

Studies on Metatherian Sex Chromosomes

III.* The Use of Tritiated Uridine-induced Chromosome Aberrations to Distinguish Active and Inactive X Chromosomes

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Abstract

The paternal X inactivation system of kangaroos has been investigated in this study by using tritiated uridine-induced chromosome aberrations to distinguish the active from the inactive X. Previous work in eutherian mammals has demonstrated that constitutive heterochromatic chromosome regions are less susceptible to breakage by tritiated uridine than euchromatic regions. The results of a comparison between the paternal X chromosome of a wallaroo $\times$ red kangaroo hybrid female and the two X chromosomes of a red kangaroo female suggested that the facultative heterochromatin of the X is also less susceptible to breakage by this treatment. However there were significantly more breaks of the paternal X in fibroblasts than in lymphocytes of the hybrid female, which agrees with biochemical findings suggesting activation of the paternal X in fibroblasts. Our results strengthen the suggestion of other workers that the reduced number of aberrations in heterochromatin occurs because such breaks occur principally when the DNA and labelled RNA are in apposition during transcription. Some evidence was found of an apparent toxicity effect of the tritiated uridine solution on the cells.

Introduction

X inactivation is the process by which all X chromosomes in excess of one are inactivated in the somatic tissues of female therian mammals early in development and become late-replicating and heterochromatic. The process is random in eutherian mammals but is one of preferential paternal X inactivation in metatherians (reviewed in Lyon 1974). The inactive X of both groups of mammals is said to be facultatively heterochromatic. This phenomenon provides an opportunity for examining control over transcription exerted at the whole chromosome level. A method for studying the RNA synthetic activity of chromosomes has been proposed by Klevecz and Hsu (1964). Beta rays from tritiated uridine can cause chromosomal aberrations. Findings by Klevecz and Hsu (1964) and Natarajan and Sharma (1971) show that active euchromatic chromosomal regions have more aberrations than constitutive heterochromatic regions. On the basis of present knowledge the most likely reason for this difference is because these aberrations are induced mainly when the uridine is momentarily in proximity to the DNA during transcription. However, a direct demonstration that transcription is necessary for most aberrations has not been made. But whatever the mechanism it is clear that the level of aberrations can be used to differentiate between actively transcribing portions of the DNA and inactive portions.

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In hybrids between some species of kangaroos the paternal and maternal X chromosomes are of different sizes and so can be distinguished cytologically (Sharman 1971). The object of this study was to use these hybrids to determine the level of breakage in the paternal X chromosome of a female hybrid kangaroo and compare this with X chromosome breakage in a normal female kangaroo. Studies on the timing of DNA replication (Sharman 1971) and on sex-linked enzymes (Cooper *et al.* 1971; Richardson *et al.* 1971) indicate that kangaroo lymphocytes have paternal X inactivation. The investigation of RNA synthesis in lymphocytes was undertaken in the anticipation that the tritiated uridine method would also support the idea of paternal X inactivation. However, findings by Cooper *et al.* (1975a, 1975b) on the expression of sex-linked enzymes in fibroblasts suggest that at least some genes on the paternal X are active. Perhaps surprisingly these cells still contain a late-labelling X, so that late DNA replication cannot be equated with inactivity for kangaroo fibroblasts (Cooper *et al.*, unpublished data). The study of tritiated uridine-induced chromosomal damage in fibroblasts was carried out because the method provides an independent and probably more direct assessment of the level of activity than timing of DNA synthesis. It also enables one to test whether the paternal X chromosome as a whole is inactive or whether it has some degree of activity.

Materials and Methods

The individual animals used were: K2 ♀, red kangaroo (*Macropus rufus* = *Megaleia rufa* Desmarest, 1822), and OK1 ♀, hybrid between wallaroo ♀ (*Macropus robustus robustus*; Gould 1841) and red kangaroo ♂ (*M. rufus*), from the marsupial collection at Macquarie University. The tritiated uridine used was [5-³H]uridine (specific activity 27.9 Ci/mmol; Radiochemical Centre, Amersham). The cell cultures used were short-term lymphocyte cultures, grown in medium 199 at 37°C for 3 days, and fibroblast cultures, established from ear explants and grown as monolayers in Ham's F10 medium at 35°C. Two hours before harvest, 5–80 µCi/ml of [³H]uridine was added to the cultures. Half an hour before harvest 0.05 ml of a 0.25 µg/ml solution of colcemid was added to arrest the cells at metaphase. Before harvesting, the fibroblast monolayer cultures were detached by treatment with 0.05% (w/v) pronase. Harvesting involved centrifugation, suspension in hypotonic KCl (0.05% w/v), and successive fixations in methanol and acetic acid (3:1). Air-dried chromosome preparations were made and stained in Giemsa solution. They were examined under 1000× magnification and the number of aberrations and the chromosomes in which they occurred were recorded.

