Primary non-random X-inactivation caused by controlling elements in the mouse demonstrated at the cellular level

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(Received 18 February 1982 and in revised form 15 March 1982)

SUMMARY

Previous studies have shown that different alleles of the mouse X chromosomal controlling element locus, Xce, cause non-random X-chromosome inactivation as judged by variegation in the coats of female mice heterozygous for X-linked coat colour/structure genes, or Cattanach's translocation (Is (X; 7) Ct), or the relative activity of biochemical variants of the X-linked enzyme PGK. This paper presents evidence using the Kanda differential staining method on 6 d.p.c. and 13 1/2 d.p.c. female mouse embryos heterozygous for the marker X chromosome Is (X; 7) Ct and carrying different Xce alleles, that the Xce locus affects the randomness of X chromosome inactivation. Furthermore the fact that a marked Xce effect is demonstrable in female embryos as early as 6 1/2 d.p.c. (i.e. very soon after the initial time of X-inactivation) is strong evidence that the Xce locus exerts its effect by causing primary non-random X-inactivation rather than by cell selection after initial random X-inactivation.

1. INTRODUCTION

In female mammals one of the two X chromosomes is genetically inactivated early in embryogenesis, thereby achieving dosage compensation for X-linked genes (Lyon, 1961). Although the Lyon hypothesis states that either X chromosome in a cell may be inactivated at random, there are very few cases of totally random inactivation. Non-randomness can be produced in two ways: (1) initial random inactivation followed by cell selection leading to later non-randomness or (2) true primary non-randomness of initial X-inactivation, or a combination of the two. Examples of random inactivation followed by cell selection are found in certain X-chromosome aberrations, such as deletions, X-autosome translocations (Cattanach, 1975; Distech, Eicher & Latt, 1979; Russell & Cacheiro, 1978) or in individuals heterozygous for X-linked enzyme deficiencies such as for HGPRT (Nyhan et al. 1970). An example of true primary non-random X-inactivation is seen

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in the preferential inactivation of the paternally derived X chromosome in certain tissues of the extraembryonic membranes in mice and rats (Takagi & Sasaki, 1975; Wake, Takagi & Sasaki, 1976; West et al. 1977; Rastan, 1981).

Cattanach described a locus on the X chromosome of the mouse which he called the X-chromosome controlling element (Xce) locus, different alleles of which appeared to affect the degree of inactivation of the autosomal insertion in Cattanach’s translocation, Is (X; 7) Ct (Cattanach & Isaacson, 1965; 1967) and certain X-linked genes (Cattanach, Pollard & Perez, 1969) as judged by the amount of variegation found in the coats of female mice heterozygous for coat colour markers. The Xce locus has been mapped very close to Ta on the proximal side of the breakpoint in Cattanach’s translocation [hereafter abbreviated to IsCt or X(7)] (Cattanach, Perez & Pollard, 1970; Cattanach, 1981). Originally alleles at the Xce locus were thought to affect the spread of inactivation into the autosomal region in IsCt, but later, when it became apparent that true X-linked genes were also affected it was suggested that alleles at the Xce locus exerted their effect by influencing the completeness or otherwise of inactivation of the X-chromosome itself. (Cattanach et al. 1969). Finally, after testing the X-chromosomes from a number of different origins which showed an Xce-type effect (Cattanach & Williams, 1972) it was postulated that alleles at the Xce locus act by influencing the probability of the whole X chromosome on which they are carried being expressed or inactivated.

As a result of this the Xce locus has been implicated as the control site or inactivation centre for X-chromosome inactivation in the mouse. Russell (1971), Grahn, Lea & Lesch (1970) and Falconer & Isaacson (1972) have since described similar controlling sites or inactivation centres which appear to map in the same region on the X chromosome, and a further mutation, Ohv, has been described in the mouse (Ohno, Geller & Kan, 1974; Drews et al. 1974) that maps very close to the Xce locus or is an allele of it. In females heterozygous for the Ohv mutation the X-chromosome carrying it is selectively expressed.

