DNA INITIATION AND CHAIN GROWTH IN SYNCHRONIZED PRIMATE CELLS

by

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ABSTRACT

Methods for determining the rate of DNA elongation in primate cells were studied. A hydrodynamic procedure was shown to overestimate rate. It is based on sequential labeling of DNA with radioactive and density tracers and analysis in neutral CsCl gradients. For accuracy this method requires a sharp transition between the two tracers. The overestimate is caused by preferential uptake of thymidine over the density analog. Reversal of the original labeling sequence largely eliminates this inaccuracy.

DNA replication was studied in CV-1 cells induced to enter S phase by the addition of serum. Rate of elongation was studied by the modified hydrodynamic method and by DNA fiber autoradiography. Autoradiography was used to determine the other parameters of DNA replication. The frequency of initiation decreases in mid S, while elongation, synchrony of initiation and replication unit size are unchanged.

SOMMAIRE

J'ai étudié les méthodes pour la détermination de la vitesse d'élongation des chaines d'ADN dans les cellules de singes. J'ai démontré qu'une méthode hydrodynamique est inexacte. Cette méthode utilise le marquage de L'ADN avec les pulses consecutifs de thymidine tritié et bromodeoyxuridine. Pour être précise, elle exige une transition aigüe entre les deux précurseurs. La suréstimation de la vitesse d'élongation est expliquée par le transport préférentiel de la thymidine comparée avec le bromodeoxyridine. L'inversion de la séquence des précurseurs peut éliminer le probleme.

La réplication de l'ADN a été étudiée dans les cellules CV-1 induites en phase S par l'addition de serum. Pour étudier la vitesse d'élongation j'ai utilisé la méthode d'ultracentrifugation modifiée et l'autoradiographie. L'autoradiographie a été aussi utilisée pour determiner la synchronisation des évenements d'initiation, la grandeur des unités de réplication et la frequence de l'initiation. Cette dernière est plus faible au milieu de la phase S que tôt et plus tard. Les autres constituants de la réplication de l'ADN ne changent pas.

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INTRODUCTION

It is now quite clear that the nearly 10^{10} base pairs of the eukaryotic genome are replicated in many shorter regions or replication units. In each of these units, DNA replication starts at an origin and proceeds by two fork-like growing points towards the outlying termini, (Huberman and Riggs, 1968).

DNA replication is regulated on many levels. This ranges from the signals enticing the cells to begin the preparative reorganization for the entry into the DNA synthetic phase (S phase) all the way down to the polymerization of the individual dNTPs. The intervening regulatory steps are the ihitiation of replication of large portions of the genome such as late replicating satellite DNA or the highly syncrohous initiation of clusters of neighbouring replication units. On the level of individual replication units, initiation is stringently regulated while the rate of replication fork movement is less tightly controlled.

I will discuss entry into S phase and the operation of individual replication units. Several recent reviews discuss eukaryotic DNA replication (Edenberg and Huberman, 1975; Sheinin et al, 1978). Reichard (1978) uses DNA replication in polyoma virus as a model for eukaryotes. Another recent article discusses the organization of DNA into chromatin by nucleosomes (Felsenfeld, 1978).

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also be noted that the task of the External Examiner is much more difficult in such cases.

Chapter 3 has been accepted for publication in the Journal of Cellular Physiology. Chapter 4 has been accepted for publication in Experimental Cell Research. The data in Chapter 3, figures 3,4,6,7 and Table 2 have been provided by Dr. Hand.

LITERATURE REVIEW

1.1 Cell Cycle -S Phase

The growth cycle of eukaryotic cells between successive mitoses can be divided into 4 phases. DNA is synthesised exclusively in S phase (Lajtha 1963). S phase is preceded by G1, the pre-DNA synthetic, preparatory phase. It is followed by G2 and mitosis. G2, M and S are of relatively constant duration while G1 varies widely between and within populations. Cells can also be in a nonproliferating, resting state called Go (Lahiri, van Putten, 1972). Go and G1 can be distinguised on the basis of biophysical and biochemical parameters (Baserga, Nicolini, 1976).

