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Heterochromatin as a factor affecting X-inactivation in interspecific female vole hybrids (*Microtidae, Rodentia*)

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Summary

Female interspecific vole hybrids were examined for the expression of the G6PD and GALA genes on the X chromosomes. When one of the parents was a species with a heterochromatin block on the X, and the other parent was *M. arvalis*, without a heterochromatin block on the X, preferential expression of the genes of the *M. arvalis* X was consistently observed. When both parental species had heterochromatin on the X, the parental forms of G6PD and GALA were in about equal proportions in the hybrid females. The results of the cytological identification of the active and inactive X on the metaphase spreads in the hybrid females are in agreement with the biochemical results. It is suggested that the observed phenomenon may be due to a nonrandom inactivation of the X chromosome containing a heterochromatin block in crosses involving *M. arvalis* and by a random inactivation in those with both parents having heterochromatin blocks on the X chromosomes. These results support our previous suggestion that heterochromatin has an effect on X inactivation in female interspecific vole hybrids.

1. Introduction

X-chromosome inactivation in female mammals is a unique phenomenon of the regulation of the activity of the genetic apparatus (Lyon, 1961, 1974). There are many models offering to explain the mechanisms of the X inactivation (Gartler & Riggs, 1983; Grant & Chapman, 1988). While not fully accounting for the phenomenon, the models do not appear to be mutually exclusive. Facts relevant to the X inactivation derived from traditional and, more importantly, new experimental models, would improve our understanding of the phenomenon and help us to gain insight into the mechanisms regulating the expression of the X chromosome genes in mammals during development.

We have previously suggested a connection between the presence of large heterochromatin regions in the X chromosome and preferential inactivation of these chromosomes in female hybrids from certain *Microtus* species crosses (Zakian *et al.* 1987). This new paper presents further evidence on this problem, based on additional crosses and biochemical and cytological tests which enable us to distinguish between active and inactive X chromosomes and to elucidate the effect of heterochromatin on the process of X inactivation in hybrid voles.

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2. Material and methods

The four vole (*Microtus*) species used in the experiments were *M. arvalis*, *M. subarvalis*, *M. kirgisorum* and *M. transcaspicus*. The wild-caught voles were maintained and bred in the vivarium of this Institute. Hybrids between the four species were produced. The number of females from each cross and the direction of the crosses are given in Table 1.

Hybrids derived from matings of *M. subarvalis* with the other vole species were analysed electrophoretically for the expression patterns of glucose-6-phosphate dehydrogenase (G6PD) in the erythrocytes, lung, liver, heart, kidney, brain, spleen and muscle; those derived from mating of *M. arvalis* with the other species, as well as the *M. transcaspicus* × *M. kirgisorum* interspecific hybrids, were examined for the electrophoretic expression of α-galactosidase (GALA) in the brain. Electrophoresis was carried out in 13% starch gels. Electrophoretic conditions and preparation of the samples for assaying G6PD and GALA, as well as the staining techniques for their visualization, were all as described elsewhere (Zakijan *et al.* 1984). Determination of the specific activity of G6PD in the erythrocytes of all the studied vole species was based on a previously described method (Serov & Zakijan, 1977). Extraembryonic tissues and embryonic organs

Table 1. *Number of hybrid vole females obtained in different crosses*

♂♂	♀♀ *M. arvalis*	*M. subarvalis*	*M. kirgisorum*	*M. transcaspicus*
M. arvalis		19 (8)*	13 (4)	5 (4)
M. subarvalis	10 (1)		5	20 (9)
M. kirgisorum	—	—		4
M. transcaspicus	—	7 (5)	—	

* Figures in parentheses are the number of voles examined cytogenetically

were taken from hybrids on the 14 days after fertilization. Cytogenetic identification of the inactive X was performed according to Kanda (1973).

