Lack of inactivation of a mouse X-linked gene physically separated from the inactivation centre

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SUMMARY

Previous evidence had shown that, when a mammalian X-chromosome is broken by a translocation, only one of the two X-chromosome segments shows cytological signs of X-inactivation in the form of late replication or Kanda staining. In the two mouse X-autosome translocations T(X;4)37H and T(X;11)38H the X-chromosome break is in the A1-A2 bands; in both, the shorter translocation product fails to exhibit Kanda staining. By in situ hybridization, the locus of ornithine carbamoyltransferase (OCT) was shown to be proximal to the breakpoint (i.e. on the short product) in T37H and distal to the breakpoint in T38H. Histochemical staining for OCT showed that in T38H the locus of OCT undergoes random inactivation, as in a chromosomally normal animal, whereas in T37H the OCT locus remains active in all cells. The interpretation is that, when a segment of X-chromosome is physically separated from the X-inactivation centre, it fails to undergo inactivation. This point is important for the understanding of the mechanism of X-inactivation, since it implies that inactivation is a positive process, brought about by some event that travels along the chromosome. It is also relevant to the interpretation of the harmful effects of X-autosome translocations and the abnormalities seen in individuals carrying such translocations.

INTRODUCTION

Valuable information concerning the process of X-inactivation has been derived from study of X-autosome translocations in the mouse and man.

In the mouse Russell (1961, 1963, 1983) and Cattanach (1961) showed inactivation of autosomal genes involved in X-autosome translocations. Russell (1963; Russell & Montgomery, 1970) showed that in some cases genes on one side of the translocation break were not inactivated, although those on the other side were. This suggested that if an X-chromosome was broken by a translocation the segment that was physically separated from the inactivation centre did not undergo inactivation (Lyon, 1963, 1966). Evidence in favour of this came from the work of Russell & Cacheiro (1978) and of Rastan (1983) who showed that only one of

Key words: mouse, X-chromosome, translocations, ornithine carbamoyltransferase, inactivation centre.
the two translocation products was late replicating (Russell & Cacheiro, 1978) or showed dark staining by the Kanda method (Rastan, 1983) in any cell.

However, although there is good evidence that only one segment of an X-autosome translocation shows the properties of late replication or Kanda staining that are associated with inactivation, so far as we are aware there are as yet no data on the actual expression of X-chromosome genes in the segment separated from the inactivation centre. An opportunity to test this point comes from the two mouse X-autosome translocations T37H and T38H reported by Searle, Beechey, Evans & Kirk (1983).

In both T37H and T38H, the translocation break is close to the locus of the gene sparse-fur, spf (Fig. 1), which DeMars, Levan, Trend & Russell (1976) showed to involve deficiency of the enzyme ornithine carbamoyltransferase (E.C.2.1.3.3) (OCT). Wareham and colleagues (Wareham, Howell, Williams & Williams, 1983; Wareham & Williams, 1986) using a histochemical method for OCT in mouse liver showed that chromosomally normal females heterozygous for OCT deficiency (+/spf) showed clear mosaicism for positive and negative cells. If the inactivation of the OCT locus was perturbed in females heterozygous for translocation T37H or T38H and also heterozygous for OCT one would expect the pattern of mosaicism in the liver to be altered. This paper describes an investigation of this point.

Searle et al. (1983), on the basis of genetic recombination of spf with the translocation break, suggested that in both T37H and T38H the locus of spf was proximal to the breakpoint. As this point was critical to the present work, the position of OCT was retested, in this case using the criterion of in situ hybridization.

![Diagram](image.png)

Fig. 1. Diagram showing the location on the X-chromosome of the spf and wild-type OCT genes in the mice carrying the translocations used in the study. The position of the Xce locus is also marked. Solid lines indicate X-chromosomal material, broken lines indicate autosomal material. The translocation product not directly relevant to the discussion has been omitted. (Chromosomes not drawn to scale.)
The expectation was that, if the OCT locus was proximal to the translocation breakpoint and therefore on the small translocation product, the wild-type allele on the translocated X would remain active in all cells. Therefore the expected mosaic pattern of histochemically positive and negative cells in the liver would not be present and all cells would appear OCT positive. On the other hand, if the OCT locus were distal to the break and therefore on the large translocation product, physical continuity with the inactivation centre would not be broken. Inactivation would then occur normally and the typical mosaicism of positive and negative cells in liver would be present. Histochemistry for OCT was therefore used to investigate these features.