Entry into S phase from Go/G1 is regulated by many factors such as serum and medium composition (Temin et al, 1972, Warburton, Poole, 1977).

a) Effect of Serum

Normal mammalian cells exhibit an absolute requirement for serum to proliferate (Brooks, 1976). When the concentration of serum is reduced to a low level, cell multiplication stops and the population becomes arrested in Go or early G1 (Holley, Kiernan, 1968). Growth may be reinitiated by adding back fresh serum. DNA synthesis begins after a

defined lag period (Bürk, 1970). The length of this lag is independent of the concentration of serum while the proportion of cells induced to enter S phase is a function of it (Temin, 1971, Clarke et al, 1970).

Addition of serum to a culture of resting 3T6 mouse fibroblasts increases the rate of ribosomal RNA synthesis within 10 minutes, while the rate of transcription of hnRNA is not directly effected (Mauck, Green, 1973). When confluent 3T3 cells are stimulated to proliferate by the addition of fresh serum, significant increases in uridine and phosphate uptake are seen (Cunningham, Pardee, 1969). These authors also observe increased synthesis and turnover of phospholipids early after stimulation. A serum factor of 100,000-150,000 daltons stimulates RNA synthesis in resting 3T3 cells (Todaro et al, 1967). When cells are deprived of serum, the intracellular concentration of cAMP increases (Oey et al, 1974). This increase occurs only when the serum concentration is below the minimum needed for growth of a particular cell line.

Human diploid fibroblasts exhibit serum dependence of the initiation of DNA synthesis. This requirement is quantitative on the concentration of serum. Analysis of this system led to the formulation of a model analogous to the Michaelis-Menten equation (Ellem, Gierthy, 1977). V_m is the maximum value for the fraction of the population of cells capable of responding to the stimulation. K'_m is an estimate of the interaction between the serum contained

mitogens and the cell surface. There is a linear relationship between the logarithms of V_m and K'_m, this is the quantitative measure of the influence of cell density on the transition probability of a cell towards replication.

b) Go/G1 Transition

Stimulation of Go cells to proliferate causes an increase in the capacity of chromatin to bind ethidium bromide (Nicolini et al, 1975). Laser flow micro-flourometric analysis can distinguish Go cells from G1 cells in culture by the increased flourescence per cell seen in G1 cells. This Go/G1 transition seems to involve a direct quantum jump. There is a change in the percentage of cells with increased flourescence intensity at different times after stimulation (Nicolini et al, 1977).

This increase may be caused by increased binding of the dye by the nuclei, by change in the cytoplasmic components of serum induced cells or by a change in membrane composition. The authors do not distinguish between the possible mechanisms.

c) Probabilistic Nature of Entry into S Phase

Smith and Martin (1973) have found upon analyzing published data that cells enter S phase with first order kinetics. They maintain that the commitment of cells to DNA synthesis is a single, random event which can be characterized by a first order rate constant, called transition probability. They suggest that the intermitotic period be divided into 2 parts: the deterministic B state which includes S, G2, M and a part of late G1, and the nondeterministic A state

which includes early G1 and in nonproliferating cells, Go. Sometime after mitosis the cell enters the A state, in which it is not progressing toward cell division. It may remain in the A state for any length of time. Throughout the A state the probability of entering the B state (or transition probability) is constant. The transition probability is a characteristic of the cell type but environmental factors may modify it.

A population of cultured fibroblasts consists of a single kinetic class which is characterized by the length of time spent in the B phase. Changes in the transition probability serve as major means of growth regulation (Shields, Smith, 1977). Since the transition probability is a key element in the regulation of cell proliferation, variants in the cell cycle are thought to be caused by changes in this parameter (Shields, 1977).

When 3T3 cells stimulated to leave Go/G1 arrest by serum are treated with low concentration of cycloheximide (33-100 ng/ml) any time after the lag phase, there is a reduction in the transition probability within 1-2 hours (Brooks, 1977). This seems to indicate that the transition probability depends on the continuous synthesis of a protein with short half life. The reduction in transition probability is proportional to the inhibition of leucine incorporation.

