

Controlling elements in the mouse IV. Evidence of non-random X-inactivation

BY P. G. JOHNSTON AND B. M. CATTANACH*

*School of Biological Sciences, Macquarie University, North Ryde,
New South Wales 2113, Australia
MRC Radiobiology Unit, Harwell, Didcot, Oxon OX11 ORD, U.K.*

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SUMMARY

The non-random X chromosome expression that has been observed with coat markers in female mice heterozygous for the *Xce* alleles, *Xce^a* and *Xce^b*, has now been investigated with the electrophoretic enzyme marker, *Pgk-1*. Because the *Xce* status of the *Pgk-1^a* marked chromosome was not known, PGK expression was assessed in *Pgk-1^a/Pgk-1^b* heterozygotes which carried either *Xce^a* or *Xce^b* on their *Pgk-1^b* chromosome. The PGK-1A allozyme was found to predominate in both genotypes but when *Xce^b* was present on the *Pgk-1^b* chromosome the expression of the two allozymes was less unequal. This effect was seen in both liver and kidney of adults and to at least the same degree in embryos aged 13.5 and 7.5 days. The results have been interpreted to mean that the non-random X expression derives from a primary non-randomness of the X inactivation process and that a new and more extreme *Xce* allele, designated *Xce^c*, was present on the *Pgk-1^a*-marked X chromosome.

1. INTRODUCTION

In previous studies (Cattanach & Isaacson, 1965, 1967; Cattanach, Perez & Pollard, 1970; Cattanach, Pollard & Perez, 1969; Cattanach & Williams, 1972) we have shown that the X chromosome of the mouse carries a locus which causes non-random X-chromosome activity (Lyon, 1961) in the somatic cells of the female. Two alleles of this X chromosome controlling element (*Xce*) locus have been distinguished, *Xce^a* and *Xce^b* (Cattanach, 1972), and the effect is seen in *Xce^a/Xce^b* heterozygotes when marker genes carried on the *Xce^b* chromosome tend to predominate (Cattanach, in preparation). Up to now it has not been possible to determine how the non-randomness is brought about. It could result from a differential cell selection operating upon the two cell populations generated by the X-inactivation process. Ample precedence for this is provided by the non-random X expression and/or late DNA replication of the X observed in the female mule (Gianelli & Hamerton, 1971; Hamerton *et al.* 1971; Hook & Brustman, 1971; Rattazzi & Cohen, 1972), in heterozygotes for certain X chromosome aberrations both in man and the mouse (Cattanach, 1975; Gartler & Andina, 1976), and in the erythrocyte populations of women heterozygous for HGPRT deficiency (Nyhan

* Reprint requests to Dr B. M. Cattanach.

et al. 1970). Alternatively, the predominance of one cell population over the other could derive from a primary non-randomness in the *X*-inactivation process itself, such as seen in the preferential inactivation of the paternal *X* in certain extra-embryonic membranes associated with the female embryo (Takagi & Sasaki, 1975; Wake, Takagi & Sasaki, 1976; West *et al.* 1977). The available evidence tends to support the latter interpretation but definite proof is still needed. Thus (1), studies upon a number of *X* chromosomes of different origins which show an *Xce*-type effect (Cattanach & Williams, 1972) all appear to implicate a single, responsible locus, i.e. *Xce*, not a range of different loci. More than one locus would have suggested the presence of diverse mutations having deleterious effects at the cell level when in an active *X*. (2) More significantly, the melanocyte variegation patterns with which the *Xce* locus was first distinguished (Cattanach & Isaacson 1965, 1967) are known to be established by the 12th–13th day of embryonic development (Mayer, 1973; Rawles, 1947). This specifies that the non-randomness is established very early (Cattanach, 1975), but does not rule out the possibility of cell selection operating over the first few cell generations after *X*-inactivation takes place (at ~ 5–6.5 days; Gardner & Lyon, 1971; Gardner, 1977; Monk & Harper, 1979; Rastan *et al.* 1980). Such evidence of early non-random *X* chromosome expression is also provided by the work of Drews *et al.* (1974) with the *X*-linked gene, *testicular feminization (Tfm)*. (3) Studies upon the coat mosaicism in the adult have not shown the departure from a binomial distribution of the two cell types which would be expected with random *X*-inactivation followed by cell selection (Cattanach & Williams, 1972).

In this communication we report on *Xce* effects observed in adult females and in embryos of different ages with the use of the *X*-linked enzyme marker, *phosphoglycerate kinase (Pgk-1)*. The findings support our previous conclusion that the *Xce* effect derives from a primary non-random *X*-inactivation. In the course of this study evidence of a new, more extreme *Xce* allele was obtained.

