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BrdU-33258 Hoechst Analysis of DNA Replication in Human Lymphocytes with Supernumerary or Structurally Abnormal X Chromosomes

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Abstract. BrdU-33258 Hoechst techniques have been used to characterize DNA replication patterns in lymphocytes from human females with supernumerary or structurally abnormal X chromosomes. Fluorescence analysis permits identification of late replicating X chromosomes in a very high proportion of cells and affords a high resolution method for determining the interchange points of X–X and X-autosome translocations. Asynchrony among terminal replication patterns of multiple late replicating X chromosomes within an individual cell can occasionally be demonstrated. The arms of isochromosomes usually exhibit symmetrical fluorescence patterns, with replication terminating in bands Xq21 and Xq23 (predominant pattern) or in bands Xq25 and Xq27 (alternative pattern) in both arms. In the vast majority of lymphocytes containing a balanced X-13 or X-19 translocation, the normal X is late replicating. However, DNA synthesis in the translocation products occasionally appears somewhat delayed relative to that expected for an early replicating X, consistent with possible position effects on replication kinetics.

Introduction

Asynchronous DNA replication is one of the cardinal features of X chromosome differentiation in mammalian cells (Lima-de-Faria and Jaworska, 1968; Miller, 1970). In somatic cells of normal human females, most of one X chromosome is genetically inactive (Lyon, 1972) and both begins and ends DNA synthesis after its homologue (German, 1962; Peterson, 1964; Priest et al., 1968). X chromosome differentiation constitutes a means of gene dosage compensation (Lyon, 1961, 1972; Russell and Bangham, 1961), and, in individuals with supernumerary X chromosomes, all but a single X exhibit late replication and are inactive (Morishima et al., 1962; Atkins et al., 1963; Rowley et al., 1963; Froland, 1967; Ricci et al., 1968; Baranovskaya et al., 1974).

Structurally abnormal X chromosomes, such as deletions (Hsu and Hirschhorn, 1970; de la Chapelle et al., 1975), ring chromosomes (Rowley et al., 1964), isochromosomes (Muldal et al., 1963; Grumbach et al., 1963; de la Chapelle et al., 1965; Ockey et al., 1965; Priest et al., 1975) and X–X translocations (Therman et al., 1972; Distèche et al., 1972; Kim et al., 1974), generally exhibit late replication. The clinical features of individuals with one normal X and one such abnormal X generally resemble those of females possessing only a single X chromosome. In contrast, the replication characteristics, as well as the phenotype changes associated with X-autosome translocations, are much more variable (Therman and Patau, 1974; Leisti et al., 1975).

In most cells of females with an X-autosome reciprocal translocation, the normal X is late replicating (Buckton et al., 1971; Lucas and Smithies, 1973; Sarto et al., 1973; Laurent et al., 1975; Gilgenkrantz et al., 1975). Primary amenorrhea is a common finding in such individuals (Sarto et al., 1974). However, if unbalanced X-autosome translocations are present, the translocation chromosome frequently exhibits late replication. The clinical features of these individuals are often consistent with genetic inactivity of the autosomal segment attached to the X (Cohen et al., 1972; Mikkelson and Dahl, 1973; Jenkins et al., 1974; Summitt et al., 1974; Crandall et al., 1974; Leisti et al., 1975). In mice, an autosomal segment adjacent to late replicating X chromatin may exhibit heteropycnosis, retarded replication, and curtailment of genetic expression (Ohno and Cattanach, 1962; Russell, 1963; Eicher, 1970). Autoradiographic data in many of the above-mentioned X-autosome translocations have been interpreted as consistent with an analogous position effect on DNA replication of the autosomal segment.

Recently developed methods utilizing fluorescent (Latt, 1973; Dutrillaux et al., 1973), Giemsa (Kim, 1974; Perry and Wolff, 1974; Korenberg and Freedlender, 1974) or immunological (Gratzner et al., 1975) methods to detect 5-bromo-deoxyuridine (BrdU) incorporation now afford improved resolution for analyzing DNA synthesis in metaphase chromosomes. For example, BrdU-33258 Hoechst techniques have been used to study X chromosome replication in normal human hymphocytes. The results obtained were consistent with autoradiographic analyses but revealed additional detail (Latt, 1974; Grzeschik et al., 1975; Willard and Latt, 1976). In the present report, these techniques have been used to characterize the DNA replication patterns in lymphocytes from nine patients with either supernumerary or structurally abnormal X chromosomes.

Materials and Methods

Cell Growth. Peripheral lymphocytes were cultured at 37° C in Eagle's minimal essential medium (MEM), to which was added 20% fetal bovine serum, 2 mM L-glutamine, and a saline extract of red kidney beans containing phytohemagglutinin (Latt, 1974b). Two protocols were followed. Most of the data were obtained with a protocol designed to highlight regions completing replication late in the DNA synthesis (S) phase. Cells were grown 44–46 h in medium containing 10^{-4} M 5-bromodeoxyuridine, 4×10^{-7} M 5-fluorodeoxyuridine (FdU), and 6×10^{-6} M uridine (U), which was replaced 5–9 h before harvest by Ham's F-10 medium containing 20% fetal bovine serum and thymidine (dT), 0.6 or 1.2×10^{-5} M. In some cultures the thymidine was tritiated (0.8 μCi/ml). In most cases, the duration of the terminal thymidine pulse in this "T-pulse" protocol was 5–7 h.

To suppress the fluorescence of regions last to complete DNA synthesis, a converse, "B-pulse" protocol was used, in which nearly 3 days of growth in control medium was followed by a 5–7 h preharvest pulse of BrdU, FdU, and U at concentrations given above. A recent modification of both protocols has been the addition of 10^{-4} M deoxycytidine (dC) (Meuth and Green, 1974) together with the BrdU (Latt et al., 1975).

Cell Harvest and Slide Preparation. Cells grown according to the above protocols were harvested by centrifugation two hours after the addition of Colcemid (0.1 μg/ml), and treated successively with KCl (0.075 M) and 3:1 methanol acetic acid fixative. Slides were prepared as described (Latt, 1974b).

