MAMMALIAN CHROMOSOMES *IN VITRO*

XVIII. DNA Replication Sequence in the Chinese Hamster

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ABSTRACT

The complete DNA replication sequence of the entire complement of chromosomes in the Chinese hamster may be studied by using the method of continuous H^3-thymidine labeling and the method of 5-fluorodeoxyuridine block with H^3-thymidine pulse labeling as relief. Many chromosomes start DNA synthesis simultaneously at multiple sites, but the sex chromosomes (the Y and the long arm of the X) begin DNA replication approximately 4.5 hours later and are the last members of the complement to finish replication. Generally, chromosomes or segments of chromosomes that begin replication early complete it early, and those which begin late, complete it late. Many chromosomes bear characteristically late replicating regions. During the last hour of the S phase, the entire Y, the long arm of the X, and chromosomes 10 and 11 are heavily labeled. The short arm of chromosome 1, long arm of chromosome 2, distal portion of chromosome 6, and short arms of chromosomes 7, 8, and 9 are moderately labeled. The long arm of chromosome 1 and the short arm of chromosome 2 also have late replicating zones or bands. The centromeres of chromosomes 4 and 5, and occasionally a band on the short arm of the X are lightly labeled.

INTRODUCTION

In the Chinese hamster, Taylor (1) found that DNA synthesis among the chromosomes is asynchronous. After pulse-labeling with tritiated thymidine (H^3-TdR), he took samples at various intervals for examination of metaphase chromosomes. The first sample that showed labeled chromosomes represented the late stage of S phase. At this stage the long arm of the X chromosome, the entire Y, and the two smallest pairs of autosomes were heavily labeled, while other autosomes were relatively lightly labeled. A number of hours later, the samples contained cells with the opposite labeling pattern: the long arm of the X and the entire Y were not labeled, but other chromosomes were heavily labeled. Taylor surmised that these cells were in early S phase when H^3-TdR was present in the medium.

Studies on human chromosomes confirmed Taylor's observation that DNA synthesis among chromosomes of a complement is asynchronous (2–5). One of the X chromosomes in the cells of the female, for example, is noticeably late replicating but the other X is not.

Most investigators have used the conventional pulse-labeling method. One disadvantage of this method is that when a chromosome shows no label the investigator is uncertain whether this chromosome, during the pulse-labeling period,

has already completed, or has not begun, DNA synthesis. This uncertainty can be removed by the continuous labeling method (5, 6). Since a cell must complete its DNA replication before entering mitosis, and since H^3-TdR has been present in the medium until the time of harvest, unlabeled chromosomes or chromosome segments must represent those which have already finished DNA replication when the precursor is introduced. Otherwise they would show radioactivity. Thus by examining the labeling pattern of samples fixed at various intervals after the introduction of H^3-TdR, one may obtain a reasonably reliable picture of the terminal stages of DNA replication in a given species.

For studies of the beginning stages of DNA synthesis, pulse-labeling is the only available method. Because of the relatively long time-span between the commencement of DNA synthesis and the start of mitosis, variation in time requirement for S and G_2 phases among individual cells may cause difficulty in determining precise substages of S phase. In cells of the Chinese hamster, the average period of S is approximately 6 hours, and that of G_2, 2 hours (7). If a 10 per cent deviation exists in the cell population, a cell at the beginning stage of DNA synthesis may enter mitosis 7 to 9 hours after pulse-labeling, and if a 25 per cent variation, 6 to 10 hours. Thus any sample taken 6 hours or longer after pulse-labeling would contain a mixture of labeled mitoses representing various stages of the S phase. In materials that require longer G_2 phase, such as human cells *in vitro*, the variation may be greater. This difficulty can be avoided by arresting a large number of cells at the beginning of S phase and relieving this blockade by pulse-labeling with H^3-TdR.

