

Controlling elements in the mouse X-chromosome

III. Influence upon both parts of an X divided by rearrangement

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SUMMARY

Data are presented which support the conclusion that in the *flecked* translocation, $T(1;X)Ct$, there is a spread of inactivation into both sides of the autosomal region inserted into the X. This would indicate that both parts of the divided X are subject to the X-inactivation process. The data also demonstrate that the inactivation of autosomal genes lying near each end of the insertion are modified by the X-chromosome controlling element system, *Xce*. Since the element modifies the heterozygous expression of X-linked genes on one side of the insertion, it would therefore be expected that it similarly modifies the heterozygous phenotypes of those on the other side. The data thus support the concept that the controlling element is the master gene, receptor site or inactivation centre which regulates the X inactivation process.

1. INTRODUCTION

Eight X-autosome translocations have been described in the mouse which give rise in the heterozygous female to variegated phenotypes for autosomal genes brought into close proximity with regions of the X (Cattanach, 1961; Russell, 1969). Genetical and cytological data have provided strong evidence that the primary cause is the X-inactivation process (Cattanach, 1963; Chu & Russell, 1965; Evans *et al.* 1965; Lyon 1961, 1963; Ohno & Cattanach, 1962; Russell, 1963, 1964) with the effect that the rearranged autosomal loci tend to become genetically inactivated when the rearranged X is inactivated.

Studies on translocations which carry two or more genes capable of expressing variegated phenotypes have demonstrated that the autosomal inactivation results from a sequential spread of the inactivating properties of the X from the break-point across the adjoining autosomal material (Cattanach, 1961; Russell, 1963). This has prompted several investigators to suggest that inactivation of the X itself results from some such spread of inactivating material, perhaps from some site of inactivation or inactivation centre (Grumbach, 1964; Lyon, 1964; Russell, 1964). The inactivation centre concept also provided a satisfactory explanation

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for the seemingly anomalous behaviour of one X -autosome translocation in which variegation for only one of two closely linked autosomal genes occurred even though the breakpoint was located between them (Russell, 1963). It was postulated that only the section of the rearranged X -chromosome material which carries the inactivation centre suffers the inactivation process and hence is capable of inactivating attached autosomal loci; the other part of the X would remain genetically active (Russell, 1963).

The *flecked X*-autosome translocation, $T(1;X)Ct$, is insertional in nature (Ford & Evans, 1964; Ohno & Cattanach, 1962); however, like Russell's (1963) translocations, which are considered to be reciprocal, the rearrangement effectively divides the X into two. Genetical evidence has clearly established that at least one part of the divided X suffers the inactivation process and that there is a spread of inactivation into one side of the autosomal insertion (Cattanach, 1961, 1963; Cattanach & Isaacson, 1965) and information now exists which suggests that there may be a spread of inactivation into the other side of the insertion also (Eicher, 1969; Cattanach, unpublished). This would indicate that both parts of the divided X are subject to inactivation, a finding that would clearly be at variance with the inactivation centre concept as originally proposed; either two inactivation centres at minimum would be required or X inactivation is not dependent upon a spread of inactivating material along the chromosome.

Recent studies with the *flecked X*-autosome translocation have demonstrated that the spread of inactivation into one side of the insertion and the heterozygous phenotype of at least two X -linked genes are under the control of an element located in the X (Cattanach & Isaacson, 1965, 1967; Cattanach, Pollard & Perez, 1969; Cattanach, Perez & Pollard 1970). The effect is considered to operate primarily upon the level of inactivation of the X -linked loci or upon the frequency of cells in which inactivation is incomplete; the influence upon the spreading effect may be a secondary consequence.

Almost all the controlling element studies concerning the autosomal gene variegation have made use of only one gene. In an attempt to obtain information which might help to elucidate whether or not there is a spread of inactivation into both sides of the autosomal insertion, we have now made quantitative studies on the levels of variegation for two other genes known to be located along the length in the insertion and investigated the influence of the controlling element system upon each. The results, presented in this communication, support Eicher's evidence of a spread of inactivation into the second side of the insertion and indicate that the variegation for each autosomal gene studied responds to the controlling element system.