Results

The red kangaroo, K2, and the wallaroo–red kangaroo hybrid, OK1, have diploid chromosome numbers of 20 and 18 respectively. Their karyotypes are shown in Figs 1a and 1b. Chromosome pairs are numbered according to their relative sizes in the red kangaroo. The types of breaks in the chromosomes caused by [³H]uridine are illustrated in Figs 2–5. While in some cases (Figs 2 and 5) the breaks were clearly distinguishable, in others, particularly those with multiple breaks or poorer metaphase spreads, identification of breaks was much more difficult (for example, Figs 3 and 4). Some difficulties were experienced in distinguishing between true breaks and achromatic regions or gaps, and it is probable that the numbers of breaks recorded here included some gaps. While gaps are known to be distinct from true chromatid breaks, their exact nature is not certain, although they are believed to represent genetic damage to the chromosome, including damage induced by radia-

tion (reviewed in Brinkley and Hittelman 1975). In this context, therefore, gaps are as legitimate a measure of transcription as are breaks. Thus the criteria used here were conservative as only breaks and obvious gaps were recorded. Because internal consistency was ensured, this problem does not affect the comparisons made here, although it may mean that the numbers may not be directly comparable with those of other workers.

The majority of aberrations scored were chromatid breaks or loss of part of a chromatid. Some isochromatid breaks—that is, breaks in the same position on both chromatids—were observed, mainly in those cells with a high dose of [^3H]uridine and many breaks. No exchanges between chromatids were observed, as would be expected if the uridine is being added after the end of the S period. These results are similar to those found by Natarajan and Sharma (1971) for the G2 phase.

X Chromosome Breaks

Both OK1 and K2 have a late-replicating red X chromosome (X_r), as assessed by [^3H]thymidine labelling (Sharman 1971; J. A. M. Graves, personal communication). In OK1 one of the small autosomes and the wallaroo X (X_w) are of similar size. In ordinary spreads these two chromosomes can be distinguished without ambiguity, but this was not found to be true for spreads made from the [^3H]uridine-treated cultures. This meant that the obvious method of comparing the breaks in X_r and X_w in OK1 to determine level of activity could not be done. Accordingly a more complex method was devised as set out below. For ease of discussion in presenting this method we make the assumption that the difference in the number of breaks between the active and inactive X is due to the high frequency of breaks which occur during transcription. The reservations attached to this assumption are outlined in the discussion.

The numbers and percentages of breaks in the red kangaroo X chromosomes, in K2 lymphocytes (K2-L), and OK1 fibroblasts (OK1-F) and lymphocytes (OK1-L) are presented in the following tabulation:

Cell type	Total No. of breaks	No. of breaks in X_r	% Breaks in X_r
K2-L	187	11	5.88
OK1-L	414	4	0.97
OK1-F	411	12	2.92

A comparison of K2 and OK1 lymphocytes was made to examine the activity of the X_r chromosomes in the two animals. The following assumptions were made: (1) the total numbers of nucleotides transcribed into RNA on the autosomes of the red kangaroo and the wallaroo are equal; (2) the effect of any difference in the amount of transcription between X_w and X_r on the total cellular transcription can be disregarded.

On the basis of those assumptions, the expected ratio of X_r breaks in K2 (with two X_r chromosomes) to OK1 (with a single paternally derived X_r chromosome) can be calculated for different hypotheses concerning states of activation.

Four possible hypotheses are considered here, each with different expectations which are given in Table 1.

- (1) *No X inactivation.* If both X chromosomes are active, K2 would have double the frequency of X_r breaks in OK1, as it has twice as many X_r chromosomes.

