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Review of Cytology

EDITED BY

G. H. BOURNE

J. F. DANIELLI

ASSISTANT EDITOR
K. W. JEON

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INTERNATIONAL REVIEW OF CYTOLOGY, VOL. 80

DNA Replication Fork Movement Rates in Mammalian Cells

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I. Introduction

The DNA replication rate is formed by two components: the number of actively operating replicating units (replicons), and the average linear rate at which the DNA replication fork moves along the parental DNA (DNA fork movement rate). The number of replicons in an average mammalian cell is about 100,000 (Painter *et al.*, 1966; Okada, 1968), and the number of replicons active at any one time during S phase appears to be the main factor affecting changes in the DNA synthesis rate (Painter and Schaefer, 1971). DNA fork movement rates vary considerably from one cell type to another; published values range from 0.1 μm/minute in human cells to 2.5 μm/minute in Chinese hamster ovary (CHO) cells. The majority of rates reported for human cells are in the range of 0.4 to 0.7 μm/minute. The reasons for the reported variations are not completely known, but some factors are species, ploidy, whether normal or transformed, growth conditions (media, serum, etc.), and the experimental techniques used.

Various aspects of DNA replication have been surveyed in recent reviews (Edenberg and Huberman, 1975; Painter, 1976; Sheinin and Humbert, 1978; Hand, 1978, 1979; De Pamphilis and Wassarman, 1980). In this article, we review DNA fork movement rates only in mammalian cells.

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II. Methodologies

The first report on fork movement rates utilized DNA fiber autoradiography. Cairns (1966), extending a technique he first used with bacteria (Cairns, 1963), incubated HeLa cells for 45 or 180 minutes with [^{3}H]thymidine. The cells were then lysed and the DNA was spread out and coated with a photographic emulsion. After a sufficiently long exposure time, the emulsion was developed, and the tracks produced by the radioactively labeled DNA were measured. Cairns found that a 45-minute labeling time produced labeled DNA lengths ranging from 10 to 30 μm, whereas the 180-minute labeling time produced lengths ranging from 50 to 100 μm. Thus, human DNA appeared to have replicated at a rate of 0.5 μm/minute or less.

Subsequently, other workers improved the DNA fiber autoradiography technique to obtain more precise results and more information about DNA replication. Huberman and Riggs (1968) first pretreated cells with fluorodeoxyuridine (FUdR) before labeling with [^{3}H]thymidine. The FUdR depleted the cells of DNA thymine precursors, so when [^{3}H]thymidine was added later it became the sole source of thymine for DNA replication. This led to an abrupt start of the labeled segment of DNA as well as a higher specific activity of thymidine, thus producing heavier grain tracks in the fiber autoradiograms. Another innovation used by Huberman and Riggs was to label cells first with [^{3}H]thymidine with a high specific activity followed by [^{3}H]thymidine with a low specific activity. This resulted in a high grain density track followed by a low grain density track. These variable grain density tracks allowed the findings that (1) replicons are arrayed tandemly (i.e., linearly or sequentially) along the DNA fiber, (2) many replicons on one fiber are of uniform size, and (3) DNA fork movement rates can be measured within each replicon. Huberman and Riggs reported that fork movement rates ranged from 0.5 to 2.5 μm/minute in CHO cells.

Recently, Yurov (1980) attempted to increase the information obtained by fiber autoradiography by isolating single labeled cells on microscope slides and then lysing the cell *in situ*. The resulting fiber autoradiograms then represented DNA from only one cell, thus allowing analysis of DNA replication in one nucleus. Examples of fiber autoradiograms are shown in Fig. 1.

The advantages of DNA fiber autoradiography in examining DNA fork movement rates are that (1) single DNA chains are resolvable, (2) information can be obtained about replicon sizes, and (3) information can be obtained about relative times of replicon initiation. It should be noted that this technique was also used to show that DNA replication occurs bidirectionally (Huberman and Riggs, 1968).