5-fluorodeoxyuridine (FUdR) is one of several agents available to inhibit DNA replication of cells by interfering with the *de novo* synthesis of thymidine monophosphate. In a growing cell population, cells are at various stages of the mitotic cycle. When FUdR is applied to the cultures, DNA synthesis ceases. Cells originally in the S phase will be arrested at their respective substages, but cells in G_2, mitosis, and G_1 phases are not poisoned until they reach the threshold of S phase at which stage they will be blocked. Therefore, when sufficient time is permitted for the FUdR treatment, most cells originally not in S phase can be accumulated at the entry of the DNA synthetic period. When sufficient amount of thymidine is introduced into the medium, all cells resume DNA synthesis and enter mitosis thereafter. Those originally in the S phase resume DNA replication from their respective positions and will enter mitosis in the usual staggered manner. Those blocked at the entry of S phase begin DNA synthesis simultaneously, and a partial synchronization of mitosis can be achieved (8).

This process was utilized in the present investigation to study the beginning step of DNA synthetic patterns. The blockade of DNA synthesis was relieved with H^3-TdR; later, radioactive thymidine was replaced with non-radioactive thymidine to permit the completion of S phase. The labeling pattern of chromosomes of these cells, showing the commencement stage of DNA replication, can be compared with that of the end stages obtained by the continuous labeling method.

MATERIALS AND METHODS

Principal material for this study was a diploid male Chinese hamster cell strain designated strain Don (9). A diploid female strain was used once only to check the late replication of the two X chromosomes. Cultures in the logarithmic growth period were treated in two ways:

1. H^3-TdR (specific activity 6.7 c/mmole, New England Nuclear Corp., Boston) was introduced into the cultures at a final concentration of 2 μc/ml. The isotope was left in the medium without washing until the time of harvest. Samples were taken at intervals of 2, 3, 4, and 6 hours after the introduction of the radioactive precursor, each with a 1 hour period of Colcemid treatment prior to fixation.

2. Cultures were treated with FUdR (courtesy of Hoffmann La Roche Laboratories, Inc., Nutley, New Jersey) at a concentration of 0.1 μg/ml for 12 hours. The cells were then labeled for 5 minutes with H^3-TdR at a final dilution of 5 μc/ml, after which the cultures were washed and allowed to grow in a medium containing 10 μg/ml of non-radioactive thymidine. Samples were fixed every hour beginning hour 7 until hour 12, each with a 1 hour Colcemid treatment. For labeling periods longer than 30 minutes, H^3-TdR was mixed with non-radioactive thymidine to reach a final dilution of 1 μc/ml. Thus the isotope would not be exhausted during the prolonged labeling periods. Other procedures were the same as just described.

Temporary preparations were made with the acetic orcein squash technique. Suitable metaphase figures were photographed. The slides were then placed on

a cake of dry ice for a minimum of 5 minutes and the coverslips were flipped off with a sharp scalpel. Kodak AR 10 stripping film was then applied to the slides and allowed to expose for 4 days. After developing for 2 minutes in D-19B developer, the slides were restained in dilute Giemsa's stain. The cells previously photographed were relocated and rephotographed. The detailed procedures of chromosome autoradiography have been described by Schmid (10).

RESULTS

The Termination Stages of DNA Synthesis

After the introduction of H^3-TdR into the Don cultures, no labeled metaphases could be detected when cells were fixed at 2 hours or less. At 3 hours the majority of metaphases were labeled. This suggests that the G_2 phase of this strain is roughly 2.5 hours. Fig. 1 represents the chromosomes of a cell fixed at 3 hours after continuous labeling. Note that the sex chromosomes and the two smallest pairs of metacentrics are heavily labeled, but other chromosomes are either partially or lightly labeled.

With the continuous labeling method, one can reconstruct the DNA replication sequence in each chromosome from autoradiographs of cells fixed at various intervals. For example, one may obtain some information by merely examining the three karyotypes presented in Fig. 2. Fig. 2 *c* shows the DNA synthetic activity of a cell at the very end stage of S phase at which time the Y chromosome alone is moderately heavily labeled. A few silver grains are still discernible on the long arm of the X chromosome. Probably such a cell would enter G_2 phase within a few minutes. Fig. 2 *b* represents the labeling pattern typical of cell samples labeled with H^3-TdR for 3 hours. Considering an average G_2 phase of 2 hours, the labeling pattern represents the DNA synthetic activity during the last hour of S phase. The long arm of the X, the Y and chromosomes 10 and 11 are heavily labeled, whereas other chromosomes are only partially labeled. Moderately heavily labeled are the short arm of chromosome 1, the long arms of chromosome 2 and chromosome 6, and the short arms of chromosomes 7, 8, and 9. Chromosomes 4 and 5 are usually free of label or are labeled lightly.

