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Genetics

Late replication in an X-autosome translocation in the mouse: Correlation with genetic inactivation and evidence for selective effects during embryogenesis

(Cattanach translocation/chromosome replication/5-bromodeoxyuridine/analysis *in vitro* and *in vivo*)

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ABSTRACT **A technique involving 5-bromodeoxyuridine, 33258 Hoechst, and fluorescence microscopy has been used to analyze replication kinetics in cells from embryonic and adult mice bearing the Cattanach [*T(X;7)ICt*] translocation in a balanced or an unbalanced form. In balanced 9- and 13-day female embryos, the translocated X was late replicating in 28 and 22% of the cells, respectively, whereas it was late replicating in only 13% of adult cells. In contrast, in unbalanced females, the translocated X was late replicating in 62 and 70% of 9- and 13-day embryos and in 70% of adult cells. Such divergent late replication frequencies suggest the operation, during development, of selection against cells with extreme genetic imbalance. Within a late-replicating translocated X chromosome, the autosomal segment itself replicated late approximately half of the time, regardless of karyotypic balance. The late replication data are consistent with the measurements of levels of mitochondrial malic enzyme (MOD-2, whose locus is on the autosomal segment) activity in these mice [Eicher E. & Coleman, D. (1977) *Genetics* 85, 647–658]. The present study also shows a dissociation between the replication timing in X chromatin distal and proximal to the autosomal segment, supporting the hypothesis of at least two inactivation centers in the X chromosome.**

Because the late-replicating X chromosome has been shown to be the inactivated X (1, 2), analysis of late replication in mammalian X chromosomes can be used to investigate both the details and phenotypic consequences of X-inactivation, a process that achieves gene dosage compensation for X chromosomal loci in females. Replication patterns in cells with X-autosome translocations often deviate from random (3–6). This could reflect either a nonrandom choice of the X to remain active or random determination (7, 8) followed by selective effects acting to minimize functional genetic imbalance. One mechanism underlying such deviations from random inactivation would be the spreading of inactivation from X to autosomal chromatin (reviewed in ref. 9), analogous to variegation position effects observed in *Drosophila* (reviewed in ref. 10).

The Cattanach translocation in the mouse, *T(X;7)1Ct*, is an excellent system in which to examine both the selection and the spreading of X inactivation. This translocation, hereafter designated *T1Ct*, involves the inverted insertion of a piece of chromosome 7 into the X chromosome (11). Heterozygous *T1Ct* females are of two chromosomal types: balanced ($X,X^{(7)},7,7^{D}$) or unbalanced ($X,X^{(7)},7,7$).‡ Expression of a biochemically assayable locus in the inserted autosomal segment of $X^{(7)}$, *Mod-2*, coding for the mitochondrial malic enzyme [MOD-2,; L-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40] has been shown to depend on the balance state of the genome (12). Inactivation of the *Mod-2* locus in $X^{(7)}$ is appreciable only in the unbalanced female carriers, the MOD-2 levels not being decreased to the extent expected if inactivation of autosomal loci in $X^{(7)}$ were random and complete. Independent assessment of the relative contribution of spreading and selection to these results can be made by analysis of replication kinetics in both mouse embryos and adults carrying *T1Ct*. 5-Bromodeoxyuridine (BrdUrd)-dye techniques for detecting DNA synthesis (13–16) are sufficiently sensitive to permit resolution of replication kinetics within the X and autosomal components of the $X^{(7)}$ chromosome.

In this study, differences in the proportion of cells containing a late replicating X or $X^{(7)}$ in female embryos and adults suggest the operation of selective factors acting against cells with functional genetic imbalance. Late replication of X chromatin is shown to be a necessary but not a sufficient condition for late replication of the autosomal segment itself. The frequency of late replication within this segment is comparable to the extent of *Mod-2* inactivation. Moreover, dissociation of replication timing in the proximal and distal X components of $X^{(7)}$ indicates that late replication and, by inference, X-inactivation is under the control of at least two loci.