Three alleles at the Xce locus have now been described, Xce\textsuperscript{a}, Xce\textsuperscript{b} and Xce\textsuperscript{c} (Cattanach, 1981; Johnston & Cattanach, 1981). Using appropriate X-chromosomal marker genes it has been shown that in Xce\textsuperscript{a}/Xce\textsuperscript{b} heterozygotes, cells with an active Xce\textsuperscript{b} chromosome tend to predominate over those with an active Xce\textsuperscript{a} chromosome, and in Xce\textsuperscript{b}/Xce\textsuperscript{c} heterozygotes cells with an active Xce\textsuperscript{c} chromosome tend to predominate over cells with an active Xce\textsuperscript{b} chromosome (Cattanach et al. 1969; Johnston & Cattanach, 1981; Cattanach, 1981).

It has not yet been finally established, however, whether alleles at the Xce locus exert their effect by biasing the randomness of primary X-inactivation or whether their effect is the result of selection of varying degrees for or against cells with a particular Xce allele active during the growth and development of the embryo. Melanocyte variegation patterns, by which means different alleles at the Xce locus were first distinguished, are not established until the 12—13th day of embryonic development in the mouse (Mayer, 1973; Rawles, 1974) which allows ample time for the operation of differential cell selection on the two populations of cells after
the initial time of \(X\)-inactivation, thought to be complete in all cells of the mouse embryo by \(5\frac{1}{2}\) days post-coitum (d.p.c.) (Rastan, 1981).

Several lines of evidence, however, suggest that cell selection is not the cause of the non-random inactivation patterns produced by alleles at the \(Xce\) locus. The first is that studies on several \(X\) chromosomes of different origins which show an \(Xce\) type effect all seem to implicate a single locus (Cattanach & Williams, 1972). If cell selection were acting on a mutation having a deleterious effect when active one would expect to find a range of different loci with similar effects. Secondly, if the \(Xce\) effect were the result of cell selection acting on two populations of cells differentiated by random \(X\)-inactivation one would expect to find a departure from a binominal distribution of the two cell types. Studies on coat mosaicism in the adult female mouse have shown no such departure from a binominal distribution (Cattanach & Williams, 1972). Finally, the study by Johnston & Cattanach (1981) using a new variant at the \(X\)-linked \(Pgk\) locus, \(Pgk-1^a\), showed that PGK expression in \(Pgk-1^a/Pgk-1^b\) heterozygotes carrying different \(Xce\) alleles on their \(Pgk-1^b\) chromosome was subject to \(Xce\) effects in both adults and \(13\frac{1}{2}\) and \(7\frac{1}{2}\) d.p.c. embryos. The fact that PGK expression in \(7\frac{1}{2}\) d.p.c. embryos was affected by \(Xce\) alleles in the same way as it was in adults is the best evidence to date that the \(Xce\) effect is unlikely to be caused by cell selection and more likely to be brought about by primary non-randomness in the \(X\)-inactivation process. However, \(X\)-inactivation is complete in all cells of the embryo by \(5\frac{1}{2}\) d.p.c. (Rastan, 1981) and so there is still possibly time for cell selection to have operated in the \(7\frac{1}{2}\) d.p.c. embryo. In order to exclude the effects of cell selection one must look at \(X\)-chromosome inactivation as near as possible to the time of initial \(X\)-chromosome differentiation.

This paper presents a cytological investigation of the effect of different alleles at the \(Xce\) locus on \(X\)-chromosome inactivation at the cellular level using a differential staining technique (Kanda, 1973), to reveal the inactive \(X\)-chromosome. The questions posed are (1) are the affects of different alleles at the \(Xce\) locus on coat colour mosaicism and biochemical expression of the enzyme PGK reflected at the cellular level by frequency of inactivation of \(X\) chromosomes? (2) If so can \(Xce\) effects be seen as early as \(6\frac{1}{2}\) d.p.c.? In order to answer these questions the modified Kanda technique (Rastan, 1981) was used on \(13\frac{1}{2}\) d.p.c. and \(6\frac{1}{2}\) d.p.c. female embryos with morphologically distinguishable \(X\)-chromosomes carrying different \(Xce\) alleles. Cattanach’s translocation, \(1sCt\) carrying known \(Xce\) alleles, was used as the morphological marker. Embryos of \(6\frac{1}{2}\) d.p.c. were chosen as this is the earliest time a practical amount of tissue is available for chromosomal analysis. It is estimated that only one full cycle of cell division will have taken place in the embryo as a whole between \(5\frac{1}{2}\) and \(6\frac{1}{2}\) d.p.c. therefore allowing little opportunity for cell selection to exert its effects (Snow, 1976). A further advantage of this cytological approach is that it provides a framework for deciding whether alleles at the \(Xce\) locus exert their influence on the expression or inactivation of the whole \(X\)-chromosome as detected by heterochromatinization.
2. MATERIALS AND METHODS