2. MATERIALS AND METHODS

Breeding stock and outline of experiments

The variant *Pgk-1^a* allele originated from feral *M. m. musculus* captured in Denmark (Neilson & Chapman, 1977) and when kindly made available to us by Dr J. D. West, Department of Zoology, Oxford University, had been introduced through 7 backcross generations on to a C3H/HeHa genetic background. At Harwell the variant was established in the homozygous condition (PGK stock) from a single male after crossing to C3H/HeH × 101/H F₁ hybrid (3H1) mice to improve reproductive performance. The *Pgk-1^b* allele derived from the C3H/HeH, 101/H and JU/FaCt inbred strains. Both the C3H and 101 inbred strains and, hence, the 3H1 hybrid carry the *Xce^a* allele at their *Xce* locus whereas the JU strain carries the *Xce^b* allele (Cattanach, in preparation; Cattanach & Williams, 1972). At the start of the experiment the *Xce* status of the PGK stock was not known but the results to be presented indicate that a new allele, designated *Xce^c*, is carried on the *Pgk-1^a*-marked *X* chromosome.

PGK expression was investigated in *Pgk-1^a/Pgk-1^b* heterozygotes carrying different *Xce* alleles on their *Pgk-1^b*-marked chromosomes. These mice were derived from reciprocal crosses between the PGK stock and the appropriate inbred strain or 3H1 hybrid. Artificial mixtures of tissue from *Pgk-1^a* and *Pgk-1^b* males were also studied to compare the activities of the enzymes of the two alleles.

When it became evident from the results that the X chromosome derived from the feral mice carried a new allele at the *Xce* locus, a test with the coat structural marker, *tabby* (*Ta*), was initiated to confirm that the pertinent *Pgk-1* results did indeed reflect the behaviour of the whole X chromosome, not just that of the *Pgk-1* locus itself. The test comprised a comparison of the *Ta*/+ progeny of *Pgk-1^a/Y* and *Pgk-1^b/Y* males mated to tester *Ta*/+ females. These males were derived from a single *Pgk-1^a/Pgk-1^b* female of PGK × 3H1 origin crossed with a *Pgk-1^b* (3H1) male. Genetic background influences upon *Ta*/+ expression were therefore controlled as far as practicable. The X chromosome of the *Pgk-1^b* males derived from the C3H strain and therefore carried the *Xce^a* allele at the *Xce* locus; that of the *Pgk-1^a* males derived from the PGK strain and therefore carried the new *Xce* allele (*Xce^c*). The *Xce* status of the *Ta*-marked chromosome was not known, but from a study of the history of the stock it is probable that only *Xce^a* was present.

As in previous experiments (Cattanach, Pollard & Perez, 1969; Cattanach & Williams, 1972) the influence of the tested X chromosomes upon the *Ta*/+ phenotype was assessed by scoring the secondary vibrissa number, which is reduced from the invariant number of 19 in non-*Ta* animals to an average of about 14–17 in the *Ta* heterozygote. The secondary vibrissa number of the *Ta*/Y sibs was also scored to check for genetic background differences between the two crosses which might influence the *Ta*/+ scores. The vibrissa number of *Ta* hemizygotes normally averages about 6–7.

Dissection of embryos and sample preparations

Embryos were dissected from the uterus on the 8th day (7.5-day embryos) and 14th day (13.5-day embryos) after mating and were placed in phosphate buffered saline (PBS). The 7.5-day egg cylinder is divided by the amnion into embryonic and extra-embryonic regions (Gardner & Papaioannou, 1975). These regions were separated with fine needles and the embryonic regions transferred to microtest tissue-culture plate wells, where the volume of buffer was reduced to approximately 2 μ l. The embryonic regions were lysed by freezing and thawing prior to electrophoresis.

The 13.5-day-old embryos were homogenized in buffer (33% w/v). The homogenates were then diluted (1 vol to 10 vols buffer) and centrifuged to remove particulate matter. Adult liver and kidney samples were homogenized in buffer (33% w/v). The kidney homogenates were diluted 1 vol to 10 vol buffer, and liver homogenates 1 vol to 15 vol buffer before centrifugation.

Electrophoretic analysis

Samples were applied to cellulose acetate ('Cellogel') strips (7.8 × 15 cm) with a draughtsman's lining pen, and electrophoresis carried out in the tris-EDTA-citric acid (pH = 7.5) buffer of Rattazzi *et al.* (1967) for 2 h with a voltage gradient of 14 V cm⁻¹. The gels were then placed in a mixture of 20 ml staining buffer, 0.5 ml PMS (3 mg/ml), 0.5 ml MTT (5 mg/ml) before being thoroughly washed in water.