Addition of serum to density inhibited Balb/c-3T3 cells induces them to proliferate by controlling an ordered sequence of events (Pledger et al, 1978). These events in Go/G1 are pointed towards cell replication. There is a transitional event which occurs as the cells enter S. This has an important role regulating the commitment of the cells to DNA synthesis. This transition is, in turn preceded by an ordered sequence of events, a series of growth arrest points. Serum derived growth factors (from platelets and plasma) regulate the cell cycle events preceding commitment to DNA synthesis. This model for the control of cell proliferation differs somewhat from the transition probability model. There are several steps envisioned to be rate limiting in this model in contrast to the single random event, the transition probability believed to be controlling in the cell cycle of Smith and Martin.

d) Entry into S

When the onset of entry into S phase is inhibited in CHO cells by hydroxyurea the results seem to indicate that the initiation of DNA synthesis in one S phase is somehow affected by the timing of the previous S period (Hamlin, Pardee, 1976). The time of entry into S was delayed up to 16 hours using hydroxyurea and the position of the subsequent S period was determined. These authors found a minimal 15 hour interval between the two S phases. This implies that the needed preparatory mechanisms for the

subsequent S cannot be triggered until the previous S has either begun or was completed.

The study of CHO cells synchronized by isoleucine deprivation and hydroxyurea showed that early replicating DNA and perhaps some event coupled to its synthesis is important for the passage through the entire S phase (Hamlin, Pardee, 1978). Euchromatin or transcriptionally active chromatin is known to be replicated predominantly in early S phase (Lima-de-Faria, 1969). This study showed that especially critical proteins are made in the first and fourth hour of S phase. Some may be implicated in the enzymology of DNA synthesis, some others may prevent reinitiation at origins already replicated.

Fusion of late S phase CHO cells with cells in G1 induces a large proportion of the G1 nuclei to DNA synthesis. The pattern of DNA synthesis in the G1 nuclei is characteristic of early S phase (Yanishevsky, Prescott, 1978). This indicates the presence of a cytoplasmically transmissable inducer of DNA synthesis in the late S phase cells. It also shows that despite the fact that the G1 cells were fused with late S phase cells the intrinsic programming for the order of DNA replication was preserved. The cytoplasmic inducer present in the late S phase cells simply set in motion the normal, temporal program present.

The addition of serum to WI38 cells in Go phase of the cell cycle causes proliferation with structural changes in

the cellular DNA. There is an increase in the amount of breaks seen in the DNA as the cells enter S (Collins, 1977). A few breaks appear as early as the middle of G1, gaps first show up later in G1. During S the level of single strandedness remains high, as the cells enter G2 the degree of single strandedness declines. This could indicate a "chromosomal" cycle inherent in the DNA of Go cells. This DNA exists as intact duplexes. Once the cells are stimulated to leave Go and re-enter the cell cycle via G1, the DNA is nicked and single stranded gaps are formed. This may provide an activated template for the DNA replication machinery.

The same system of quiescent WI38 cells stimulated to proliferate was used to study the DNA intermediates formed after induction of DNA synthesis (Rawles, Collins 1977). Alkaline sucrose sedimentation showed that 4.5 S pieces are converted to 11 S then to 50-60 S pieces in 1 minute. The same results have been found by others (Tseng, Goulian, 1975). The 50-60 S material is rapidly converted to larger molecules of 212S and 275 S. These two classes of DNAs are precursors to the chromosomal complex.

e) Ordered Replication of Subclasses

In synchronized African green monkey cells rapidly reassociating component α DNA is replicated late in S phase (Tobia et al, 1972). Rapidly reassociating, A-T rich mouse sattelite DNA is also replicated late in S phase (Tobia et al, 1970).

When SV40 was used to induce host cell DNA replication in contact inhibited African green monkey cells, the synthesis of α component DNA did not precede the replication of bulk DNA (Parker et al, 1975). This indicates that the temporal replication order of distinct subclasses of sequences is stringently preserved even on induction of DNA synthesis by SV40.

The examination of mouse sattelite DNA replication in synchronized 3T3 cells showed that the replication of this class of DNA is not a time dependent event. Instead, it requires the previous replication of a certain fraction of non-satellite, main band DNA (Dooley, Ozer, 1979). The main band sequences adjacent to satellite are replicated at the same time as satellite DNA. This means that the replication of a specific class of DNA is not automatic, dependent solely on the time elapsed since entry into S. It requires the previous replication of certain other main band sequences having perhaps regulatory functions.