**Mice**

Fertile adult male mice which were Type II (unbalanced or duplication form) carriers of Cattanach's translocation, IsCt or $X^{(7)}$, known to be carrying either $Xce^a$ or $Xce^b$ alleles on the rearranged $X$-chromosomes ($X^{(7)}$) were used. Male mice of the so-called 'high' c-variegation line, which had been extensively progeny tested, were carriers of the $Xce^a$ allele, and male mice of the 'low' c-variegation line, again which had undergone extensive progeny testing, were carriers of the $Xce^b$ allele (Cattanach, 1972; Cattanach, 1981).

Adult female mice of the inbred strain C3H/HeH, known to be carrying the $Xce^a$ allele on both their $X$-chromosomes (Cattanach & Williams, 1972; Cattanach, 1981) were used for mating with these males.

**Embryos**

Embryos were obtained by mating fertile male carriers of Type II IsCt carrying either $Xce^a$ or $Xce^b$ on their $X$-chromosome to spontaneously ovulating C3H/HeH female mice carrying the $Xce^a$ allele. The two types of cross were thus (1) $X^{(7)} Xce^a/Y \times X^N Xce^a/X^N Xce^a$ and (2) $X^{(7)} Xce^b/Y \times X^N Xce^a/X^N Xce^a$. Mating was ascertained by the presence of a copulation plug, and the morning of finding the plug was designated day 5.

Female mice were sacrificed by cervical dislocation on day 6½ and day 13½ of pregnancy and embryos recovered. The extraembryonic membranes of the 13½ d.p.c. embryos were removed and discarded, the embryos sexed by dissection of the gonad, which has visibly differentiated by this age, and males discarded. Reichert's membranes, trophectoderm and ectoplacental cone were removed from the 6½ d.p.c. embryos and discarded.

The 13½ d.p.c. embryos were incubated in tissue culture medium 199 with colchicine (4 $\mu$g ml$^{-1}$ final concentration) for 1 h at room temperature, and the 6½ d.p.c. embryos for 2 h at room temperature, to accumulate cells at metaphase.

**Chromosome preparation by the modified Kanda method**

After the period in colchicine the embryos were treated by the modified Kanda method for revealing the inactive $X$ at metaphase, as previously described (Rastan et al. 1980). Briefly, the embryos were transferred to hypotonic 0.5% potassium chloride solution at room temperature and incubated in a waterbath at 50 °C for 15 min, fixed in 3:1 absolute ethanol/acetic acid solution, disaggregated in 60% acetic acid and slides made on a hotplate at about 40 °C by allowing small drops of acetic acid mixture to evaporate off leaving the metaphase chromosomes spread on the slide. All slides were stained in 2% Giemsa buffered at pH 6.8 for 20 min.
Metaphase chromosomes from 2 cells from a 13½ d.p.c. female embryo heterozygous for IsCt and RB1Ald (metacentric fusion) between chromosomes 6 and 15. (a) Cell in which $X^N$ is the inactive dark staining chromosome (arrow). (b) Cell in which $X^{(7)}$ is the inactive dark staining chromosome (arrow).
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Analysis

All female embryos from the matings described above are necessarily heterozygous for the $X^{(7)}$ chromosome and will be of the type $X^{(7)}X\text{ce}^a/X^N X\text{ce}^a$ or $X^{(7)}X\text{ce}^b/X^N X\text{ce}^b$, depending on the Xce genotype of the father.