- (2) *Random X inactivation.* In OK1, X_r should be inactivated randomly in half the cells. K2 will have twice the background X_r breakage as it has two X_r chromosomes compared to one in OK1.
- (3) *Maternal X inactivation.* In this case the paternal X_r in OK1 would not be affected and the only difference between K2 and OK1 would be in the amount of background radiation absorbed by the two X chromosomes in K2.
- (4) *Paternal X inactivation.* This ratio should be large provided background, b , is small. If the breakage caused by background radiation is considerable then the method will have a low sensitivity and lose its usefulness.

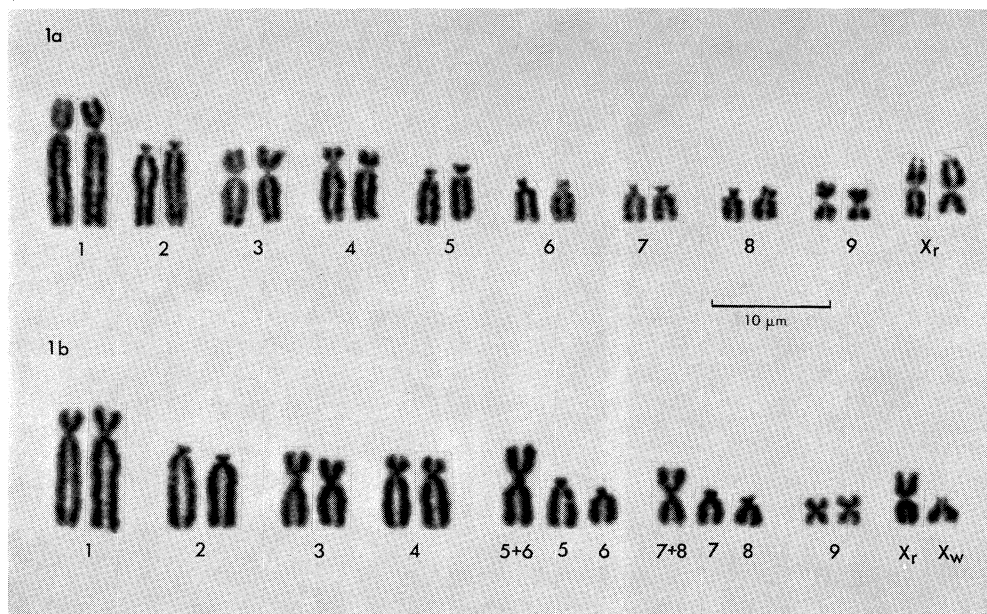


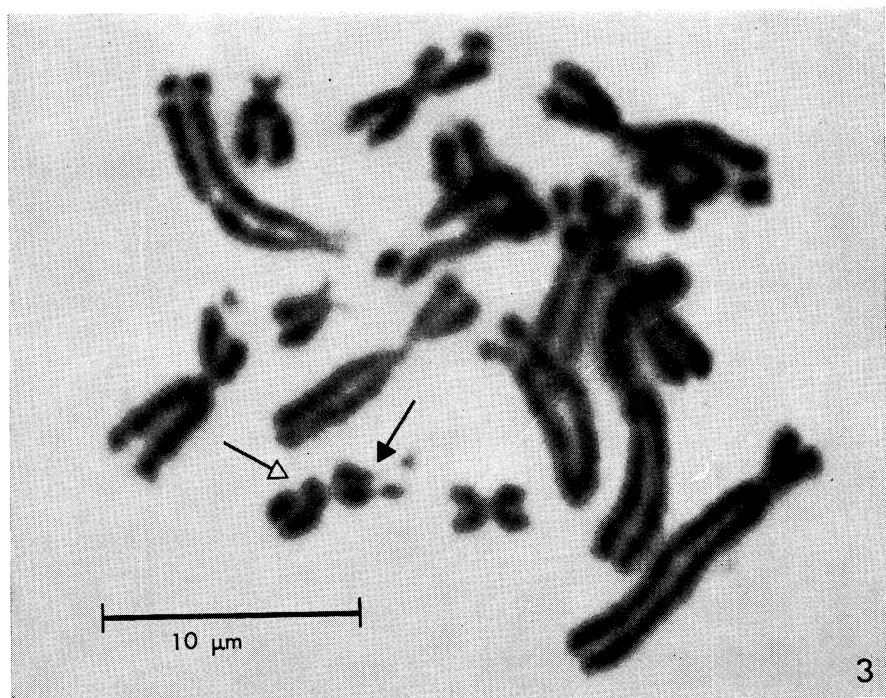
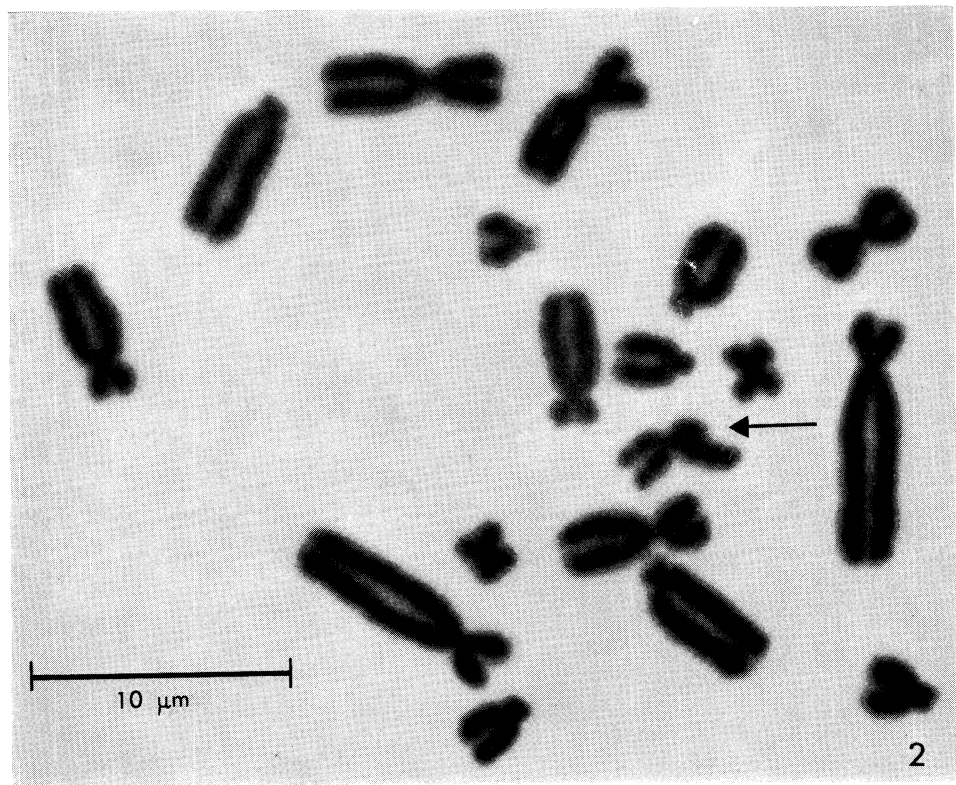
Fig. 1. (a) The karyotype of a female red kangaroo (K2) used in the investigation. (b) The karyotype of the wallaroo $\times$ red kangaroo hybrid (OK1) used in the investigation. The female parent was the wallaroo. The wallaroo has $2n = 16$ and the red kangaroo $2n = 20$; the hybrid has $2n = 18$. Data on this karyotype were kindly supplied by Professor G. B. Sharman.

