountered in the Rb(1.3)1Bnr series. This meant that one of the initial aims, namely to investigate if there was a link between developmental potential and the X-inactivation pattern, could not be resolved satisfactorily, because of the relatively small number of advanced somite-stage embryonic stages recovered. Furthermore, because of the poorer development of the conceptuses from this strain combination, the amount of tissue available to work with was small, and only a relatively low number of metaphase spreads were suitable for analysis following Kanda staining.

Our preliminary findings would seem to indicate that there was no obvious relationship observed between the sex chromosome constitution of the tetraploid conceptuses and their developmental stage, or gross morphological appearance, at the time of their isolation. Similar findings have previously been reported in connection with triploid embryos recovered from LT/Sv strain mice at a similar stage of gestation (Speirs & Kaufman, 1989; Speirs *et al.* 1990). This is in contrast to the very recent observation (Kaufman, 1992) that in the developmentally most advanced tetraploid embryos analysed from Rb(1·3)-1Bnr × F1 matings there appears to be a correlation between the sex of the embryo and the presence of certain postcranial vascular abnormalities. Thus three out of four of the male embryos analysed histologically that were developmentally equivalent to normal diploid embryos of about 13–14·5 days p.c., possessed aortic arch abnormalities, while the fourth male embryo possessed other possibly related vascular abnormalities. Curiously, none of three female embryos analysed which were of a similar developmental age displayed any cardiovascular abnormalities.

While we were undoubtedly considerably hampered by the relatively poor postimplantation developmental potential of the homozygous tetraploid embryos analysed in this study, because of the necessity of using the Rb(X.2) marker chromosome to establish the parental origin of the inactivated X chromosomes, we believe that our investigation has nevertheless shed new light on the X-inactivation status of the various tissues of this interesting group of embryos.

The authors thank Dr Peter de Boer, Genetics Department, Agricultural University, Wageningen, for helpful discussions, and T. J. de Vries thanks the Wageningen Fund for financial support.

References

Adler, I. D., Johannisson, R. & Winking, H. (1989). The influence of the Robertsonian translocation Rb(X.2)2Ad on anaphase I non-disjunction in male laboratory mice. *Genetical Research* **53**, 77–86.

Boué, J., Boué, A. & Lazar, P. (1975). The epidemiology of human spontaneous abortions with chromosomal anomalies. In *Aging Gametes: Their Biology and Pathology* (ed. R. J. Blandau), pp. 330–348. Basel: S. Karger.

Dyban, A. P. & Baranov, V. S. (1987). *Cytogenetics of Mammalian Embryonic Development*. Oxford: Clarendon Press.

Eglitis, M. A. (1980). Formation of tetraploid mouse blastocysts following blastomere fusion with polyethylene glycol. *Journal of Experimental Zoology* **213**, 309–313.

Eglitis, M. A. & Wiley, L. M. (1981). Tetraploidy and early development, effects on developmental timing and embryonic metabolism. *Journal of Embryology and Experimental Morphology* **66**, 91–108.

Endo, S., Takagi, N. & Sasaki, M. (1982). The late replicating X chromosome in digynous mouse triploid embryos. *Developmental Genetics* **3**, 165–176.

Evans, E. P., Burtenshaw, M. D. & Ford, C. E. (1972). Chromosomes of mouse embryos and newborn young, preparations from membranes and tail tips. *Stain Technology* **47**, 229–234.

Frels, W. I. & Chapman, V. M. (1980). Expression of the maternally derived X chromosome in the mural trophoblast of the mouse. *Journal of Embryology and Experimental Morphology* **56**, 179–190.

Frels, W. I., Rossant, J. & Chapman, V. M. (1979). Maternal X chromosome expression in mouse chorionic ectoderm. *Developmental Genetics* **1**, 123–132.

Golbus, M. S., Bachman, R., Wiltse, S. & Hall, B. D. (1976). Tetraploidy in a newborn infant. *Journal of Medical Genetics* **13**, 329–332.

Graham, C. F. (1971). Virus assisted fusion of embryonic cells. *Acta Endocrinologica*, Suppl. **153**, 154–165.

Harper, M. I., Fosten, M. & Monk, M. (1982). Preferential paternal X-inactivation in extra-embryonic tissues of early mouse embryos. *Journal of Embryology and Experimental Morphology* **67**, 127–135.

Hassold, T., Chen, N., Funkhouser, J., Jooss, T., Manuel, B., Matsuura, J., Matsuyama, A., Wilson, C., Yamane, J. A. & Jacobs, P. A. (1980). A cytogenetic study of 1000 spontaneous abortions. *Annals of Human Genetics* **44**, 151–178.

Kanda, N. (1973). A new differential technique for staining the heteropycnotic X-chromosome in female mice. *Experimental Cell Research* **80**, 463–467.

Kaufman, M. H. (1991). Histochemical identification of primordial germ cells and differentiation of the gonads in homozygous tetraploid mouse embryos. *Journal of Anatomy* **179**, 169–181.

Kaufman, M. H. (1992). Postcranial morphological features of homozygous tetraploid mouse embryos. *Journal of Anatomy* (in the press).

Kaufman, M. H & Webb, S. (1990). Postimplantation development of tetraploid mouse embryos produced by electrofusion. *Development* **110**, 1121–1132.