The 13½ d.p.c. embryos had been sexed prior to chromosome preparation and males discarded. The 6½ d.p.c. embryos were sexed cytologically by the presence or absence of a $Y$ chromosome (Rastan, 1981) and slides from male embryos discarded.

The remaining slides from female embryos heterozygous for IsCt were coded and metaphase cells scored for whether the normal X chromosome ($X^N$) or the marker $X$ ($X^{(7)}$) was dark-staining. As noted elsewhere (Rastan, 1981) after the modified Kanda treatment differential pale-staining within the $X^{(7)}$ chromosome corresponding to the region of the autosomal insertion can be seen in about 55% of cells in which the $X^{(7)}$ chromosome is dark-staining (Rastan, 1981). However, even in those cells in which differential staining within the $X^{(7)}$ chromosome does not occur, the two chromosomes can be morphologically distinguished on the basis of size (Eicher, 1970).

The following criteria were used to decide whether the dark-staining inactive chromosome in a cell was $X^N$ or $X^{(7)}$.

1. If a differential pale-staining area corresponding to the region of the autosomal insertion could be seen within the otherwise dark-staining chromosome the inactive chromosome was classified as $X^{(7)}$.
2. If no differential staining within the chromosome could be seen the chromosome was initially classified as $X^N$ or $X^{(7)}$ on the basis of size.
3. This decision was double checked by looking for the presence or absence of a single pale-staining chromosome which was longer than any of the others in the set. If the dark-staining chromosome had been initially classified as $X^N$ and a single pale-staining chromosome which was longer than any other could be seen the classification was corroborated and taken to be correct. Similarly, if the dark-staining chromosome had been initially classified as $X^{(7)}$ and no single pale-staining chromosome longer than any of the others could be seen, the classification was taken to be correct.

Plate 1 (a and b) show cells from a 13½ d.p.c. female embryo heterozygous for IsCt and also carrying a metacentric chromosome, Rb1Ald, produced by Robertsonian fusion of chromosomes 6 and 15 (Miller et al. 1971), which have the $X^N$ and the $X^{(7)}$ chromosome respectively dark-staining and inactive after treatment by the modified Kanda technique. Although differential staining within this particular $X^{(7)}$ is not seen in this case the two chromosomes are nevertheless morphologically distinguishable after heat/hypotonic treatment. A comparison of the relative lengths of the dark-staining chromosome with the length of the arms of the metacentric chromosome show that in Plate 1a, the inactive normal X chromosome appears about the same size as the long arm of the metacentric (chromosome 6) whereas in Plate 1b the $X^{(7)}$ dark-staining chromosome is
considerably longer than the long arm of the metacentric. In addition in Plate 1a the pale-staining long marker chromosome can also be distinguished.

3. RESULTS

Four fertile male Type II IsCt mice of the 'high' c-variegation line (carrying Xce\(^a\)) and four of the 'low' c-variegation line (carrying Xce\(^b\)) were used to produce 13\(\frac{1}{2}\) d.p.c. embryos. In each group one male died before the completion of the experiment, so the 6\(\frac{1}{2}\) d.p.c. embryos were sired by three males of the original four in each case. Since the mothers of the embryos were all members of an inbred stock their genotypes were identical. Therefore, use of the same males to sire both 13\(\frac{1}{2}\) and 6\(\frac{1}{2}\) d.p.c. embryos ensures that the embryos from the two age groups were genotypically matched as far as possible. The male mice carrying Xce\(^a\) on their X-chromosome sired a total of 59 embryos at 13\(\frac{1}{2}\) d.p.c., of which 27 were female, and a total of 21 embryos at 6\(\frac{1}{2}\) d.p.c. of which 8 were female. The male mice carrying Xce\(^b\) on their X-chromosome sired a total of 56 embryos at 13\(\frac{1}{2}\) d.p.c. of which 29 were female and 24 embryos at 6\(\frac{1}{2}\) d.p.c. of which 10 were female.

