Genet. Res., Camb. (1988), **51**, *pp.* 103–109 *With 3 text-figures Printed in Great Britain*

The relationship between position and expression of genes on the kangaroo *X* chromosome suggests a tissue-specific spread of inactivation from a single control site

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(*Received 13 July 1987 and in revised form 24 August 1987*)

Summary

In marsupials, *X* chromosome inactivation is paternal and incomplete. The tissue-specific pattern of inactivation of *X*-linked loci (G6PD, PGK, GLA) has been attributed to a piecemeal inactivation of different regions of the *X*. We here propose an alternative hypothesis, in which inactivation of the marsupial *X* is a chromosome-wide event, but is differentially regulated in different tissues. This hypothesis was suggested by the relationship between the positions and activity of genes on the kangaroo paternal *X*. In the absence of an HPRT polymorphism, we have used somatic cell hybridization to assess the activity of the paternal HPRT allele in lymphocytes and fibroblasts. The absence of the paternal *X*, and of the paternal forms of G6PD or PGK, from 33 cell hybrids made by fusing HPRT-deficient rodent cells with lymphocytes or fibroblasts of heterozygous females, suggests that the HPRT gene on the paternal *X* is inactive in both tissues and therefore not selectable. Since HPRT is located medially on the *Xq* near GLA, which shares the same characteristics of activity, we suggest that the locus-specific and tissue-specific patterns of activity result from a differential spread of inactivation from a single control locus, located near HPRT and GLA, outwards in both directions to G6PD and PGK. The nucleolus organizer region on the short arm does not seem to be part of the inactivated unit.

1. Introduction

X chromosome inactivation in marsupials (mammalian infraclass Metatheria) is both qualitatively and quantitatively different from *X* chromosome inactivation in placental mammals (infraclass Eutheria) from which they diverged 130 million years ago (Air *et al.* 1971). Rather than random inactivation of one or other *X* in clones of somatic cells (Lyon, 1961), inactivation is observed only for alleles borne on the paternally derived *X* (reviewed by Cooper *et al.* 1975; Graves, 1983). Also, inactivation is incomplete; expression of alleles on the paternal *X* depends on the tissue and the locus under study (reviewed by VandeBerg *et al.* 1987). Paternal *X* chromosome inactivation is also observed in extra-embryonic tissues of rodents (Takagi, Wake & Sasaki, 1978); and inactivation may be incomplete in human chorion (Migeon *et al.* 1985), suggesting that the ancestral mechanism of mammalian dosage compensation may have been paternal and incomplete.

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Most of the information on marsupial *X* chromosome inactivation has been obtained from studies of isozyme expression in cells from heterozygous females, and is therefore limited to investigations of *X*-linked enzymes in species polymorphic for electrophoretically distinguishable forms, or in hybrids between interbreeding species or subspecies. Expression of glucose-6-phosphate dehydrogenase (G6PD), phosphoglycerate kinase A (PGK-A) and α-galactosidase (GLA) has been studied in several different kangaroo species (reviewed by VandeBerg *et al.* 1987). All three loci show paternal inactivation, but have different patterns of activity in different tissues. The paternal GLA locus seems not to be expressed in any tissue *in vivo*, nor in cultured fibroblasts (Cooper *et al.* 1983), paternal G6PD is expressed partially or fully in cultured fibroblasts, but not in any somatic tissue, and paternal PGK is expressed partly in cultured fibroblasts (Cooper *et al.* 1977) and in several somatic tissues, not including lymphocytes or erythrocytes (VandeBerg, Cooper & Sharman, 1977). The limited number of markers provide too few data to distinguish whether the pattern of inactivation reflects a patchwork of loci (or regions) under independent control, as has been

suggested (VandeBerg *et al.* 1987), or whether there is some kind of chromosome-wide control of inactivation in the marsupial *X* chromosome.

It is therefore important to study the activity of other *X*-linked loci, and to correlate the pattern of gene activity with the position of genes on the *X* chromosome. Using somatic cell genetic techniques, we have recently assigned the three loci discussed above (G6PD, PGK and GLA) as well as the hypoxanthine phosphoribosyltransferase (HPRT) locus, to *Xq* in *Macropus robustus*, and have provided a tentative gene order CEN–G6PD–HPRT–GLA–PGK (Dawson & Graves, 1986). Since no electrophoretic HPRT variant is known in marsupials, it has not been possible to study directly the activity of the HPRT allele on the paternal *X*. We report here studies which provide indirect evidence that the paternal HPRT allele is inactive in lymphocytes and fibroblasts, as well as cytological studies showing that the nucleolus organizer on both *X* chromosomes is active. We present a model for a chromosome-wide control of *X* chromosome activity in marsupials.