Kubiak, J. Z. & Tarkowski, A. K. (1985). Electrofusion of mouse blastomeres. *Experimental Cell Research* **157**, 561–566.

Levak-Svajger, B., Svajger, A. & Skreb, N. (1969). Separation of germ layers in presomite rat embryos. *Experientia* **25**, 1311–1313.

Lin, C. C., De Braekeleer, N. & Jamro, H. (1985). Cytogenetic studies in spontaneous abortions: the Calgary experience. *Canadian Journal of Genetics and Cytology* **27**, 565–570.

Lyon, M. F. (1961). Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* **190** 372–373.

Lyon, M. F. (1962). X-chromosome inactivation and developmental patterns in mammals. *Biological Reviews* **47**, 1–35.

Monk, M. (1978). Biochemical studies on mammalian X chromosome activity. In *Development in Mammals*, vol. 3 (ed. M. H. Johnson) pp. 189–224. Amsterdam: North-Holland.

Monk, M. (1981). A stem-line model for cellular and chromosomal differentiation in early mouse development. *Differentiation* **19**, 71–76.

Monk, M. & Harper, M. I. (1979). Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. *Nature* **281**, 311–313.

O'Neill, G. T., Speirs, S. & Kaufman, M. H. (1990). Sex-chromosome constitution of postimplantation tetraploid mouse embryos. *Cytogenetics and Cell Genetics* **53**, 191–195.

Ozil, J. & Modliński, J. A. (1986). Effects of electric field on fusion rate and survival of 2-cell rabbit embryos. *Journal of Embryology and Experimental Morphology* **96**, 211–228.

Pajares, I. L., Delicado, A., Diaz de Bustamente, A., Pellicer, A., Pinel, I., Pardo, M. & Martin, M. (1990). Tetraploidy in a liveborn infant. *Journal of Medical Genetics* **27**, 782–783.

Quinn, P., Barros, C. & Whittingham, D. G. (1982). Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *Journal of Reproduction and Fertility* **66**, 161–168.

Pitt, D., Leversha, M., Sinfield, C., Campbell, P., Anderson, R., Bryan, D. & Rogers, J. (1981). Tetraploidy in a liveborn infant with spina bifida and other anomalies. *Journal of Medical Genetics* **18**, 309–311.

Rastan, S., Kaufman, M. H., Handyside, A. H. & Lyon, M. F. (1980). X-chromosome inactivation in extra-embryonic membranes of diploid parthenogenetic mouse embryos demonstrated by differential staining. *Nature* **288**, 172–173.

Sheppard, D. M., Fisher, R. A., Lawler, S. D. & Povey, S. (1982). Tetraploid conceptus with three paternal contributions. *Human Genetics* **62**, 371–374.

Snow, M. H. L. (1973). Tetraploid mouse embryos produced by cytochalasin B during cleavage. *Nature* **244**, 513–515.

Snow, M. H. L. (1975). Embryonic development of tetraploid mice during the second half of gestation. *Journal of Embryology and Experimental Morphology* **34**, 707–721.

Snow, M. H. L. (1976). The immediate postimplantation development of tetraploid mouse blastocysts. *Journal of Embryology and Experimental Morphology* **35**, 81–86.

Speirs, S., Cross, J. M. & Kaufman, M. H. (1990). The pattern of X-chromosome inactivation in the embryonic and extra-embryonic tissues of post-implantation digynic triploid LT/Sv strain mouse embryos. *Genetical Research* **56**, 107–114.

Speirs, S. & Kaufman, M. H. (1989). Analysis of the sex chromosome constitution of digynic triploid mouse embryos. *Cytogenetics and Cell Genetics* **52**, 151–153.

Surti, U. (1987). Genetic concepts and techniques. In *Gestational Trophoblastic Disease* (ed. A. E. Szulman and H. J. Buchsbaum), pp. 111–121. Berlin: Springer.

Surti, U., Szulman, A. E., Wagner, K., Leppert, M. & O'Brien, S. J. (1986). Tetraploid partial hydatidiform moles: two cases with a triple paternal contribution and a 92.XXXY karyotype. *Human Genetics* **72**, 15–21.

Takagi, N. & Sasaki, M. (1975). Preferential inactivation of the paternally derived X chromosome in the extra-embryonic membranes of the mouse. *Nature* **256**, 640–642.

Takagi, N., Wake, N. & Sasaki, M. (1978). Cytologic evidence for preferential inactivation of the paternally derived X chromosome in XX mouse blastocysts. *Cytogenetics and Cell Genetics* **20**, 240–248.

Tarkowski, A. K., Witkowska, A. & Opas, J. (1977). Development of cytochalasin B-induced tetraploid and diploid/tetraploid mosaic mouse embryos. *Journal of Embryology and Experimental Morphology* **41**, 47–64.

West, J. D., Frels, W. I., Chapman, V. M. & Papaioannou, V. E. (1977). Preferential expression of the maternally derived X chromosome in the mouse yolk sac. *Cell* **12**, 873–882.

Whittingham, D. G. (1971). Culture of mouse ova. *Journal of Reproduction and Fertility*, Supplement **14** 7–21.

Wilson, G. N., Vekemans, M. J. J. & Kaplan, P. (1988). MCA/MR syndrome in a female infant with tetraploidy mosaicism: review of the human polyploid phenotype. *American Journal of Medical Genetics* **30**, 953–961.