**MATERIALS AND METHODS**

**Breeding**

Females carrying translocations T37H or T38H, kindly provided by Dr A. G. Searle, were crossed to males hemizygous for *spf*. The female offspring of this cross were necessarily heterozygous for *spf*, but had various possible chromosomal constitutions. The chromosomal types were determined by means of cultures of tail blood, according to the method of de Boer *et al.* (1977). Heterozygotes, T37H+/+*spf* or T38H+/+*spf*, were crossed again to *spf/Y* males.

Animals for the histochemical studies were T37H+/+*spf* or T38H+/+*spf* females and their +/+*spf* sisters, from the first generation of crossing to *spf/Y*. Those used for *in situ* hybridization were from subsequent generations.

**In situ hybridization**

Air-dried mitotic preparations were obtained from 2-day LPS (Lipopolysaccharide W, Difco)-stimulated spleen cultures. Since we were unsuccessful in our attempts to induce replication G banding by the technique of Zabel *et al.* (1983), some of the slides were G-banded by a modification of the ASG/trypsin method (Galimore & Richardson, 1973) and up to 60 suitably spread and banded metaphases photographed and their positions recorded. The remainder of the slides were left untreated.

For *in situ* hybridization we followed the technique of Buckle *et al.* (1985). Slides were treated with 100 µg ml⁻¹ of RNAase in 2 × SSC (0-3 M-NaCl–0-03 M-sodium citrate) at 37°C in a moist chamber for 1 h, washed in four changes of 2 × SSC and dehydrated by 1 min rinses in an ethanol series of 10, 50, 75, 95 and 100 %. After air drying, the chromosomes were denatured in 70 % formamide and 0-1 mM-EDTA in 2 × SSC at pH7 at 65°C for 4 min, washed in four changes of 2 × SSC, dehydrated through the ethanol series and air dried. The slides were acetylated in 0-25 % acetic anhydride in 0-1 M-triethanolamine at pH8 for 10 min at room temperature, washed in four changes of 2 × SSC, dehydrated through the ethanol series and air dried.

A human cDNA probe for ornithine carbamoyltransferase was kindly provided by Dr Kay Davies.

Three separate samples were labelled with tritium (Amersham) by nick translation using a mixture of labelled nucleotides (³H-ATP, ³H-CTP, ³H-TTP, all at 1 mCi ml⁻¹) to a specific activity in the range of 1.5–5.0 × 10⁶ cts min⁻¹ µg⁻¹ DNA.

For the metaphases, the probe was brought to a final concentration of between 300–500 ng ml⁻¹ in 50 % formamide, 5 × Denhardt’s solution, 5 × SSPE (0-9 M-NaCl–50 mM-Na₂H₂PO₄–5 mM-EDTA) at pH7-2, 10 % dextran sulphate and 200 µg ml⁻¹ salmon sperm DNA. Before applying to the slides, the mixture was denatured by boiling for 2–3 min and plunging into ice. 30 µl of the hybridization mixture was applied per slide under a 22 × 50 mm cover slip which was sealed with rubber solution and the slides were incubated at 42°C for 16–20 h in a sealed chamber moistened with 2 × SSC. After hybridization, the cover slips were removed in 5 × SSC, the slides washed for 1–2 h in 2 × SSC at room temperature followed by stringency washes consisting of two changes of 2 × SSC at 65°C over 1 h and ½ h washes at room temperature in
0·2 × SSC and 0·1 × SSC. Slides were dehydrated through an ethanol series and air dried. For autoradiography, they were dipped in total darkness in Ilford Nuclear Emulsion L4 diluted 1:1 with distilled water and warmed to 55°C. Dipped slides were stored in light-proof boxes with desiccant at 4°C and left to expose for between 8 and 35 days, after which time they were developed in Kodak D19 at 20°C for 5 min, rinsed in a stop bath, fixed in Amfix diluted 1:4 with added hardener and washed in gently running water for 1 h. The developed slides were stained for 10–20 min in a 1:10 dilution of Giemsa (Merck) in 6·8 pH buffer, rinsed in buffer, air dried and examined.

**Histochemical studies**

Multiple sections from each lobe of the liver from two T37H and two T38H mice were stained for the presence of normal OCT using a modification of the technique of Mizutani described by Wareham *et al.* (1983).

Sections were incubated for 15 min at room temperature in metal-salt precipitation medium containing carbamyl phosphate (3 mg), ornithine (5 mg), sucrose (0·8 g) and 1% lead nitrate (1 ml) buffered by 0·05 M-triethanolamine pH 7·2 (4 ml) and water (5 ml). The final reaction product was visualized by immersion of the section into deionized water presaturated with H₂S gas. Liver sections from chromosomally normal +spf/++, ++/+++ and +spf/y mice were also processed at the same time.