2. METHODS AND MATERIALS

In the *flecked X*-autosome translocation, $T(1;X)Ct$, a piece of linkage group I bearing the wild-type alleles of at least three coat colour genes, *albino* (c), *pink-eye* (p) and *ruby-eye-2* ($ru-2$), has been inserted into the X (Cattanach, 1961; Eicher, 1967, 1970; Ford & Evans, 1964; Ohno & Cattanach, 1962). The linkage distances

between the three genes are *c-12-16-p-3-ru-2* (Dunn & Bennett, 1967; Eicher, 1970*a*). One autosomal break lies between the *haemoglobin-beta chain locus* (*Hbb*) and *shaker-1* (*sh-1*) (Eicher, 1967; Wolfe, 1967) and, hence, about 4–7 crossover units away from the *c* locus. The second break lies between *ru-2* and *quivering* (*qv*) which are about 9 crossover units apart (Eicher, 1970*a*).

All the translocation-bearing animals employed in the experiments to be described carried the unbalanced, duplication form, *Dp(1;X)Ct*, of the rearrangement and either *c*, *p* or *ru-2* was present on both normal linkage group I chromosomes. The heterozygous females thus showed *c*-, *p*- or *ru-2*-variegated phenotypes. All the animals were homozygous for *non-agouti* (*a*) and hence *c*- and *p*-variegations were seen against a solid black background colour. *Albino* (*c*) hair is, of course, white but a *p* hair which is normally a lilac colour also appears quite white-looking when contrasted against the black background hair in variegated animals. A different situation exists with *ru-2*; the single wild-type allele in the rearranged X is incompletely dominant over the two recessive alleles located in the normal chromosomes (Eicher, 1970*a*) and hence *ru-2*-variegation was seen as chocolate-coloured *ru-2* areas against a dark chocolate intermediate-*ru-2* background. As in recent communications, the abbreviated term Dp will be used to describe *Dp(1;X)Ct* heterozygotes and homozygotes and the genotypes of various Dp females produced will be described in the following manner; *Dp/+*; *pc^{ch}/pc^{ch}* indicates a Dp female carrying *p* and *c^{ch}* on both normal linkage group I chromosomes.

The alternative 'states' of the X-chromosome controlling element are distinguishable by the levels of translocation-induced *c*-variegation they permit and by their influence upon the heterozygous phenotypes of at least two X-linked genes, *Tabby* (*Ta*) and *Viable-brindled* (*Mo^{vbr}*) (Cattanach & Isaacson, 1967; Cattanach *et al.* 1969, 1970). The 'state' designated 'high' permits a near-50% level of *c*-variegation or high levels of expression of *Ta* or *Mo^{vbr}*, and that designated low, a near-30% level of *c*-variegation or lower levels of expression of *Ta* or *Mo^{vbr}*. The difference is attributed to the frequency of cells in which the *c⁺*, *Ta⁺* or *Mo⁺* genes are inactivated when the rearranged X is inactivated (Cattanach *et al.* 1969). Since the alternative 'states' of the element have now been isolated on normal X-chromosomes (Cattanach, 1970) and shown to modify the heterozygous phenotypes of the X-linked genes, the element has been formally given the name, *X-chromosome controlling element*, and symbol *Xce*. The low and high 'states' are thought to permit incomplete and near-complete inactivation of the X-linked loci, respectively, and thus may be provisionally designated *Xceⁱ* and *Xce^e* respectively.

Xceⁱ and *Xce^e* are carried in coupling with the insertion in two sublines of an inbred strain, JU/FaCt (Cattanach & Isaacson, 1967; Cattanach *et al.* 1969, 1970) and these are maintained by repeated backcrosses of Dp females to males of the inbred strain. The *c* allele is present on the linkage group I chromosomes and hence all the Dp females show *c*-variegated phenotypes. Animals with *p*- or *ru-2*-variegated phenotypes and carrying *Xceⁱ* or *Xce^e* in their rearranged X-chromosomes

were produced by substituting *p* and *ru-2* for *c* in derivatives of the two sublines. This was done in such a way that all the variegated animals to be studied were progeny of Dp males. The parental-source effect which causes the levels of translocation-induced *c*-variegation to differ in reciprocal crosses (Cattanach & Perez, 1970) does not therefore have to be considered when interpreting the data.