2. Materials and methods

(i) *Cell lines and cell fusion*

Marsupial cells (lymphocytes or fibroblasts cultured from earpunch or fascia) were derived from female animals (Table 1) having heteromorphic *X* chromosomes, and/or heterozygous for electrophoretically distinguishable forms of G6PD or PGK-A. Material was kindly supplied by Professor D. W. Cooper and Dr P. G. Johnston, Macquarie University. Rodent cells were the HPRT-deficient mouse lines PG19 and Na and the HPRT-deficient Chinese hamster line V79/129. These lines, as well as media and culture conditions, have been described previously (Graves *et al.* 1979; Dawson & Graves, 1986). Lymphocyte fusions were performed in suspension with inactivated Sendai virus, and fibroblasts were fused in a mixed monolayer with polyethylene glycol (PEG, 50% w/v, MW 1000, Ajax Australia). Lymphocyte hybrids were selected in HAT medium (Szybalski, Szybalska & Ragni, 1962), containing 10^{-4} M hypoxanthine (Merck, 4×10^{-7} M aminopterin (Sigma), and $1{\cdot}6 \times 10^{-5}$ M thymidine (Merck), and fibroblast hybrids in HAT medium containing 10^{-6} M ouabain (Sigma), to which marsupial cells are sensitive (Hope & Graves, 1978). Hybrids were propagated in HAT.

(ii) *Biochemical studies*

Isozymes of hypoxanthine phosphoribosyltransferase (HPRT, E.C. 2.4.2.8), glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49), phosphoglycerate kinase (PGK-A, E.C. 2.7.2.3) and α-galactosidase (GLA, E.C. 3.2.1.22) were separated as described previously (Graves *et al.* 1979; Dawson & Graves, 1984).

(iii) *Cytological procedures*

Cell hybrids to be examined for the presence of a euro, wallaroo, or red kangaroo *X* chromosome or *X* chromosome fragment were subjected to all or some of C-banding, G-banding, chromomycin A3 and methyl green staining, and Hoechst 33258 staining as described previously (Dawson & Graves, 1984). Nucleolar organizer regions were stained with the silver technique (Goodpasture & Bloom, 1975), which reveals NOR activity (Miller *et al.* 1976).

3. Results

(i) *Nucleolus organizer activity*

The nucleolus organizer region (NOR) is located on the short arm of the *X* in this and other kangaroo species. We have compared the activities of the NOR regions on the maternal and paternal *X* in fibroblasts from a 032 (wallaroo × euro) female with a heteromorphic sex pair. In all suitably stained cells from 032, we observed that the NOR regions of the maternal (wallaroo) and paternal *X* (euro) were equivalently

Table 1. *Source of marsupial parent cells*

Animal no.	Parents		Tissues studied	Chromosome markers	Gene markers
	Maternal	Paternal			
031	Euro	Wallaroo	Lymphocytes	X_e (X_w)	G6PD S (F)
032	Wallaroo	Euro	Lymphocytes, fibroblasts (skin)	X_w (X_e)	G6PD F (S)
0K1	Wallaroo	Red	Lymphocytes, fibroblasts (skin)	X_w (X_r)	G6PD F (S)
G9	Grey	Grey	Fibroblasts (skin)	—	PGK VE (N)
G60	Grey	Grey	Fibroblasts (fascia)	—	PGK N (VE)

Wallaroo is *Macropus robustus robustus*, euro is *M. r. erubescens*, red kangaroo is *M. rufus* and grey kangaroo *M. giganteus*.
Chromosome and gene markers are represented as maternal (paternal); X_e, X_w and X_r refer to the euro, wallaroo and red kangaroo *X* chromosomes.

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Fig. 1. Activity of nucleolar organizing regions of the maternal (X_e) and paternal (X_w) *X* chromosomes.