Limitations of this technique are: (1) Nonrepresentative sampling. Since the average mammalian cell contains approx. 10^5 replicons and most autoradiographic data represent 150–300 tracks, only a very small percentage of the replicating DNA is represented. (2) Low resolution. The limit of resolution in

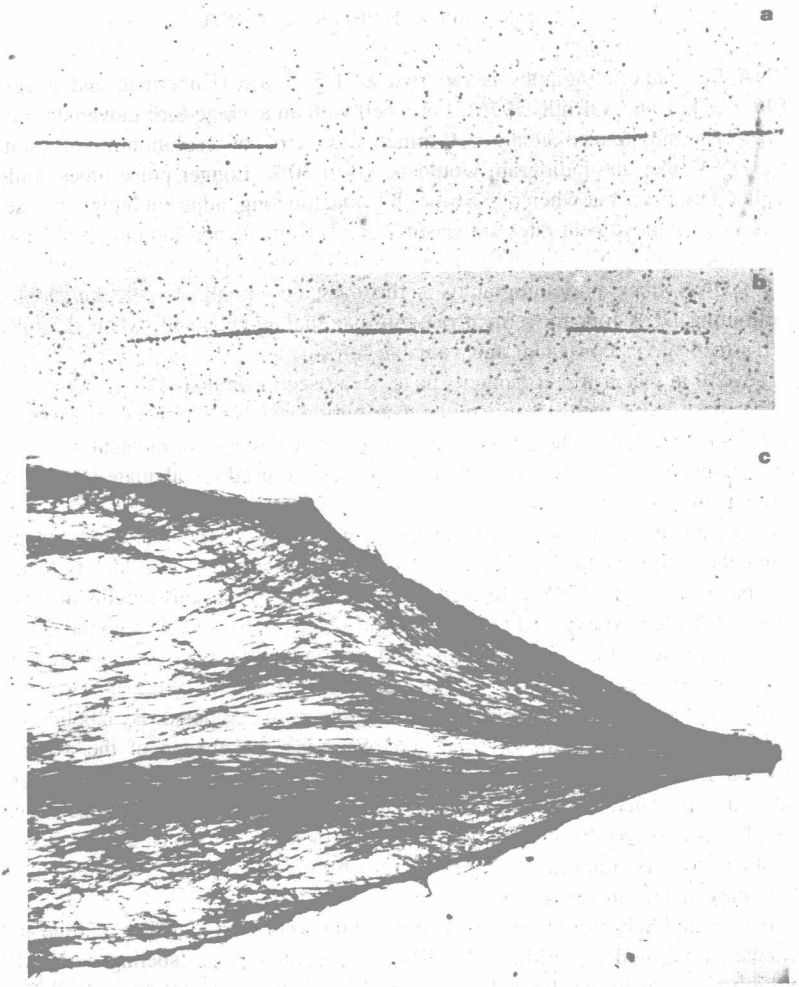


FIG. 1. Autoradiograms from mouse L5178Y cells. (a) Thirty-minute pulse-labeling. Fibers show tandem arrays of replicons. (b) Labeling with high specific activity [^{3}H]thymidine (hot pulse) for 30 minutes followed by labeling with low specific activity [^{3}H]thymidine (warm pulse) for 30 minutes. The two dense unbroken tracks represent the origin regions of replicons that initiated operation after the beginning of the labeling period (i.e., a postpulse figure), whereas the gap represents the origin of a replicon that initiated operation before the beginning of the labeling period (i.e., a prepulse figure). The dense areas are the result of labeling by high specific activity [^{3}H]thymidine, whereas the lighter trailing tracks represent the DNA labeled by low specific activity [^{3}H]thymidine. The direction of replication was from the high to low specific activity areas. (c) A comet of tangled DNA. This may represent DNA from one cell or a portion of one cell. To measure the fiber lengths, it is necessary to find a region of the slide or comet where the tracks are sufficiently clear and separated. These autoradiographs were provided by S. Sawada, Kumamoto University, Kumamoto, Japan.