MATERIALS AND METHODS

Mice. The *T1Ct* translocation is maintained at the Jackson Laboratory by a crisscross breeding scheme involving the mating of chromosomally balanced *T1Ct*/+ females to C57BL/6J c^{2J}/c^{2J} (c^{2J} = albino-2J, an allele at the albino, *c*, locus) males for one generation to C3H/HeJ-p^J/p^J (p^J = pink-eyed dilution-J, an allele at the pink-eyed dilution, *p*, locus) males for the next. Both types of matings were used to provide female adults and embryos for replication kinetics analysis. When mated to normal males, balanced *T1Ct*/+ females produce three different types of live female offspring: X,X,7,7 (normal), $X,X^{(7)},7,7^{D}$ (balanced), and $X,X^{(7)},7,7$ (unbalanced). A fourth type, $X,X,7,7,^{D}$, presumably dies *in utero*. Balanced and unbalanced females are distinguishable on basis of their coat color mosaicism (9).

Bone Marrow and Spleen Cell Preparation *In Vivo* from Adult Mice. To label late-replicating chromosomal regions, a modified T-pulse protocol (13, 15) was performed as follows. A tablet of BrdUrd weighing 55 mg was implanted under the abdominal skin (17) and then removed 3½ hr later. Females were injected with 0.4 ml of a Colcemid solution (200 μg/ml) 3 hr after the tablet was removed and cells were harvested 2 hr

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Abbreviations: *T1Ct*, Cattanach translocation, *T(X;7)1Ct*; BrdUrd, 5-bromodeoxyuridine.

‡ $X^{(7)}$ designates chromosome X with the inserted piece of 7; the deleted chromosome 7 is represented by 7^{D}.

after that. Bone marrow cells were collected by flushing a hypotonic solution (75 mM KCl) through the femurs. The spleen was minced in 75 mM KCl. After fixation in acetic acid/methanol, 1:3 (vol/vol), the cells were dropped onto slides and the slides were air dried.

Preparation of Mouse Embryo Cells. Nine- or 13-day-old (counting from day 0 based on presence of a vaginal plug) embryos were removed from the uterus and dissected free of the fetal membranes. All embryos were processed separately; embryo sexing was based on subsequent chromosome analysis. Two different culture methods were used.

(*i*) The embryos were minced in trypsin solution [0.25 g of trypsin (Difco 1:250) per liter of 0.01 M phosphate-buffered saline containing 50 μM EDTA, pH 7]. Pieces of embryo were cultured in Eagle's minimal essential medium supplemented with 2 mM L-glutamine and 20% fetal bovine serum. BrdUrd (final concentration, 0.1 mM) and deoxycytidine (final concentration, 0.1 mM) were added on day 3 of culture. Twelve hours later, the cultures were rinsed with Hanks' solution and the medium was changed to supplemented minimal essential medium containing thymidine (final concentration 10 μM). After 4 hr, Colcemid (0.6 μg/ml) was added to the culture. The cells were harvested 2 hr later by trypsinization, exposed to hypotonic solution (75 mM KCl) for 10 min, fixed in acetic acid/methanol, 1:3 (vol/vol), and spread on slides by flame or air drying.

(*ii*) In order to reduce possible cell selection during the 3 days of culture in method (*i*), whole embryos were incubated in supplemented minimal essential medium containing 0.1 mM BrdUrd. After 11–12 hr, the embryos were transferred to fresh supplemented minimal essential medium containing 10 μM thymidine but no BrdUrd. Five hours later, Colcemid was added to a final concentration of 0.6 μg/ml. After 3 hr, each embryo was cut in two or three pieces and incubated in a hypotonic solution (75 mM KCl) for 30 min. The tissue was transferred to fixative [acetic acid/methanol, 1:3 (vol/vol)] and left overnight at −20°C. To dissociate the cells, the fixed pieces of embryo were incubated for 2 min in 60% acetic acid, rinsed several times in fresh acetic acid/methanol fixative, and spread on slides as described above.