(Table 2), although the wallaroo F form is well separated from the mouse and the euro S form. Animal 032 was derived from the reciprocal cross; this animal and 0K1 (a wallaroo–red kangaroo hybrid) both possess a maternal wallaroo X, carrying the G6PD-F allele (Table 1); only this allele was ever expressed in rodent–0K1 cell hybrids (Table 2). The grey kangaroo G9 was heterozygous for the normal (N) and Eastern variant (VE) allele of PGK; the maternal VE allele was the only one ever detected in mouse-G9 cell hybrids (Table 2). Thus in each case the allele expressed was of maternal origin.

Ten hybrids (6 lymphocyte, 4 fibroblast), retained a

Table 2. *Hybridization of rodent and marsupial cells*

Cells hybridized				Characteristics of cell hybrids			
Marsupial				Numbers expressing marsupial isozymes			Numbers retaining identifiable marsupial *X*
Animal no.	Tissue	Rodent	Total no.	G6PD	PGK	GLA	
031	Lymphocytes	PG19	32	16[a]	19	.	3[a]
032	Lymphocytes	PG19	9	7[a]	7	.	2[a]
032	Fibroblasts	PG19	2	2[a]	2	.	2[a]
032	Lymphocytes	Na	10	0	5	.	0
0K1	Lymphocytes	PG19	10	3[a]	5	.	1[a]
0K1	Lymphocytes	Na	12	0	3	.	0
0K1	Fibroblasts	V79	4	2[a]	2	2	2[a]
G60	Fibroblasts	Na	5	0	0	.	0
G60	Fibroblasts	PG19	10	0	0	.	0
G9	Fibroblasts	Na	7	0	1[a]	.	0
G9	Fibroblasts	PG19	9	1	4[a]	.	1

. The mouse and kangaroo forms of GLA could not be reliably separated by electrophoresis.
[a] In every case where the parental source of the isozyme or *X* chromosome could be distinguished, it was the maternal isozyme expressed and the maternal *X* retained.

stained (Fig. 1), demonstrating that they are both active to approximately the same extent.

(ii) *HPRT activity*

Table 2 lists the total of 110 hybrids isolated from fusions of HPRT-deficient rodent cells with cells from hybrid or heterozygous female kangaroos. All expressed the marsupial form of the selected marker HPRT. Hybrids were also screened for expression of the other sex-linked marsupial markers G6PD and PGK; in addition, hamster–kangaroo hybrids were typed for GLA. Those positive for HPRT and at least one other marker were karyotyped. As previously reported (Graves *et al.* 1979; Dawson & Graves, 1984), most hybrids expressed only the selected marker and retained no intact marsupial chromosome.

Thirty-five hybrids (26 lymphocyte, 9 fibroblast) expressed a marsupial form of the enzyme for which the parent was heterozygous. Animal 031 is heterozygous for the euro (slow, S) and the wallaroo (fast, F) forms of G6PD (Table 1); only the maternal S (euro) form was ever detected in rodent–031 cell hybrids

kangaroo *X* chromosome, or a derivative of it whose parental origin could be determined cytologically. The euro and wallaroo *X* chromosomes are differentiated by the size of the satellited short-arm; the euro *X* contains a heterochromatic (C-banding, G-band negative, Hoechst-dull, chromomycin-bright) region proximal to the nucleolus organizer, which is considerably larger than that of the wallaroo *X*. The red kangaroo *X* has a very large heterochromatic (chromomycin-bright) region on the satellited arm (Fig. 2*a*), which is particularly obvious in the rodent–red kangaroo cell hybrids described by Donald & Hope (1981). In all hybrids, the retained *X* was of maternal origin (Fig. 2*b*, *c*; Table 2).

4. Discussion

In order to determine whether the activity of genes is related to their position on the paternal marsupial *X*, we examined the activity of the HPRT locus on the long arm of the *M. robustus* X and the nucleolus organizer region (NOR) on the short arm.