The number of metaphase cells from female embryos with either X\(^m\) or X\(^N\) inactive from the two types of cross, X\(^7\)Xce\(^a\)/Y \(\times\) X\(^N\)Xce\(^a\)/X\(^N\)Xce\(^a\) or X\(^7\)Xce\(^b\)/Y \(\times\) X\(^N\)Xce\(^a\)/X\(^N\)Xce\(^a\) producing female embryos of the type X\(^7\)Xce\(^a\)/X\(^N\)Xce\(^a\) and X\(^7\)Xce\(^b\)/X\(^N\)Xce\(^a\) respectively, are shown in Table 1. The data from individual embryos were analysed statistically in the form of \(m \times 2\) contingency tables by an extension of Fisher’s exact test and found to be sufficiently homogeneous overall, both between embryos from the same father and between fathers of the same genotype, to justify comparison between overall group percentages.

Table 1 shows that males of the genotype X\(^7\)Xce\(^a\)/Y produce female embryos at both ages with a higher percentage of cells with X\(^7\) inactivated than X\(^7\)Xce\(^b\)/Y males when mated to female mice homozygous for Xce\(^a\). A dark-staining inactive X\(^7\) chromosome was seen in 56.9% of cells of 13\(\frac{1}{2}\) d.p.c. female embryos from X\(^7\)Xce\(^a\)/Y fathers compared with 31.5% of cells of embryos from X\(^7\)Xce\(^b\)/Y fathers. This difference is very highly statistically significant. \(\chi^2 = 257, P = 6 \times 10^{-58}\) (1 D.F.). A similar difference is observed in cells from female embryos at 6\(\frac{1}{2}\) d.p.c., with X\(^7\) inactivated in 65.9% of cells from embryos with X\(^7\)Xce\(^a\)/Y fathers compared with 27.0% for female embryos from X\(^7\)Xce\(^b\)/Y fathers. Again this difference is very highly significant, \(\chi^2 = 79, P = 5 \times 10^{-19}\) (1 D.F.). Clearly the proportion of cells with X\(^7\) inactivated is affected by the Xce allele carried on the chromosome, in a way which is compatible with the preferential expression of an X chromosome carrying an Xce\(^b\) allele over an X-chromosome carrying an Xce\(^a\) allele, i.e., preferential inactivation of an X-chromosome carrying an Xce\(^a\) allele over an X-chromosome carrying an Xce\(^b\) allele. The fact that the difference between embryos from the two types of father at 13\(\frac{1}{2}\) d.p.c. is reflected in a similar, in fact, more extreme, difference in embryos as early as 6\(\frac{1}{2}\) d.p.c. is strong evidence that alleles at the Xce locus exert their effect by biasing the randomness of initial X-chromosome inactivation.
4. DISCUSSION

The results presented in this paper show that the proportion of cells with \(X^{(7)}\) inactivated in female heterozygous embryos is affected by the \(Xce\) genotype in such a way that a chromosome carrying an \(Xce^a\) allele is more likely to be inactivated than a chromosome carrying an \(Xce^b\) allele. Furthermore, this difference is apparent as early as 6½ d.p.c., indicating that the effect is likely to be the result of \(Xce\) alleles biasing the randomness of initial \(X\)-chromosome inactivation.

Table 1. Effect of \(Xce^a\) and \(Xce^b\) alleles on distribution of cells with \(X^{(7)}\) inactive in 6½ d.p.c. and 13½ d.p.c. female embryos heterozygous for \(IsCt\).

<table>
<thead>
<tr>
<th>Genotype of embryo</th>
<th>(X^{(7)}Xce^a/X^N)Xcea</th>
<th>(X^{(7)}Xce^a/X^N)Xce(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(X^{(7)}Xce^a/)Xce(^a)</td>
<td>No. of female embryos</td>
<td>Metaphases with a dark-staining chromosome</td>
</tr>
<tr>
<td>Age</td>
<td>13½ d.p.c.</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>6½ d.p.c.</td>
<td>8</td>
</tr>
</tbody>
</table>