DNA fiber autoradiographs is reported as 1.5–5 μm (Huberman and Riggs, 1968; Ockey and Saffhill, 1976). For a cell with an average fork movement rate of 0.5 μm/minute incubated for 30 minutes, the error of resolution in the resulting DNA fiber autoradiogram would be 10 to 30%. Longer pulse times could reduce this error, but when pulse times become too long, adjacent replicons fuse, making fork movement rates unmeasurable. (3) Coincidence counting problems. All tracks scored may not be from separate DNA fibers. Despite these shortcomings, DNA fiber autoradiography is the most commonly used technique for examining DNA fork movement rates in mammalian cells and, when carefully performed, gives consistent and valuable information.

Several ultracentrifugal methods have been used to estimate DNA fork movement rates. These methods utilize the distribution of DNA along a gradient in an ultracentrifuge tube. The gradient can measure differences in molecular weight or differences in density, and these differences are used to calculate DNA fork movement rates.

Lehmann and Ormerod (1970) calculated DNA fork movement rates by examining the sedimentation of labeled DNA on alkaline sucrose gradients. They incubated mouse L5178Y cells with [^{3}H]thymidine for various lengths of time. The cells were next exposed to X rays to introduce random breaks into the DNA, lysed on the top of a 5–20% alkaline sucrose gradient to release single-stranded DNA, and then centrifuged. From the resulting distributions of the labeled DNA on the gradients, the average molecular weights were computed. By labeling the cells for different lengths of time and observing the change of the average molecular weights in the resulting gradients, the DNA replication fork movement rate was calculated. Similar approaches, utilizing the increase in DNA molecular weights as observed by sucrose gradient centrifugation, have been used by several other workers (Lanotte *et al.*, 1977; Laughlin and Taylor, 1979) to calculate fork movement rates (Fig. 2).

Painter and Schaefer (1969, 1971) utilized incorporation of [^{3}H]thymidine and unlabeled bromodeoxyuridine (BUdR) in sequential pulse-labeling and CsCl equilibrium density gradients to measure DNA fork movement rates. With this technique, cells were first incubated with [^{3}H]thymidine for a short time (10 to 30 minutes), followed by incubation with BUdR for 1 to 2 hours. The DNA was then isolated and sheared, and one aliquot was analyzed by velocity sedimentation to determine the average molecular weight of the sheared fragments. A second aliquot was analyzed on CsCl equilibrium density gradients to determine the fraction of DNA that was distributed on the heavy side of the normal-density DNA, i.e., that which contained molecules that had ^{3}H at one end and BUdR at the other. From this shift, DNA fork movement rates were calculated using published equations (Painter and Schaefer, 1971; Roti-Roti and Painter, 1977) (Fig. 3).