The level of variegation for each gene was determined in the manner standard for *c*-variegation (Cattanach & Isaacson, 1967); the proportion of the coats of individual females showing the recessive phenotype was estimated to the nearest 5%, scoring as large groups of animals as possible and without knowledge of their identity with respect to the controlling element. The method overestimates the true levels of *c*-variegation by about 10–15% (Cattanach & Isaacson, 1967) and this will almost certainly be true for the very similar *p*-variegation. Variegation for *ru-2* is more difficult to assess because the two colours in the coat are so similar, but it is unlikely that the scoring bias is of a different magnitude. Since *c*-variegated animals are routinely scored at 3–6 weeks of age, *p*-variegated animals were also scored at this time. However, *ru-2*-variegation could be assessed with more confidence in 10-day-old animals, i.e. when the hair is short and non-overlapping. Again, this is unlikely to bias the estimated levels of *ru-2*-variegation relative to that for the other two genes for it is known that scores on *c*-variegation made at this age differ little from those determined later (Cattanach, unpublished).

3. RESULTS

The levels of *ru-2*-, *p*- and *c*-variegation observed in Dp females carrying *Xceⁱ* and *Xce^c* in their rearranged X-chromosomes are shown in Table 1. Each set of females was the progeny of at least eight Dp males, and since there appeared to be no heterogeneity between males within each type of cross the data have been summed as shown. The levels of *c*-variegation are taken from some of the progeny-test data of Cattanach & Perez (1970); the *c*-variegated females studied were scored at the same time as most of the *p*- and *ru-2*-variegated animals.

The first finding that should be noted is that with either 'state' of the element the levels of variegation differ with each autosomal gene. In accord with the earliest observations (Cattanach, 1961), *p*-variegation is clearly less extensive than *c*-variegation, and since the two variegations are so similar it is most improbable that the difference is an artifact of the admittedly subjective scoring procedure. The comparison of the levels of *ru-2*-variegation with those of *p* and *c* must be made with more caution since the coat colours studied are so different. Nevertheless there is little doubt that *ru-2*-variegation is considerably more extensive than *p*-variegation. If the level of variegation for each gene is dependent upon its proximity to the breakpoint as established with the V-type position effects in *Drosophila* (see reviews by Baker (1968) and Lewis (1950)) and demonstrated with other mouse X-autosome translocations (Russell, 1963), then it may be concluded from the data presented that the *p* locus must be more remote from the breakpoint and hence less frequently inactivated than either the *c*- or *ru-2* loci. Since *p* is

known to lie between *ru-2* and *c* in the linkage group (Eicher, 1970*a*) and since the rearrangement is known to be insertional (Ford & Evans, 1964; Ohno & Cattanach, 1962), the relative levels of *ru-2*-, *p*- and *c*-variegation may be taken to indicate that the inactivation of the autosomal genes results from the presence of X-chromosomal material to either side of them. The data are thus in agreement with the observation that the coats of *Dp/+; ru-2p/ru-2p* females contain *ru-2* areas in addition to *ru-2 p* and *ru-2+p+* (Eicher, 1969; Cattanach, unpublished). They suggest that there is a spread of inactivation into the *ru-2* side of the insertion as well as that into the *c* side. If this is correct the very similar levels of variegation for *ru-2* and *c* would indicate that the two genes are located at approximately the same distance from the breakpoints in the autosomal material. This is in accord with the genetic evidence available on the location of the breakpoints in the linkage group I chromosome (Eicher, 1967, 1970*a*; Wolfe, 1967).

Table 1

'State' of element in X^T	Levels of variegation		
	<i>ru-2</i>	<i>p</i>	<i>c</i>
Xce^i	$48.36 \pm 2.14\%$	34.89 ± 1.97	44.03 ± 0.55
Xce^c	$58.44 \pm 2.20\%$	43.61 ± 2.15	57.30 ± 0.82

Levels of autosomal gene variegation in *Dp* females carrying Xce^i and Xce^c in their rearranged (X^T) X-chromosomes.