3. Results and discussion

Voles as experimental material suffer from the lack of laboratory strains, paucity of vole genetics, difficulties in obtaining hybrid offspring and sterility of these hybrids; but they have the advantage that the sex chromosomes are easily identifiable in all four species, because of differences in the amount and location of heterochromatin in their X chromosomes (see Figs. 1, 2). These differences are equally visible in hybrid females.

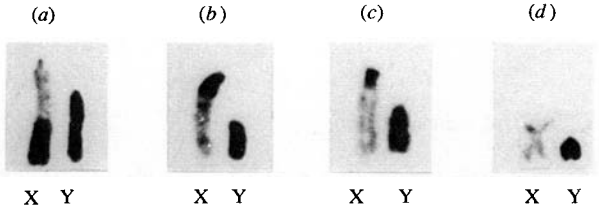

Fig. 1. C-banding of the sex-chromosomes in the four vole species: (*a*) *M. subarvalis*; (*b*) *M. transcaspicus*; (*c*) *M. kirgisorum*; (*d*) *M. arvalis*. Bar = 10 μm.

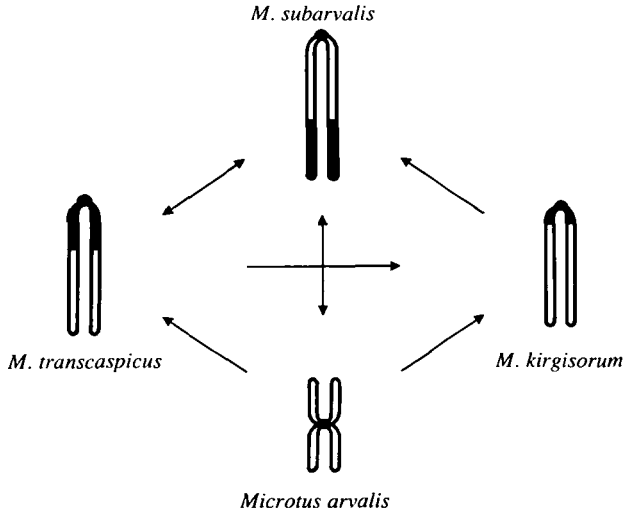

Fig. 2. Cross combinations of voles having different heterochromatin blocks on the X chromosomes. Arrows indicate direction of cross combination.

Another merit of this experimental material is the differences in electrophoretic mobility of at least one of the enzymes, G6PD or GALA, whose genes are on the X. It was expected that these differences would allow us to observe the expression of the genes in question in the hybrid females. We could thus examine the inactivation of the X chromosome in female vole hybrids by means of both cytological and biochemical markers.

Based on a survey of G6PD and GALA electrophoretic patterns obtained from reciprocal *M. arvalis* × *M. subarvalis* crosses and also those between ♀ *M. kirgisorum* × ♂ *M. arvalis*, we have previously suggested that the preferential inactivation of the X in these hybrids is due to the presence of heterochromatin blocks on the X in *M. subarvalis* and *M. kirgisorum*, which is large in the former (Zakian *et al.* 1987). To verify this assumption, another vole species, *M. transcaspicus*, was involved in the crosses. To reiterate, *M. transcaspicus* has a large heterochromatin block on the X comparable in size with that of *M. subarvalis* which is pericentromeric, however. The hybrids from *M. transcaspicus* × *M. arvalis* crosses, we supposed, would have a preferential inactivated X-bearing heterochromatin like those from *M. arvalis* × *M. subarvalis* and *M. arvalis* × *M. kirgisorum* crosses, i.e. an X derived from *M. transcaspicus*. Indeed, in analysis of G6PD and/or GALA patterns, when one partner was a species having a heterochromatin block on the X and the other was *M. arvalis*, we consistently observed predominance of the expression of the *M. arvalis* derived X (Figs. 3, 4).