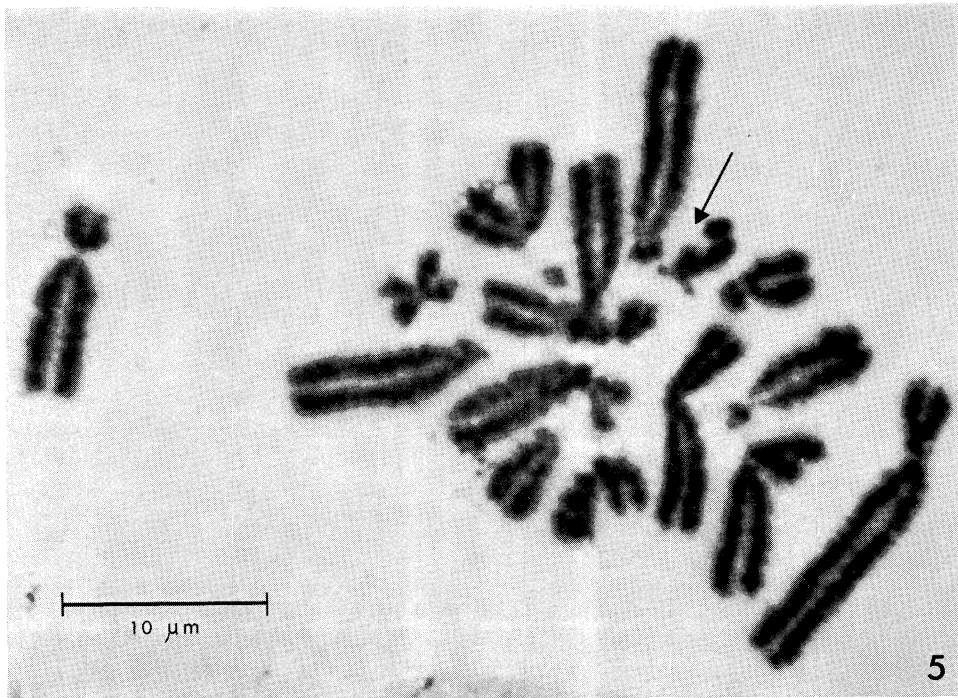
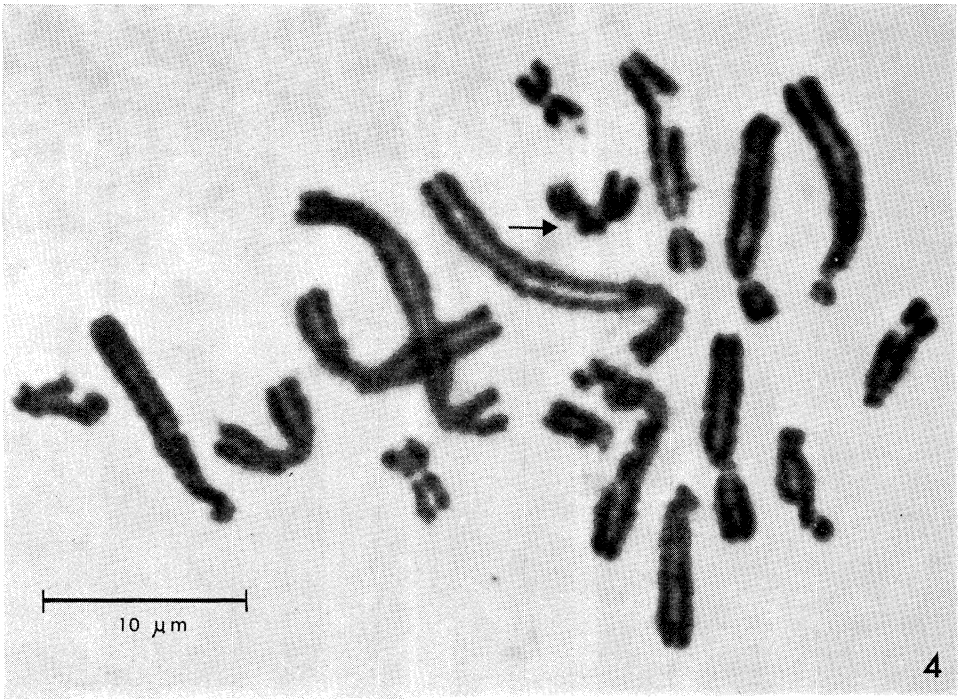
From these expected ratios it is apparent that an observed ratio significantly greater than 2 : 1 can be compatible only with paternal X inactivation. The hypothesis of a 2 : 1 ratio can be tested by a maximum likelihood procedure. The expectations for the proportion of breaks in the autosomes to the proportion in the X chromosomes are $1-p:p$ for K2-L and $1-p/2:p/2$ for OK1-L. The log likelihood is

$$1761 \log(1-p) + 11 \log(p) + 410 \log(1-p/2) + 4 \log(p/2).$$

Differentiating with respect to p and equating the differential to zero gives the

Figs 2 and 3. [^3H]Uridine-treated cultures of OK1. The arrow in Fig. 2 indicates a deletion of the non-satellited arm of X_r ; the break is easily seen. In Fig. 3 the open arrow points to a break in the non-satellited arm whilst the solid arrow indicates a break at the nucleolar organizer of X_r ; this cell was one where scoring the aberrations was more difficult.





Figs 4 and 5. [³H]Uridine-treated cultures of OK1. The arrow in Fig. 4 indicates a break in the non-satellited arm of X_r; the cell has multiple breaks, some of which were not easily scored. In Fig. 5 the arrow indicates one of the few easily scorable breaks in X_w.

maximum likelihood estimate of $p = 0.03827$. When the observed values (tabulation on p. 105) are tested against the expected ones on the basis of this value of p , $\chi^2_1 = 4.13$, $P < 0.05$ is obtained. This indicates that the ratio of K2 : OK1 was just significantly greater than 2 : 1 and thus supports paternal X inactivation.