More recently, Povirk and Painter (1976) pulse-labeled cells with [^{3}H]BUdR

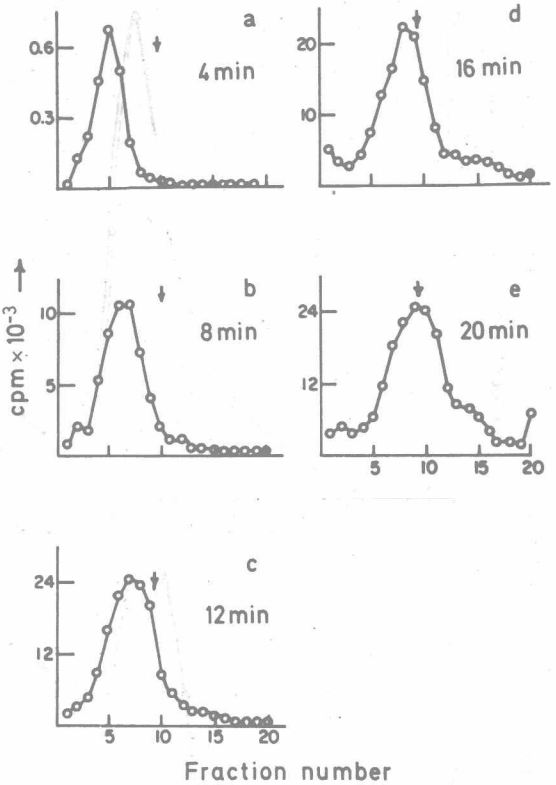
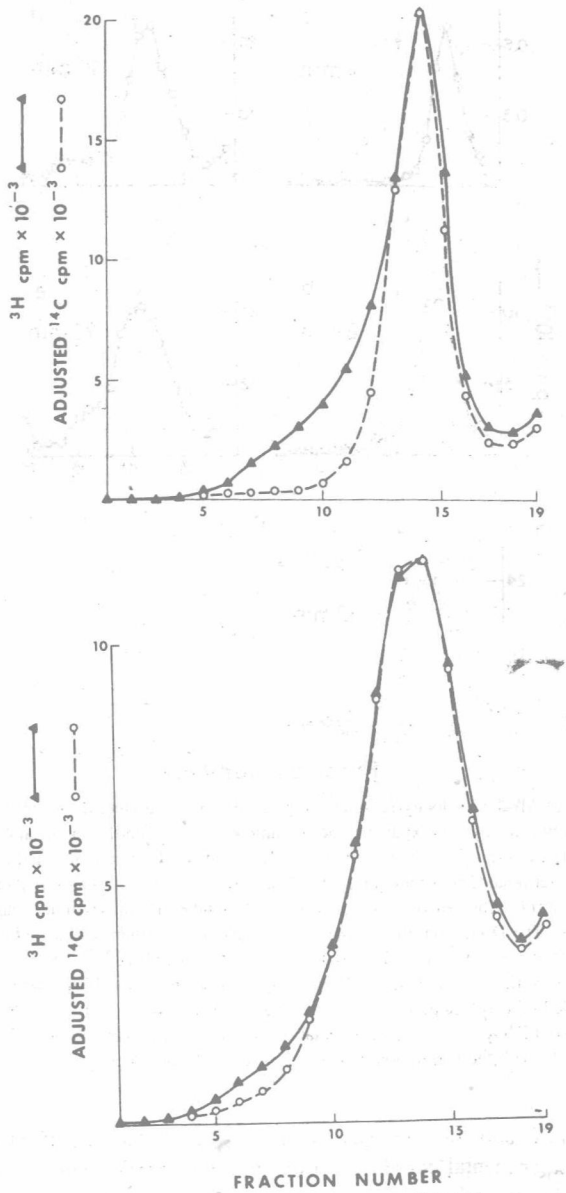


FIG. 2. (a–e) Alkaline velocity sedimentation profiles of pulse-labeled nascent DNA segments from CHO cells blocked by hydroxyurea at the beginning of S phase until 14 hours after division. The pulse times were (a) 4 minutes, (b) 8 minutes, (c) 12 minutes, (d) 16 minutes, and (e) 20 minutes. Each pulse was terminated by submerging the cultures in SSC (0.15 *M* sodium chloride, 0.015 *M* sodium citrate) at 0°C. The cells were lysed at 0°C with standard lysing solution containing 500 μg/ml proteinase K. The DNA from these lysates was denatured and then sedimented in alkaline NaI velocity gradients 9 hours after lysis. The gradients were centrifuged at 20,000 rpm for an $\omega^2 t$ of 10^{11} rad^2/second. The vertical arrows indicate the position of the center of the T7 marker DNA band. The T7 DNA included in these samples sedimented in each case as expected for a homogeneous population of phage-sized DNA molecules. Sedimentation was from left to right. Reprinted from Laughlin and Taylor (1979) with the permission of the authors and Springer-Verlag, Inc.

for short times, and then exposed them to several fluences of 313 nm light. Under the experimental conditions used, the only breaks induced in the DNA were caused by the action of the 313 nm source on the BUdR-substituted residues in the DNA. The cells were then lysed on the top of an alkaline sucrose gradient and centrifuged. The resulting DNA gradient profiles showed that increasing the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exposure to 313 nm light caused an increased shift of the labeled DNA toward low molecular weights. This shift can be quantified and used to estimate the length of DNA labeled with BUdR. Dividing the length of labeled DNA by incubation time with [^{3}H]BUdR yields the average fork displacement rate (Fig. 4).