The 9-day-old embryo data were obtained by using method (*ii*). Because no significant differences were found between the replication patterns of 13-day-old embryos by method (*i*) or (*ii*), these data are pooled.

Staining and Chromosome Analysis. The 33258 Hoechst staining procedure and the conditions for microscopy and photography have been described (13, 16). The karyotype of each adult or embryo was established by examination of several cells. Selection of cells for photography was based on metaphase chromosome morphology and appropriate overall labeling (see *Results*). About 20% of the bone marrow and noncultured embryo metaphases and 40% of the cultured embryo metaphases were usable. Thirty-five cells were analyzed per female to determine the relative number of cells with the normal X [or $X^{(7)}$] late replicating. For each metaphase, the fluorescence intensities among the two Xs and chromosome 1s were compared in order to obtain a more accurate visual comparison of the two X chromosomes and the autosomal region within $X^{(7)}$ relative to other autosomal chromatin. Chromosome 1 was chosen for a reference because it is unambiguously recognizable in each metaphase (chromosome 7 is more difficult) and its bands 1A5 to 1C1 display replication kinetics similar to those of the segment of chromosome 7 inserted into the X in the *T1Ct* translocation. Chromosome band nomenclature is that described by Nesbitt and Francke (18).

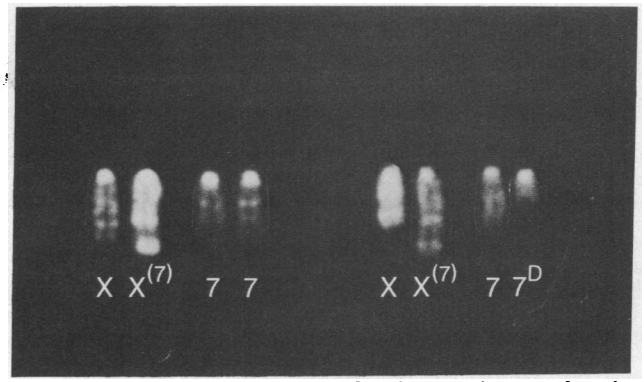

FIG. 1. Chromosomes from female mice carrying translocation *T1Ct* in an unbalanced form, $X,X^{(7)},7,7$ (*Left*), and in a balanced form, $X,X^{(7)},7,7^{D}$ (*Right*). The 13-day-embryo cells were cultured so that the bright fluorescence after staining with 33258 Hoechst indicates late replication. In these examples, the X chromatin in the $X^{(7)}$ of the unbalanced female is late replicating and the inserted segment is early; in the balanced female, the normal X is late replicating.

RESULTS

X-Replication Patterns in Adult *T1Ct*/+ Female Mice. Examples of chromosomes X and 7 are presented for a *T1Ct*/+ female with an unbalanced chromosome constitution and a *T1Ct*/+ female with a balanced one (Fig. 1). Consistent with previous autoradiographic (19, 20) and fluorescence data (16), the late-replicating regions corresponded to the bright Q-bands and the centromere regions, which are the latest replicating of any of the chromosomal regions. In female mouse cells, both X chromosomes replicate relatively late compared to the autosomes. The so-called late-replicating or inactive X is the last chromosome to finish its replication and thus is the brightest chromosome in the complement.

Fluorescent intensities in X and $X^{(7)}$ were compared by considering only the proximal regions of both Xs (Fig. 2, regions XA, XB, XC, XD, and XE) above the inserted autosomal segment in $X^{(7)}$; the distal material in $X^{(7)}$ (region XF) showed great variability, as described below. To determine optimally whether the inserted autosomal segment (Fig. 2, regions 7C, 7D, 7E) in