Table 1. Expected ratios of breaks in the two X_r chromosomes of K2 to those in the one paternally derived X_r of OK1 under four hypotheses of X-inactivation
b denotes the breaks due to background radiation, i.e. those not caused by transcription. The derivations of the ratios are discussed in detail in the text

Hypothesis of X-inactivation	Breaks in 2 X_r chromosomes of K2 : breaks in X_r of OK1
(1) None	$2+2b : 1+b$
(2) Random	$1+2b : 0.5+b$
(3) Maternal	$1+2b : 1+b$
(4) Paternal	$1+2b : b$

The OK1 fibroblast data were collected to test whether the activation of the paternal X fibroblasts found by Cooper *et al.* (1975a, 1975b) for enzyme markers is also evident with this method. If the X_r is not activated in either fibroblasts or lymphocytes, the OK1-L and OK1-F values should be equal. If X_r is totally activated in OK1 fibroblasts and not activated in K2 lymphocytes, the value for X_r breakages in the OK1 fibroblasts should be almost that for K2 lymphocytes—a ratio of $(1+2b) : (1+b)$. The two comparisons were carried out using 2×2 contingency χ^2_1 tests. For OK1-L = OK1-F, $\chi^2_1 = 4.14$, or 3.85 with Yates correction for continuity, for both of which $P < 0.05$. Therefore the levels of X_r breakages in the OK1 lymphocytes and fibroblasts are just significantly different, suggesting an activation of the paternal X in fibroblasts. For K2-L = OK1-F, $\chi^2_1 = 3.05$, $P > 0.05$. Therefore K2-L and OK1-F are not significantly different. This is consistent with activation of the paternal X_r in the fibroblasts.

Distribution of Breaks Among Chromosomes

The number of breaks in any particular chromosome pair is dependent on the amount of transcription taking place. If the level of transcription is equal in all chromosomes, the number of breaks in any pair should be dependent on the amount of DNA present, which corresponds approximately to the relative size of the pair. To test this, the expected proportion of breaks in each chromosome pair on the basis of their relative size was compared with the observed breaks (Table 2). The data on percentage size in the karyotype of individual chromosome pairs were provided by G. B. Sharman (unpublished data), based on karyotype measurements. Chromosomes 6, 7 and 8 were grouped together for the comparison, as it was often difficult to distinguish these three small acrocentrics from each other under the microscope although they can be distinguished when measurements are made on photographs of the spreads.

The χ^2 test showed a significant difference between the observed breaks and those expected on the basis of size, with more breaks found in the larger chromosomes and fewer breaks in the smaller chromosomes. This may be accounted for by the

fact that breaks and deletions are much easier to see in large chromosomes than in small ones, where a greater proportion are terminal and hence more difficult to distinguish. Thus the bias shown in Table 2 is consistent with observed difficulties in detecting breaks in small chromosomes, although we cannot exclude the possibility that the bigger chromosomes are transcribed more often per unit length than the smaller ones.

Table 2. Distribution of breaks among chromosome pairs of the female red kangaroo (K2)

Chromosome	% Karyotype	Expected	Observed	(O-E) ² /E
1	21.43	40.07	59	8.94
2	13.94	26.07	35	3.06
3	12.53	23.43	28	0.89
4	11.92	22.29	24	0.13
5	8.53	15.95	15	0.06
6	6.55	12.25 ^A	13 ^A	12.00
7	5.79	10.83 ^A		
8	5.23	9.78 ^A		
9	5.27	9.85	2	6.26
X	8.81	16.47	11	1.82
Totals	100	187	187	$\chi^2_7 = 33.16$

^A Chromosomes 6, 7 and 8 not distinguished when collecting the data. Therefore, they were treated as a single value when calculating the χ^2 value. See text for further explanation.

Dose-Response Relationships

The number of breaks per cell was counted for the different cell types at several different concentrations of [³H]uridine. Results are shown in Fig. 6. Only for the OK1 fibroblasts were sufficient data obtained to plot a full dose-response curve.