Fig. 2 *a* represents the labeling pattern in a cell during the final 2 hours of S phase (continuous labeling for 4 hours). Here few chromosomal segments are without label. Among the first to complete DNA replication are the long arms of chromosomes 5 and 9, the distal portions of the long arms of 7 and 8, and parts of chromosome 4.

From over 100 autoradiographs examined, all of which were photographed before and after stripping, several general conclusions can be made regarding the terminal stages of DNA synthesis.

1. In each sample, variation in labeling patterns representing several substages of S phase did occur, but one pattern generally predominated. The lack of uniformity in non-synchronized populations is anticipated, not only because of natural variation in time requirement among cells but also because of Colcemid treatment which arrested mitoses during the last hour prior to fixation. However, it is not difficult to reconstruct the sequence of events that took place by comparing the labeling patterns of various cells.

2. There is a definite pattern of H^3-TdR incorporation on each chromosome. Although deviations from the general pattern were found, the late replicating regions were characteristic. This observation confirms that made by Schmid on chick (6) and on human chromosomes (5).

3. Although disparity in labeling pattern between homologous chromosomes was found, homologous chromosomes generally showed similar labeling pattern in the same cell, indicating that the homologs reached similar stages of DNA replication at the same time. Disparity may be due to several possibilities: (*a*) one chromosome more advanced than the homolog, (*b*) a real difference between the homologs, or (*c*) random decay of the isotope. The most obvious example of asynchrony between homologs is the X chromosomes of female cells. In cells of the female Chinese hamster, one of the X chromosomes is late replicating (similar to the behavior of the Y chromosome in the cells of the male) whereas the other behaves like the X of the males, that is, only the long arm is late replicating.

Fig. 3 presents cut-out photographs from various cells to illustrate the completion sequence of DNA replication. From left to right, the pictures depict progressive completion of DNA replication, the figure to the far right for each chromosome representing the last step before the chromosome enters G_2 phase. It should be emphasized that the replication stages of one chromosome seen in Fig. 3 does not necessarily correspond to those of

