THE PATTERN OF DNA SYNTHESIS IN THE CHROMOSOMES OF HUMAN BLOOD CELLS

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ABSTRACT

The sequence in which various regions of the chromosomes of human blood cells complete DNA synthesis *in vitro* has been studied through the use of H^3-thymidine labeling and autoradiography. Certain of its aspects have been defined, and these may serve as a basis for comparing the pattern of synthesis in cells of other tissues. In general, the long chromosomes continue replication later than the short ones. Variability of the sequence has been prominent. One pair from Group 13–15 and pair No. 17 complete replication early. In certain other chromosomes, replication is very active late in the S period, *e.g.* one X of the female cell, the Y of the male cell, two of Group 4–5, two of Group 13–15, the Nos. 16, and the Nos. 18. In the normal human female a striking correlation exists between the late replication of one of the X chromosomes, condensation during the intermitotic period, and presumed genetical inactivation. The pattern of replication characterizes certain chromosomes whose structural features alone are non-distinctive, and it may be useful in studies of cells in which a chromosomal aberration occurs.

Recent advances in the understanding of nucleic acid metabolism and in techniques for the handling of chromosomes make possible a combined study of chromosomal function and structure (2–19). The reproduction of somatic cell chromosomes is completed before the onset of cell division. This particular chromosomal function, DNA synthesis, can be studied using isotopic labeling of the newly forming DNA followed by autoradiography. The replication of DNA is asynchronous in the chromosomes of the human complement (3 *a*). Near the termination of the S period,[1] when the timing of synthesis is more readily studied than at the onset (19) or midportion of the period, the time of completion of DNA replication is different in different chromosomes (3 *b*). The timing in one X chromosome of cells derived from the normal human female is the most remarkable, extensive replication continuing late in the S period (3 *b*, 3 *c*, 5). Studies of the mammalian X chromosome (20–22) may lead to further understanding of the mechanism of gene activation and inactivation, since one of the two X's is already known to remain heavily condensed (23–25) and, in large part, genetically inactive (5, 22, 26–28) in the interphase nucleus. Certain autosomal regions in the lymphocyte also have been shown (3 *b*) to be unusually late replicating, but their relation to autosomal heterochromatin and inactive genetic material remains speculative. The early duplication of certain genes and very late duplication of others is at the present time of unknown significance in relation to cellular differentiation.

The experiments to be described provide information concerning the sequence in which the various chromosomes of the human nucleated

[1] S is the period of DNA synthesis, using terminology of Howard and Pelc (1). G_2 is the time interval between the completion of DNA synthesis and the onset of prophase. G_1 is the time between completion of telophase and the onset of S (See Fig. 5).

blood cell complement complete replication. The pattern in these cells can, in future experiments, be compared with that existing in cells of other tissues. The pattern also provides characterization of specific chromosomal pairs and groups, data applicable to the detection and definition of numerical and structural aberrations.

METHODS

CELL CULTURES: Bean extract (Difco phytohemagglutinin batch No. 120184) was added to fresh heparinized venous blood. The agglutinated erythrocytes were removed by gentle centrifugation, and the plasma containing the nucleated cells was combined with TC medium 199, giving a final plasma concentration of 15 to 30 per cent and cell concentration of 1.0 to 1.5 $\times 10^3/mm^3$. The cell suspension was divided into multiple 6 to 9 ml cultures and placed in a constant temperature room at 37°C. Blood cells were obtained from 2 male and 4 female humans, normal, healthy individuals who were between the ages of 20 and 40.

ISOTOPIC LABELING: The experimental approach is based on principles and techniques devised and used by Taylor *et al.* (29) in the study of chromosomes of various species of plants and animals including man (5). The final concentration of H^3-thymidine (obtained from Schwartz Bioresearch, Inc., as a sterile aqueous solution, specific activity 1.9 c/mmole, conc. 0.5 mc/ml) in the cultures was 30 μc/100 ml medium, and of desacetylmethylcolchicine (colcemide, Ciba Pharmaceutical Products, Inc.) 4 mg/100 ml medium. In the 6 experiments reported in this paper, the cultures contained H^3-thymidine during the final 6 hours and colcemide during the final 3 hours unless otherwise indicated. Temperature change of the cultures was avoided during addition of these materials. At the end of 3 days' incubation, the cells were transferred to 0.95 per cent sodium citrate (37°C) for 20 minutes and fixed with a freshly prepared 1:3 mixture of acetic acid and anhydrous methanol. They were suspended in fixative and placed on a cold, wet microscope slide and dried within 30 seconds by fanning or gentle warming. The slide was placed in freshly filtered 1 per cent synthetic orcein in 45 per cent acetic acid solution for several hours, following which it was quickly passed through alcohols to xylol and then allowed to dry.

DUPLICATE - PHOTOGRAPHY TECHNIQUE: With immersion oil directly on the slide, mitotic figures were observed through a high-power objective. Many of those considered potentially suitable for chromosomal analysis were photographed, and the slide location of each was recorded. Following this preliminary photography, the oil was removed from the slide in several fresh xylol baths, and autoradiographic stripping film (Kodak AR. 10) was applied to the slide. After exposure for 9 days at 4°C, the film was developed *in situ* in Kodak D-19 developer for 4.5 minutes at 18°C. Each cell previously photographed was then relocated, and if radioactive its autoradiograph was examined and photographed. Prints of the cells and of their autoradiographs were made at the same magnification. These were stapled together, the autoradiograph exactly in place beneath the picture of the chromosomes. Each chromosome and its underlying autoradiograph were cut out, and the karyotype was prepared. The pictures of the chromosomes were lifted from the underlying picture of the autoradiograph only after the former had been arranged in the karyotype, and both were then mounted in the same order (see Figs. 7 to 12). This technique was devised (3 *b*) so that knowledge of the structural features of the various chromosomes, often obscured by grains of the autoradiograph, would be available during the preparation of the karyotype, and the distribution of grains would not be considered in the selection of homologous chromosomes. Eighty-seven cells from 4 females and 33 cells from 2 males have been analyzed in this manner.

RESULTS AND COMMENTS

Examination of these radioactive cells and their autoradiographs (Fig. 1) shows that the grain distribution is limited to the area directly overlying the interphase nuclei or the chromosomes of the dividing cells. The β-irradiation accurately delimits the margins of the orcein-stained structures with little scatter beyond. It is, therefore, possible to identify localized radioactivity of portions of short chromosomes. Radioactivity of a chromosomal region provides evidence that DNA synthesis had been in progress in that region during the period that H^3-thymidine was present in the culture medium.

The degree of radioactivity of nuclei varies because the division cycles of cells in these cultures are not synchronized. The wide variation in number of grains per cell, ranging from a few scattered ones to massive confluent blackness, indicates that from a minimal to an extensive degree of synthesis had been in progress during the time a given cell had access to the tritium. In the non-dividing nucleus the radioactivity may be diffuse (Fig. 1 *a*) or, as reported by Lima-de-Faria and Reitalu (4 *b*), localized or concentrated in certain areas (Fig. 1 *b*). This latter finding suggests a degree of chromosomal localization within the nucleus at the time when most chromosomes assume the uncoiled, extended state characteristic of the intermitotic period. Non-dividing

nuclei exhibiting one localized heavily radioactive area are found commonly in cultures of cells derived from the female. Such cells probably were near the termination of the S period when the isotope became available. Thus, heavy labeling in these cells would have occurred predominantly in the late replicating X chromosome that during period. Furthermore, the cell cycles in the cultures are non-synchronized, and, as will be seen, the G_2 period is variable. The more lightly labeled cells in metaphase are assumed to be those which were nearer the termination of the S period when the isotope was added to the medium. It has become apparent, from observation of many