The positions of four genes G6PD, HPRT, GLA

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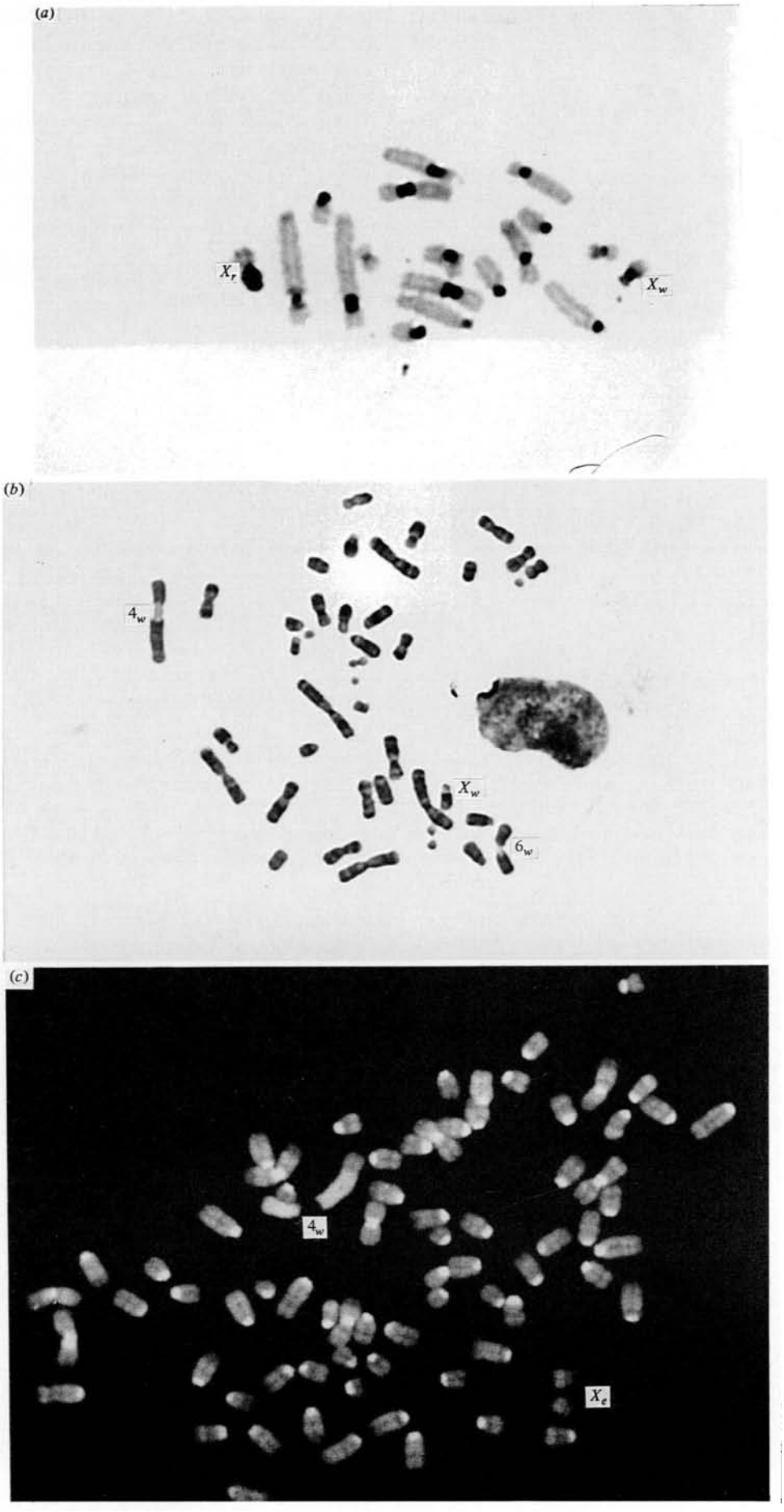

Fig. 2. For legend see opposite.

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and PGK-A on the long arm of the *M. robustus X* have already been determined, and the activity of three of these loci on the paternal *X* in this or related species has been reported for several tissues. We have used indirect means to assess the activity of a fourth locus, HPRT, by determining whether the paternal HPRT locus is selectable in cell hybrids made with HPRT-deficient rodent cells. For these experiments we used fibroblasts or lymphocytes from animals with cytologically or genetically distinguishable maternal and paternal *X* chromosomes, and screened them for the retention of maternal and paternal alleles or *X* chromosomes.