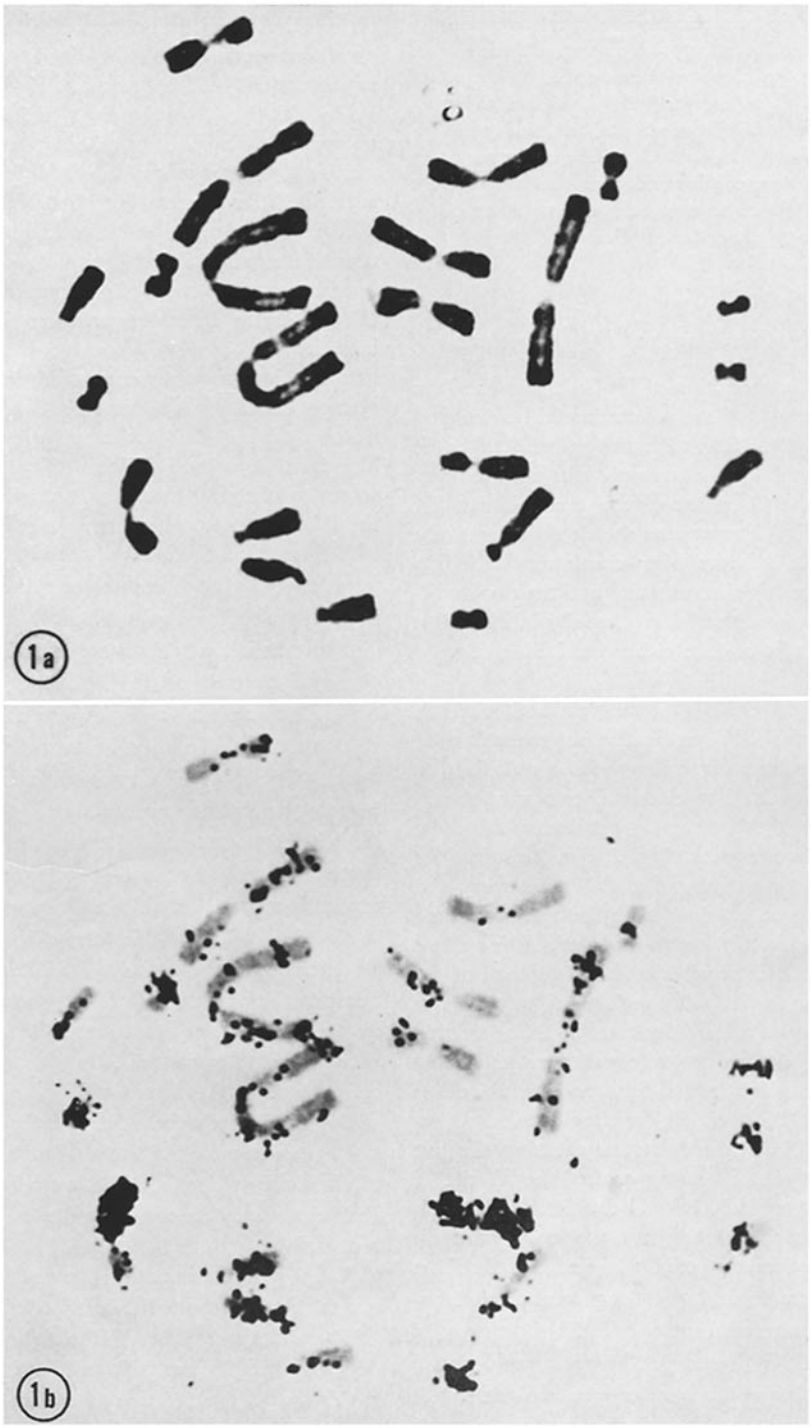


FIGURE 1 *a* A metaphase figure of male Chinese hamster. × 2700.

FIGURE 1 *b* Autoradiograph of the same, showing late replicating chromosomes and chromosome segments. Continuous tritiated thymidine labeling, 3 hours; Colcemid, 1 hour. × 2700.