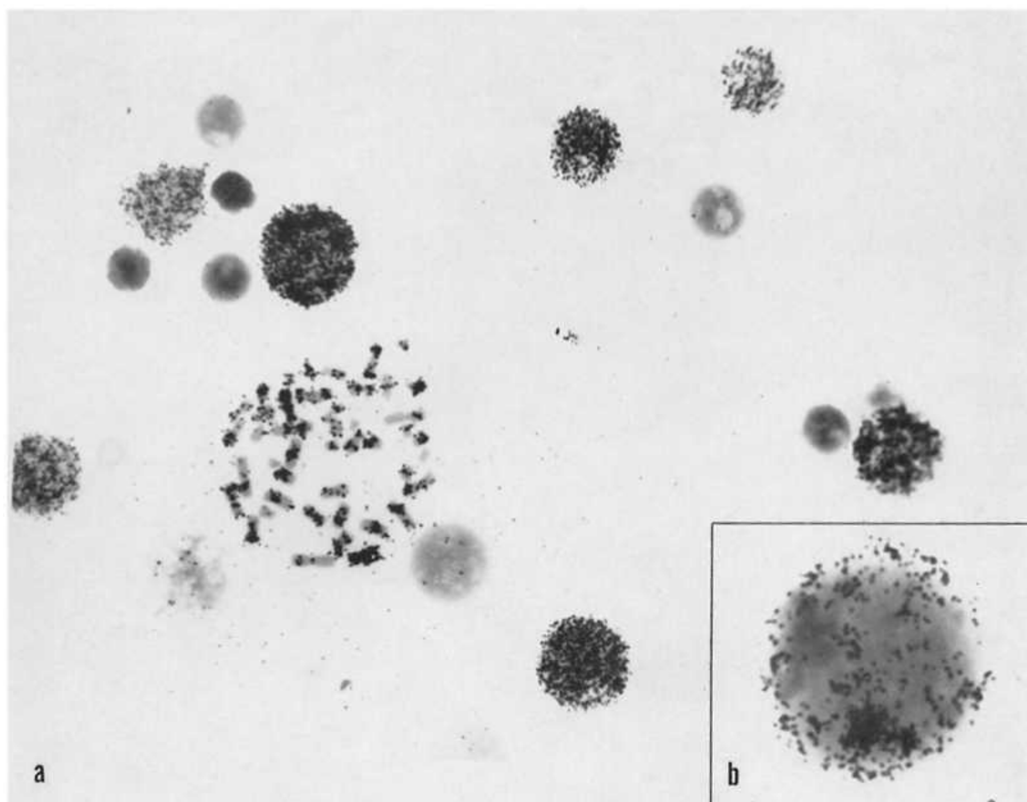

FIGURE 1 *a.* Autoradiograph of orcein-stained cells from normal human female after culture for 3 days in presence of bean extract; 10-minute pulse with H^3-thymidine 4 hours before fixation. × 720.
b. Insert under higher magnification showing a localized area of heavy radioactivity in an interphase nucleus. × 1360.

interphase constitutes the sex chromatin mass of Barr.

The degree of radioactivity of cells fixed in metaphase also varies. Some cells (Fig. 2 *a*) exhibit many grains over the entire length of every chromosome, and in these there are increased numbers of chromatid breaks in comparison to non-radioactive or lightly radioactive cells (3 *d*). Others (Fig. 2 *f*) have only an occasional grain over a few chromosomes. This variation in total cellular radioactivity results in part from the accumulation of *c*-metaphases over a 3-hour metaphase figures with varying degrees of radioactivity, that there are recognizable and repeating patterns of grains overlying the chromosomes. These patterns can be defined, and an attempt has been made to coordinate them into a sequence to be described.

In these cultures, some lymphoid cell nuclei do not engage in the synthesis of DNA. Furthermore, the proportion of radioactive nuclei at the 3rd day in cultures derived from different individuals varies considerably (Table I). It is now known that there are cells in which the synthesis of DNA is in

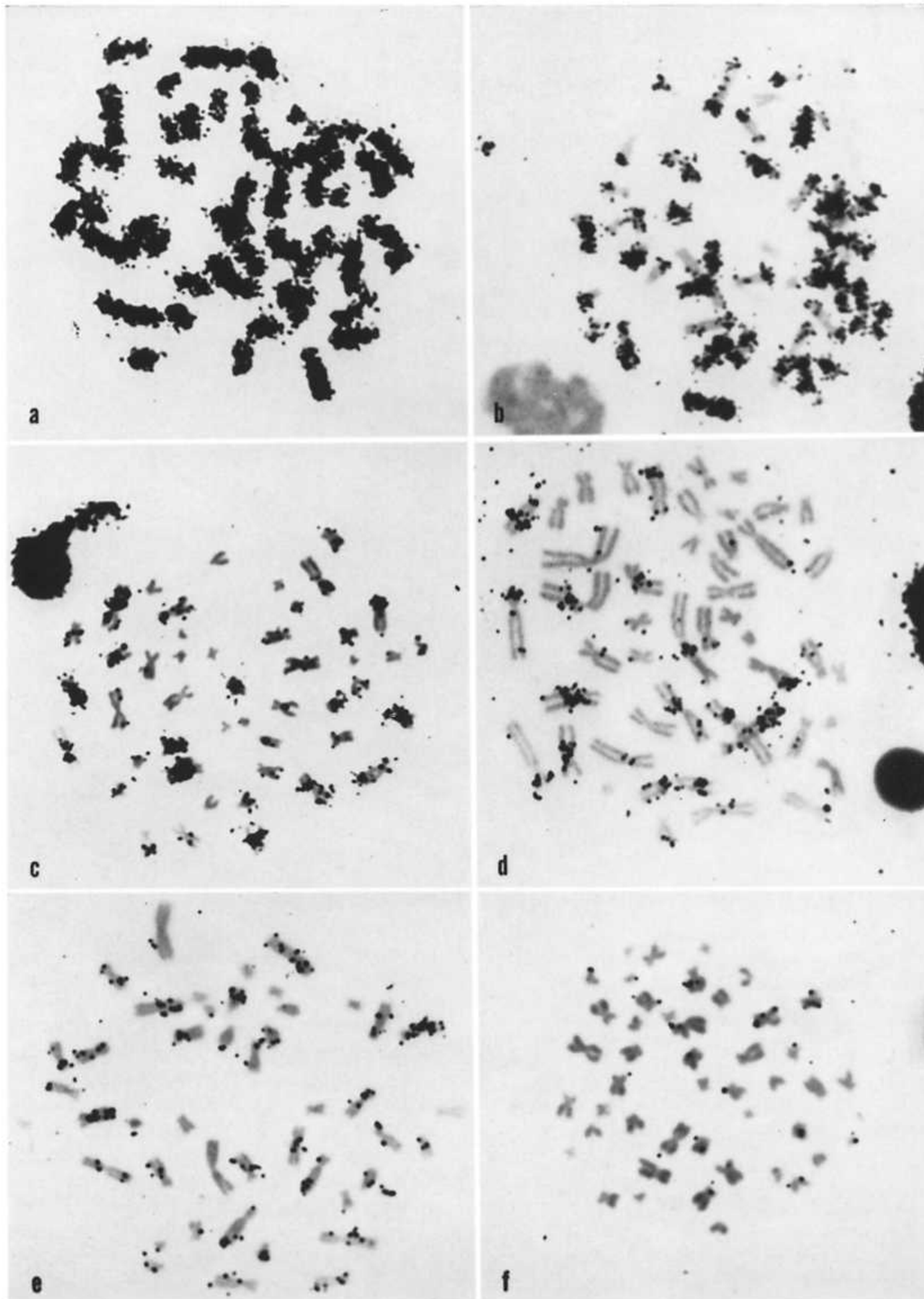

FIGURE 2 A series of six radioactive cells in metaphase from a single experiment (normal human male). During the 6 hours before fixation, H^3-thymidine was present in the medium, and during the final 3 hours, colcemide also. Film exposure 9 days. The decreasing degree of radioactivity in the series indicates that more of the S period had been completed at the time the isotope became available to the cells. × 1350.

progress as they circulate in the blood of normal individuals (30) (Fig. 3). These circulating cells develop an extensive cytoplasm in comparison to the ordinary lymphocytes, and their nuclei commonly are lobulated and enlarged. Synthesis does not usually begin in the other cells in cultures to which no mitogenic agent has been added, although after several days of incubation synthesis infrequently can be detected in some cells (Fig. 4). However, the addition of the mitogen extracted from the beans of *Phaseolus* stimulates a large proportion of nucleated blood cells to enter the S period (Fig. 4) and then, as discovered by Nowell *et al.* (31), to enter mitosis. H^3-thymidine incorporation has not been observed in cells of the granulocytic series in these cultures. The numerous metaphase figures observed 3 days after addition of bean extract are predominantly of lymphocytic origin.

The G_2 period varies in different blood samples (Table I) as well as in different cells from the same sample, but, in general, is of 3 to 4 hours' duration. The usual duration of G_2 in a given experiment was determined in the following way. Radioactivity of a cell in metaphase indicated that the G_2 in that cell had been 3 hours or less if both colcemide and H^3-thymidine had been placed in the culture for 3 hours preceding fixation. A cell with G_2 longer than 3 hours was indicated by non-radioactivity of its metaphase in an experiment in which H^3-thymidine had been available for the final 6 hours, colcemide the final 3 (5 and 32 per cent of cells in the two experiments of Table I).