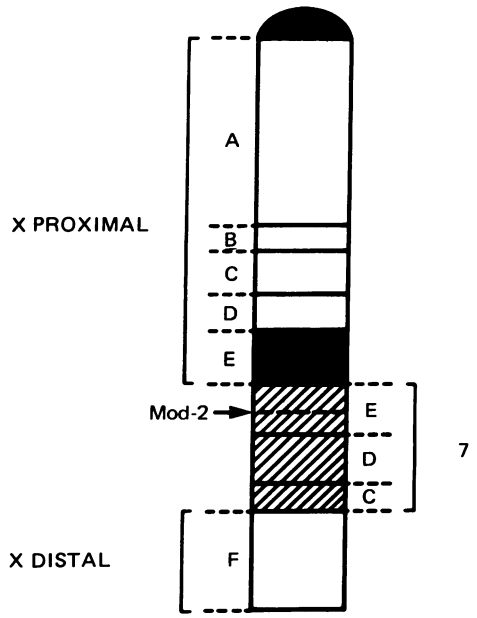

FIG. 2. Chromosome $X^{(7)}$ (from ref. 11). Note that the *Mod-2* locus is near the proximal breakpoint bounded above by the chromosome region XE.

Table 1. $X^{(7)}$ late replication in *T1Ct/+* female mice

Development	Unbalanced translocation: Animals, no.	Cells, no.	% late: $X^{(7)}$*	% late: Autosomal segment†	Balanced translocation: Animals, no.	Cells, no.	% late: $X^{(7)}$*	% late: Autosomal segment†
Embryo								
9-day	4	119	61.7 ± 5.1	35.8 ± 4.2	2	52	27.5 ± 3.5	17.5 ± 11.5
13-day	4	150	69.7 ± 6.5	36.2 ± 2.7	3	91	22.3 ± 1.8	13.7 ± 3.2
Adult								
Bone marrow	5	118	63.2 ± 5.7	29.8 ± 5.3	7	244	12.4 ± 2.6	3.9 ± 1.5
Spleen	4	74	75.0 ± 6.9	26.7 ± 13.2	2	58	14.0 ± 5.0	4.5 ± 4.5
Total	5	192	69.8 ± 2.8	29.0 ± 6.2	8	302	13.1 ± 2.4	3.6 ± 1.4

* Shown as mean (±SEM) of all cells in which the distinction between the replication kinetics of the two X chromosomes could be achieved and in which the $X^{(7)}$ was late replicating.

† Shown as mean (±SEM) of all cells with the inserted autosomal segment replicating late, considering cells in which differentiation between the X chromosomes was possible.

$X^{(7)}$ was late replicating in each metaphase, it was compared with the chromosome 1s in the same metaphase. In about 10% of the well-spread metaphases, the autosomes had typical late-replicating patterns, but distinction between the fluorescence intensities of the two X chromosomes was not possible. These metaphases are not included in the present study.

Comparison between the banding patterns observed in early-replicating chromosomes $X^{(7)}$, 7, and 7^{D} permits confirmation of the breakpoints involved in *T1Ct* (11, 21). In particular, it can be seen that the autosomal segment (regions 7C, 7D, and 7E) is inserted distal to region XE, which makes the proximal breakpoint easy to locate (Fig. 2).

Differences in X Replication between Unbalanced and Balanced *T1Ct* Female Mice. In *unbalanced T1Ct/+* females, the $X^{(7)}$ was late replicating in about 70% of the cells in which differentiation between the fluorescence levels of the two X chromosomes was distinguishable. However, different cells exhibited various X and $X^{(7)}$ replication patterns (Fig. 3). The autosomal segment within $X^{(7)}$ can be totally late replicating (set 1), partially late replicating (set 2), or early replicating (sets 3 and 4). In this last case, the very distal X material (region XF) can be either late replicating (set 3) or early replicating (set 4). By comparison with bands 1A5 to 1C1 of chromosome 1, the inserted autosomal segments in the late replicating $X^{(7)}$ shown in sets 3 and 4 were classified as early replicating. The autosomal segment in $X^{(7)}$ was found to be late replicating in about 29% of all the cells (Table 1), suggesting that the spread of inactivation from X chromatin into the autosomal material occurs in approximately half of the inactive (late replicating) $X^{(7)}$ chromosomes.