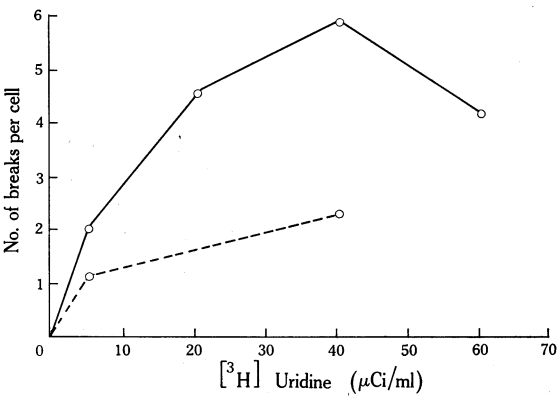


Fig. 6. Number of breaks per cell for OK1 fibroblasts (—) and OK1 lymphocytes (---) treated with different levels of [³H]uridine.

It is apparent that the different cell types showed quite different responses to [³H]uridine in terms of number of breaks. An important feature is the drop in frequency of breaks in OK1-F at a concentration of 60 μCi/ml. This is in contrast to the results found by Klevecz and Hsu (1964) for Chinese hamster fibroblasts. In

their study, the dose-response curve levelled off to about 0.5 breaks per cell, beginning at a concentration of approximately 50 $\mu\text{Ci/ml}$, and there was no evidence of the drop apparent in Fig. 6.

It appears that for the OK1 fibroblasts, at least, high concentrations of [^3H]uridine solution are toxic to the cells, and some form of cell selection may be operating in favour of those cells that have absorbed less [^3H]uridine and hence have a lower level of breakage. Further results connected with this apparent toxicity effect have been obtained.* There are signs that, particularly at higher concentrations, [^3H]uridine may act to lower the mitotic index. In addition, the distributions of numbers of breaks per cell showed considerable heterogeneity that could have been caused by a selective cell mortality effect. However, considerably more complete data, including full dose-response curves for all treatments, are necessary before any conclusions can be drawn about this apparent toxicity effect of the [^3H]uridine solution.

Discussion

It had previously been shown from enzyme and DNA replication data that kangaroo lymphocytes possessed paternal X inactivation. The data for kangaroo lymphocytes given in the tabulation on p. 105 support the conclusion that paternal X inactivation occurs and results in a decreased amount of RNA synthesis by the inactive X chromosome. This conclusion depends on the validity of several assumptions, all of which appear reasonable for these experiments. Two of these are mentioned in deriving the expected ratios (on p. 105). Firstly, comparison between OK1 and K2 lymphocyte ratios rests on the assumption of equal transcription of red kangaroo and wallaroo autosomes. There is no evidence for or against this, but as the two species are very closely related, one would not expect a significant difference in rates of transcription. Secondly, whether or not there is a greater amount of RNA synthesis in the larger X_r than the small X_w will not affect the validity of the results, as any difference due to size would only decrease the OK1 : K2 ratio. Other considerations concern the basis of the method. There should be a linear relationship between the exposure of a chromosome to the [^3H]uridine—that is, transcription plus background—and the number of breaks. This holds as long as there is no chance of incorporation of the tritium into DNA, where it would cause greater breakage. This was achieved here by restricting the treatment period to 2 h, which is the minimum length of G2 for all the *in vitro* mammalian cells listed in Mitchison (1971). The lymphocytes of grey kangaroos, which are closely related to the two species used here, have a G2 period of 3–7 h (Graves 1967). The consequence of this is that all conclusions concerning the relative activities of the X chromosomes can be applied only to the last 2 h of the cell cycle. Whether the same characteristics of activity are shown in the rest of the cell cycle remains to be determined.

The evidence that the aberrations occur as a result of incorporation into RNA during transcription is largely circumstantial. It is possible that other factors may be involved in determining the level of breakage observed, as well as direct radiation damage from the [^3H]uridine during transcription. These background effects could

* These results are lodged as an Accessory Publication with the Editor-in-Chief, Editorial and Publications Service, CSIRO, P.O. Box 89, East Melbourne, Vic. 3002. Copies are available upon request.

include production of other tritiated products, the size of the cellular uridine pool, *de novo* pyrimidine synthesis, and other factors. However, none of these factors should affect the relative break frequencies of the X chromosomes, and hence they are not relevant to a comparison such as in this study. In addition to transcription the only other factor which could plausibly account for differential effects would be a difference in the condensation of the DNA between maternal and paternal X chromosomes, and hence the susceptibility of these chromosomes to radiation damage. Of course decondensation of DNA is usually associated with transcription so that whilst the aberrations may not be occurring during transcription, their incidence on this hypothesis is very likely a direct reflection of the level of transcription. Another less plausible suggestion is that active DNA is less likely to undergo repair than inactive DNA.