Other techniques or variations of some of the described techniques have also been used to calculate fork movement rates. For example, Planck and Mueller (1977a) labeled cells briefly with BUdR, and then with]^{3}H]thymidine, and then exposed the labeled cells to 313 nm light. The light ruptured the DNA at the BUdR-labeled regions and released the ^{3}H-labeled DNA. The [^{3}H]thymidine incubation times were varied and the growth of labeled DNA chains was analyzed by velocity gradient sedimentation. Taylor (1968) used variable incubation times with [^{3}H]BUdR and estimated the ratio of ^{3}H in completely substituted DNA to that in partially substituted DNA as a function of incubation time. The rate of fork movement was estimated from the changes in this ratio.

Gradient methods have the advantage of speed (most determinations take only 1 to 5 days) when compared to autoradiography, which often requires developing times of up to 1 year. In addition, gradient techniques also yield results that reflect an average of all the replicating DNA in the S phase cells. However, they give no information about distributions of fork movement rates within a cell.

Several workers have directly compared various techniques for measuring fork movement rates. Laughlin and Taylor (1979) compared an alkaline velocity gradient method with fiber autoradiography in CHO cells. The alkaline gradient method gave an estimated fork movement rate of 0.5 to 0.6 μm/minute. Fiber autoradiographic data from the same cells were in excellent agreement with the sedimentation results, indicating that two independent techniques can both result in the same findings.

In another comparison of sedimentation and autoradiographic techniques, Richter and Hand (1979b) measured fork movement rates in monkey CV-1 cells. Fiber autoradiography gave a value of 0.56 μm/minute, whereas the equilibrium density method described by Painter and Schaefer (1971) gave a value of 0.36 to

FIG. 3. CsCl equilibrium density gradient profiles of HeLa S3 DNA labeled for 30 minutes with [^{3}H]thymidine and then for 2 hours with BUdR and sheared (upper panel) to produce number-average molecular weight DNA (B) of 1.3×10^7 and fraction of [^{3}H]DNA at densities greater than normal (F) of 0.225 or sheared (lower panel) with ultrasound to produce B of 0.26×10^7 and F of 0.026. ▲, ^{3}H radioactivity; ○, ^{14}C radioactivity (adjusted). The ultrasound F value is considered the minimum possible and is primarily due to thymidine pool mixing of [^{3}H]thymidine and BUdR. This is subtracted from the F value for the 12,000 rpm shearing to give F_{net}. From B and F_{net}, L, the average molecular weight of DNA labeled during the pulse with [^{3}H]thymidine, can be estimated. Since about one-twelfth of the total DNA must be synthesized in 30 minutes (S period ≅ 360 minutes), the total number of sites replicating DNA ≅ DNA molecular weight per cell/12 × L. Reprinted from Painter and Schaefer (1969) with permission of the authors and Academic Press, Inc.

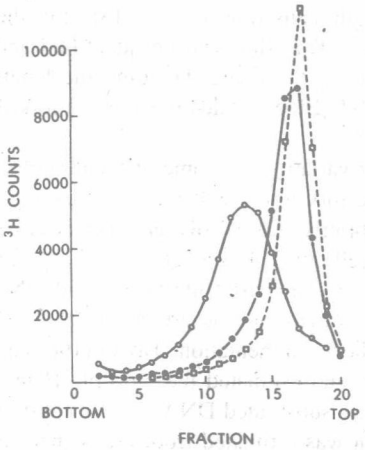

FIG. 4. Alkaline sucrose gradient profile of DNA from pulse-labeled E-11 human diploid cells that were exposed to various fluences of 313 nm light, lysed on a 5–20% alkaline sucrose gradient, and centrifuged. Cells were exposed to 0 (○), 60 (●), or 180 (□) seconds of 313 nm light. Such gradients allow calculations of parameters that can be used to estimate fork movement rates. Reprinted from Kapp and Painter (1978) with permission of the Biophysical Society.