In the present paper, special consideration is given to the terminal portion of the S period, the U through Z intervals diagrammed in Fig. 5. This is the portion of the cell cycle between the point at which synthesis of DNA is completed in a detectable segment of any chromosome and the final moment of the synthesis period after which H^3-thymidine is no longer incorporated into the nucleus. In cultures containing isotope for the final 6 hours and colcemide for the final 3 hours prior to fixation, the chromosomal localization of radioactivity in cells in metaphase delineates the areas which synthesize DNA during the final 180 minutes of S (Figs. 6 and 7). A small proportion of the labeled cells in metaphase have a large amount of radioactivity over the entire length of every chromosome (Fig. 2 *a*). In such experiments, if G_2 should be of 180 minutes' duration consistently, the percentage of all radioactive metaphase figures this extensively labeled would, in turn, indicate the percentage of the final 180 minutes of the S period during which replication still continues throughout the entire length of all chromosomes. This portion of the S period is then followed by the U interval which, in cell populations such as Population I of Table I, would begin approximately 170 minutes before the termination of the S period. Thereafter, the various regions of the entire complement become completely duplicated in succession and then no longer incorporate isotope. Six intervals may be identified from the distribution of non-radioactive chromosomal regions during this time. These are referred to in this paper as the U through Z intervals, using the final letters of the alphabet to name the final stages of synthesis of DNA (Figs. 5 and 7, Table II). It is possible, from the grain distribution over the chromosomes, to determine at which

TABLE I

Lymphocyte Populations from 2 Human Subjects Cultured Simultaneously under Identical Conditions

These show variation in (1) the proportion of round-nucleus cells undergoing DNA replication and (2) the duration of G_2. During the final 6 hours prior to fixation, H^3-thymidine was available to the cells, and during the final 3 hours, colcemide. Cells fixed 78 hours after mitogen addition.

	Population I (normal ♂, blood type O+)	Population II (normal ♀, blood type O+)
	per cent	*per cent*
Cells synthesizing DNA (radioactive nuclei)	76	40
Degree of radioactivity in dividing cells:		
(1) None (G_2 > 3 hours)	5	32
(2) Light to minimal (Y and Z intervals)	23	16
(3) Moderate (W through Z intervals)	47	43
(4) Heavy (U through Z intervals)	18	7
(5) Massive (every chromosomal region)	6	1

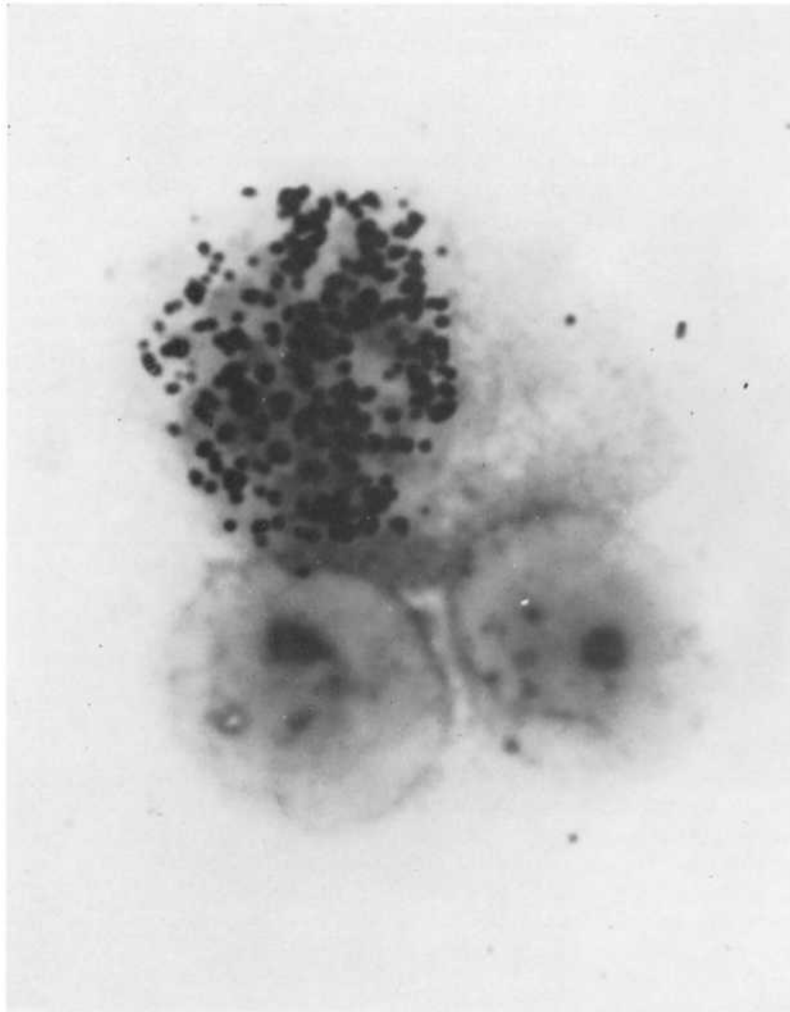

FIGURE 3 Autoradiograph showing DNA synthesis in one of three nucleated blood cells. Whole blood of a normal human male had been incubated with H^3-thymidine for 10 minutes immediately after removal from the vein, following which the cells were fixed and stained with orcein. Circulating cells which are synthesizing DNA sometimes increase in number after booster injections of certain antigens into individuals having immunity against those antigens. Such cells also circulate in increased numbers in individuals with active lupus erythematosus and certain other diseases of immunity (3 *d*). × 3760.

interval the isotope had become available to a given radioactive cell in metaphase.

DEFINITION OF THE U THROUGH Z INTERVALS OF THE S PERIOD

U INTERVAL: For the first time in the S period, synthesis becomes complete in a few detectable chromosomal regions. This is indicated by absence of grains and, therefore, absence of radioactivity over these regions. Areas free of grains appear first over the Nos. 17, one or two of Group 19–20, one or two of Group 21–22, and one or two of Group 13–15 (frequently the shortest pair of Group 13–15, thus probably the Nos. 15). Replication of almost the entire length of these chromosomes is completed during the U interval. Of the long chromosomes (Nos. 1 to 12), the Nos. 1 are unusual because of a relatively light grain distribution indicating that much of

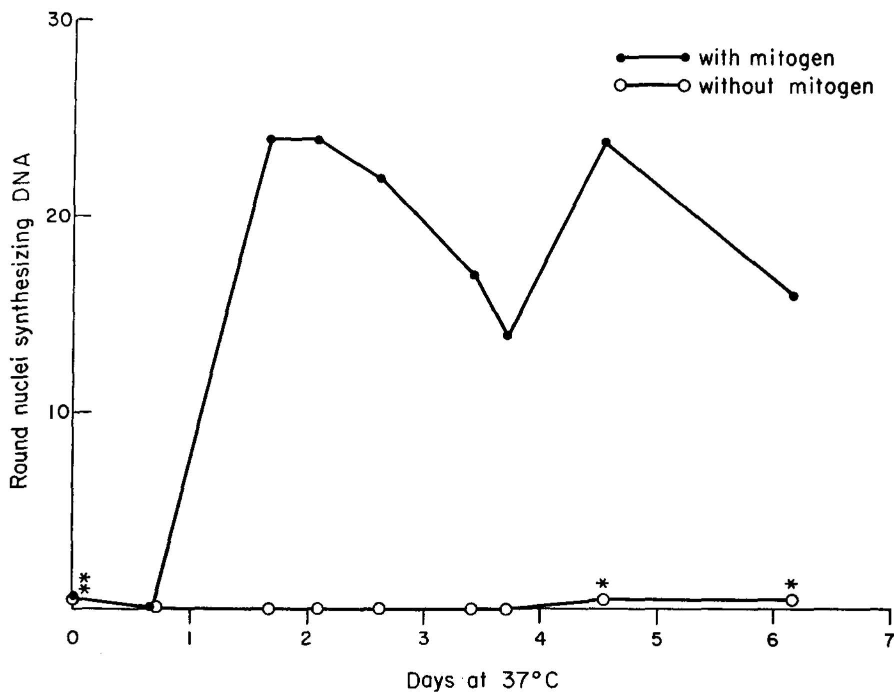

FIGURE 4 Human blood cells *in vitro*. Stimulating effect of a bean extract (*Phaseolus vulgaris*) on the synthesis of DNA in cultured lymphoid cells of blood from a normal human male. At the various times indicated, the percentage of cells engaged in synthesis was determined using a 10-minute pulse of H^3-thymidine to one of 18 identical cultures. An asterisk indicates that fewer than 0.1 per cent nuclei were radioactive. In the absence of mitogen, only an occasional cell enters DNA synthesis.