Staining and Photomicroscopy. Slides were stained with 0.5 μg/ml 33258 Hoechst in 0.14 M or 0.40 M NaCl, 0.004 M KCl, 0.01 M Phosphate, pH 7.0. Fluorescence photomicroscopy of well spread metaphases was performed with slides mounted in pH 7.0 or 7.5 McIlvaine's buffer. Some slides exposed to the intense illumination associated with fluorescence microscopy, but not subjected to autoradiography, were incubated 30 min at 65° C in 2 × SSC, (0.30 M NaCl, 0.03 M Na citrate, pH 7.0) and then stained 20–30 min with 2% Gurr's R-66 Giemsa (pH 6.8). For autoradiography, slides were dipped in Kodak NTB-2 emulsion (diluted 1:1 with water), exposed 2–3 weeks, and developed with Dektol. 33258 Hoechst was the gift of Dr. H. Loewe, Hoechst A.G., Frankfurt, Germany.

Cases. The patient were all females who initially presented either with multiple congenital or developmental abnormalities or with symptomatology suggestive of an abnormality in sexual development. The list below includes their age at the time of study and their principal clinical features:

I. (47,XXX) – a 2 year old with short stature and delayed development.

II. (48,XXXX) – a 10 year-old with a dislocation of the head of the right radius and a surgically corrected patent ductus arteriosus.

III, IV and V. – 15, 21, and 21 year olds with phenotypic features of Turner's syndrome (including retarded skeletal development, primary amenorrhea and gondal dysgenesis). Virtually all lymphocytes of III had a 46 X, i(Xq) karyotype. IV and V exhibited both 46,X,i(X)q and 45,X cells (92% and 68% of the latter, respectively), while V had in addition a few (2%) 46,XX cells.

VI. 46 X,t(X;X) – a 22 year old with gondal aplasia, relative short stature, and lupus erythematosis.

VII. 46 X,t(X;X)/45,X – a 31-year old with short stature, secondary amenorrhea, small ovaries, and mild hirsutism.

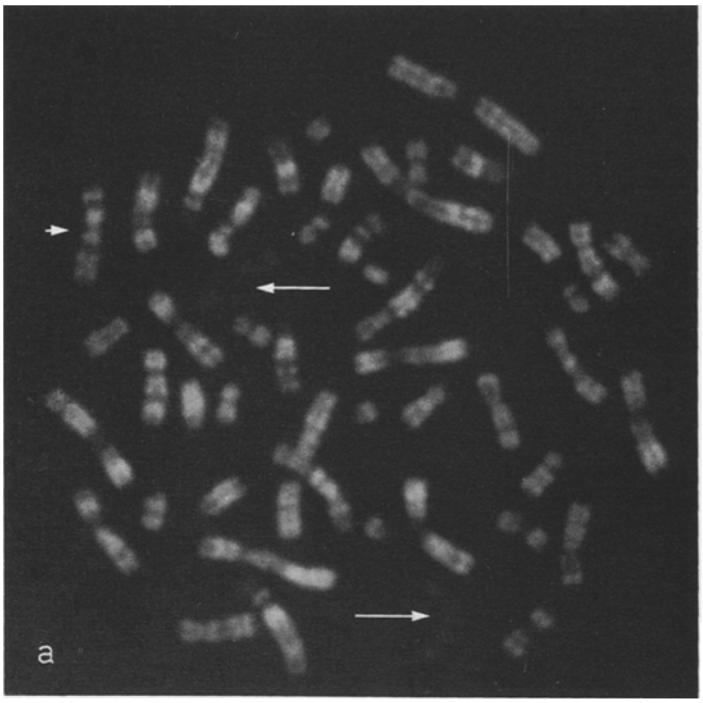
VIII. 46,X, rcp(X;13) – a 12 year old with short stature and a form of pseudohypertrophic muscular dystrophy.

IX. 46,X, rcp(X;19) – a 16 year old with spastic paraplegia, scoliosis, mental retardation, and primary amenorrhea.

Results

Supernumerary X Chromosomes. Growth of cells according to either pulse protocol permits differentiation between early and late replicating chromosomes based