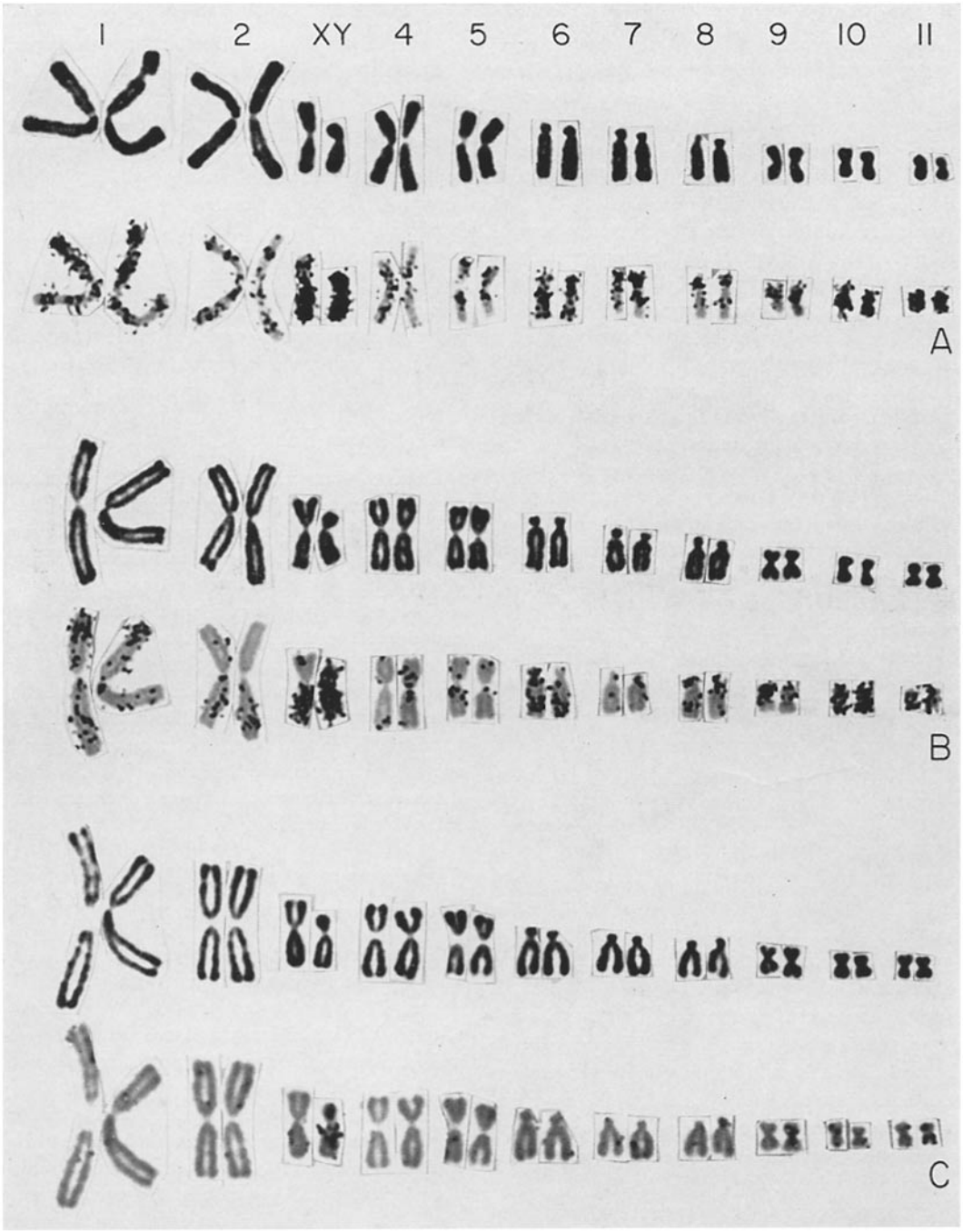


FIGURE 2 Karyotypes (before and after autoradiography) of male Chinese hamster. Continuous tritiated thymidine labeling; Colcemid treatment, 1 hour. Fig. 2 *a*, H^3-TdR labeling for 4 hours; Fig. 2 *b*, H^3-TdR labeling for 3 hours; and Fig. 2 *c*, H^3-TdR labeling for 2.5 hours. × 3600.

others arranged in the same column. Briefly, the termination process of each took place as follows:—

CHROMOSOME 1: A lightening of label appeared at first in the middle-lower portion of the long arm 4 to 5 hours before the cell reached metaphase. At approximately 3 hours before metaphase, many regions became free of label except the following areas which remained as labeled zones: the distal half of the short arm, the centromeric region, the upper middle portion, and a region near the distal end of the long arm. Progressively, the long arm completed replication earlier than the short arm, leaving narrow bands or spots of radioactivity.

CHROMOSOME 2: The very distal portions of both arms were the first to complete DNA replication. However, as replication progressed, the majority of the long arm remained actively synthesizing DNA while only a broad band of radioactivity was detected on the short arm.

THE X CHROMOSOME: The short arm completed replication earlier than the long arm in cells of the male and in one of the two X's in cells of the female. During the last hour of S phase (3 hours before metaphase), the long arm was still heavily labeled while no radioactivity or only scattered silver grains were seen on the short arm. In females, the second X chromosome was the last to complete replication.

THE Y CHROMOSOME: The Y chromosome was the last replicating element of the entire male complement. Three different ending stages (Fig. 3) were noted. Most commonly, the label faded rather uniformly throughout the length; but occasionally, either the short arm or the long arm finished DNA replication before the other component.

CHROMOSOMES 4 AND 5: Both pairs completed replication early. At approximately 4 to 5 hours before mitosis, radioactivity was limited in the central portion, on both sides of the centromere. At 3 hours before mitosis, the entire chromosome was practically devoid of label except for occasional grains at the centromere.