In about 30% of the cells of the unbalanced *T1Ct/+* females, the normal X chromosome exhibited a fairly consistent late-replication pattern; bright fluorescence was distributed uniformly along its length, sometimes with a faint banding pattern (Fig. 3, set 5). The very distal region (XF) sometimes appeared less fluorescent than the remainder of the chromosome.

In contrast to unbalanced females, *balanced T1Ct/+* females had the normal X chromosome replicating late in most of the metaphases (e.g., Fig. 4, sets 1–3). The $X^{(7)}$ chromosome was late replicating in only 13% of the cells (Table 1). In these cells, the inserted autosomal segment replicated either early (Fig. 4, set 4) or late (set 5). Only 4% of all of the cells analyzed showed a spread of late replication from the X chromosome into the autosomal segment (Table 1). The length of $X^{(7)}$ exhibited considerable intercellular variation. When $X^{(7)}$ was early replicating (Fig. 3, set 5), it was clearly longer than chromosome 1 and therefore the longest chromosome in the metaphase.

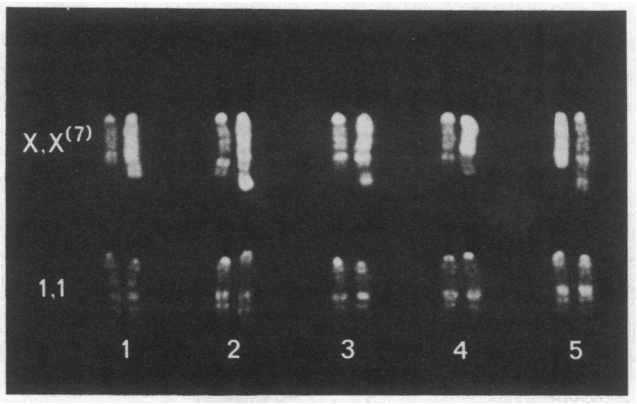

FIG. 3. Examples of chromosomes from female cells containing the *T1Ct* in an unbalanced form. Each set shows the X, $X^{(7)}$ and chromosome 1 pairs from the same cell. Staining utilized 33258 Hoechst such that the brightly fluorescent regions are late replicating. Sets 1–4 show late-replicating $X^{(7)}$; these were found in a majority of the cells from unbalanced *T1Ct/+* females. Set 5 shows material from a cell in which the normal X replicates late. The autosomal segment inserted in $X^{(7)}$, classified by comparison with chromosome 1, is late replicating in sets 1 and 2 and early replicating in sets 3, 4, and 5. Sets 2, 4, and 5 contain chromosomes from 13-day-old *T1Ct/+* female embryos cultured as described in Fig. 1. Chromosomes shown in sets 1 and 3 were from a bone marrow cell and a spleen cell, respectively, collected from adult *T1Ct/+* mice 5 hr after removal of the BrdUrd tablet that had been implanted subcutaneously for 3½ hr.

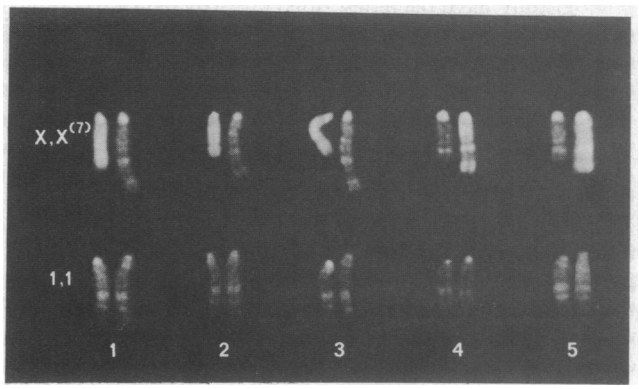

FIG. 4. Examples of chromosomes from cells of balanced *T1Ct/+* females. Chromosomes were prepared and arranged as in Fig. 4. Set 1 shows chromosomes from an adult bone marrow cell; the chromosomes in sets 2–5 are from 13-day embryos. The first three sets show a late-replicating normal X; $X^{(7)}$ replicates late in sets 4 and 5. The inserted autosomal segment replicates late only in set 5.