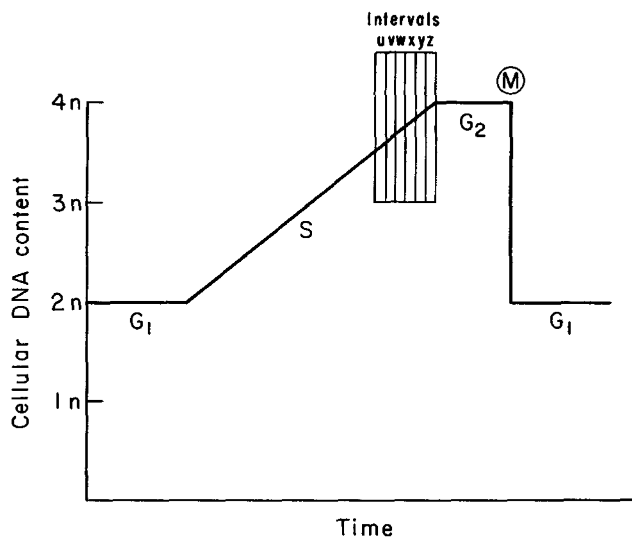

FIGURE 5 Cell division cycle. The U through Z intervals during which various chromosomal regions sequentially complete replication.

the DNA of this pair completes replication early. Telomeric areas of this pair become free of grains very early, and, in a third of the cells, the centromeres and pericentromeric areas also. Localized areas in the long arms of the Nos. 3 often complete synthesis at this time.

V INTERVAL: Multiple short segments of chromosomes Nos. 1 to 12 complete replication,

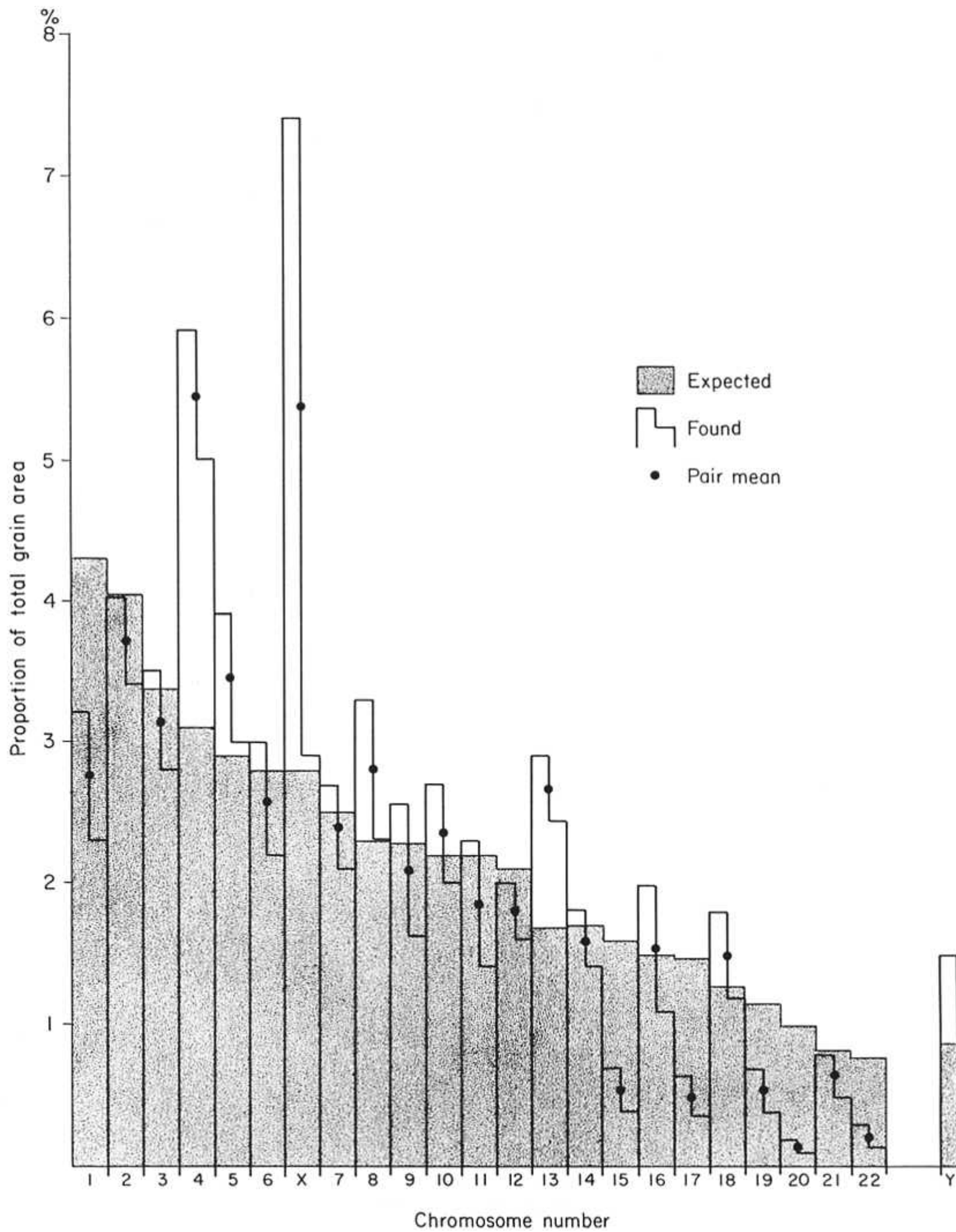

FIGURE 6 Analysis of the pattern of radioactivity of 87 metaphase figures derived from blood cells of 4 normal human females. The graph includes all analyzed cells which had a distribution of grains indicating that isotope had become available to the cells at some time during the terminal intervals of the S period. The radioactivity of the various chromosomes of the complement of each cell was estimated by direct grain count, if possible, or by approximation from the area of blackness when large numbers of grains were present. (The presence of heavy grain masses frequently encountered in these experiments prevents ac-

although, in general, these chromosomes continue their activity. The Nos. 1 cease in large areas. In two of Group 4–5, one before the other, areas in the long arms near the centromeres cease synthesis; the remaining two chromosomes of the group remain very active throughout their entire length. The telomeres and distal areas of both arms of the Nos. 6 cease synthesis. A chromosome of Group 19–20, one or two of Group 21–22, and the Y continue.

W INTERVAL (FIG. 8): The late replicating X chromosome of female cells becomes prominent during this interval because of continued massive replication signified by a dense confluence of grains over its entire length. At this time, the other chromosomes of Group 6-X-12 show multiple patchy areas of radioactivity adjacent to non-radioactive areas. Other chromosomes prominently active at this time are: the two late replicators of Group 4–5, one more than the other; two or three of Group 13–15; the Nos. 16, the Nos. 18, and the Y in most cells from the male. The centromeres and juxtacentromeric areas of one or both Nos. 1 become prominent in some cells because of continued replication at a time when much of the remainder of the chromosome has ceased. The conspicuous juxtacentromeric bulb sometimes observed in one of the No. 1 pair has shown later replication than the homologous area on the other of the pair in the few informatively labeled cells found in which the area was present. In two chromosomes of Group 4–5 there is extensive replication in progress in the short arms, but little or none in the long arms. One chromosome of Group 21–22 often continues a moderate degree of synthesis into this interval.

X INTERVAL (FIG. 9): This is characterized by multiple, small, well localized areas of synthesis in many of the longer chromosomes, whereas in most short chromosomes synthesis has ceased. Prominent among the chromosomes because of relatively heavy synthesis are the following: two of Group 4–5, one more than the other; the late X of the female; two or three of Group 13–15; one or both of the Nos. 16; one or both of the Nos. 18; and the Y of most male cells.

Y INTERVAL: Minimal synthesis in scattered areas continues with the following exceptions. (The pattern characteristic of the Z interval, described below, has in general, been reached). A prominent degree of replication continues in one X chromosome of the female. Variable, but generally light degrees of synthesis may continue in the late replicator(s) of Group 4–5, the two latest replicators of Group 13–15, and sometimes in a No. 16, a No. 18, one of Group 21–22, and the Y.