The proportions of the two PGK-1 allozymes were assessed by a quantitative method involving photography of the PGK-1 phenotype of each sample and making tracings from the negatives using a densitometer. The areas under the curves represented the quantity of PGK-1A and 1B allozyme present in each sample. These areas were then calculated using a 'Quantimet' instrument. The proportions of PGK-1A to total allozyme were calculated for offspring from the different mating types and data were analysed by the Mann-Whitney non-parametric test as described by Pollard (1977).

Table 1. *The mean proportion of PGK-1A allozyme to total allozyme in artificial 50:50 mixtures of liver homogenates from different strains*

Liver mixture	No. of tests	Mean proportion of PGK-1A to total allozyme (% ± s.e.m.)
1. C3H♂ + Pfk1 ^a /Y	13	52.2 ± 1.3
2. JU♂ + P _{gk} -1 ^a /Y	13	51.5 ± 1.4

3. RESULTS

Table 1 shows the mean proportion of PGK-1A allozyme to total allozyme in homogenates made by mixing liver from PGK (*Pgk-1^a*) males with equal amounts of liver from JU (*Pgk-1^b*) males, mixture 1, and C3H (*Pgk-1^b*) males, mixture 2. The mean proportions of the two allozymes were not significantly different in either mixture, neither did the mean proportion of PGK-1A allozyme in mixture 1 significantly differ from that in mixture 2. These results indicate that there is no major difference in PGK-1 activity between the two alleles or between the strains of mice investigated.

The results obtained with PGK-1AB heterozygotes derived from the reciprocal crosses between the PGK stock and the JU and C3H inbred strains are shown in Table 2, and it can be seen that they differ markedly from those of the artificial mixtures (Table 1). Attention should be given to four aspects of the data.

(1) In 15 of the 16 tests the mean proportion of PGK-1A allozyme exceeded the proportion of PGK-1B allozyme and the difference from a 50:50 expectation was statistically significant in each case. Since the studies on mixtures of male tissue (Table 1) had shown the activities of the two allozymes to be similar, the unequal proportions observed in the heterozygous female tissue studied suggest that the proportion of cells with the *Pgk-1^a*-marked X genetically active was generally greater than the proportion with the *Pgk-1^b*-marked X genetically active. The un-

equal proportions of the two allozymes in both C3H and JU crosses provide the first indication that a new *Xce* allele is present on the *Pgk-1^a*-marked chromosome.

(2) Comparison of the data obtained from the C3H and JU crosses shows that in all eight sets of tests the proportion of PGK-1A allozyme was lower when the *Pgk-1^b*-marked X was introduced from a JU parent. This clearly demonstrates the expected effect of the known *Xce* alleles of the *Pgk-1^b*-marked chromosomes; when set against a single common X chromosome an *Xce^b* chromosome (JU) is more likely to be the genetically active X than is an *Xce^a* chromosome (C3H).

Table 2. Proportion of PGK-1A allozyme in *Pgk-1^a/Pgk-1^b* heterozygotes (mean % \pm S.E.), when *Pgk-1^b* is associated with different *Xce* alleles

Tissue studied	Genotype of heterozygotes (strain of origin of X chromosome)		Significance of <i>Xce^a</i> - <i>Xce^b</i> difference
	<i>Pgk-1^a Xce^c</i> (PGK) <i>Pgk-1^b Xce^a</i> (C3H)	<i>Pgk-1^a Xce^c</i> (PGK) <i>Pgk-1^b Xce^b</i> (JU)	
(a) <i>Pgk-1^a</i> maternally derived			
Adult Kidney	71.4 \pm 2.9 (20)	63.2 \pm 1.9 (20)	0.02 < <i>P</i> < 0.05
Adult Liver	71.1 \pm 3.2 (20)	63.1 \pm 3.7 (20)	0.1 < <i>P</i> < 0.02
13.5 d embryo	73.9 \pm 2.4 (26)	65.1 \pm 2.1 (28)	0.005 < <i>P</i> < 0.01
7.5 d embryo	82.9 \pm 2.3 (25)	73.0 \pm 2.0 (17)	0.002 < <i>P</i> < 0.01
(b) <i>Pgk-1^a</i> paternally derived			
Adult kidney	69.3 \pm 2.6 (20)	60.7 \pm 2.3 (20)	0.02 < <i>P</i> < 0.05
Adult liver	79.7 \pm 2.5 (20)	67.6 \pm 3.6 (22)	0.01 < <i>P</i> < 0.02
13.5 d embryo	71.9 \pm 2.0 (26)	63.2 \pm 1.9 (28)	0.005 < <i>P</i> < 0.01
7.5 d embryo	71.7 \pm 3.8 (17)	59.5 \pm 2.8 (19)	0.01 < <i>P</i> < 0.02

Number in parentheses = number of animals investigated.