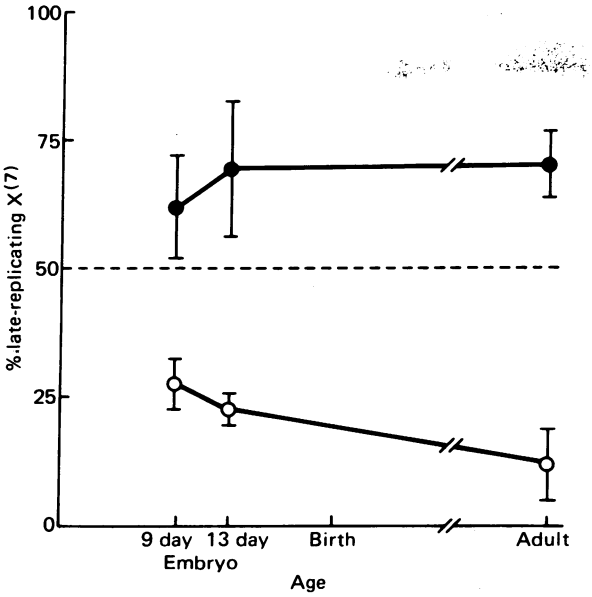

FIG. 5. Age dependence of the replication pattern of $X^{(7)}$ in *T1Ct*/+ female mice. Data are shown as mean ± SD. ●, Unbalanced females; ○, balanced females.

However, condensation of chromatin in the late-replicating $X^{(7)}$ can reduce it to the same size as the normal X.

Tissue and Age Influence on Replication Patterns in Adult *T1Ct*/+ Females. At most, small differences were observed between the replication patterns in the two actively dividing adult tissues studied, bone marrow and spleen (Table 1). Although Cattanach and Isaacson (22) had reported that coat color variegation in unbalanced females decreased with age, we did not find any significant change in the replication patterns in spleen and bone marrow cells and from animals 1 month of age compared to those 15 months of age.

X Replication Patterns in *T1Ct*/+ Female Embryos. Compared to adult *T1Ct*/+ females, 9- and 13-day *T1Ct*/+ embryos showed patterns closer to random, with a preponderant number of cells with a late-replicating $X^{(7)}$ in the unbalanced state and a late-replicating normal X in the balanced state (Table 1; Fig. 5). The deviation from random appeared to increase with age, approaching the replication patterns found in the adults (Fig. 5) and suggesting the operation of a cell selection mechanism. Also, it appeared (Table 1) that the spread of late replication from X chromatin into the autosomal segment of late-replicating $X^{(7)}$ was slightly more frequent in 9-day embryos than in the adults.

DISCUSSION

In cells from adult females heterozygous for the *T1Ct* translocation, the $X^{(7)}$ chromosome was found to replicate later than its normal homologue in 70% of cells from mice with an unbalanced karyotype but in only 13% of cells from mice with a balanced karyotype (Table 1). Compared with previous autoradiographic approaches (23–26), BrdUrd/dye techniques provide more accurate identification of the late-replicating X and permit resolution of replication kinetics within the inserted autosomal segment of $X^{(7)}$, which was classified as late-replicating in 29 and 4% of cells with unbalanced and balanced karyotypes, respectively (Table 1).

The proportion of adult cells with a late-replicating $X^{(7)}$ chromosome may reflect selective processes operative during embryonic development. Compared with cells from adults, cells from 9-day embryos show $X^{(7)}$ late replication frequencies closer to random in both balanced and unbalanced *T1Ct*/+ mice (Fig. 5); in 13-day embryos the frequencies approach those found in adults. Late replication in embryonic tissues from unbalanced mice obtained somewhat earlier than any described here (6.5 and 8.5 days after fertilization) showed a near-random pattern (27). These data support Lyon's hypothesis that the choice of the X to be inactivated is random; in the case of X-autosome translocations, cell survival or proliferation would then depend on which X remained active.