1.2 DNA Replication

a) Organization of the Eukaryotic Genome

During the 6 - 8 hour S phase the eukaryotic genome is replicated by the initiation of DNA synthesis on many tandemly joined replication units. Replication proceeds from the initiation points bidirectionally until neighbouring replication units fuse (Huberman, Riggs, 1968). The rate of replication fork movement varies between 0.5 μ/min. to 2.0 μ/min. (Huberman, Riggs, 1968; Painter, Schaefer, 1969; Weintraub, 1972). In cultured mammalian cells the average size of replication units is less than 60 μm (Huberman, Riggs, 1968; Hand, Tamm, 1974).

In order to duplicate the large number of replication units within the genome, temporal as well as spatial organization of replication units exists (Tobia et al, 1970, 1971; Huberman, Riggs, 1968; Hand, Tamm, 1974; Hand, 1975a). Adjacent replication units are apt to initiate synchronously (Hand, 1975a), while certain fractions of the genome such as highly reiterated satellite DNA is always replicated late in S phase (Tobia et al, 1970).

When total DNA synthesis is measured during S, bursts of tritiated thymidine incorporation are observed (Klevecz, Kapp, 1973; Remington, Klevecz, 1973). These bursts are thought to be synchronous initiation of clusters of replication units alternating with a reduction in the number of new initiation events.

Clusters of replication units synthesized during the early part of S are spatially separate on the genome from those replicated during mid-S (Kowalski, Cheevers, 1976). It was also found that in HeLa cells DNA molecules replicated in the first 2h of S phase were preferentially replicated in the first 3h of the later S phase (Mueller, Kajiwara, 1966). In CHO cells there is programmed synthesis of DNA throughout S which is reproduced in a later S. The stringency of control is not great, especially for DNA made very early in S. DNA replicated later in S is more likely to be replicated late in the subsequent S (Adegoke, Taylor, 1977).

b) Measurement of DNA Synthesis in S

1. Rate of Total DNA Synthesis

The rate of overall DNA synthesis changes during S. It is initially rapid, slows down in mid S then speeds up at the end of S (Collins, 1978).

There are several methods for determining the rate of total DNA replication as well as the onset of S phase. Total isotope incorporation in pulse and continuous labeling experiments gives indication of the total DNA synthesis.

When a synchronous cell population is allowed to enter S, several questions may be asked about DNA replication. The initiation of S phase, the rate of DNA synthesis and the pattern of DNA accumulation can be studied. In V79 and CHO cells autoradiography was shown to be the most sensitive method for determining the onset of S. Experiments determining

DNA fluorescence/cell using a fluorescent Feulgen assay showed a saltatory increase in DNA content during S. This increase began to be detectable a few hours later in the cell cycle than the onset of S phase as determined by autoradiography. Fluctuations in the rate of ^{3}H-thymidine incorporation were seen, these coincided with the stepwise increases in fluorescence. Total thymidine incorporation and DNA specific fluorescence studies showed that in these two cell lines the bulk of DNA synthesis does not begin until 2 hours after the beginning of the autoradiographically detectable S phase (Klevecz et al, 1975).

2. Rate of Replication Fork Movement

The rate of replication fork movement may be measured by several methods. DNA fiber autoradiography gives the direct length of the newly synthesized DNA chains (Huberman, Rigg, 1968). The replicating DNA is pulsed with a radioactive label and the DNA fibers are spread on microscope slides after cell lysis. By measuring the length of the labeled fragments obtained after a radioactive pulse of known length, the rate of replication fork movement (or chain growth) can be calculated. Very long exposure times are needed to adequately visualize the extended DNA chains, the collection of data is slow and tedious and there is a risk of examining only a handful of cells from the population. This last drawback is eliminated in the use of the hydrodynamic methods for the measurement of rate of chain growth. These methods allow sampling of large cell populations. In