**RESULTS**

**In situ hybridization**

The mitotic chromosomes of T37H and T38H have been previously described and the translocation breakpoints determined as being in the 4D3 and XA2 bands for T37H and in 11E1 and XA2 for T38H (Searle *et al.* 1983). Both translocations give long and short marker chromosomes and in T37H these consist of an estimated 87% of chromosome 4 plus 81% of the X-chromosome (4X) and 13% of chromosome 4 plus 19% of chromosome X (X⁴). In T38H, they consist of an estimated 92% of chromosome 11 plus 86% of the X-chromosome (11X) and 8% of chromosome 11 plus 14% of chromosome X (X¹¹) (Searle *et al.* 1983). Since the translocations are so unequal, the translocated chromosomes are easily recognized and furthermore, in the majority of unbanded mitotic cells, the long marker breakpoints can be identified by the change in morphology, or a constriction, at the point at which the autosomal chromatin adjoins X chromatin. The short markers are not thus differentiated but they can be approximately apportioned into X and autosomal segments by reference to the previously determined percentage content.

Slides were scored under a ×100 oil-immersion lens by counting the total number of grains overlying all the chromosomes in a metaphase spread and recording the number and distribution of grains overlying the long and short markers. For convenience of recording, grains observed at, or closely distal to, the breakpoint in the long marker or closely proximal to the breakpoint in the short marker, were referred to as being in the XA2 region. In cases in which G-banded metaphases had been photographed before *in situ* hybridization, the cells were rephotographed and silver grain distribution compared in relation to the bands.
If the positions of the silver grains bore no relationship to the location of the OCT sequence but were randomly distributed over all the 40 mouse chromosomes, the numbers of grains overlying the long and short markers would be in proportion to their lengths. The proportions of the translocated segments of the X-chromosome and chromosomes 4 and 11 in T37H and T38H are given above and if these are multiplied by the length of each normal chromosome as a percentage of the total mouse diploid genome \( [X = 6.18\%; 4 = 5.89\%; 11 = 4.63\% \text{ (Nesbitt & Francke, 1973)}] \) for the haploid genome, and therefore half these values for the diploid genome, one obtains values of 5.06% and 4.79% silver grains expected to overlie the long markers in T37H and T38H, respectively, and 0.97% and 0.62% to overlie the short markers. The grain counts obtained are summarized in Table 1. Clearly, in T37H the numbers expected from a random distribution were observed overlying the long marker (expected 5.06%, observed overall mean 5.05%; \( \chi^2 = 0.000437, P = 0.983 \)) whereas three times the expected numbers (expected 0.97%, observed overall mean 3.01%; \( \chi^2 = 86.18, P = 1.6 \times 10^{-20} \)) were observed overlying the short marker. In T38H, the reverse was observed, a slightly higher than expected number were observed overlying the short marker (expected 0.62%, observed overall mean 0.9%; \( \chi^2 = 2.74, P = 0.098 \)) but over twice as many overlying the long marker (expected 4.79%, observed overall mean 10.76%; \( \chi^2 = 189.62, P = 3.8 \times 10^{-43} \)). (Yates' correction was used throughout in calculating \( \chi^2 \).)

These observations become even more significant if the silver grain distribution over the markers is considered. In the long marker of T37H, only 0.87% of the total of 5.05% were observed overlying the XA2 region but 2.91% of a total grain score of 3.01% overlay this region in the short marker. In the XA2 region of the long marker of T38H, however, 6.9% out of a total of 10.76% were observed whereas, in the short marker, 0.74% were observed out of a total of 0.90%. It is therefore concluded that the OCT sequence must lie on the short marker in T37H but on the long marker in T38H.

**Histochemical observations of OCT activity**

The sections from chromosomally normal +spf/++, ++/+ and +spf/Y mice displayed the expected staining pattern: OCT normal females showed staining in all hepatocytes (Fig. 2A), heterozygous females were mosaics for OCT (Fig. 2B), all hepatocytes in sections from spf/Y males lacked staining (Fig. 2C).

In the T37H+/+spf mice the staining resembled that seen in the ++/+ controls expect that, very rarely, negative cells were seen, at least one negative cell being found in 13 out of 68 sections (Fig. 2D).