(3) Comparison of sections (a) and (b) of Table 2 shows no indication of a difference between reciprocal crosses. This is a surprising finding in view of the fact that differences between the heterozygous phenotypes of females derived from reciprocal crosses have been found with other X-linked markers studied (T(7;X)Ct, Cattanach & Perez, 1970; *Ta*, Kindred, 1961; *Mo^{br}*, Falconer & Isaacson, 1972). It is perhaps possible that numbers of animals used in the present experiments were too low to show up the very small reciprocal cross differences that were observed with the other markers.

(4) Finally, the main result of the experiment is provided by comparisons of the PGK expression in adults and embryos within each genotypic group. The key observation is that both the overall predominance of PGK-1A and the influences of the *Xce^a* and *Xce^b* alleles carried on the different *Pgk-1^b*-chromosomes evident in the adult are also evident in both embryonic stages studied. These observations therefore strongly support the view that the *Xce* effect is brought about by a primary non-randomness in the X-inactivation process. The similar early and then constant predominance of PGK-1A allozyme activity in all tests provides a second line of evidence that there is a new *Xce* allele on the feral mouse-derived *Pgk-1^a* chromosome.

One other feature of the data requires comment. It may be noted that in Table 2, section (a) the proportion of PGK-1A allozyme was considerably higher at the 7.5 day foetal stage in both JU and C3H crosses than at 13.5 days. The differences in each case were statistically significant, but since there was no indication of any such trend among the data from the reciprocal crosses (section b) it seems unlikely that this finding is meaningful. Certainly, this anomalous finding in no way casts doubt on the general conclusion that non-random X inactivation is responsible for the general predominance of the PGK-1A allozyme in embryos and adults. Were cell selection the responsible mechanism, less predominance would have been expected in the earlier embryos.

Table 3. *Vibrissa scores of Ta/+ females (mean \pm S.E.) when the non-Ta X chromosome carries different Xce alleles*

Genotype of <i>Ta/+ ff</i> (strain of origin of tested X chromosome)	No.	Mean vibrissa score	Significance
<i>Pgk-1^b Ta Xce^a</i>	37	18.35 \pm 0.21	<i>P</i> < 0.001
<i>Pgk-1^a + Xce^c (PGK)</i>			
<i>Pgk-1^b Ta Xce^a</i>	42	14.72 \pm 0.34	
<i>Pgk-1^b + Xce^a (C3H)</i>			

The results of the *Ta* test crosses are shown in Table 3, and it can be seen that the mean vibrissa scores of *Ta/+* females carrying the feral mouse-derived *Pgk-1^a* chromosome were higher than those carrying the C3H-derived *Pgk-1^b* chromosome and were in fact little reduced from the normal number of 19. By contrast the scores for the *Ta/Y* males produced in the two sets of crosses (7.00 ± 0.23 , $n = 33$ and 7.09 ± 0.22 , $n = 46$, respectively) did not differ significantly. The female differences cannot therefore be attributed to genetic background influences; the non-*Ta* X chromosomes of different origin appear to be responsible. The important point of the data is that the *Pgk-1^a*-marked feral mouse X which gives a predominance of PGK-1A allozyme activity in PGK-1AB heterozygotes also causes a higher expression of its *Ta⁺* allele in the *Ta/+* females than does the C3H X chromosome. This was also evident from the banding pattern in the coat of the *Ta* heterozygotes. Females possessing the *Pgk-1^a* X chromosome regularly showed little of the standard *Ta* striping; genetic and/or cytological tests were necessary to distinguish them from patroclinous XO females. These findings provide a third line of evidence that the feral mouse X chromosome carries the new *Xce* allele.

4. DISCUSSION

West & Chapman (1978) have assayed PGK activity spectrophotometrically and found no significant difference in activity in liver, brain and kidney among four inbred strains or between *Pgk-1^a/Y* and *Pgk-1^b/Y* males. Our experiment in which we assayed PGK activity in artificial mixtures of liver homogenates from

different strains (Table 1) supports their findings and allows us to conclude that the differences observed in PGK allozyme activity in PGK-1AB heterozygotes (Table 2) are not due to allelic or strain differences. Rather, these results suggest that the proportions of cells with one or other genetically active X are unequal, as indicated by the results of previous studies with other X-linked marker genes (Cattanach & Williams, 1972).