the procedure developed by Painter and Schaefer (1969) the replicating DNA is labeled with ^{3}H-thymidine followed by a BrdUrd label. The fraction of greater than normal density DNA is then determined by equilibrium-centrifugation in neutral CsCl. The rate of chain growth is inversely proportional to the fraction of density substituted DNA. In theory the rate obtained this way is independent of the length of the ^{3}H thymidine pulse (Painter, Schaefer, 1969; Roti Roti, Painter, 1977). There are other methods to measure replication fork movement. One uses velocity gradient analysis of DNA after BrdUrd and ^{3}H thymidine labeling and 313 nm photolysis. The radioactivity is distributed over a wide range of molecular weights after labeling. When the cells are irradiated with 313 nm light, there is a loss of label from large DNA molecules. The fraction of label remaining attached to the large molecules is a function of segment length and it can be used to determine the elongation rate (Povirk, Painter, 1976).

3. Initiation

There are several methods for measuring the relative frequency of initiation. When synchronized cells are grown in BrdUrd and the DNA is centrifuged in neutral CsCl, the size of the heavy density DNA peak is proportional to the percentage of cells that replicated their DNA in the presence of the analog (Meyn, Hewitt, Humprey, 1973). Knowing the DNA content of the cells, the amount of DNA replicated in different time

intervals can be determined. Since the rate of replication fork movement is known, the number of operating replication units may be calculated and hence the relative frequency of initiation.

DNA fiber autoradiography can also be used to determine the relative frequency of initiation. When DNA is labeled for fiber autoradiography with ^{3}H-thymidine of high specific activity (S.A.) followed by a label of low specific activity, two types of autoradiograms are yielded. On replication units where initiation occured before the high S.A. pulse, a small gap is bordered on each side by areas of high grain density. These stretches are followed by tails with lower grain density. These are prepulse autoradiograms. Units initiating during the high S.A. pulse have an unbroken stretch of heavy density trailing into lower density tails. These are post pulse units. To obtain the relative frequency of initiation, the ratio of prepulse to postpulse units is measured. This gives the relative number of units initiating before and during the high S.A. pulse. If the length of the pulse is constant, a change in the ratio indicates a change in the relative frequency of initiation (Hand, 1975a).

The rate of replication fork movement and the frequency of initiation of new chains are the main factors determining the overall rate of DNA synthesis.

When DNA fibers were examined for autoradiograms, a striking synchrony of initiation was noted on adjacent replication units (Hand, 1975b). This synchrony of initiation

is an index of the normal state of the cellular DNA synthetic machinery since it is disrupted by the inhibition of DNA synthesis.

Although most of the replication units seen by DNA fiber autoradiography are bidirectional, there is always a measurable proportion of unidirectional replication. Increase in the frequency of unidirectional replication indicates a perturbation at the initiation site preventing one of the two forks from firing off.

4. Replication Units

Using DNA fiber autoradiography, replication unit size may be determined by measuring inter-initiation distances (Huberman, Riggs, 1968).

Another method combines the known initiation inhibitory action of X-irradiation with BrdUrd pulse labeling and 313 nm photolysis to give the length of the replication units (Kapp, Painter, 1978). Cells are labeled with ^{3}H-thymidine and BrdUrd. The length of the labeled region (L_{obs}) is determined in alkaline sucrose gradients by the method of Povirk and Painter (1976). This involves the measurement of the molecular weight change induced in the newly replicated DNA chains by 313 nm light. Exposure of the cells to 100 rads of X-rays inhibits initiation of new replication units without affecting chain growth (Painter, Young, 1975). If the cells are irradiated immediately before the pulse label, increase in L_{obs} is seen only until all operating replication

units have finished elongating. As long as the initiation of new replication units is inhibited, L_{obs} does not change and it represents the average size of replication units operating before the X-irradiation.

The second hydrodynamic method uses sequential labeling of DNA with ^{3}H-thymidine and BrdUrd. In this procedure the degree of density substitution is determined in neutral CsCl gradients using the protocol of Painter and Schaefer (1969). The size of replication units is determined from the fraction of greater than normal density DNA in samples sheared to different molecular size (Roti Roti, Painter, 1977).

c) Changes in DNA Replication Parameters

After having described the measurable parameters of DNA replication, I now will discuss the effectors of these parameters. These may be natural ones (such as the developmental stage of a cell or its position in the cell cycle) or artificial ones (such as irradiation, inhibitors of protein synthesis or virus infection). I will discuss each parameter separately but some of the effectors cause changes in several components of DNA replication.