In two sequential sections from the right ventral lobe of one animal approximately 0.01% OCT-negative cells, restricted to the same small region in both sections, were present. It is suggested that these OCT-negative cells result from the loss of the small translocation marker. These findings therefore indicate that the OCT locus on the short translocation product is remaining active in all cells, despite the essentially random inactivation of the normal X or large translocation
Table 1. Comparison of grain count over T37H and T38H marker chromosomes with random expectation

<table>
<thead>
<tr>
<th>Translocation</th>
<th>Probe sample</th>
<th>Days development</th>
<th>Total grains per 100 mitotic cells</th>
<th>Total over ‘A&lt;sub&gt;1&lt;/sub&gt;–A&lt;sub&gt;2&lt;/sub&gt;’ bands of long marker</th>
<th>Total over ‘A&lt;sub&gt;1&lt;/sub&gt;–A&lt;sub&gt;2&lt;/sub&gt;’ bands of short marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>T37H</td>
<td>1</td>
<td>12</td>
<td>390</td>
<td>10 (2-56%)</td>
<td>2 (0-51%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>19</td>
<td>416</td>
<td>11 (2-64%)</td>
<td>3 (0-72%)</td>
</tr>
<tr>
<td></td>
<td>2(a)</td>
<td>8</td>
<td>670</td>
<td>56 (8-36%)</td>
<td>9 (1-34%)</td>
</tr>
<tr>
<td></td>
<td>2(b)</td>
<td>12</td>
<td>291</td>
<td>13 (4-47%)</td>
<td>2 (0-68%)</td>
</tr>
<tr>
<td></td>
<td>2(b)</td>
<td>19</td>
<td>294</td>
<td>14 (4-76%)</td>
<td>2 (0-87%)</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td></td>
<td>2061</td>
<td>104 (5-05%)</td>
<td>18 (0-87%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4-12*</td>
<td>5-06 %†</td>
</tr>
<tr>
<td>T38H</td>
<td>1</td>
<td>12</td>
<td>529</td>
<td>46 (8-70%)</td>
<td>22 (4-16%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12</td>
<td>434</td>
<td>52 (11-98%)</td>
<td>30 (6-91%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>19</td>
<td>448</td>
<td>37 (8-23%)</td>
<td>26 (5-80%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>19</td>
<td>358</td>
<td>41 (11-45%)</td>
<td>33 (9-22%)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>35</td>
<td>544</td>
<td>75 (13-79%)</td>
<td>49 (9-01%)</td>
</tr>
<tr>
<td></td>
<td>2(b)</td>
<td>12</td>
<td>252</td>
<td>25 (9-92%)</td>
<td>17 (6-75%)</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td></td>
<td>2565</td>
<td>276 (10-76%)</td>
<td>177 (6-90%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4-27*</td>
<td>4-79 %†</td>
</tr>
</tbody>
</table>

* Mean per cell.
† Expected value.
product found by Rastan (1983). In the T38H mice a quite different pattern of staining was seen. In this case the staining in each section resembled that seen in spf heterozygous control animals (Fig 2E). The proportion of OCT-positive cells in each lobe was estimated using a point counting technique. In one animal, the mean proportion of positive hepatocytes was $70 \pm 15\%$, while in the other the mean was $32 \pm 14\%$. These proportions fall within the range seen in chromosomally normal spf heterozygotes; in a study of 12 animals the overall proportion of positive cells was $45\%$, the means for the liver of each animal varied between $29\%$ and $77\%$, while the means for the lobes varied between $4\%$ and $91\%$ (Wareham & Williams, 1986). The results in the T38H mice suggest that, when the OCT locus is on the long marker chromosome so that the physical continuity with the inactivation centre is maintained, normal inactivation of the OCT locus occurs.

**DISCUSSION**

The results of the *in situ* hybridization show clearly that in T37H the OCT locus is on the small translocation marker and in T38H it is on the long marker. This is in contrast to the view of Searle *et al.* (1983) who, on the basis of genetic recombination, assumed that OCT was proximal to the breakpoint in both translocations. The *in situ* results must be regarded as more conclusive than those of recombination. In addition, the revised location of the break in T38H is in line with the cytogenetic studies of Searle *et al.*, since the small marker of T38H is smaller than that of T37H.