These hybrids were analysed cytogenetically by Kanda's technique (Kanda, 1973) to differentiate the active from the inactive X. The inactive X stains dark in contrast to the rest of the chromosomes on the metaphase spreads (Fig. 5). Figure 5(*a–f*) presents fragments of the metaphase spreads from these hybrids. The inactive X of *M. arvalis* derivation occurs in a small percentage of the cells (Table 2). In the majority of the cells (79–88 %), the *M. arvalis* X is active; the inactive X is the chromosome containing a heterochromatin block. The ratio of the active to the inactive X chromosomes of the parental species supports our biochemical data.

It is known that the paternally derived X in some

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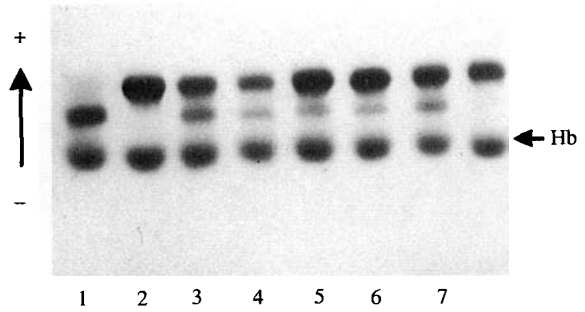

Fig. 3. Electrophoretic patterns of G6PD in the erythrocytes of *M. subarvalis* (1), *M. arvalis* (2, 8) and in the F_1 hybrids from *M. subarvalis* × *M. arvalis* (3–7). Hb, haemoglobin.

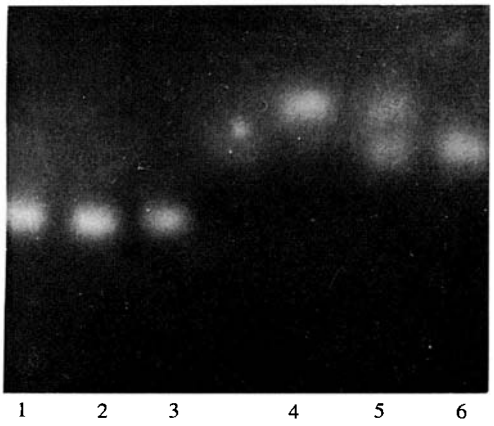

Fig. 4. Electrophoretic GALA patterns from brain tissue of *M. arvalis* (1), *M. kirgisorum* (4), *M. transcaspicus* (*M. subarvalis* has the same mobility) (6), and their F_1 female hybrids from the crosses: ♀ *M. transcaspicus* × ♂ *M. arvalis* (2), ♀ *M. kirgisorum* × ♂ *M. arvalis* (3), ♀ *M. transcaspicus* × ♂ *M. kirgisorum* (5).

extraembryonic membranes of the mouse is preferentially inactivated (Takagi & Sasaki, 1975). From this point of view it appeared of interest to observe how the maternally derived X containing a heterochromatin block would behave in the yolk sac of vole hybrids. Which mechanism would be given preference: nonrandom inactivation of the paternally derived X or preferential inactivation due to the presence of the heterochromatin block? We studied the electrophoretical patterns of G6PD in the yolk sacs of 14-day-old embryos in females obtained from crosses between ♀ *M. subarvalis* and ♂ *M. arvalis*. Preferential expression of the *M. subarvalis* (maternal) form of G6PD was found (Fig. 6). In contrast, in analysis of the electrophoretic G6PD patterns in the amnion, liver, lung, heart, brain and muscle of these embryos a preferential expression of *M. arvalis* chromosome was observed just like in the organs and tissues of adults. We believe that these observations are consistent with the hypothesis of nonrandom inactivation of the paternally derived X in some extraembryonic tissues of mammalian cells (Gartler & Riggs, 1983). Within the framework of this hypothesis the mechanism of nonrandom inactivation of the paternal X in some extraembryonic tissues of rodents is presumably different at the molecular level from the random inactivation characteristically operating in the somatic cells of mammals. This is achieved by imprinting of the parental chromosomes during gametogenesis. Perhaps as a consequence of different methylation, the paternal and maternal Xs become nonidentical by the time of fertilization and the paternal X is preferentially inactivated in those tissues that differentiate early. A *de novo* methylation occurs in the embryo, and, by the time when X inactivation starts in the primitive ectoderm, imprinting by hypomethylation is effaced.