Fig. 1a–d. Terminal replication patterns in lymphocytes from a 47,XXX individual (Patient I). The first cell (**a, b**) was grown nearly two divisions in control medium except for a six-hour terminal pulse of BrdU. The second cell (**c, d**) was administered a six-hour preharvest pulse of dT after 44 h of growth in medium containing BrdU. Late replicating X chromosomes (long arrows) in the first cell exhibit weak 33258 Hoechst fluorescence (**a**) and, following incubation in 2 × SSC, faint Giemsa staining (**b**) relative to the early replicating X (short arrow). A complementary pattern is found in the T pulse cell, with the late replicating X chromosomes (arrows) exhibiting bright fluorescence (**c**) and dark Giemsa staining (**d**). (Bar: 10 μ)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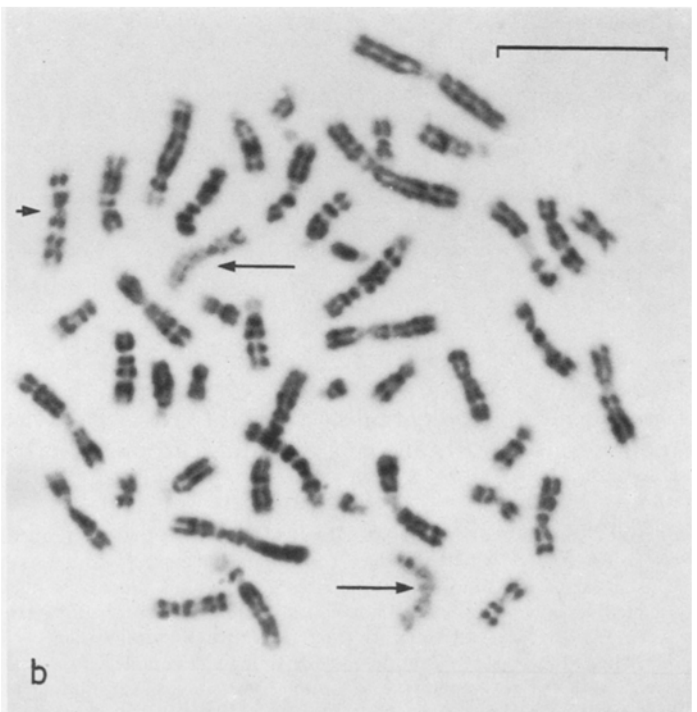
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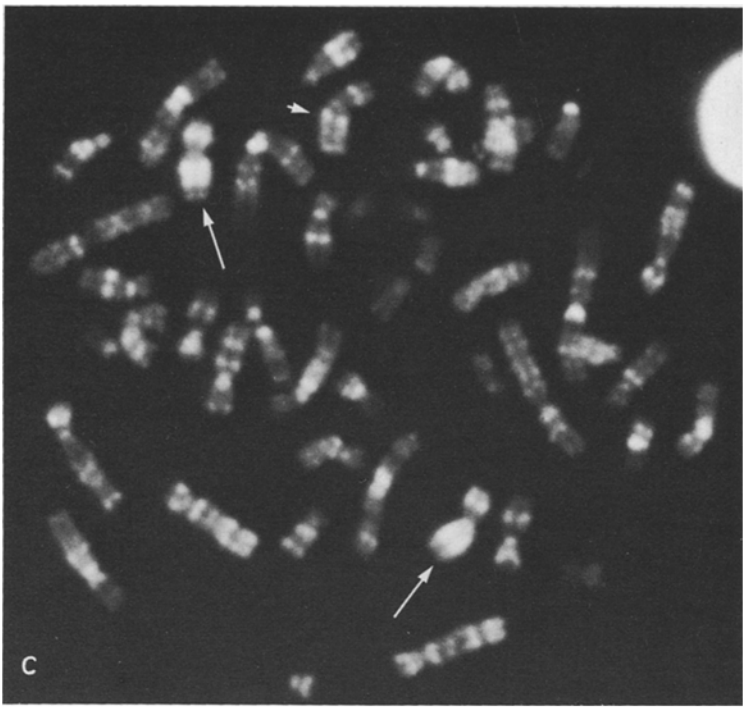
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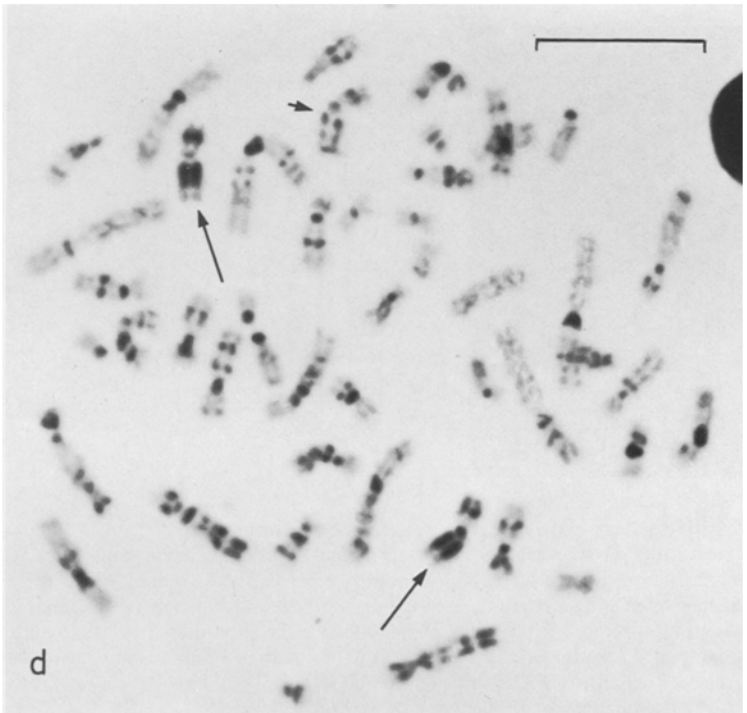
d

both on the localization and timing of DNA synthesis (Willard and Latt, 1976). For example, the two late replicating X chromosomes of 47,XXX lymphocytes cultured according to the B pulse protocol can be detected either by weak 33258 Hoechst fluorescence (Fig. 1a) or, following treatment with $2 \times$ SSC, by faint Giemsa staining (Fig. 1b). In a cell grown according to the T pulse protocol, a converse pattern is apparent, with more intense staining in the two late X chromosomes (Fig. 1c and d). As described previously for cells of normal females (Willard and Latt, 1976), replication in late X chromosomes terminates in bands p21, q21, and q23, while bands p21, q21 and q27 are last to complete replication in the early X. The companion Giemsa staining patterns of such cells generally parallel the results of the preceding fluorescence microscopy, although subtle differences in the intensity of Giemsa staining (relative to that of 33258 Hoechst fluorescence) can be observed.

While late replication patterns such as those of Figure 1 were found in most lymphocytes from this 47,XXX individual (patient I), variations in these patterns were occasionally observed. In the most common alternative replication pattern, late replication accentuated the terminus of the long arm of the X (bands q25 and q27) rather than the proximal part (bands q21 and q23) (Fig. 2). Out of 281, 47,XXX cells exhibiting differential chromosome arm fluorescence following the T pulse protocol, 33 possessed a single X chromosome with this latter pattern, (e.g. Fig. 2c, d), while 6 cells exhibited two such chromosomes (e.g. Fig. 2e, f). One X with the fluorescence pattern expected for an early replicating, active X was found in *all* of these cells. Other replication patterns

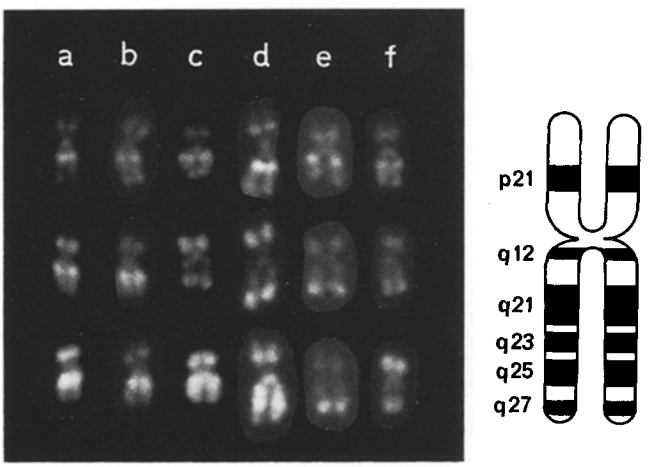

Fig. 2a–f. Heterogeneity in the terminal replication patterns of X chromosomes from a 47,XXX individual. Lymphocytes cultured according to a six hour T pulse protocol. Each column of X chromosomes was obtained from a separate cell. The top member of each column is the early replicating X. Typical late replicating patterns are found in the remaining members of columns **a** and **b** and in the bottom chromosomes of columns **c** and **d**. Chromosomes in which bands Xq25 and Xq27 are highlighted are shown in the middle row of columns c and d and the middle and bottom rows of the two columns **e** and **f**. The approximate locations of the X chromosome bands referred to in this and accompanying figures is shown at the right