0.38 μm/minute. Richter and Hand concluded that the equilibrium density method provided an objective measurement of fork movement rates, although it underestimated the actual rate. However, if these authors had used the equilibrium density gradient method exactly as described by Painter and Schaefer (1969, 1971) (one necessary step was omitted), the results from the two methods would have been in closer agreement.

Kapp and Painter (1979) compared two sedimentation techniques: the equilibrium density method of Painter and Schaefer (1971) and the BUdR–313 nm photolysis method described by Povirk and Painter (1976). It was found that for asynchronous CHO cells, both techniques gave the same average value and range (about 1 μm/minute). The same methods were used with synchronous CHO populations and resulted in the same conclusions: a constant rate of about 1 μm/minute throughout S phase. Rates in synchronous HeLa cells were also measured using the BUdR–313 nm photolysis method and a 2- to 3-fold increase in rate from early to late S was found. This confirmed the earlier report of Painter and Schaefer (1971) for HeLa cells using the equilibrium density method. From this work, it appears that different sedimentation techniques using different approaches can be in excellent agreement.

The work discussed above indicates that most of the techniques currently in use give comparable results. Therefore, it appears possible that the reported variations in fork movement rates actually are due to differences in the rates in

different cell types or in the same cell type under different culture conditions, rather than to inappropriate techniques.

To examine this question in more detail, Kapp and Painter (1981) split a single cell culture into two equal subcultures and measured fork movement in them using the BUdR–313 nm photolysis method. All measurements were made in duplicate, portions of each cell culture being run in separate centrifuge rotors. The idea was to determine, when cell cultures were as identical as possible, how much variation in fork rate measurements was inherent in the experimental techniques. There was less than 10% variation between the most extreme values in this set of measurements. However, under normal experimental conditions, rates measured in any single cell type vary by about 30%. Again, these results suggest that the differences measured with different techniques are largely due to variations in cell types or culture conditions.

III. DNA Replication Fork Movement Rates

DNA fork movement rates have been examined in a wide variety of mammalian and human cells (Tables I and II). The values shown in the tables are the average values reported in the papers. As mentioned above, large variations were seen around the mean values in most of the reports. Since not all means and variances were shown in the same manner, they are omitted in the tables for the sake of uniform presentation and comparison. In addition, some reports discussing fork movement rates presented comparisons but did not present data on actual fork movement rates, and thus are not listed here (Wickremasinghe and Hoffbrand, 1979; Giannelli *et al.*, 1977; Hand, 1975a).

For human cells (Table I) the population mean and standard deviation is 0.60 ± .021 μm/minute. For other mammalian cells (Table II), it is 0.75 ± 0.33 μm/minute. Considering the variety of cell types, culture techniques, and measurement techniques, this is a surprisingly narrow range. In contrast, fork rates in bacteria are reported to be up to 15 μm/minute (Cairns, 1963), and workers examining amphibians have found relatively low values of 0.02 μm/minute (Hyodo and Flickinger, 1973).

A. Fork Rates in Human Cells

Kapp and Painter (1981) measured fork movement rates in 20 human cell types using a single technique (BUdR–313 nm photolysis) and uniform culture conditions. The overall mean fork rate was 0.53 ± 0.08 μm/minute (population mean and population standard deviation). For individual cell types the means and standard deviations were much larger. For example, GM637, an SV40-transformed cell line, had an average fork movement rate of 0.75 ± 0.20 μm/minute,