Table 2. *Proportion of cells from bone marrow with active and inactive X chromosomes of parental derivation in interspecific female vole hybrids*

Cross (female × male)	No. of hybrids	Number of metaphase spreads (% ± S.E.M.)				
			Dark chromosomes			
		Total	Xa	Xs	Xt	Xk
M. subarvalis × *M. arvalis*	8	2843	447 (14·8 ± 2·45)	2396 (85·2 ± 2·45)	—	—
M. arvalis × *M. subarvalis*	1	342	80 (23·4)	262 (76·6)	—	—
M. transcaspicus × *M. arvalis*	4	1535	315 (20·9 ± 2·51)	—	1220 (79·0 ± 2·51)	—
M. kirgisorum × *M. arvalis*	4	1140	126 (11·1 ± 0·77)	—	—	1014 (88·9 ± 0·77)
M. transcaspicus × *M. subarvalis*	9	3624	—	1914 (52·9 ± 0·85)	1710 (47·14 ± 0·85)	—
M. subarvalis × *M. transcaspicus*	5	1815	— —	978 (53·7 ± 0·96)	837 (46·3 ± 0·96)	—

Derivation of the X chromosome: Xa, *M. arvalis*; Xs, *M. subarvalis*; Xt, *M. transcaspicus*; Xk, *M. kirgisorum*

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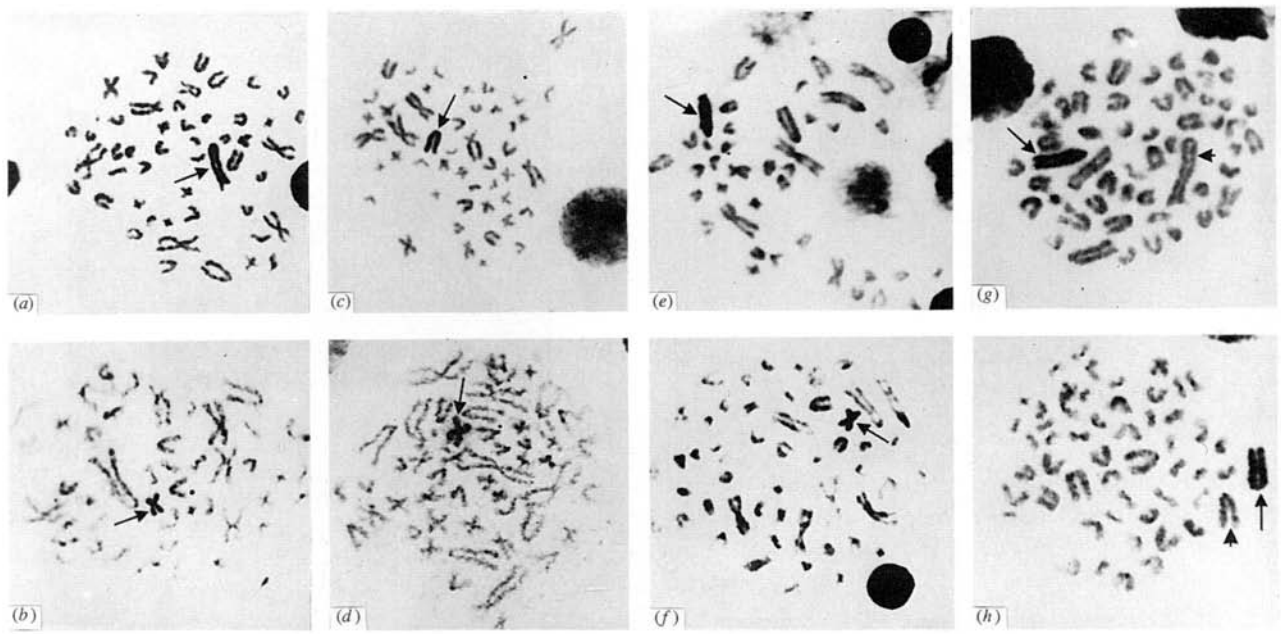