The results shown in Table 2 further show that it is the *Xce* genotype of the X chromosomes which is important in determining the expression of the PGK-1 allozymes in PGK-1AB heterozygotes. The PGK-1 allozyme activity from the *Pgk-1^a* chromosome was greater than that from the JU X chromosome which in turn was greater than that from the C3H X chromosome, and more recent experiments have shown a JU-like response with the C57BL/GoH X chromosome. West & Chapman (1978) also noted that the proportion of PGK-1B allozyme was consistently lower in PGK-1AB heterozygotes when the *Pgk-1^b* allele was derived from the C3H/HeHa strain than when derived from C57BL/6Ha, DBA/2Ha or JBT/Jd strains. However, they did not recognize the overall predominance of PGK-1A allozyme activity in PGK-1AB heterozygotes found in the present study.

The effects of *Xce* genotype upon PGK expression observed in the present experiment and also that of West and Chapman (1978) are in full accord with the results obtained with other X-linked markers (Cattanach, in preparation; Cattanach, Pollard & Perez, 1969; Cattanach & Williams, 1972) and so validate the rationale for the present investigation. These studies have shown that in *Xce^a/Xce^b* heterozygotes marker genes present on the *Xce^b* chromosome tend to predominate. The present results now show that when set against the *Pgk-1^a*, feral mouse X, the *Pgk-1^b* allele on an *Xce^b* (JU) chromosome is more likely to be expressed than when on an *Xce^a* (C3H or 101) chromosome. The anticipated *Xce* effect was therefore clearly indicated.

The unexpected finding in these experiments was the apparent predominance of the feral mouse X in both crosses, and this suggested that a new, more extreme *Xce* allele was present on this chromosome. This possibility is more or less established when all the data are considered together. We have: (1) the predominance of PGK-1A activity in both JU and C3H crosses, as discussed above; (2) the predominance observed with regard to the *Ta* locus (Table 3), which together with the PGK data suggests that the effect is brought about by a preponderance of cells with the feral mouse X genetically active; (3) the fact that the effect is established early in embryonic development, like that attributable to the *Xca^a-Xce^b* substitutions (Table 2); and (4) from the history of the feral mouse chromosome, the responsible locus must be very closely linked to the *Pgk-1^a* marker and, therefore, located in precisely the same limited region of X as that indicated for *Xce* (Cattanach, in preparation; Cattanach, Perez & Pollard, 1970). Although these data do not entirely establish allelism they do provide reasonable evidence that a new *Xce* allele, which we have called *Xce^c*, is present in the feral mouse-derived region of X containing the *Pgk-1* locus.

Prior to this study the earliest age that *Xce* effects have been distinguished has

been the 12th–14th day of gestation. This was established from the melanocyte variegation patterns seen in the coats of the adult mice (Cattanach, 1974), as these are laid down at the above stage of embryonic development (Mayer, 1973; Rawles, 1947) and West & Chapman (1978) have also noted that the strain influences upon PGK expression they observed in their experiments were established by the fourteenth day. Neither observation, however, has made it clear whether cell selection or non-random *X* inactivation is responsible for the non-random *X* expression detected. Some evidence against cell selection has been provided by the studies of Drews *et al.* (1974) with *Tfm*, however. The present finding that the *Xce* effects are apparent as early as 7.5 days of gestation, and do not change significantly thereafter, now effectively rules out the possibility that selection is taking place throughout foetal and early adult life, and suggests that the non-randomness is established very early. Since there is no evidence of selection after 7.5 days and it is known that *X*-inactivation occurs only 1–2.5 days earlier (Gardner & Lyon 1971; Monk & Harper, 1979; Rastan *et al.* 1980) it seems highly unlikely that selection operating in the short intervening period is responsible for the effects observed at all later ages. We therefore favour non-random *X* inactivation as the mechanism by which the non-random *X* expression is brought about in *Xce* heterozygotes. If the *Xce* locus is indeed concerned with the *X*-inactivation process itself, it may serve as the primary site for *X*-inactivation (Cattanach and Isaacson, 1967) and thus represent the long-postulated *X*-inactivation centre (Grumbach, 1964; Lyon, 1964; Russell, 1964). In this connexion it is of interest that in man the postulated *X*-inactivation centre is thought to be located proximally in the long arm of the *X* chromosome (Therman *et al.* 1974, 1979) and therefore, like *Xce* in the mouse, close to the *Pgk* locus (Franke & Taggart, 1980).

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