+, $t_{96} = 3.28; P < 0.01$. ++, $t_{86} = 2.99; P < 0.01$. + + +, From Cattanach & Perez (1970); $t_{869} = 13.46; P < 0.001$.

Table 1 also shows the levels of *c*-, *p*- and *ru-2*-variegation obtained with the rearranged X-chromosomes carrying the two 'states' of the controlling element. It can be seen that no matter which autosomal gene is considered, *Dp* females carrying Xce^i in their rearranged X exhibit lower mean levels of variegation than do those carrying Xce^c . The variegation for *ru-2* and *p* thus appear to respond to the controlling element in the same manner as the *c*-variegation.

4. DISCUSSION

The significance of the observation that the levels of variegation for *ru-2*, *p* and *c* all appear to respond in the same way to the controlling element system is dependent upon whether or not the variegations observed result from the proximity of X-chromosomal material to either side of the autosomal insertion. The evidence rests on the relative frequencies with which the three genes become inactivated when the rearranged X is inactivated, and this can be deduced from the variegated phenotypes compared singly, as described in the present communication, or when two or more genes are variegating together within single animals. There is little doubt that the *c* locus is inactivated more frequently than the *p* locus for, as originally observed (Cattanach, 1961) and as confirmed in this paper, *c*-variegation is considerably more extensive than *p*-variegation. In addition, females hetero-

zygous for either the balanced or unbalanced forms of the translocation and carrying both p and c^{ch} on their linkage group I chromosomes clearly show three colours in the coat. This can be observed the more readily with the balanced form of the translocation on a a background for the third colour is that of the brownish $c^{ch}c$ (equated to c^{ch} /deficiency) and hence quite distinguishable from the whitish pc^{ch} and black $p^{+}c^{+}$. The evidence that the $ru-2$ locus is inactivated more frequently than the p locus is similarly based on the observation that $ru-2$ -variegated is clearly more extensive than p -variegation (this communication). The detection of $ru-2$ areas, in addition to $ru-2p$ and $ru-2^{+}p^{+}$ in the coats of $Dp/+ ; ru-2p/ru-2p$ females also indicates this to be the case though here doubts could be raised as to the correct identification of the third colour as $ru-2$. Conceivably the $ru-2$ -like areas could result from the admixture of the hair of the other two colours, or could even be that of p on the intermediate $ru-2$ background.

Another piece of evidence that inactivation spread into the $ru-2$ side of the insertion as well as into the c side has been provided by Eicher (1969). Convincing areas of p cannot normally be detected in $Dp/+ ; pc^{ch}/pc^{ch}$ females—only c^{ch} , pc^{ch} and $p^{+}c^{+}$ (Cattanach, 1961). This is true whether the variegation is studied on an a^{+} or a a background and with either 'state' of the controlling element (Cattanach, unpublished). However, Eicher (1969) has found a variant line in which p -variegation may be expressed without c^{ch} -variegation. The evaluation of this finding is difficult since the nature of the 'mutational' event responsible is not known but unless some other major chromosome change is involved, the observation requires a spread of inactivation from the $ru-2$ side of the insertion.

The above data provide fairly good evidence that there is a spread of inactivation into both sides of the insertion and this is what would be expected if both parts of the divided X -chromosome were subject to inactivation. Unfortunately the distribution of X -linked genes in the rearranged chromosome does not allow this to be determined by genetical means (Cattanach & Isaacson, 1965) but the cytological evidence would indicate this is so. Thus, if heteropyknotic behaviour and late DNA replication are taken as evidence of genetic inactivation, both parts of the divided X are inactivated (Evans *et al.* 1965; Ohno & Cattanach, 1962). Until recently the evidence from the labelling studies were weakened by the fact that the mouse X is not as conspicuously late-replicating as that of some other mammals and the distal part replicates its DNA later in the cell cycle than the proximal part. However, Nesbitt & Gartler (1970) have now been able to demonstrate late DNA replication of the mouse X -chromosome much more satisfactorily by the study of cells labelled early in S. When the long X of $T(1;X)Ct$ heterozygotes was investigated it was found that the whole chromosome replicates its DNA late in the cell cycle. There thus would seem to be little doubt that both parts of the divided X are subject to inactivation.