Nonrandom X inactivation has been observed in extraembryonic membranes, in which the X involved is predominantly of paternal origin (27). Although the DNA synthesis data in the present study were derived from females in which the $X^{(7)}$ was of maternal origin, analysis of late replication in cells from one mouse in which the $X^{(7)}$ was of paternal origin yielded comparable results (67% late replication versus an average of 70% for the other mice). If cell selection within the embryo proper were responsible for the changes observed in $X^{(7)}$ replication data, it probably would operate predominantly between gestation days 8 and 13. Remarkably, the average yield of embryos per pregnancy decreased significantly during that interval (data not presented), a result expected if lethal effects of genetic imbalance were realized (12, 28, 29). The existence of a low proportion (29%) of unbalanced *T1Ct*/+ adult female cells with a late-replicating $X^{(7)}$ autosomal segment, together with the virtual lack of the same cells in balanced *T1Ct*/+ female mice, suggests that selection is greater against functionally monosomic rather than against functionally trisomic states. Examination of replication kinetics in cells from mice containing deletions within the autosomal segment of $X^{(7)}$ (30) should help test this hypothesis.

When compared with genetic activity at the *Mod-2* locus which lies in the inserted autosomal segment, data on the distribution of late replication within the $X^{(7)}$ chromosomes further strengthen the correlation between late replication and inactivation of X chromatin. MOD-2 activity in unbalanced *T1Ct*/+ females is decreased by 11% from the value of 150% (relative to diploid levels) expected if all three gene copies are fully active (12). Such a reduction is equivalent to 22% inactivation of one *Mod-2* allele, a value closer to the percentage (29%) of late-replicating autosomal segments but significantly less than the percentage (70%) of cells in which the proximal X component of $X^{(7)}$ replicated late. Similarly, in balanced *T1Ct*/+ females, essentially no inactivation of the *Mod-2* locus on $X^{(7)}$ could be demonstrated (12), a result in better agreement with the late-replication data if late replication of the autosomal segment (4%) rather than the proximal X chromatin (13%) is considered.

Semiquantitative analysis of coat color variegation has permitted estimation of genetic inactivation of another locus (albino, *c*) within the autosomal segment of $X^{(7)}$. In unbalanced *T1Ct*/+ females, inactivation of the *c* locus ranged between 30% (31) and 50% (26, 31), whereas we found 22% of inactivation of the *Mod-2* locus and 29% of late-replicating autosomal segments. Locus-specific variations in genetic inactivation might reflect differences in the tissues examined or in the locus proximity to inactive X chromatin (*Mod-2* being closer to the proximal breakpoint than *c*) or both.

The observation of cells in which late-replicating X chromatin occurred both proximal and distal to an early replicating autosomal segment implies the existence of at least two X-linked loci controlling late replication of the X chromosome. The two loci must be on opposite sides of the autosomal insertion. This result is cytological evidence supporting Eicher's hypothesis (9) that at least two X inactivation centers exist, one on each side of the autosomal segment. Differences in the amount of var-

iegation for the pigment loci *c*, *p*, and *ru-2* (ruby-2) had suggested that the spread of X inactivation occurs from both sides of the inserted autosomal segment (9, 32). The proximal X region seems to have a predominant role in replication because late replication in $X^{(7)}$ was most frequently observed in the regions proximal to the translocation (XA to XE). Late replication in the distal region (XF) occurred in approximately 70% of cells with late-replicating proximal $X^{(7)}$ regions.

Analogous studies of late-replication patterns during embryonic development in another mouse translocation, *T(X; 16)16H*, in which genetic expression data imply total inactivation of the normal X in adults (3), should provide an indication of the generality of the above selective effects, the spreading of X inactivation and the existence of at least two inactivation centers.

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