All the above factors must be taken into account when assessing the results. Nevertheless, the data do support the conclusion that paternal X inactivation results in decreased transcription by the inactive X chromosome. The extent of this decrease, as expressed in the $K2(X_r) : OK1(X_r)$ ratio, would be expected to be very large if inactivation is complete. However, the observed ratio is approximately 6 : 1. The reasons for this could either be the factors discussed above, especially the amount of background, or incomplete inactivation at the transcriptional level. In order to determine the reasons, the background breakage must be estimated and, in addition, further results for the $K2 : OK1$ comparison obtained.

A more direct test of paternal X inactivation than the one used above would be to compare the maternal and paternal X chromosomes. This must be done in an animal in which the two X chromosomes are distinguishable cytologically, which usually means a species hybrid. However, it cannot be done in OK1 because of the size difference of the two X chromosomes and the consequent scoring problems. The best alternative to a direct comparison would be to compare reciprocal hybrids, where one X chromosome could be tested in maternal and paternal states. Unfortunately a reciprocal hybrid to OK1 could not be obtained for this study, although such hybrids do exist (W. E. Poole, personal communication).

The results for fibroblasts in the tabulation on p. 105 show a significant difference between the level of X_r breaks in OK1 lymphocytes and fibroblasts, that of the fibroblasts being not significantly different from the K2 lymphocyte level. Taken alone, the data are not sufficient for these results to be conclusive. However, they must be assessed in conjunction with fibroblast results obtained by other methods. The reports of Cooper *et al.* (1975a, 1975b) of partial activation of biochemical markers on the paternal X chromosomes in fibroblasts, their further evidence from cloning showing activity of both alleles in individual cells (Cooper *et al.*, unpublished data), and findings of twice the level of G6PD enzyme activity in female fibroblasts as compared to males for one species (Raphael 1974) all suggest some degree of activation of the paternal X. In the light of these findings, the fibroblast data from this study provide another piece of evidence for activity of the paternal X chromosome in fibroblasts.

In general, the results have supported the validity of the method of using [3H]uridine-induced chromosomal aberrations as an index of transcriptional activity. The principal difficulty encountered in this study is the apparent toxicity effect, which is at present unresolved. The lymphocyte data are consistent with findings by other biochemical and cytological methods, and provide information on another

component of the X inactivation system. In contrast to the situation for lymphocytes which is well characterized, the fibroblast problem is poorly understood, and in this case the [^3H]uridine results suggest partial paternal X inactivation, particularly when interpreted in the light of the enzyme data.

The value of this method is that it provides another means of examining X inactivation, with advantages not possessed by existing methods. DNA replication studies do not provide a direct measure of activity. It is true that so far the relationship between late DNA synthesis and inactivity in eutherians has been found to be absolute, but in marsupials this is not so (Cooper *et al.* 1975a, 1975b, and unpublished data). Enzyme studies do measure activity directly, but these findings apply to only a few selected gene loci. By contrast, the method developed in this study can be used to examine the X inactivation system directly at the level of transcription of the chromosome as a whole. However, it is not sufficiently sensitive to determine accurately levels of partial activity, and its greatest usefulness is in detecting gross changes in the level of transcriptional activity.

Other workers have sought to examine transcription using [^3H]uridine autoradiography. Comings (1966), Back and Dormer (1967), Mann and Mukherjee (1970) and Schneider (1970) found a high level of labelling of both X chromosomes, whilst Fujita *et al.* (1966) found one X chromosome was totally unlabelled. The time interval between application of the [^3H]uridine to the cell and fixing ranged from 20 min to several hours. These results must be questioned in the light of findings, discussed by Darnell *et al.* (1973), that the RNA is released within 15 min from the chromosome, and much of it is degraded and subsequently reused. Thus the RNA may not necessarily have been transcribed from the chromosome on which autoradiography reveals it. The breakage method is not open to this criticism and at present seems to be the only candidate for a relatively direct method of measuring transcription, however insensitive it may be.

Acknowledgments

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