Z INTERVAL (FIG. 2 *F*): This interval lasts about 20 minutes after the late replicating X has ceased the greater part of its synthesis. The Z interval is characterized by minimal DNA synthesis, thus a few scattered single grains over several chromosomes which under the experimental conditions described here assume a random distribution.

Intragroup Patterns

The replicational pattern of chromosomes of certain groups provides characterization of the individual chromosomes which are indistinguishable by structural features alone.

GROUP 4–5: Extensive synthesis throughout the entire length of two chromosomes of this group continues into the X and Y intervals. A major portion of the long arms of the other two

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curate scoring). In the graph, the length of the heavy line bar for each chromosome represents the proportion of all grains over all cells which were contributed by that particular chromosome in the same group of cells. Within a pair of chromosomes, the homologue with the greater radioactivity is represented on the graph by the left one of the two bars, the mean of the two by a dot. Thus, in the X pair one chromosome has more extensive DNA synthesis than the other during the terminal portion of S. A less striking difference is found in other pairs when plotted in this manner. The stippled area represents the radioactivity to be expected if the degree of synthesis were proportional to chromosomal length. Certain pairs are found to have less radioactivity than expected (No. 1, No. 15, No. 17, and Nos. 19 to 22), while others have more (No. 4, No. 13, and the late replicating X chromosome).

The bar length of the Y chromosome is also indicated (analysis of 33 cells from 2 human males). Often there is extensive synthesis in the Y during the terminal intervals in comparison to autosomes of similar size.

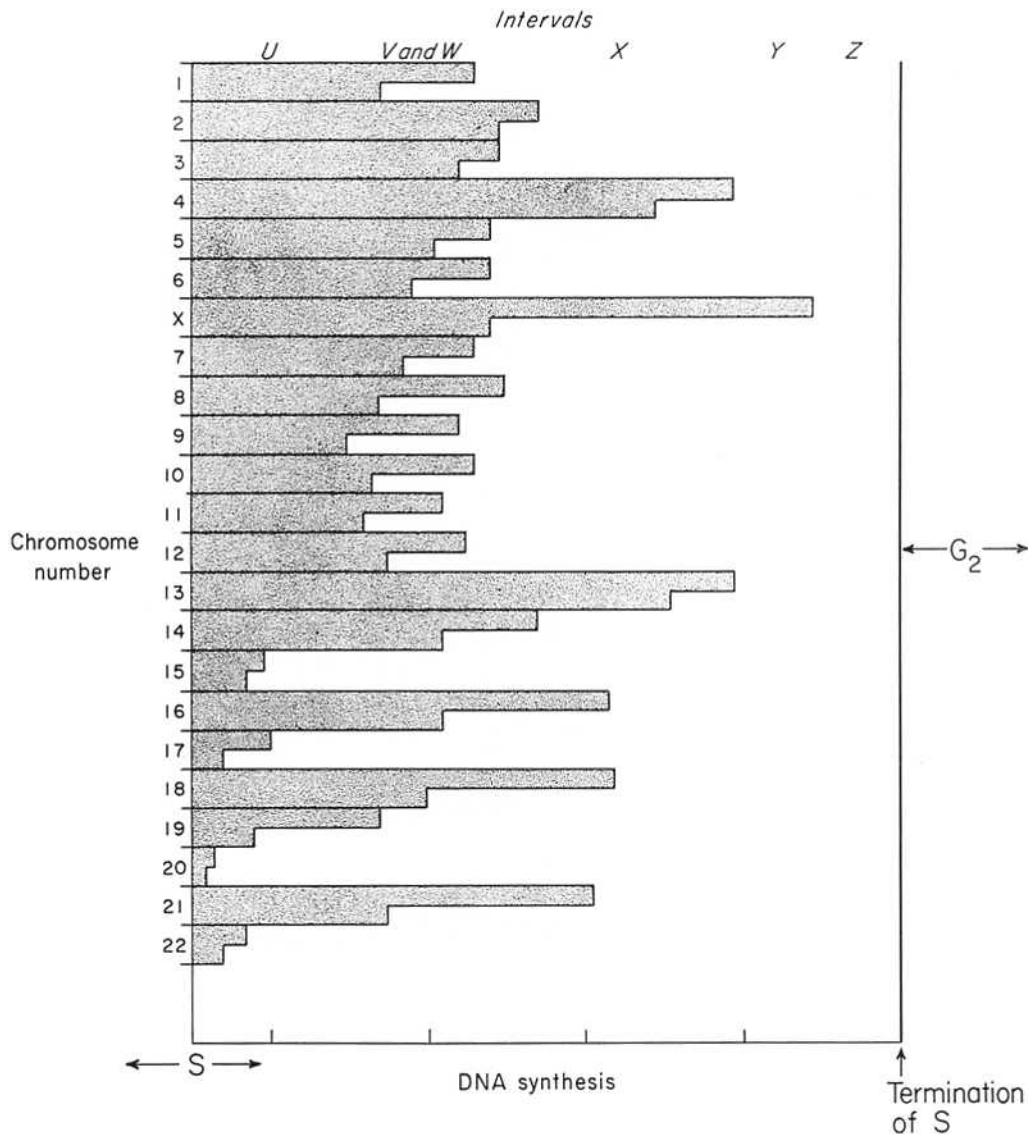


FIGURE 7 Further analysis of the 87 cells of Fig. 6 indicating the time during the terminal intervals of the S period at which an extensive degree of DNA replication ceases in the various chromosomes. Graph derived by scoring the degree of radioactivity of each chromosome of each metaphase figure, the total score for each determining the length of the bar. A chromosome in a cell was given a score of 1 if its grain distribution was moderate to heavy and covered at least one-third of the chromosomal length; it was given no score if less than one-third of its length was covered by grains. Certain chromosomes complete the greater part of their replication earlier than others, while others continue unusually late. From the distribution of radioactive chromosomes present in a given cell, it may be determined when the isotope became available to the cell.

complete synthesis relatively early (Fig. 8). It is probable that this pattern delineates homologues, although this is not established at the present time. (Homologue replicational synchrony is often seen in clearly recognizable pairs such as those of Group 1–3 and Group 16–18).

GROUP 13–15: Two chromosomes are among the earliest of the complement to complete

replication (U interval), while two others are among the last. The remaining two are intermediate, one of these later than the other. In this group the final portion of a given chromosome to replicate is variable, infrequently the satellite, sometimes the end of the long arm, and more often an intermediate area of the long arm.

GROUP 21–22–Y: The intragroup grain pattern of chromosomes of this group is less striking, possibly because of their small size. However, two of the group complete replication early in the U interval, whereas two others often continue with light radioactivity into the V or W intervals, one later than the other (Fig. 9). The Y chromosome in most cells from the male shows a heavy grain mass later than others of the group (Fig. 11). The Y frequently continues replication as late as the Nos. 16 and Nos. 18 but, possibly because of its size, its lateness is not so prominent as that of the late replicating X of female cells.

Homologue Asynchrony

In autosomes, homologue synchrony is commonly observed when the cells have prolonged access to the H^3-thymidine. However, as first demonstrated using pulse-labeling (3 *b*), there are striking examples of asynchrony in large areas of homologous chromosomes. In the present experiments using prolonged labeling, 10 per cent of cells had marked differences in the number of grains over comparable areas of two chromosomes of an identifiable pair (Figs. 8 to 11), differences which cannot be accounted for by the variations predicted on the basis of decay of the isotope. Areas which often display asynchrony include: the centromeric areas of the Nos. 1 (Fig. 9); the late replicating pair of Group 4–5 (Figs. 9 and 10); the two later pairs of Group 13–15 (Figs. 8 and 9); the Nos. 16; the Nos. 18 (Fig. 8); and the later pair of Group 21–22.

It may be concluded that the autosomal homologue asynchrony in most instances represents relatively short difference in timing because (*a*) it is observed much more frequently in cells which have received 10-minute pulse labeling (3 *b*), and (*b*) with prolonged labeling it is not seen repeatedly in the majority of cells in any given autosomal region. In the two X chromosomes, in contrast, it is observed regularly.