The 35 hybrids which expressed the marsupial marker for which the female donor was heterozygous (G6PD or PGK), and the 10 which retained an identifiable marsupial *X*, all showed evidence of retention of material only from the maternal *X*. The presence of the maternal *X* and the absence of the paternal *X* in the hybrids which retained marsupial chromosomes provides unequivocal evidence for selection of only the maternal HPRT allele. However, since intact marsupial chromosomes were retained by only 10 hybrids, the conclusion that only the maternal HPRT allele is selectable depends also on the expression of only maternal enzyme markers. Since the paternal G6PD and PGK alleles are both expressed in fibroblasts, they would be expected to be expressed also in cell hybrids, and their presence in a hybrid should therefore have been detected; thus the absence of paternal G6PD or PGK from fibroblast hybrids demonstrates unequivocally that the paternal HPRT was never selected in fibroblast hybrids. In lymphocytes, however, paternal G6PD and PGK are not expressed; thus the absence of these markers in lymphocyte hybrids does not eliminate the possibility that silent paternal G6PD or PGK loci are present in at least some of the hybrids which have retained a portion of the paternal *X* bearing an active HPRT allele. Since segregation of marsupial chromosomes from hybrids is rapid and extreme, it seems most unlikely that hybrids would have retained independently an HPRT-bearing fragment from the paternal *X*, as well as a G6PD-bearing fragment of the maternal *X*; thus hybrids which do express maternal G6PD or PGK have probably retained a single fragment derived solely from the maternal *X*. We cannot exclude the possibility that among the 59 hybrids which expressed marsupial HPRT but neither PGK nor G6PD, some harboured inactive paternal PGK and/or G6PD alleles. However, none of these hybrids examined retained identifiable marsupial chromosomes, consistent with their retention of only a small fragment of the maternal *X*.

Thus the best interpretation of our results is that the selected HPRT locus in all these hybrids derives from the maternal *X*. This suggests that the HPRT allele on the paternal *X* is silent in kangaroo fibroblasts and lymphocytes. Because of the low numbers of informative hybrids, and their derivation from kangaroos of different species, it is always possible that the paternal HPRT allele is active in a species or a tissue which is poorly represented among these hybrids. In particular, we obtained no hybrid from lymphocytes of heterozygous grey kangaroos; however, since no allele borne on the paternal *X* has been found to be active in this tissue in any species, we can probably assume that the paternal HPRT, too, is not expressed in grey kangaroo lymphocytes. Obviously it would be an advantage to study more hybrids that retained marsupial chromosomes. However, the hybrids described in this report are the products of more than ten years' work; the poor recovery of rodent–marsupial hybrids and their extreme segregation of marsupial chromosomes (Graves *et al.* 1979; Dawson & Graves, 1984) discourages extension of these studies.

The NOR region is located on the heterochromatic short arm of the *X* in this and other kangaroo species (Hayman & Rofe, 1977). This arm replicates late in the S phase, but does not appear to be asynchronous either in lymphocytes or fibroblasts (Cooper *et al.* 1977). It has been supposed, therefore, that the NOR-bearing arm of the *X* does not participate in inactivation in either tissue. This hypothesis received direct support from observations that both *X* chromosomes in lymphocytes of female *Macropus eugenii* are silver-stained and that interphase nuclei may have two nucleoli (Hayman & Rofe, 1977; Robins, Hayman & Wells, 1984). We have confirmed that the NOR located on the paternal *X* (which can be distinguished by morphology in the hybrid females we investigated) is silver-stained to the same extent as the NOR on the maternal *X* in fibroblasts. We conclude, therefore, that at least the NOR region, and probably the entire heterochromatic short arm, is not inactivated in any tissue. Since the *X* chromosome of most other groups of marsupials does not include the NOR region, it seems likely that the heterochromatic, NOR-bearing arm is not a part of the region of the marsupial *X* whose gene content is conserved (Dawson & Graves, 1986; Dobrovic & Graves, 1986), but has been rather recently translocated from an autosome to the *X* in an ancestral phalangerid, as suggested by Hayman & Rofe (1977), and has not been included in the inactivated region.

It now becomes possible to consider the relationship between the positions and the expression of genes on the kangaroo *X*, in order to distinguish the alternative

Fig. 2. Chromosomes of marsupial parent cells and hybrids. (*a*) c-Banded metaphase of OK1 fibroblast, showing the heteromorphic maternal *X* (X_w) and paternal *X* (X_r). (*b*) g-Banded metaphase of a V79 × OK1 cell hybrid, showing the marsupial chromosomes 4_w, 6_w and the maternal X_w. (*c*) Hoechst-stained chromosomes of a PG19 × O31 cell hybrid showing two copies of the maternal X_e, and a derivative of the wallaroo 4_w.