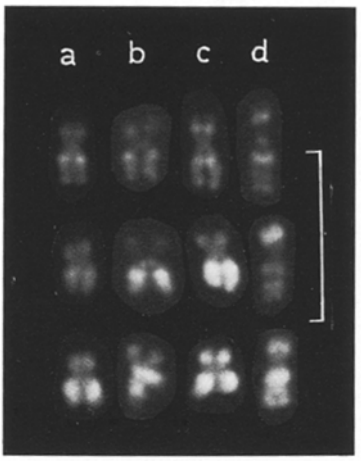

Fig. 3. Asynchrony in late X chromosome replication in lymphocytes from a 47,XXX individual. Culture conditions and X chromosome arrangement (early vs. late) are the same as those of Figure 2. (Bar: 10 μ)

illustrating asynchrony between the two late replicating X chromosomes were less frequently observed (Fig. 3). The most marked differences of this type tended to occur in the short arm (band Xp21).

Terminal replication (T-pulse) analysis of 48,XXXX lymphocytes provides further evidence of asynchrony among multiple late replicating X chromosomes. Consistent with previous observations (Giannelli, 1970), such cells usually contained three chromosomes with the fluorescence pattern of a late replicating

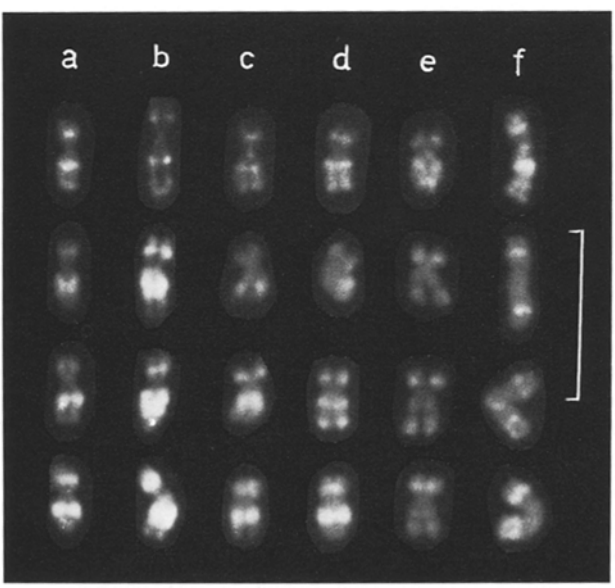

Fig. 4a–f. Heterogeneity in the terminal replication patterns of X chromosomes from a 48,XXXX individual (Patient II). Cell growth was identical to that of Figures 2 and 3. Columns **a** and **b** illustrate cells displaying the characteristic early X (top row) and predominant late X (bottom 3 rows) patterns. Various alternative late X replication patterns are shown for one (**c**), two (**d**), or three X chromosomes (**e, f**). (Bar: 10 μ)

Table 1. Replication characteristics of structurally abnormal X chromosomes

Patient:	Isochromosomes			X–X translocations		X-autosome translocations	
	III	IV	V	VI	VII	VIII	IX
Number of cells	127	268	81	288	247	268	41
Number of cells with abnormal X[b, c]	122	22	24	284	63	268	41
Normal X late-replicating	0	0	0	0	0	263	39
Alternative late X pattern[d]	–	–	–	–	–	55	1
Abnormal X late-replicating	122	22	24	284	62[f]	5	2
Alternative late X pattern[d, e]	10	1	3	0	0	0	0

[a] Both T- and B-pulse protocols were employed (see Methods section). Cells were scored only if chromosomes incorporated significant amounts of the pulse material (dT or BrdU) as judged by 33258 Hoechst fluorescence

[b] Fluorescence assignment of late replication was based primarily on the appearance of bands q21, q23 and p21 (when present) and secondarily on overall fluorescence (Willard and Latt, 1976)

[c] Cases III–VII had populations of 45,X cells (see descriptions in Methods section)

[d] In these cells, DNA replication in the late X terminated in bands Xq25 and Xq27, rather than Xq21 and Xq23

[e] Two late-replicating isochromosomes from III and one from V were asymmetrical (see text). All others exhibited symmetrical alternative replication pattern

[f] In one cell, assignment of the late replicating X by fluorescence was not possible

X (Fig. 4). In most cells, three X chromosomes displayed the predominant terminal pattern, in which bands p21, q21, and q23 are accentuated. However, one late X chromosome exhibited the pattern highlighting the terminus of the long arm in 4 of 103 cells cultured according to the T pulse protocol (Fig. 4c), while in 5 cells from this group, all three late X chromosomes displayed this pattern (Fig. 4e, f). In one cell (Fig. 4d), three distinct late X terminal replication patterns were observed.

Structurally Abnormal X Chromosomes. In every cell with a normal X and an isochromosome for the long arm of the X, the isochromosome exhibited a fluorescence pattern indicative of late replication (Table 1). Subsequent autoradiographic analyses were consistent with the fluorescence results (Fig. 5), although they afforded less detail. Late replication patterns in these isochromosomes are strikingly symmetrical (Fig. 5). Alternative terminal replication patterns highlighting bands q25 and q27 in at least one arm were observed in 14 isochromosomes (Table 1). In 11 of these, the fluorescence patterns were highly symmetrical, with bands q25 and q27 highlighted in both arms (Fig. 6, the two columns at the right). In one isochromosome (Fig. 6, middle column), late replication also involved band q21 in one arm. In two other cells (not

shown) one arm exhibited the alternative pattern, while the other arm displayed either the predominant late replication fluorescence pattern or uniformly dull fluorescence.