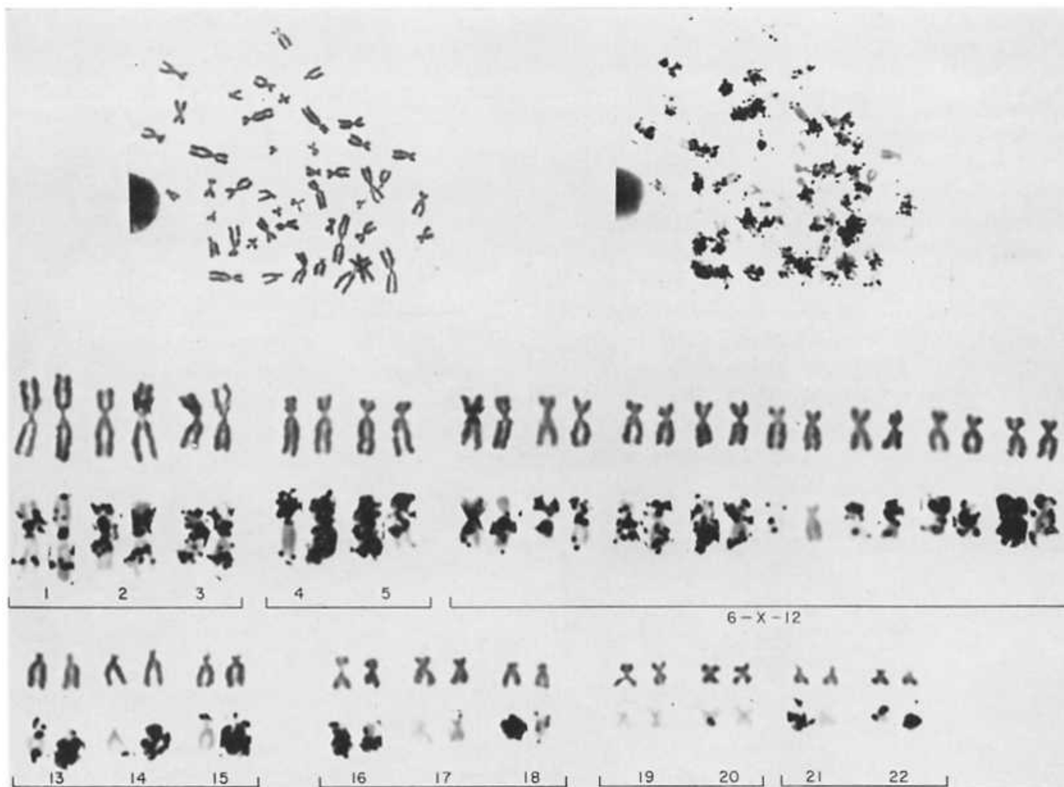
TABLE II

Tentative Classification of Various Intervals of the Terminal 2.5 to 3.0 Hours of the S Period, as Described in Text

Interval	Description
U	Earliest point in S period at which any detectable (1 to 2 μ length) chromosomal regions have completed replication.
{V W	Many regions of multiple chromosomes complete replication; pattern appears complex. In the W interval, the late X of the female becomes apparent.
X	Chromosomes prominent because of continued extensive replication: one X of female cells; two of Group 4–5; two of Group 13–15; the 16's; the 18's; two of Group 21–22; the Y of male cells. Scattered smaller localized areas in other chromosomes.
Y	In female cells, the late X continues extensive replication.
Z	Minimal synthesis, indicated by rare, scattered, single grains in several chromosomes.

Variability

The pattern of synthesis is variable in these cells. In a given cell the distribution of radioactivity is usually consistent with the sequence just described. The diagram of Fig. 7 is a composite derived from the analysis of the entire dividing cell population. However, the pattern



FIGURES 8 through 12 Magnifications given in the legends for these figures are for the two upper circular groupings only.

FIGURE 8 Duplicate-photography study of a radioactive normal human female cell in metaphase. The karyotype was prepared from the photograph of the orcein-stained chromosomes (upper left) without knowledge of the grain distribution of the autoradiograph (upper right). The autoradiograph of individual chromosomes indicates the regions in which DNA synthesis was in progress during the W through Z intervals of the preceding S period. In this cell there was extensive synthesis in two chromosomes of Group 4–5, the late replicating X, three of Group 13–15, the Nos. 16, a No. 18, and two of Group 21–22. Note homologue asynchrony in the X pair, Group 13–15, and the Nos. 18. The variable position in the karyotype of the late replicating X is demonstrated, here mounted as a No. 12. This shift of the X to the right in the karyotype has been observed more often in cells exposed to colcemide for 3 hours than in those exposed for 15 to 30 minutes (3 *d*). × 960.

(Experimental conditions of Figs. 8 to 12 are those described for Fig. 2.)

in any one cell may be slightly or even strikingly different. For example, the Nos. 18 may cease replication before the Nos. 16 in one cell but later in another. The variability in the centromeric area of the Nos. 1 has been described. The Y chromosome ordinarily continues to replicate throughout its length after the other short acrocentric units, but occasionally it may cease at an earlier time. Infrequently, radical departure from the usual pattern is found and many autosomes of a cell behave in atypical fashion. Sometimes, a female cell in metaphase is found without a late replicating X chromosome although the grain distribution otherwise indicates that the cell had been labeled during the X through Z intervals. The variability as well as homologue asynchrony is obviously most readily detected in easily identifiable chromosomal regions. Probably it occurs in all regions, although certain chromosomes exhibit a less variable pattern than others; these include the Nos. 2, 3, 16, and 17. Variability is, in part, responsible for the difficulty

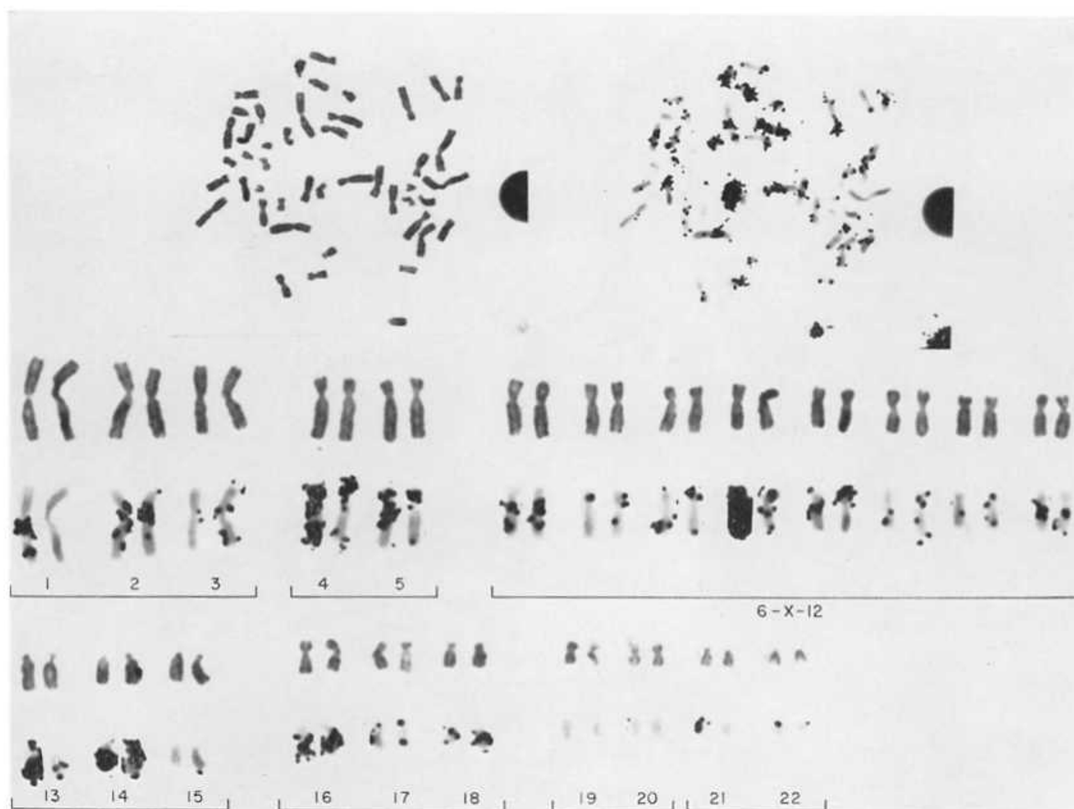


FIGURE 9 The grain distribution delineates the areas of synthesis in this cell during the X through Z intervals. A prominent degree of replication had continued in two chromosomes of Group 4–5, the late X (mounted in the No. 8 position), three of Group 13–15, the Nos. 16, and a No. 18. Autosomal homologue asynchrony in the pericentromeric region of the Nos. 1, in the long arms of the late replicating chromosomes of Group 4–5, and in the two of Group 13–15 which are intermediate in their timing of replication. × 1120.

encountered in defining the sequence of replication in the chromosomes.