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Table 3. *Position and activity of the loci on the kangaroo paternal* X

	Activity of paternal allele in		
	Lymphocytes	Various somatic tissues	Cultured fibroblasts
NOR	+	+	+
G6PD	−	−	+
HPRT	−		−
GLA	−	−	−
PGK-A	−	−,+	+

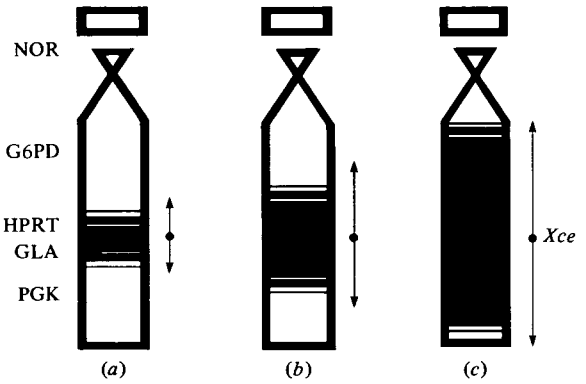

Fig. 3. Model for the tissue-specific spreading of inactivation from a controlling element (*Xce*) on the long arm of the paternal *X*; the position of *Xce* is uncertain, but must be located in or near the region containing HPRT and GLA.

hypotheses that *X* chromosome inactivation in kangaroos is a piecemeal or a chromosome-wide event. We have recently mapped HPRT, G6PD, PGK and GLA to the *Xq* of *M. robustus* and tentatively determined the order CEN–G6PD–HPRT–GLA–PGK (Dawson & Graves, 1986). Our conclusion that the paternal HPRT allele is inactive in fibroblasts as well as lymphocytes is of particular interest, because HPRT is located near the GLA locus, which is also paternally inactive in both tissues (Fig. 3). This suggests that the inactivation pattern shared by these loci is a property of the region of the paternal *X* in which both genes are located. The G6PD and PGK loci, which escape inactivation in fibroblasts, are located on either side of this region. The positions and activity of the four loci are summarized in Table 3. The gene order G6PD–HPRT–PGK on the *M. giganteus X* (Dawson & Graves, 1984) and the pattern of inactivation are consistent with the observations for *M. robustus*.

We suggest that these tissue-specific and locus-specific patterns of activity may result from a chromosome-wide control mechanism (shown in Fig. 3), in which inactivity spreads in both directions from a control centre in the medial segment of *Xq* (containing HPRT and GLA) to the proximal region (containing G6PD) and the distal region (containing PGK). We suggest that the extent of spread is tissue-specific, being least in cultured fibroblasts (in which the two loci farthest removed from the putative centre escape inactivation), and greatest in lymphocytes (in which inactivation is complete). Spreading to an intermediate degree could account for the patterns of inactivation in many body tissues, in which the expression of the paternal PGK, but not G6PD, allele is detected. Variable spreading may also account for the partial expression of paternal PGK observed in fibroblasts (Cooper *et al.* 1977).

Our model could be tested by examining the relationship between gene position and expression in other marsupial groups in which gene order is different (e.g. two dasyurid species have G6PD–HPRT–PGK–GLA; Dobrovic & Graves, 1986), or the tissue-specific pattern of expression is different (e.g. paternal G6PD but not PGK is expressed in some tissues of the American opossum, Samollow, Ford & VandeBerg, 1987).

Our suggestion of a spreading of inactivation from a single controlling element on the kangaroo *X* has features in common with *X* chromosome inactivation in eutherian mammals. There is genetic and cytological evidence for a single controlling element (*Xce*) in the mouse and the human *X* chromosome (Cattanach, 1975; Therman *et al.* 1979), from which inactivation spreads into autosomal material translocated to the *X* (Russell, 1963). A similar effect may account for the unequal expression of the STS alleles on the active and inactive human *X* (Migeon, 1985). We suggest that the mammalian *X* chromosome may have contained such a controlling element before the divergence of marsupials and eutherian mammals more than 130 million years ago, and that spreading inactivation may be a property of a mechanism of inactivation which has been conserved over this period. Spreading may even have been involved in a progressive recruitment of newly unpaired loci as the *Y* was gradually reduced during mammalian evolution, as we have suggested elsewhere (Wrigley & Graves, 1988).

We thank Mrs Iole Barbieri for expert technical assistance, and Dr P. G. Johnston for confirming many of our allozyme typings. This study received support from the Australian Research Grants Scheme.

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