Two patients in the present study (VI and VII) possessed cells with one normal X and an X–X translocation (Fig. 7). In no lymphocyte from either individual was the structurally normal X late replicating by fluorescence analysis (Table 1). The nature of the structural abnormalities precluded detection of the previously described alternative replication patterns (e.g. Fig. 2, 4, 6) although asymmetry between the fluorescence patterns of the two moieties of these compound X chromosomes was occasionally observed (e.g. Fig. 7, VII). The B-pulse protocol was especially useful for determining the amount of material in these chromosomes distal to band q23 (Fig. 8). Only a small, early replicating interstitial segment, probably corresponding to q24, was seen in the abnormal X from cells of patient VI, and the translocation is interpreted as Xpter → Xq23:: Xq24 → Xpter or Xpter → Xq24:: Xq24 → Xpter. On the other hand, two such regions plus an intervening segment (presumably part of band q25) were frequently observed in chromosomes from patient VII, and this translocation chromosome is interpreted as Xpter → Xq24:: Xq25 → Xpter or Xpter → Xq25:: Xq25 → Xpter. Results of trypsin banding on cells from these patients (not shown) were consistent with the above conclusions, although the BrdU-Hoechst patterns provide much greater resolution. C banding analyses

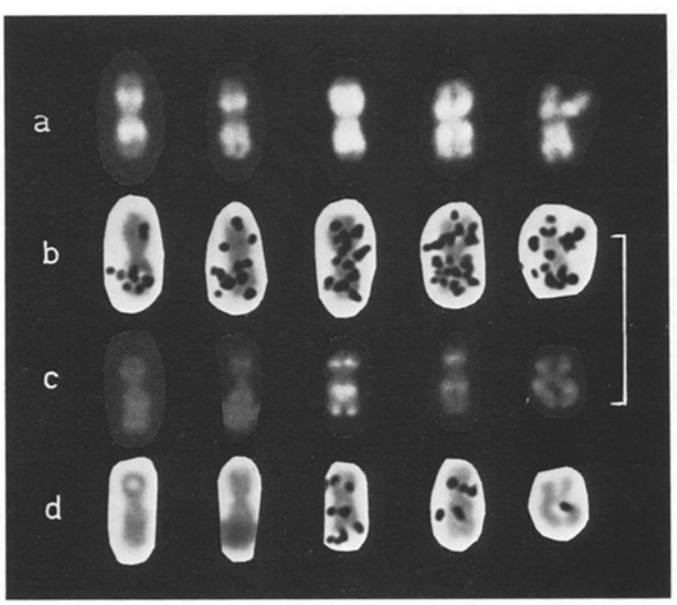

Fig. 5a–d. Fluorescent and autoradiographic analysis of X chromosome terminal replication in 46,X,i(Xq) lymphocytes. Cells from patient grown according to a 7 hour T pulse protocol, using (^{3}H)dT was employed. Each column illustrates late replicating isochromosome (**a, b**) and structurally normal early X (**c, d**) from a single cell after fluorescence microscopy (**a, c**) or autoradiography (**b, d**). (Bar: 10 μ)

show two, symmetrically located (centromeric) heterochromatic regions, although, in agreement with Therman et al. (1974), the mitotic metaphase configuration suggested that only one functional centromere was present.

X-Autosome Translocations. The late replication patterns of X-autosome translocation products similarly provide an accurate estimate of the translocation break points as well as an indication of possible position effects on replication kinetics. In the X-13 translocation (patient VIII) (Fig. 9 and 10), the break point is positioned between q21 and q23 on the X and between q21 and q31 on the #13. In 263 cells out of 268 cells grown according to either the T-pulse or B-pulse protocols (Table 1), the structurally normal X was unequivocally late replicating. In 78% of these 263 cells, the normal X displayed the predominant late replicating pattern, while in 22% an alternative replication pattern (with late replication in Xq25 and Xq27) was detected (Table 1). In the

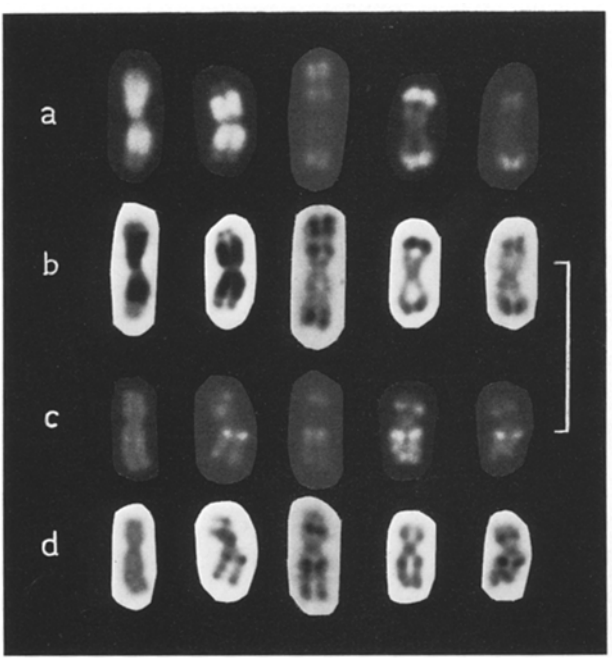

Fig. 6a–d. Heterogeneity in the terminal replication patterns of isochromosomes for the long arm of the X. Lymphocytes from a 45,X/46,X,i(Xq) female (patient III) were cultured as described in the caption to Figure 1 and the chromosomes were then stained with 33258 Hoechst. Shown are the isochromosome X (rows **a** and **b**) and the normal X (rows **c** and **d**) from each of five 46,X,i(Xq) cells. Cells were first photographed to obtain the 33258 Hoechst fluorescence patterns (rows **a** and **c**). They were then washed sequentially with xylene and water, incubated 30 minutes in 2 × SSC at 65°, and stained with Giemsa (rows **b** and **d**). In all 5 cells, the isochromosome exhibits a fluorescence pattern characteristic of late replication. Predominant patterns are shown in the two left hand columns, while less frequent, alternative patterns accentuating the distal part of the long arms of the isochromosome are shown in the other columns. Identical regions in each arm are highlighted in all but the isochromosome of the middle column, in which the proximal region of only one arm is late replicating. (Bar: 10 μ)