1. Rate of Fork Movement

The rate of replication fork movement may change during S phase, but this depends on the synchronized cell line examined. In CHO cells the rate (as measured by DNA fiber autoradiography) is slow very early in S, this is then followed by a rise to a much higher level. This rate is then

maintained through the rest of S (Housman, Huberman, 1975). Also in CHO cells the rate was measured using the hydrodynamic method (Painter, Schaefer, 1969) and BrdUrd photolysis (Povirk, Painter, 1976). This study showed no change in the rate of fork movement during S (Kapp, Painter, 1979).

In HeLa cells, the same study showed the rate increases during S phase. This agrees with a previous report on this cell line using the hydrodynamic method (Painter, Schaefer, 1971).

When diploid human cells were examined by photolysis, the rate of fork movement rose and fell with the peaks seen in total thymidine incorporation.

Inhibition of protein synthesis causes a decline in DNA synthesis (Mueller et al, 1962).

In a detailed study of 8 different methods of inhibiting protein synthesis including drugs, amino acid analogs and a temperature sensitive tRNA synthetase in mouse L, CHO and HeLa cells, a consistent reduction in DNA synthesis was seen (Stimac et al, 1977). They found the rate of DNA synthesis to be inhibited by the same extent as protein synthesis.

When the rate of replication fork movement was measured by DNA autoradiography early after inhibition of protein synthesis, the decline in it paralleled the decline in total DNA synthesis. At later time points the reduction in the rate of fork movement does not entirely account for the inhibition of total DNA synthesis. These authors proposed

that reduction in the frequency of initiation of new replication units may be an additional consequence of the inhibition of protein synthesis at later time points. These results agree with other experimental data on L cells using the protein synthesis inhibitors puromycin and cycloheximide (Hand, 1975a). The rate of fork movement (measured by autoradiography) was reduced along with other parameters.

An earlier examination of the inhibition of DNA replication by cycloheximide in CHO cells showed total correlation between the reduction in total DNA synthesis and the reduced rate of chain growth early after the inhibition of protein synthesis (Gautschi, Kern, 1973).

The nucleotide analog 5,6 dichloro-1-β-D ribofuranosyl benzimidazole (DRB) - inhibits DNA synthesis in L cells (Hand, Tamm, 1977). This effect is secondary to the inhibition of HnRNA synthesis. The rate of fork movement as well as the transport of exogeneous thymidine into the cells are inhibited.

2. Initiation of new Replication Units

Stimac et al (1977) have postulated the inhibition of replication unit initiation to partially account for the reduction of total DNA synthesis seen late after inhibition of protein synthesis. Direct fiber autoradiographic measurements in mouse L cells showed that the frequency of initiation is reduced up to 1/3 after 10 minutes of addition of the inhibitor (Hand 1975c). Other characteristics of normal

initiation are also perturbed by the inhibition of protein synthesis as shown in this study. There is a reduction in the synchrony of initiation of adjacent replication units as well an increase in the proportion of unidirectional replication units seen.

2.4 dinitrophenol (DNP), another inhibitor of protein synthesis, which acts by uncoupling oxidative phosphorylation, modifies initiation patterns in HeLa cells (Gautschi et al, 1973). When these authors looked at the rate of fork movement by the hydrodynamic method, no reduction was seen in the presence of DNP. This is in direct contrast with the findings of Stimac et al (1977). They report a substantial decrease in the rate of chain growth. These authors mention the different modes of measurement (autoradiographic versus hydrodynamic) as the possible source of error. They also point out, however, that when cycloheximide was used as protein synthesis inhibitor, both methods indicated a decrease in rate (Stimac et al, 1977 ; Gautschi, Kern, 1973).

When mouse L5178Y cells were X-irradiated, the results showed that the rate of total DNA synthesis was immediately inhibited. Low doses of X-rays inhibit DNA synthesis by depressing initiation, while high doses are needed to retard chain elongation (Watanabe, 1974). Irradiation of HeLa, CHO and L cells by moderate X-ray doses causes inhibition of DNA synthesis. This can be accounted for by the inhibition of initiation (Painter, Young, 1975).