DISCUSSION

Following the demonstration of late replication of one X chromosome in normal human female somatic cells, it was presumed that a genetically determined pattern of replication would be detectable in autosomes. Such a prediction is supported by data presented here as well as those of Patau (7), Schmid (8), Gilbert *et al.* (11), Muldal *et al.* (15), and Kikuchi and Sandberg (19).

Throughout most of the S period, DNA synthesis is intermittently in progress in different regions of each chromosome. Experiments using pulse-labeling with H^3-thymidine provide information concerning the location of synthesis at various times (3–6, 9, 12, 19), and the pattern has been found to be complex. The different chromosomes follow no consistent pattern such as replication beginning at the centromeres and continuing to the telomeres. However, during the final 150 to 180 minutes of the S period, one chromosomal region after another becomes fully replicated although the pattern at this time also is complex and different in each chromosome. In the experiments described in this paper the cell had prolonged access to the isotope. Delineation of the sequence in which each region is completed has been begun, and the general pattern to be found in nucleated blood cells has become apparent (Figs. 6 and 7). The significance of an established sequence remains unknown. It may be taken as a

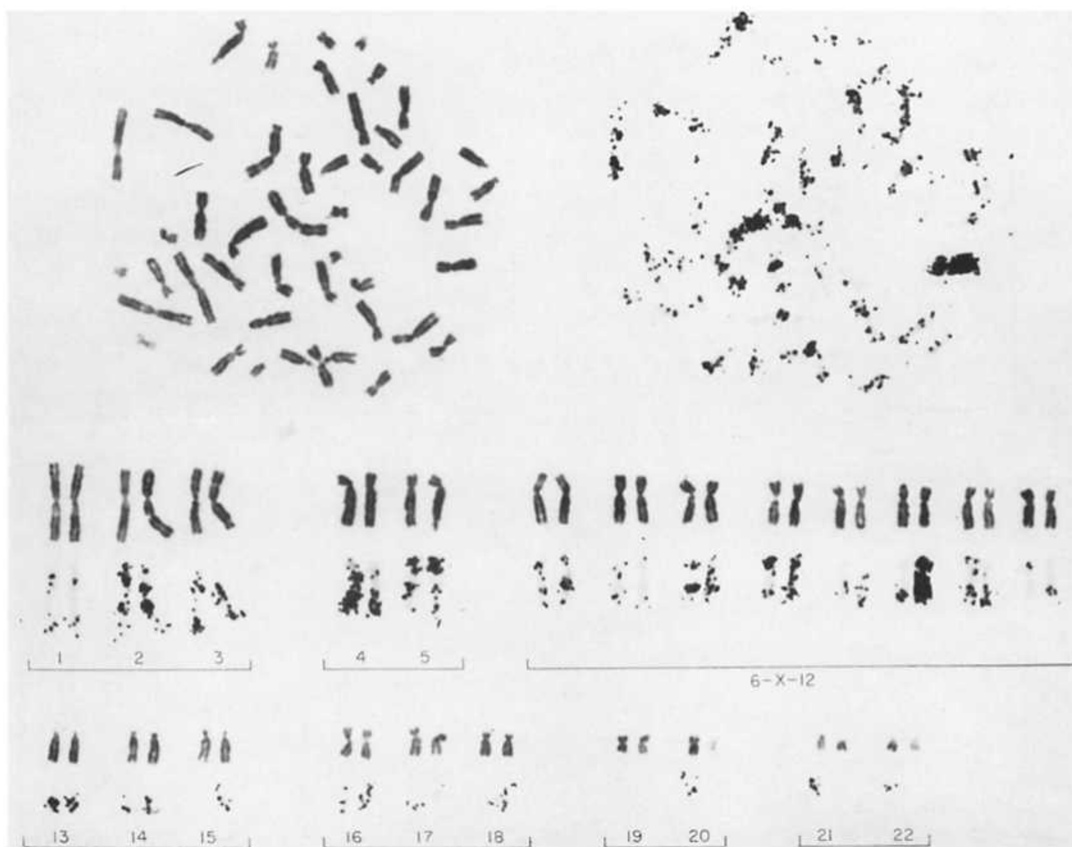


FIGURE 10 DNA synthesis during the X through Z intervals has been labeled. Scattered small areas; extensive replication in the late X; a lesser degree in regions of the Nos. 2 and the chromosomes of Group 4–5. Chromatid breakage present. × 1200.

baseline for further observations, including comparison with the pattern in other types of cells and in cells suspected of having an occult chromosomal aberration.

One striking finding strongly suggests that the pattern of DNA replication may be of functional significance. This is the very late replication of one X chromosome of the female cell (Figs. 8 to 10). This is still present, after many months *in vitro*, in fibroblasts derived from various human somatic tissues (3 *d*). It is known that the female somatic cell contains one X which is almost completely inactive genetically (22, 26–28) and also one X which during interphase is densely heteropyknotic (25). Since the male cell has no inactive X, no extensively heteropyknotic X, and no late replicating chromosome of Group 6-X-12, it is assumed that, in the case of the X, genetical inactivity is associated with excessive interphase contraction as well as late replication of DNA. Taylor (29 *d*) and Prescott and Bender (32) have shown that in certain cells nuclear and nucleolar RNA synthesis declines in early prophase and ceases in mid-prophase. It has been suggested that this cessation may be due to the inability of DNA to act as a template for RNA synthesis because of the marked chromosomal coiling of mitosis. Areas of interphase heterochromatin formation might, by mechanisms similar to those in effect in all chromosomes during mitosis, result in relative inactivity of localized chromosomal regions during interphase and also in delayed DNA replication. Although the mechanisms by which heterochromatin itself is produced remain uncertain, the pattern of DNA replication would become an index of function if the phenomenon

so strikingly detected in the X's, in relation to gene inactivation, is one utilized by the cell generally. Homologue replicational asynchrony, seen most prominently in the X's, is present also in autosomes (3 *b*, 5), but here it involves less extensive chromosomal lengths, and the timing differences are less marked.

derived and (*b*) whether the same chromosome (the early, intermediate, or late replicator) is involved in different families. The pattern in the late S period in cells containing an abnormal chromosomal complement is under investigation in several laboratories. Schmid (8) has reported that both the extra chromosome in Down's syn-

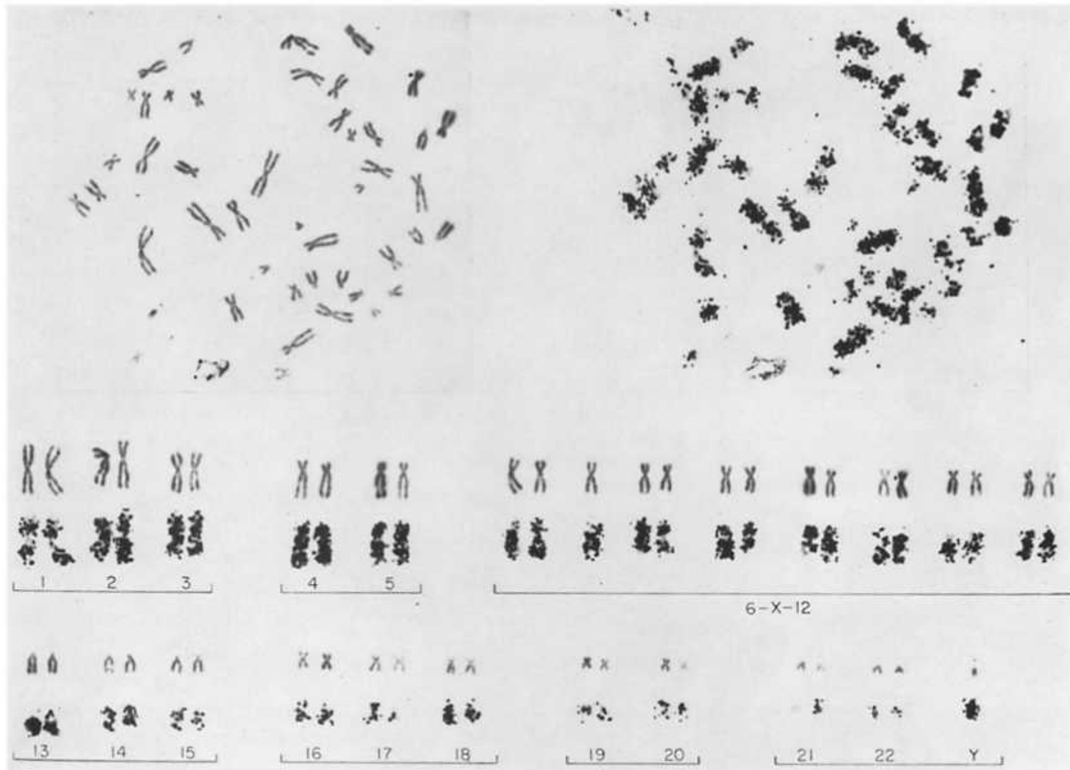


FIGURE 11 Normal human male cell studied by duplicate photography technique. The DNA synthesized during the W through Z intervals was labeled. The Y chromosome demonstrates extensive replication later than others from Group 21–22–Y. Unusually late synthesis in a No. 17 is an example of variability (see text). × 920.