Several points, however, require more detailed consideration. For example, female embryos from \(X^{(7)}Xce^a/\)Y fathers mated to C3H/HeH females are of the genotype \(X^{(7)}Xce^a/X^N\)Xce\(^a\), i.e. homozygous for the \(Xce^a\) allele. One would therefore expect, all other things being equal, the ratio of cells with \(X^{(7)}\) inactive to \(X^N\) inactive to be 1 : 1. In fact, as shown in Table 1, on average 65.9 % of cells had the \(X^{(7)}\) chromosome inactive at 6½ d.p.c. and 56.9 % at 13½ d.p.c. These figures both represent statistically significant departures from a 1:1 ratio, \(\chi^2 = 45.5, P = 1.5 \times 10^{-11}\) (1 d.f.) and \(\chi^2 = 21.9, P = 2.8 \times 10^{-5}\) (1 d.f.) respectively. Cell selection for a maximally balanced genotype in Type II heterozygotes would produce an excess of cells with \(X^{(7)}\) inactive, which would explain the departure from a 1:1 ratio at 13½ d.p.c., by which age there will have been ample time for cell selection to have operated. However, the contention in this paper is that cell selection will not have had time to operate as early as 6½ d.p.c., so for these embryos an alternative explanation must be found. One consideration is that the 6½ d.p.c. embryos used in this experiment included the extraembryonic part of the egg cylinder, which eventually contributes to the extraembryonic membranes in which there tends to be preferential inactivation of the paternally derived \(X\) chromosome (Takagi & Sasaki, 1975). In 13½ d.p.c. embryos these membranes were removed prior to chromosome preparation. However, one would expect a similar non-random component in the 6½ d.p.c. embryos of the genotype \(X^{(7)}Xce^b/X^N\)Xce\(^a\), whereas in fact no such evidence is found, the proportion of cells with \(X^{(7)}\) inactive at this stage being as low as 27 %. One possibility is that the magnitude of the effect of the \(Xce^b\) allele on \(X\)-inactivation over the \(Xce^a\) allele is sufficient to override the...
non-random inactivation of the paternally derived X in the extraembryonic part of the 6\textsubscript{1/2} d.p.c. embryo. This possibility is at present being investigated.

A further possibility is that a scoring error could be responsible for the unexpected results of the 6\textsubscript{1/2} d.p.c. embryos. However, this explanation is considered unlikely as the criteria used to decide whether X\textsuperscript{(7)} or X\textsuperscript{N} was inactive in a cell were chosen so as to eliminate scoring error as far as possible, and even if a scoring error had occurred it would be constant for embryos of both genotypes, and cannot therefore be used to explain the differences between embryos of different genotypes.

Reciprocal crosses using adult females heterozygous for IsCt with known Xce alleles on the rearranged chromosome would be informative on this point, but unfortunately at the time of the experiment no such females which had had their Xce genotype characterized by progeny testing were available.

In spite of this departure from expected results for 6\textsubscript{1/2} d.p.c. embryos the basic difference between embryos from the two groups is quite clear, namely that there is a highly statistically significant difference in the proportion of cells with X\textsuperscript{(7)} inactive in female heterozygous embryos dependent on the Xce allele carried on the X\textsuperscript{(7)} chromosome and such that a chromosome carrying Xce\textsuperscript{a} is more likely to be inactivated than a chromosome carrying an Xce\textsuperscript{b} allele. This difference is in accord with the preferential expression of an X-chromosome carrying an Xce\textsuperscript{b} allele over an X-chromosome carrying an Xce\textsuperscript{a} allele. The fact that it can be detected as early as 6\textsubscript{1/2} d.p.c. is very strong evidence that alleles at the Xce locus exert their effect by biasing the initial choice of which X-chromosome to inactivate, rather than by causing cell selection for or against cells with a particular Xce allele. This supports the interpretation for the mode of action suggested by Johnson & Cattanach (1981).

This work is the first cytogenetic demonstration of the effects of alleles at the Xce locus on the non-randomness of X inactivation. Furthermore it finally proves that alleles at the Xce locus exert their effect by altering the probability of inactivation of the whole X-chromosome as detected by heterochromatization.

I would like to thank Mr Papworth for statistical advice.

REFERENCES


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