The above conclusion is at variance with the hypothesis that X -inactivation results from a spread of inactivating material along the chromosome. However, although such a spread of inactivation has been amply demonstrated in autosomal

material attached to the X, there is as yet no evidence of such a process in the X itself. Similarly, the concept that regions of X isolated from the postulated inactivation centre are not inactivated is based on the finding that in one of Russell's (1963) X-autosome translocations variegation for only one of two autosomal genes occurs even though the breakpoint is located between them. It has not, in fact, been established that any other gene in the vicinity of the breakpoint is so affected, nor has it been demonstrated that genes on one part of the divided X are free of the inactivation process. An alternative mechanism to account for the lack of variegation for the single autosomal gene has been proposed by Eicher (1969). At least for the *flecked* translocation the balance of the available data indicate that both parts of the divided X are subject to the inactivation process. This and the spread of inactivation into each side of the insertion are thus in accord with observations on the equivalent type of rearrangement in *Drosophila*, e.g. in the insertional rearrangement *Dp(1;3)(w^m)264-58a* evidence has been obtained which indicate there is a spread of inactivation into both sides of a euchromatic region inserted into a region of heterochromatin (Baker, 1968). The difference between the levels of autosomal gene variegation observed in Dp females carrying *Xceⁱ* and *Xce^c* in their rearranged chromosomes may now be considered.

The data presented in Table 1 clearly demonstrate that the levels of *ru-2*- and *p*-variegation expressed in Dp females differ according to whether they have inherited a rearranged X carrying *Xceⁱ* or *Xce^c*. That the levels of each variegation are modified in the same way as the *c*-variegation provides strong presumptive evidence that the inactivation of these loci, like that of the *c* locus, is under the influence of the controlling element system, i.e. the spread of inactivation into both sides of the insertion is under the control of the one element. The alternative possibility is that there are two or more controlling elements—at least one to the region of X on each side of the insertion. This would seem to be highly improbable for it would require that the element responsible for the spread of inactivation into the second side of the insertion should have two alternative 'states' equivalent to *Xceⁱ* and *Xce^c* and the corresponding 'states' of each of the elements should be present in each tested rearranged X chromosome.

The *ru-2* and *p* loci are separated by a distance of about 3 crossover units (Eicher, 1970*a*) and the *c* locus lies 12–14 units beyond *p* (Dunn & Bennett, 1967). If the spreads of inactivation into each side of the insertion are equivalent, the levels of *ru-2*- and *c*-variegation would indicate that the two breakpoints are located about the same distance away from each gene. This is in agreement with the genetic evidence available on the location of the two breakpoints in the linkage group I chromosome (Eicher, 1967, 1970; Wolfe, 1967). The *p* locus must therefore lie closer to the *ru-2* side of the insertion than to the *c* side and it would then be expected that the *p*-variegation results primarily from the spread of inactivation through *ru-2* and less from the spread through *c*. The variegation in Dp/+; *ru-2c^{ch}/ru-2c^{ch}* has not so far been investigated, but the fact that convincing *p* areas are not normally produced in Dp/+; *pc^{ch}/pc^{ch}* females suggests that the spread of inactivation into each side of the insertion is fairly rigidly controlled, i.e. they do

not occur independently of each other. This would further suggest that a single control system is operating.

Several investigators have suggested that X-inactivation may result from the action of inactivators scattered along the length of the X which respond to the control of a master gene or receptor site. Lyon (1968) and Eicher (1969) have suggested that the controlling element system may be one of the subsidiary inactivators while, in consideration of the fact that the element influences both the translocation-induced *c*-variegation and the heterozygous expression of two X-linked genes, we have proposed that it is the master gene or inactivation centre which controls inactivation of the whole X chromosome (Cattanach *et al.* 1970). The data presented would tend to support the latter interpretation.

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