With more detailed understanding of the replicational pattern and its variability, detection of small and otherwise obscure chromosomal aberrations may be anticipated in cells of abnormal as well as normal individuals. The use of autoradiography of tritium-labeled chromosomes will, in certain instances, indicate the chromosomal origins of an abnormal unit in the complement. In the case of familial Down's syndrome (33), for example, in which a translocation chromosome is inherited, it might be determined (*a*) from which chromosome of Group 13–15 the long arm is

drome and the deleted unit of chronic myelogenous leukemia cells complete replication at the time characteristic of the later pair of Group 21–22. We have found no evidence that the extra chromosome in Down's syndrome or in the trisomy 18 syndrome ceases replication at an anomalous time. In cells from one patient with the latter syndrome, the extra chromosome continued to replicate later than the U interval; it, therefore, has a pattern characteristic of the No. 18 pair (3 *d*) (Fig. 12). Morishima *et al.* (5) and Rowley *et al.* (13) have shown late replication of all but one X

in cells containing multiple X chromosomes. Miller *et al.* (10) reported that a structurally abnormal X was the late replicator in 100 per cent of cells observed. Similar findings have been reported by others (15, 16). Rowley (18) has found that a ring-X chromosome was late-replicating in almost all cells from a human female whose complement includes a ring and a normal X. In these few cases examined, therefore, the structurally abnormal X has been relegated to late replication and presumably to genetical inactivity, probably through selection for the cell in which the more normal genome is active. Certain females with a major aberration of one X, therefore, may be functionally hemizygous for the greater proportion of genetical determinants on the normal unit. The replicational pattern of X heterochromatin translocated to an autosome, or of the autosomal material adjacent to the translocation, has not yet been determined. If genetical function is associated with the timing of DNA replication, the timing may be affected by such position alterations.

In these experiments, the grain masses overlying the chromosomes were obviously frequently too heavy for accurate grain counting. However, such autoradiographs were preferable to light grain distributions for purposes of demonstrating relatively heavy and light areas of chromosomal radioactivity. Fine detail of the pattern within single chromosomes may be delineated in future experiments by varying the design of the experiment so that grains may be counted over very small regions of radioactivity. The experimental conditions, therefore, will be of importance when comparing the pattern in one cell with that in another, or the cells of one experiment with those from another experiment. The specific activity of the isotope in the culture and especially the time of exposure of the radioactive metaphase cell to film will be of importance in determining the grain distribution. In the work presented here, condi-

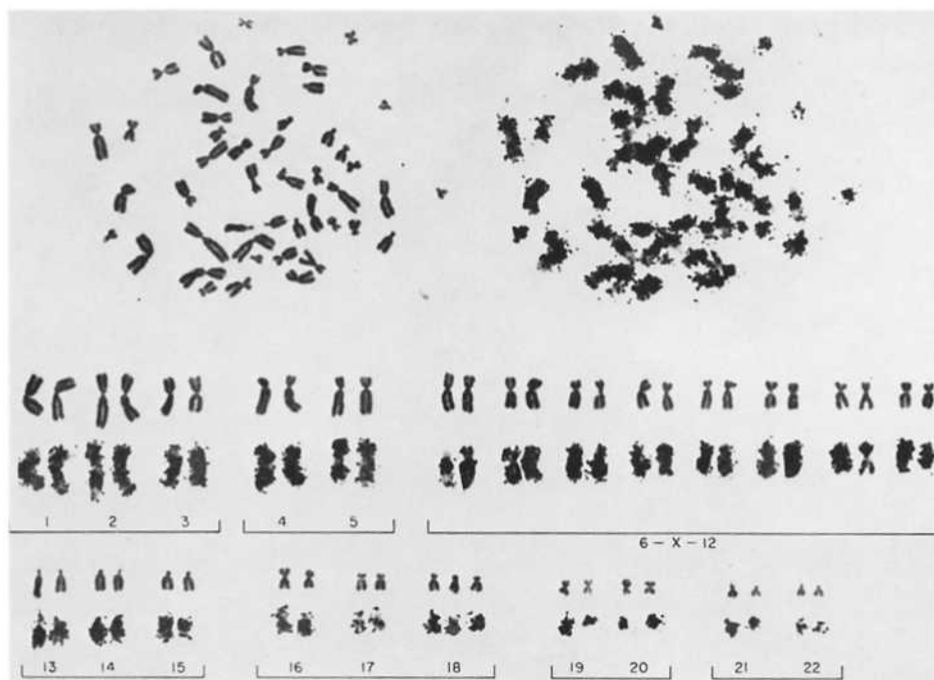


FIGURE 12 Duplicate-photography study of a nucleated blood cell derived from a female infant with trisomy 18 syndrome. Isotope had become available to the cell during the early U interval so that most chromosomes are extensively radioactive. Two of the five chromosomes of pairs No. 17 and No. 18, however, have relatively light labeling, while three are heavily labeled. Characteristically, in the U interval of the normal cell the Nos. 17 have almost ceased replication while there is extensive replication in progress in the Nos. 18. × 1100.

tions were kept constant in each experiment. The distribution of radioactivity of the various chromosomal areas observed would be expected only under the same experimental conditions. It seems probable (3 *d*, 17) that more detailed study of the Y and Z intervals will be useful in the search for small chromosomal aberrations and for variations among different tissues. The seemingly random grain distribution during these very late intervals may assume a clear pattern when the cells are exposed to autoradiographic film for prolonged periods. However, the approach used in the present experiments is suitable for demonstrating an established pattern and for elucidating some of the characteristics of the pattern within groups, such as the striking difference in timing of chromosomes of Group 4–5, Group 13–15, and Group 16–18.

The degree of variability in the distribution of chromosomal radioactivity in cells labeled at comparable times during late S is unexplained. If extensive chromosomal lengths cease replication simultaneously, and if the time at which this occurs in each chromosome should vary somewhat, independently of the progress of replication in other chromosomes, variable distributions would be demonstrated in the present type of experiments. The observed autosomal homologue asynchrony might be explained similarly. In further analysis of the data, detailed interrelationships between two or more pairs, for example No. 2 and No. 3, may indicate the time range in the S period over which the period of cessation of replication in a given chromosome may extend. The variability may reflect either a true normal variability of the *in vivo* state or, perhaps, only an abnormality resulting from the cultural conditions. In designing these experiments, it was assumed that cellular incorporation of the H^3-thymidine would be continuous as long as it was available in the medium. Evidence that in certain cells incorporation ceases after a short period (20 minutes), although the H^3-thymidine remains in the medium, has recently been presented by Rubini *et al.* (34) and could account for variation of the pattern. It is also possible that the variability is a reflection of the heterogeneity of the blood cell population. The study of the pattern of DNA replication in cloned diploid cell lines may provide answers to some of these questions concerning the variability.

Regardless of the variability of sequence in which the various chromosomes complete replication, these experiments have provided further characterization of certain chromosomes of the complement which are structurally indistinguishable from others in their group. The final naming of these chromosomes awaits more study, but already it is clear that two in Group 4–5, assumed to be a pair, are distinctively late in completing replication. The three presumed pairs of Group 13–15 and the two pairs of Group 21–22 also demonstrate distinctive sequences. In the chromosomes of Group 6-X-12 and Group 19–20, with the exception of the late replicating X of the female, the pattern is as yet poorly defined. Further experiments will provide more understanding of the intragroup patterns and aid in defining further the human chromosomal complement.

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