CHROMOSOME 6: Chromosome 6 was relatively late replicating, especially when compared with the other two morphologically similar pairs, chromosomes 7 and 8. A late replicating band was usually prominent near the distal end of the long arm. Sometimes the short second arm would also bear late label. However, near the end stage, only the distal band would show radioactivity, a property useful for the identification of this pair.

CHROMOSOMES 7 AND 8: These two chromosomes behaved similarly in respect to late replication pattern. Generally the distal half of the long arm was the first segment to complete replication. During the last hour of the S phase, heavy label could be seen only on the short arm. These two pairs are difficult to distinguish.

CHROMOSOME 9: The short arm was late replicating, especially its proximal portion.

CHROMOSOMES 10 AND 11: These two pairs of chromosomes were late replicating, especially the centromeric region.

The Commencement of DNA Synthesis in Male Chinese Hamster Cells

Cultures were treated with FUdR for 12 hours. The cultures were then labeled with H^3-TdR for 5 minutes; then the isotope was replaced with nonradioactive thymidine for an additional 10 hours. Autoradiographs of metaphase figures revealed a uniform labeling pattern in several hundred cells examined. Fig. 4 shows the pattern of such a cell before and after exposure to stripping film. Note that the labeling pattern of this cell is exactly the reverse of the terminal stage illustrated in Figs. 1, 2, and 3. Here the Y and the long arm of the X are unlabeled, and chromosomes 10, 11, and the short arms of the acrocentrics and of chromosome 9 are lightly labeled.

The Commencement of DNA Synthesis in Late Replicating Chromosomes

To determine the precise time at which the late replicating chromosomes (such as the Y) began DNA replication, cultures were treated with FUdR for 12 hours and relieved with nonradioactive thymidine (10 μg/ml) mixed with H^3-TdR at a final dilution of 1 μc/ml. Every 30 minutes the labeling medium of one culture was decanted and medium containing non-radioactive thymidine (10 μg/ml) was added to permit completion of DNA synthesis. All cultures were fixed at 8 hours after the introduction of the labeled medium. The mitotic figures thus represented DNA synthetic patterns of the first 30, 60, 90, . . . minutes up to continuous labeling from the beginning to harvest. As anticipated, continuous labeling yielded completely labeled chromosomes in the entire complement. The Y chromosome