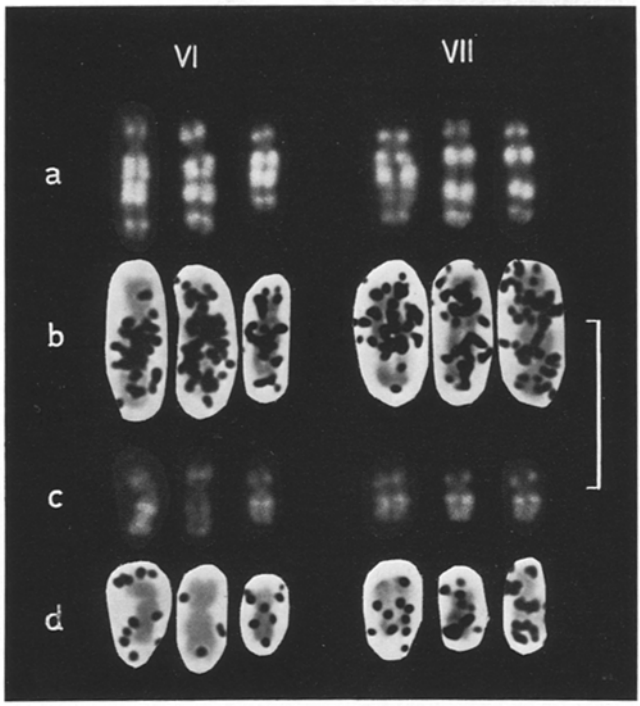

Fig. 7. Fluorescence and autoradiographic analysis of X chromosome terminal replication in 46,X,t(X;X) lymphocytes. Culture conditions (7 or 9 hour T-pulse protocol), chromosome arrangement and processing were similar to those of Figure 5. Chromosomes were derived from patient VI (left) or from patient VII (right). (Bar: 10 μ)

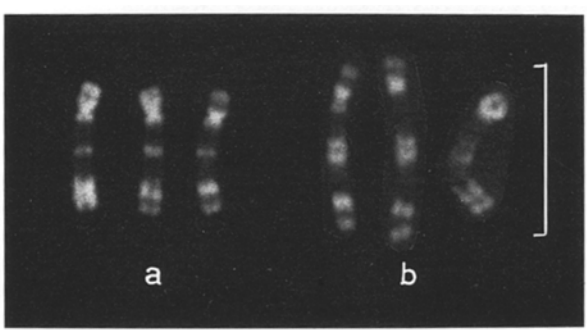

Fig. 8a and b. Early replication in X–X translocations. Lymphocytes from patients VI(**a**) and VII(**b**) were cultured in control medium except for a 5 or 7 h preharvest pulse of BrdU. Dull regions reflect BrdU incorporation at the end of S, while bright fluorescence indicates early replication. Note that there is a greater amount of early replicating chromatin in the mid portion of (**b**) than in (**a**). (Bar: 10 μ)

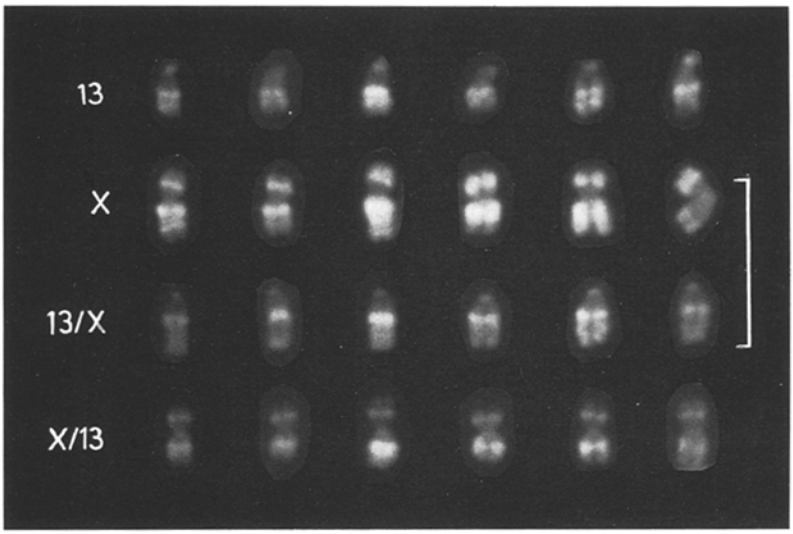

Fig. 9. Late replication in #13, X, and the X-13 translocation products from a 46, X, rcp(X;13) patient (VIII). Lymphocytes were grown as described in the caption to Figure 2 and stained with 33258 Hoechst. The chromosomes in each column are from the same cell. Bright fluorescence is evidence of late replication. For all six cells shown, the structurally normal X was late replicating, as judged from its fluorescence. In the first five columns, the predominant late replication pattern was observed for this X. In the right-most column, an alternative late replication pattern is evident. (Bar: 10 μ)

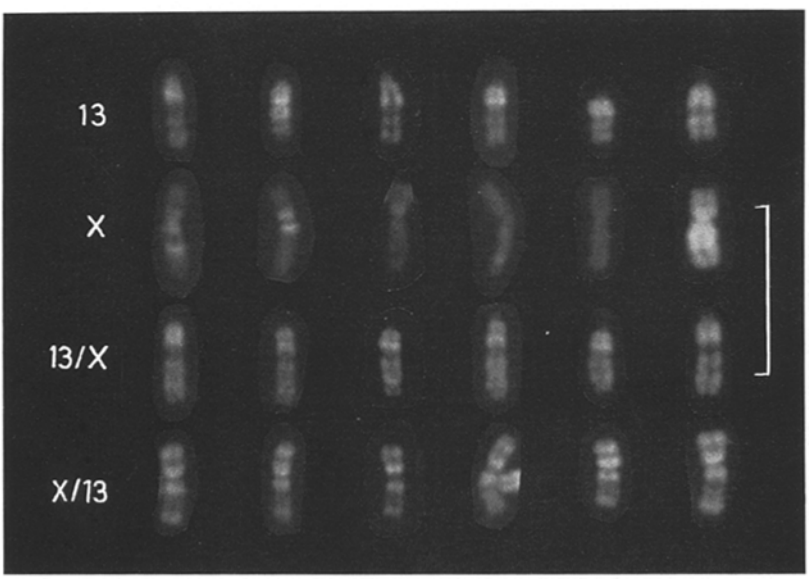

Fig. 10. Early replication in #13, X, and the X-13 translocation products from a 46, X, rcp(X:13) patient. Lymphocytes were grown according to the B pulse protocol described in the caption to Figure 1a and b. The chromosomes in each column are from the same cell. The fluorescence of late replicating regions is suppressed by incorporated BrdU, with resultant accentuation of early replicating segments. The column at the extreme right demonstrates an alternative late replication pattern in the structurally normal X. (Bar: 10 μ)