Fig. 5. Metaphase chromosomes from the bone marrow cells of hybrid female voles: *M. arvalis* × *M. subarvalis* (*a*, *b*), *M. kirgisorum* × *M. arvalis* (*c*, *d*), *M. transcaspicus* × *M. arvalis* (*e*, *f*), *M. transcaspicus* × *M. subarvalis* (*g*, *h*). Note the dark staining inactive X (long arrow): *M. arvalis* (*b*, *d*, *f*), *M. subarvalis* (*a*, *h*), *M. kirgisorum* (*c*), *M. transcaspicus* (*e*, *g*), and the light staining active X (short arrow): *M. subarvalis* (*g*), *M. transcaspicus* (*h*).

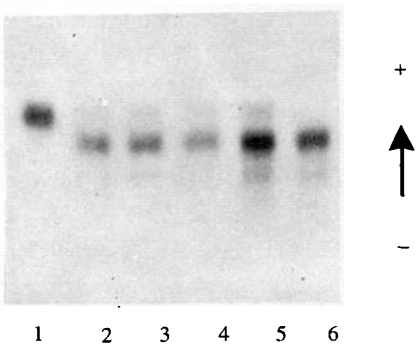

Fig. 6. Electrophoretic patterns of G6PD in the erythrocytes of *M. arvalis* (1), *M. subarvalis* (6) and in the yolk sacs of hybrid females from *M. subarvalis* × *M. arvalis* crosses (2–5).

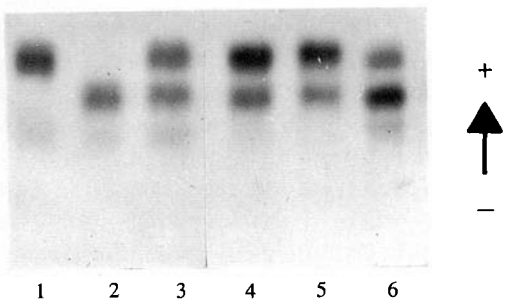

Fig. 7. Electrophoretic patterns of G6PD in the erythrocytes of *M. transcaspicus* (1), *M. subarvalis* (2), in *M. transcaspicus* × *M. subarvalis* hybrids in heart (3), in brain (4), in kidney (5) and in *M. kirgisorum* × *M. subarvalis* hybrids in lung (6).

The salient finding of the present experiments is that heterochromatin has no effect on X inactivation in the extraembryonic tissues of hybrid voles. This is in contrast to what we have observed for their somatic tissues in this and previous experiments (Zakian *et al.* 1987).

The involvement of *M. transcaspicus* in the crosses allowed us to examine the X-inactivation process in a situation where both parental species have heterochromatin blocks on the X. To test our expectation of random inactivation of either parental X chromosome, the following crosses were performed: *M. subarvalis* × *M. transcaspicus*, ♀ *M. transcaspicus* × ♂ *M. kirgisorum*, and ♀ *M. kirgisorum* × ♂ *M. subarvalis*. In analysis of the electrophoretic patterns of G6PD and GALA in the hybrids from these crosses, we found no significant preference for the activity of one of the parental enzymes of the kind we observed for crosses with *M. arvalis* (Figs. 4, 7). The proportion of the G6PD parental forms was almost 1:1 in the hybrids from the *M. transcaspicus* × *M. subarvalis* cross. The same was observed for the GALA parental forms in the brain of hybrids from the ♀ *M. transcaspicus* × ♂ *M. kirgisorum* cross. In ♀ *M. kirgisorum* × ♂ *M. subarvalis* hybrids, there was a marked shift towards predominance of the parental G6PD transmitted from *M. subarvalis*.