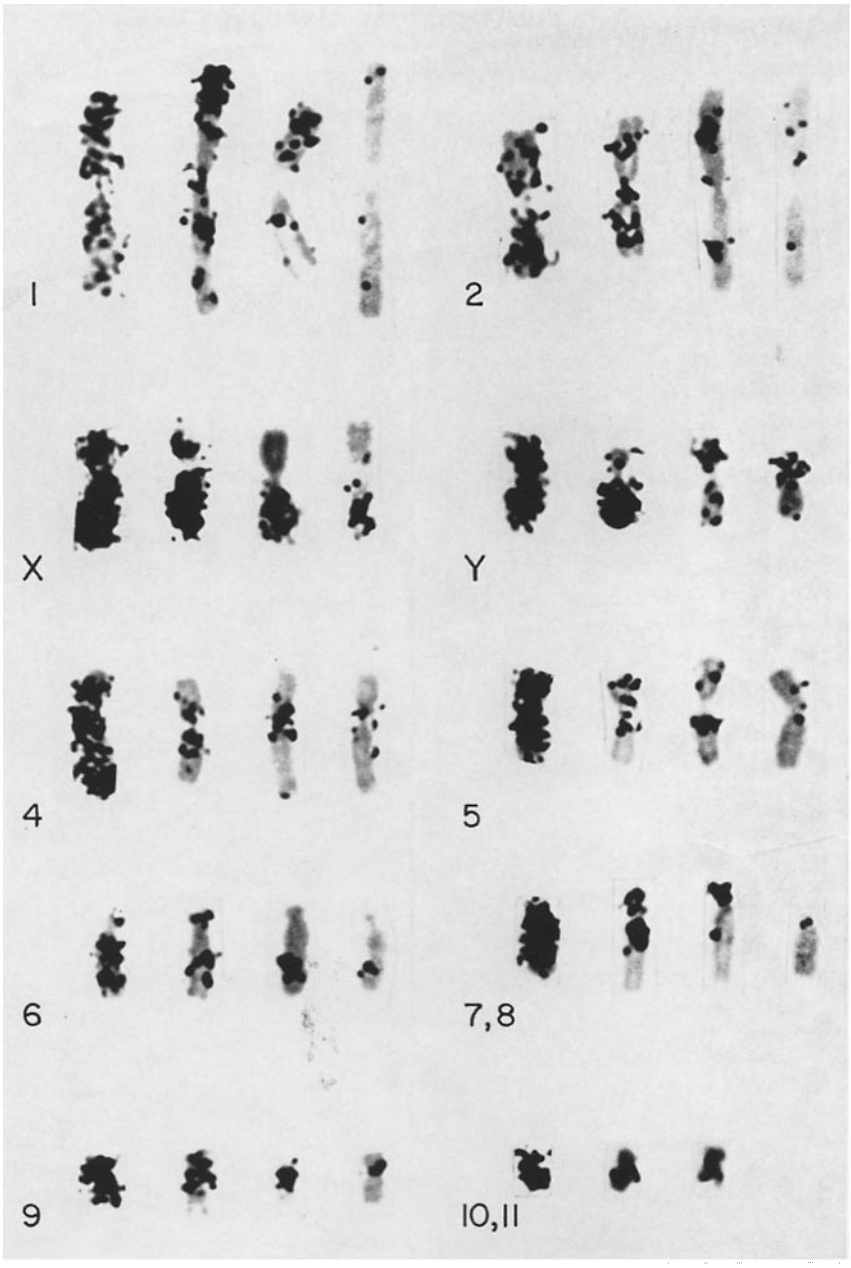
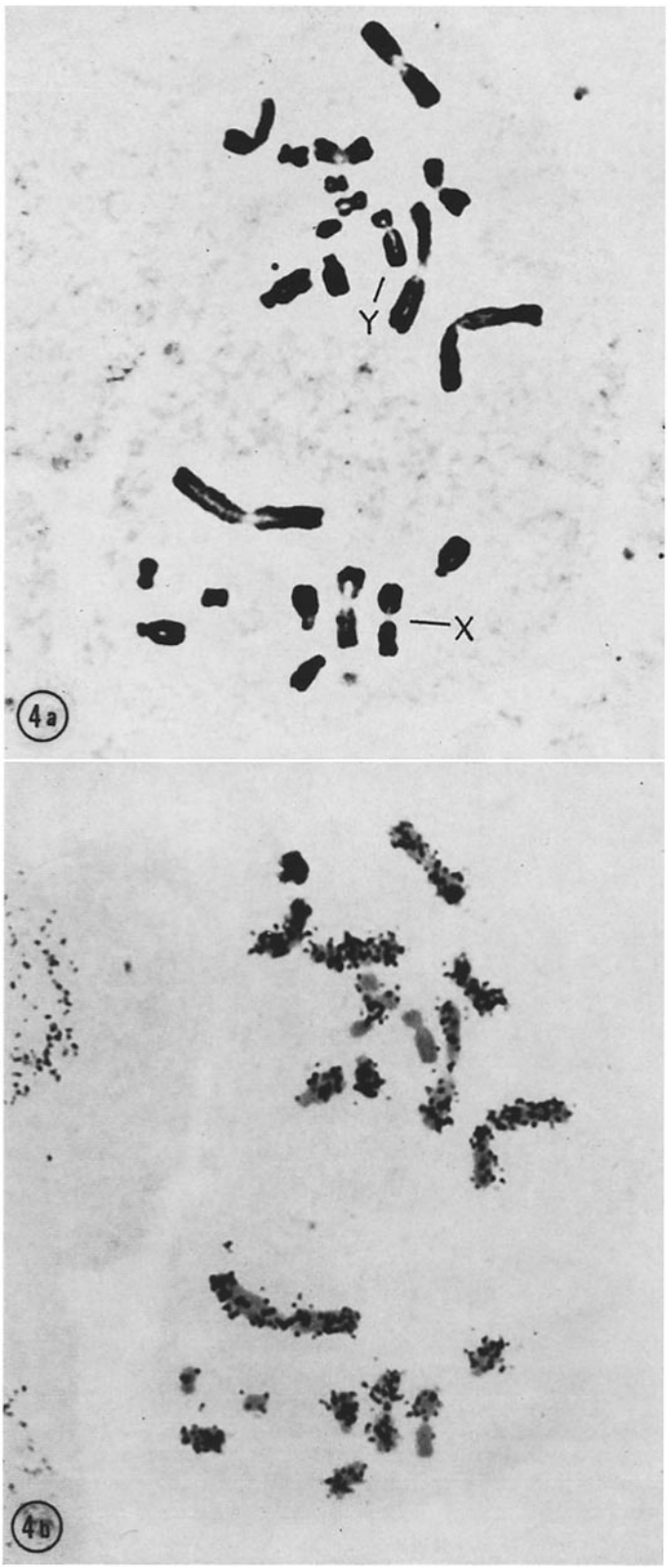