The histochemical studies indicate that in heterozygotes for T37H the OCT gene remained active in all cells, whereas in T38H inactivation of OCT occurred more or less randomly, as in chromosomally normal spf/+ heterozygotes. For both translocations there is cytogenetic evidence, from Kanda staining, that the long marker chromosome undergoes near random inactivation. Rastan (1983) found that with T37H 29% of cells and with T38H 36-5% of cells had the long marker chromosome dark staining. Furthermore, in T37H there is evidence of spread of inactivation from the X into the autosome of the long marker, since variegation occurs for the chromosome 4 coat colour gene brown, b (Searle *et al.* 1983). By contrast in both translocations the small marker never shows dark Kanda staining (Rastan, 1983). Thus, the cytogenetic evidence suggests that the long marker and the normal X-chromosome undergo inactivation almost randomly, but the short marker escapes inactivation. The fact that the OCT locus, when on the short marker in T37H, remains active in all cells, provides evidence at the level of the gene product that this is indeed the case. The random inactivation of the locus, when on the long marker in T38H, makes it unlikely that the failure of inactivation in T37H is due to any effect of proximity to a translocation breakpoint. Rather, it appears to be due to physical separation of the locus from the inactivation centre.

Evidence for the location of the inactivation centre on the mouse X comes from the recent work of Rastan & Robertson (1985). They studied a series of embryo-derived cell lines, each carrying one normal and one deleted X-chromosome, the
length of the deleted chromosome differing between the lines. In those lines in which the deleted X extended to band D or more distally, cytological evidence of inactivation of one or other X could be induced by appropriate culture conditions, whereas in the lines with a shorter deleted X no sign of inactivation could be induced. The authors concluded that those deleted X-chromosomes with band D carried inactivation centres and the others did not. Rastan had previously presented evidence that in Searle's translocation, T(X;16)16H, the centre was carried in the distal segment. Together with the deletion data, this placed the centre in the distal part of band D (Rastan & Robertson, 1985). This location is consistent with the centre being identical with the Xce locus (Fig. 1), different alleles of which determine the probability of inactivation of the X on which they are carried (Cattanach, 1975; Johnston & Cattanach, 1981).

The evidence that a segment of X separated from the inactivation centre remains active in all cells has important implications concerning the mechanism of X-inactivation. First, it suggests that inactivation is a positive process. Theoretically, since all X-chromosomes except one become inactive, one could have envisaged that the X had an inherent tendency to become inactive, unless it received a signal to remain active, emanating from the centre on the single active X. This appears not be the case. It seems that X-chromosomal material remains active unless it receives an inactivating signal from the centre. On the active X, as suggested by Rastan (1983) the centre must be nonfunctional. Second, this work provides further evidence that inactivation entails the travel of some process along the chromosome, so physical continuity with the inactivation centre is required. Some evidence of travel of inactivation was already provided by the inactivation of attached autosomal material in X-autosome translocations. However, Cattanach (1974) had argued that this autosomal inactivity could in fact be a position effect due to the proximity of the autosomal material concerned to heterochromatic X-chromosomal material. Whether or not this is indeed the explanation of autosomal inactivation, there is now clear evidence of the need for physical continuity and hence for travel of the inactivation process in the X-chromosome itself.

Further significance of this work arises from the new insight it provides into the deleterious effects of X-autosome translocations. It was already known that if the translocated X became inactive the spread of inactivation into attached autosomal material would lead to functional monosomy for the autosomal genes concerned and hence to deleterious effects. We now know that, in addition, there is excess dosage of X-chromosomal genes on the noninactivated translocation product.

Fig. 2. Fixed, frozen cryostat sections (5 μm) from mouse liver showing OCT staining (×240). (A) +/+ mouse. The perportal/centrilobular gradient is typical of this and other liver enzymes. (B) +spf/+ mouse, showing the mosaic pattern of staining due to X-chromosome inactivation. (C) +spf/y mouse. Normal OCT activity is absent. (D) T37H+/+ mouse. Virtually all cells are positive for the enzyme. The single small group of negative cells in the centre of the field is presumed to have lost the short translocation marker chromosome (X4). (E) +T38H/spf+ mouse showing the mosaic staining pattern typical of random X-chromosome inactivation.
Thus, incorrect X-chromosome dosage may be a partial explanation of abnormalities seen in individuals heterozygous for X-autosome translocations in which cells with the translocated X inactive and normal X active are present. Finally, it provides an additional explanation for the finding that in most human X-autosome translocations only cells with the translocated X active are present.

We are grateful to Elaine Moore and Peter Glenister for assistance with the animals, to Veronica Buckle and John Whittaker for advice and assistance, to David Papworth for statistics, and to the Cancer Research Campaign for partial support of this study.

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*(Accepted 19 April 1986)*