The specific activity of G6PD in the erythrocytes was measured in the parental species. They showed significant deviations in *M. kirgisorum* compared with the other species. Thus, G6PD specific activity was almost equal in *M. arvalis*, *M. subarvalis* and *M. transcaspicus* (175, 171 and 193 mU/mg of protein,

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Table 3. *Summary of relative expression of maternally- and paternally-derived X chromosomes (X^m and X^p)*

Cross (female × male)	Marker	Inactive X chromosome: Adult tissue	Fetus	Yolk sac
arvalis × *subarvalis**	Kanda	$X^p > X^m$ (77% X^p)		
	G6PD	$X^p > X^m$	$X^p > X^m$	
	GALA	$X^p > X^m$	$X^p > X^m$	
*subarvalis** × *arvalis*	Kanda	$X^m > X^p$ (85% X^m)		
	G6PD	$X^m > X^p$	$X^m > X^p$ $X^p \geqslant X^m$	
	GALA	$X^m > X^p$	$X^m > X^p$	
*subarvalis** × *transcaspicus**	Kanda	$X^m = X^p$ (54% X^m)		
	G6PD	$X^m = X^p$		
*transcaspicus** × *subarvalis**	Kanda	$X^m = X^p$ (47% X^m)		
	G6PD	$X^m = X^p$		
*kirgisorum** × *arvalis*	Kanda	$X^m > X^p$ (89% X^m)		
	GALA	$X^m > X^p$		
*kirgisorum** × *subarvalis**	G6PD	$X^m \geqslant X^p$		
*transcaspicus** × *arvalis*	Kanda	$X^m > X^p$ (79% X^m)		
	GALA	$X^m > X^p$		
*transcaspicus** × *kirgisorum**	GALA	$X^m = X^p$		

*X chromosome has blocks of constitutive heterochromatin
Data from Table 2, Figs. 3, 4, 6, 7, text, and from Zakian *et al.* 1987

respectively) but was about 1·5-fold lower (103 mU/mg of protein) in *M. kirgisorum*. Consequently, the observed unequal expression of the parental forms of G6PD in *M. kirgisorum* × *M. subarvalis* appears to be due to differences in the specific activity of the enzyme in their parental species rather than to preferential X inactivation in one of the parents.

The results of cytological investigation of the active and inactive Xs in the metaphase spreads of hybrid females from the *M. subarvalis* × *M. transcaspicus* cross are given in Table 2. The inactive X of *M. transcaspicus* and *M. subarvalis* occurs in almost equal proportions. Here again, there is a good agreement between the results of cytological and biochemical analyses. All results obtained in this and the previous paper (Zakian *et al.* 1987) are summarized in Table 3.

We attempted to explain the present results in terms of the various hypothetical mechanisms of X inactivation proposed in the literature. The results seem to be best accounted for by the replication-expression model of gene regulation advanced by Grant & Chapman (1988). According to this model the genes replicating early in the S phase have more chance to express themselves. This model does not consider, however, the key event of inactivation – initiation – making replication asynchronous and producing a general repression of all the genes of the X chromosome. We would rather suggest that the homologous chromosomes (or genes) presumably replicate asynchronously to a degree that is sufficient to retain the earlier replicating X active, and the other (or others) X(s) inactive. It is known that heterochromatin replicates late in the S phase. For this reason, the lagging replication of an X chromosome carrying heterochromatin may possibly be a factor provoking X inactivation. If both Xs carry heterochromatin blocks, we should expect inactivation to become a matter of chance.

When seeking to explain our results we are at the crossroads of two different problems, X inactivation and heterochromatin contribution to the process. Each problem deserves consideration in its own realm. We would rather describe only the phenomenon we observed. Our following reports will deal with the putative mechanisms of the effect of heterochromatin on X inactivation in female vole hybrids.

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