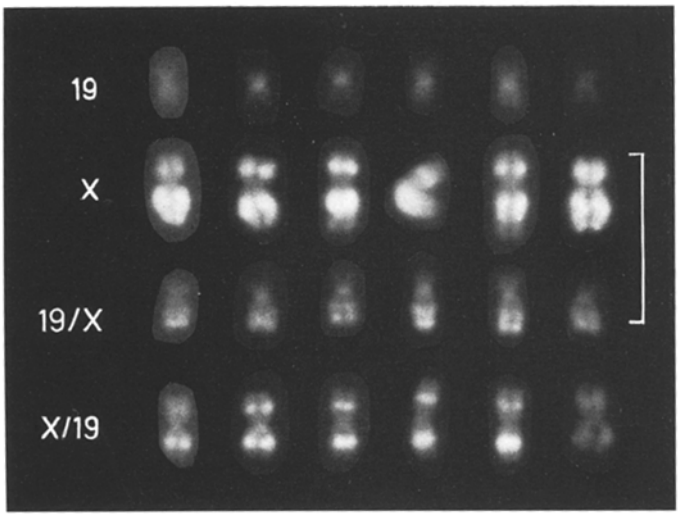

Fig. 11. Late replication in #19, X, and X-19 translocation products from a 46, X, rcp(X;19) patient (IX). Lymphocytes were grown as described in the caption to Figure 1c and d and stained with 33258 Hoechst. The chromosomes in each column are from the same cell. The normal X is late replicating in all cells shown. Extensive bright fluorescence is also apparent in the long arm of the translocation product in the bottom row (X/19). (Bar: 10 μ)

Xpter → Xq::13q → 13qter chromosome, bands Xq21 and 13q31 exhibited somewhat later replication (Fig. 9 and 10) than the corresponding regions of an early X or a structurally normal #13. However, in the complementary product (13pter → 13q::Xq → Xpter), there was no detectable influence of the late replicating band 13q21 on the adjacent X chromosomal material. In 4 out of the 5 cells in which the Xpter → Xq::13q → 13qter product was classified as late replicating, the long arm of the structurally normal X exhibited negligible incorporation of the terminal pulse of dT, while, in the other cell, the normal X displayed a fluorescence pattern characteristic of an early replicating X.

In the X-19 translocation (patient IX), the interchange occurred between q23 and q25 on the X and within q13 on #19 (Fig. 11). The normal X could be assigned as late replicating by its 33258 Hoechst fluorescence pattern in 95% of the cells (Table 1). In one cell, the structurally normal X displayed a long arm replication pattern accentuating q25 and q27 rather than q21 and q23 (Table 1). Late replication near Xq21 was unusually prominent in the Xpter → Xq::19q13 → 19qter chromosome, while late replication in 19q13 of the 19pter → 19q13::Xq → Xqter chromosome appeared somewhat more pronounced than in the structurally normal chromosome #19.

Discussion

This paper illustrates the application of BrdU-33258 Hoechst techniques for the analysis of DNA synthesis in lymphocytes from patients with X chromosome

abnormalities. Previous studies of X chromosome replication kinetics in normal individuals (Latt, 1974; Grzeschik et al., 1975; Willard and Latt, 1976) provide a basis for assessing variations associated with supernumerary or structurally abnormal X chromosomes. Conversely, the results of the present study assist in the interpretation of fluctuations observed in the replication patterns of normal cells.

Consistent with previous autoradiographic studies (Grumbach et al., 1963; Atkins et al., 1963; Rowley et al., 1963; Froland, 1967; Ricci et al., 1968) supernumerary X chromosomes in 47,XXX and 48,XXXX cells exhibit 33258 Hoechst fluorescence patterns characteristic of the late replicating X (Fig. 1–4). However, fluorescence permits definite assignment of late replicating X chromosomes in a higher proportion of cells than does autoradiography, and asynchrony among terminal replication patterns can be demonstrated much more clearly. Differences in the terminal labelling of the short arms of multiple X chromosomes, which had been detected by autoradiography (Froland, 1967; Ricci et al., 1968), are especially striking using BrdU and 33258 Hoechst (Fig. 3), and additional, less common fluctuations can also be noted. One alternative replication pattern, in which the distal region of the long arm (Xq25–Xq27) terminates DNA synthesis later than Xq21 and Xq23 in the late X chromosome (Latt, 1974; Willard and Latt, 1976) is often observed (Fig. 2 and 4).

The observed heterogeneity in the DNA replication patterns of late replicating supernumerary and structurally abnormal X chromosomes is consistent with the hypothesis that control of X chromosome replication is multifocal (Eicher, 1970; Willard and Latt, 1976). Asynchronous terminal replication was observed in cells with multiple late replicating X chromosomes (Figs. 2–4), isochromosomes for the long arm of the X (Fig. 6), and in the duplicate segments of two X–X translocations (Fig. 7). The frequency with which replication asynchrony was observed between two separate late X chromosomes within an individual cell was somewhat greater than the found when duplicate X chromosome segments were joined near the ends of their long arms (Fig. 7) and much greater than that observed between the two arms of isochromosomes (Fig. 5). Previous autoradiographic analyses (Muldal et al., 1963; Ockey et al., 1965) had suggested that replication in isochromosomes was symmetrical. The present study demonstrates the extent of this symmetry in three patients. Interpretation of the structure specific differences in the DNA synthesis kinetics of duplicate late replicating X chromosome segments will require more information about the molecular basis by which replication kinetics are controlled.