The irradiation by 313 nm light of DNA partially substituted with bromodeoxyuridine inhibits the replication of the substituted stretches of DNA without affecting the unsubstituted portions (Povirk, 1977). The inhibition of DNA synthesis is by the inhibition of replicon initiation.

The rate of replication fork movement and initiation of new chains are the elements most stringently controlled in eukaryotic DNA replication. Their operation is sensitive to the inhibition of protein synthesis. They are also affected by breakage of X-rays or the effect of 313 nm light on BrdUrd containing DNA.

3. Replication Unit Size

Replication units are quite heterogenous in size depending on the cell line, the growth conditions and the developmental stage of the cells examined.

When the size of replication units in cleavage embryoes of Drosophila was compared with those in adult cells, a fivefold increase in unit size in adult cells was noted (Blumenthal et al, 1973).

Comparison of DNA replication rates in Triturus somatic cells and premiotic spermatocytes showed that the increased length of the premiotic S was not due to slower rate of chain growth. Instead, an increase in the size of replication units can account for the difference (Callan, 1972).

Examination of SV40 transformed Chinese hamster lung cells showed that these cells have shorter replication units

than their untransformed parent cells (Martin, Oppenheim, 1977). When the cells were tranformed with tsA class of mutants of SV40, they displayed smaller replication units at the permissive temperature when other transformed characters were also expressed. At the non-permissive temperature the cells displayed the larger replication units as well as other non-transformed phenotypic characters. The tsA class of viral mutants are known to be temperature sensitive for the initiation of viral DNA synthesis.

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2. AIM

This study is devoted to the examination of DNA replication in primate CV-1 cells. I have examined and contrasted the methods available for the measurement of DNA chain growth. A modification of a published procedure was contrasted with the original method developed by Painter and Schaefer (1969). This hydrodynamic method may overestimate the rate of fork movement at short pulse times due to the preferential uptake of thymidine over bromodeoxyuridine. The modifications introduced into the experimental protocol largely eliminate this preferential incorporation. DNA fiber autoradiography was used also to measure the rate of DNA chain growth.

The second part of the study uses the modified hydrodynamic method and autoradiography to measure the DNA replication parameters in CV-1 cells stimulated to enter DNA synthesis by the addition of serum.

3. DNA SYNTHESIS: EVALUATION OF A HYDRODYNAMIC METHOD FOR MEASURING THE RATE OF REPLICATION FORK MOVEMENT

ABSTRACT When replicating DNA is labeled sequentially with radioactive and density tracers and analyzed by equilibrium centrifugation, the fraction banding at heavier than normal density is inversely proportional to the rate of replication fork movement if there is a sharp transition from one tracer to the other on the newly synthesized chains (Painter and Schaefer 1969). Primate CV-1 DNA labeled for 5 to 30 min. with ^{3}H-dThd and then for 3 h with BrdUrd in the presence of FdUrd bands in a bimodal distribution in alkaline CsCl, rather than in a continuous distribution with a skew toward heavier density seen when FdUrd is omitted and centrifugation is in neutral CsCl. The heavy density peak represents interspersion of both tracers in the DNA and is caused by slow transition from dThd to BrdUrd incorporation when the tracers are switched in the labeling medium. This may result from preferential uptake and incorporation of dThd over BrdUrd. Because of the interspersion, calculation of the rate of replication fork movement is inaccurate. Reversal of the labeling sequence with administration of long density pulse before the radioactive pulse reduces the problem of interspersion. Using this sequence of labeling, estimates of the rate of fork movement of 0.36 to 0.38 μm/min are obtained when the ^{3}H pulse time is long enough to allow accurate

measurement of the fraction of heavy DNA. Analysis by fiber autoradiography yields a rate of 0.56 μm/min in the same cell line. This estimate may be too high because of slow transition from high - to low-specific-activity tracer in the labeled DNA. If appropriate precautions are taken to minimize mixing of the two tracers in the precursor pool and to ensure that the fraction of heavy DNA is measured accurately, the hydrodynamic technique provides an objective method of measuring rate of fork movement.

Eukaryotic cells replicate their DNA during a discrete part of the cell cycle, the S phase. Replication takes place on many individual replication units, each of which has a central initiation site for new chain synthesis. From these sites, the chains are elongated at fork-like growing points (Edenberg and Huberman, 1975).