FIGURE 3 Cut-out autoradiographs representing terminal sequences of DNA replication, arranged from left to right for each chromosome. One stage of one chromosome does not necessarily correspond to that of another chromosome in the same column. × 3600.

and the long arm of the X remained unlabeled until 4.5 hours after the commencement of the DNA synthesis of the cell. Since the entire S phase lasted approximately 6 hours, the late replicating elements apparently began DNA synthesis when other elements had almost completed the replication process and finished the process within 2 hours.


Y
X
4a
4b

DISCUSSION

The data presented herein confirm the findings of Taylor (1) that DNA replication among chromosomes of the Chinese hamster is asynchronous, and of Schmid (5, 6) that many chromosomes possess late replicating zones or bands which are characteristic for each element. The combined use of FUdR inhibition and continuous labeling methods reported in this study allows the determination of the complete replication sequence as well as the precise timing of every step. Furthermore, the FUdR method employed here ensures that the observed DNA synthesis pattern represents the very beginning of, not simply early, S phase.

As mentioned, the duration of individual phases of the cell cycle varies from cell to cell. Using the DNA synthesis pattern of the first 5 minutes as a guide, investigators may estimate the variability in time requirement for the combined S and G_2 phases. In our experiments, this pattern prevailed in metaphases sampled from 7 to 12 hours, although the frequency of mitosis was the highest at hour 10. This fact also explains why attempts to synchronize divisions in the Chinese hamster cell populations have not been very successful (11).

Another application of this technique is in the study of the variation in time requirement for the G_1 phase by varying the time of FUdR treatment. Our preliminary data showed that when FUdR inhibition lasted for 12 hours all metaphase figures harvested 10 hours following relief were labeled. However, when FUdR inhibition lasted for 8 hours only and H^3-TdR relief lasted for 5 minutes, unlabeled metaphases were observed in the 10 hour sample. This indicates that some cells were still in late G_1 phase when H^3-TdR was applied.

Of special interest to cell biologists is the fact that DNA replication seems to start among many chromosomes at multiple sites. The 5 minute pulse was indeed short compared with the entire S phase. Yet, with the exception of the late replicating areas, all chromosomes appeared to have many replicating centers operating simultaneously. If the DNA molecule of each mammalian chromosome represents a multiple of DNA molecules similar to that of bacteria (12), there may be numerous pivot points from which replication starts. One should, however, keep in mind that FUdR may have considerably compressed the regular process so that the information obtained might not strictly reflect what happens under ordinary circumstances (13).

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FIGURE 4 *a* A metaphase figure of male Chinese hamster. × 2700.

FIGURE 4 *b* Autoradiograph of the same. The cultures were originally treated with FUdR (0.1 μg/ml) for 12 hours, pulse-labeled with H^3-TdR (5 μc/ml) for 5 minutes, and grown in non-radioactive thymidine (10 μg/ml) for 10 hours. Colcemid treatment for 1 hour before fixation. × 2700.

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