Termination of replication in bands Xq25 and Xq27 rather than bands Xq21 and Xq23 occurred in a proportion of lymphocytes from patients I–V, VIII and IX (e.g. Figs. 2, 4, 6, 9, 10). This pattern had previously been observed in 3–15% of normal female lymphocytes (Latt, 1974; Grzeschik et al., 1975; Willard and Latt, 1976). Since a second X exhibiting fluorescence typical of early replication was present in all such cells, the alternative pattern was attributed to the late replicating X (Latt, 1974; Willard and Latt, 1976). In patients III–V, this pattern is observed only in the isochromosome X, while in patients VIII and IX, it occurs only in the normal X. Observation of the pattern reflecting termination of replication in q25 and q27 only in those chromosomes which

are both morphologically distinguishable and characteristically late replicating confirms the previous assumption based on results with 46,XX cells.

Remarkably, eleven out of the fourteen isochromosomes in which late replication highlighted Xq25 and Xq27 exhibited this pattern in both arms (Fig. 6, Table 1). In one of the three exceptional chromosomes, fluorescence asymmetry in the proximal long arm region was observed, while in only one of the remaining two cells was a mixture of replication patterns found in the arms of a single isochromosome. The marked correlation between the replication patterns in the two arms of isochromosomes could be due to a common replication control point. However, inasmuch as concordance between these arms was not complete, an equally plausible antecedent would be an identical structural origin of the component arms of the isochromosome.

Concordance for the alternative replication pattern among the multiple late replicating X chromosomes of 47,XXX and 48,XXXX cells was weaker than that between the arms of isochromosomes, although correlation with individual cells was significantly greater than that expected were occurrence of this pattern totally random. One possible mechanism for the clustering of this discrete pattern in individual cells is the existence of a subpopulation of lymphocytes with inherently different replication kinetics. However, the observation of both typical and alternative late replication patterns within individual 47,XXX or 48,XXXX lymphocytes (Figs. 2 and 4) suggests additional complexity.

The replication patterns of the translocations studied here do not conclusively establish the existence of position effects on the timing of DNA synthesis, although some of the data on the X-autosome translocations are at least suggestive of such influences. The segment of the long arm of the X distal to the band q23 in the X–X translocations continues to exhibit relatively early replication, even when bordered on its other side by a duplicate late replicating q23–q21 region (Fig. 7). The two X–X translocation chromosomes examined possess late replicating regions of comparable size, although the intermediate, earlier replicating junction region is larger in cells from patient VII than in cells from patient VI (Fig. 7). Examination of these chromosomes with the B-pulse protocol provides a precise analysis of this intervening region (Fig. 8). Late replication patterns similarly afford a convenient way of characterizing the interchange points of the X-autosome translocations (Figs. 9–11).

In cells with X-13 or X-19 translocations (Figs. 9–11), late replication in band Xq21 of the Xpter → Xq::autosome product is somewhat more prominent than is characteristic of this region in a structurally normal early X. Fluorescence in this region is especially pronounced in the X-13 translocation, due at least in part to the very late replicating attached autosome segment (13q31). Interestingly, these possible alterations in timing of DNA synthesis involve the early-replicating, and presumably, therefore, genetically active, portions of the translocations. The possibility exists, then, that position effects, directly or indirectly affecting genetic expression and replication kinetics, may play a role in many if not all translocations, even in those not involving constitutive or facultative heterochromatin. While the changes in chromosome replication kinetics observed thus far are subtle, they point to the desirability of an examination of the

genetic expression of X and autosome markers located near exchange points in both the X-13 and X-19 translocations.

In both X-autosome translocation patients, as in previously reported cases with balanced X-autosome translocations (Buckton et al., 1971; Lucas and Smithies, 1973; Sarto et al., 1973; Laurent et al., 1975; Gilgenkrantz et al., 1975), the normal X was late replicating in the vast majority of cells (Table 1). This observation may reflect selection operating against cells in which the translocation chromosomes exhibit late replication, resulting in a population of cells in which X chromosome expression is largely restricted to loci on the translocation products. Such virtual hemizygosity may account for certain clinical features of these and similar patients. The unusually high occurrence of X chromosomes terminating replication in q25 and q27 in X-13 translocation cells and its infrequent occurrence in X-19 cells might similarly reflect selection effects with possible indirect physiological consequences.

In the X-13 and X-19 translocation patients described (Table 1), as well as in the X-1 and X-2 translocation patients analyzed by a BrdU-acridine orange technique (Laurent et al., 1975; Gilgenkrantz et al., 1975), a small fraction of lymphocytes exhibit fluorescence patterns interpretable as reflecting late replication in the translocation product instead of the structurally normal X. The existence of such cells would suggest that selection for inactivation of the structurally normal X could be incomplete, perhaps because of counter selective pressure associated with functional hemizygosity. The resulting distribution of cells might then, as has been suggested (Leisti et al., 1975), minimize overall genetic imbalance.

Future examination of position effects on chromosome replication might take two additional directions. First, the Cattanach translocation of the mouse constitutes a system in which position effects on genetic expression are associated with insertion of an autosomal segment into the X (Cattanach, 1961; Ohno and Cattanach, 1962). A recent report on this translocation using BrdU and acridine orange (Takagi and Sasaki, 1975) determined the proportion of cells in which this compound chromosome exhibited asynchronous replication. Detailed analysis of the replication of the inserted autosome segment should now be possible. Second, BrdU fluorochrome analysis might also be applied to other types of human X-autosome translocations. Of particular interest would be unbalanced, late replicating translocation products in which spread of inactivation from the X to the attached autosome segment has frequently been hypothesized (Crandall et al., 1974; Summitt et al., 1974; Leisti et al., 1975; Cohen et al., 1972; Jenkins et al., 1974). It may prove especially informative to examine those X-autosome translocations which are present in both the early- and the late-replicating states in different family members (Buckton et al., 1971; Grzeschik et al., 1971; Summitt et al., 1974; Leisti et al., 1975; Cann et al., 1975).

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