The overall rate of DNA synthesis varies during the S phase. Recent evidence suggests that it is rapid initially, slow in mid S, and then rapid again toward the end of S (Collins, 1978). There are two factors that determine the rate, the frequency of initiation on replication units, and rate at which the replication forks elongate the new chains. The frequency of initiation is probably the more important of the two for regulating the overall rate. In invertebrates, embryonic

cells replicate their DNA faster than adult cells and the changes in rate are due to changes in initiation frequency (Callan, 1972,; Blumenthal et al., 1973; Lee and Pavan, 1974). In mammalian cells, transformation by simian virus 40 results in activation of new initiation sites (Martin and Oppenheim, 1977; Oppenheim and Martin, 1978). Also in mammalian cells, the frequency of initiation is higher in early and late S phase than in mid S phase (Richter and Hand, 1979). This correlates well with the changes in overall rate during S phase (Collins, 1978).

Replication fork movement, although less important as a regulator, also varies during the S phase. In some mammalian cells, such as Chinese hamster ovary, it is slow at the very beginning of S and then increases and remains constant through the remainder of S (Housman and Huberman, 1975). In the human diploid strain, WI-38 (Kapp and Painter 1979), and in the simian line, CV-1 (Richter and Hand, 1979), the rate remains constant through S phase. Other cell lines, such as HeLa (Painter and Schaefer, 1971; Kapp and Painter, 1979), show an increase in rate as the cells progress through S phase. Replication forks on the same units and on adjacent units move at similar rates (Hand 1975). Inhibition of protein synthesis by a variety of agents causes an immediate reduction (Weintraub and Holtzer 1972; Gautschi and Kern 1973; Hand and Tamm 1973; Stimac et al. 1977). Lower temperature also slows the rate (Hand and Gautschi, 1979). Aside from these observations on the requirement for ongoing protein

synthesis and effect of temperature, little is known of factors altering the rate of replication fork movement.

There are presently several methods for measuring fork displacement in mammalian cells. The most direct is fiber autoradiography (Huberman and Riggs 1968), in which the lengths of new DNA chains synthesized during a radioactive pulse are measured after the DNA fibers have been spread on a glass slide. Although it is simple and gives reproducible results, there are drawbacks to it, such as the sampling bias inherent in any microscopic technique, and the requirement for long exposure time. Other methods, based on hydrodynamic techniques, have been described. Of these, the most widely used is that of Painter and Schaefer (1969) in which DNA is sequentially labeled with ^{3}H-dThd and BrdUrd and the fraction of density-substituted DNA is measured by equilibrium centrifugation. The fraction is inversely proportional to the rate of fork movement. The advantages of this method are its objectivity and its ability to sample large numbers of cells compared to fiber autoradiography. However, in at least 2 published studies (Painter and Schaefer, 1969; Martin and Oppenheim, 1977), the rates were slower with longer ^{3}H pulse times. Theoretically, the calculated rate should be independent of pulse time (Painter and Schaefer, 1969; Roti Roti and Painter, 1977).

In the present experiments, we have looked in detail at this hydrodynamic method. We have found that preferential uptake of dThd over BrUrd may interfere with the measurement

of rate of fork movement. We also propose a modification in the technique that may overcome this drawback.

MATERIALS AND METHODS

Cell line

The CV-1 cell line, a continuous line derived from African green monkey kidney, was a gift from Dr. E. Gershey of the Rockefeller University, New York. The cells were maintained in monolayers in minimal essential medium supplemented with 10% fetal calf serum and 25 μg/ml of gentamicin (a gift from the Schering Corporation of Canada, Ltd.), 0.25 μg/ml of amphotericin B and 60 μg/ml of tylocin. The cells were seeded into plastic flasks or Petri dishes one or two days prior to use, at a concentration that would give a density of 1.3×10^4 cells/cm^2 on the day of the experiment.

Experimental conditions

All experiments were performed in a walk-in warm room at 37°C. Flasks were gassed with 5% CO_2 in air and closed tightly after each experimental manipulation and petri dishes were kept in a closed container with an atmosphere of 5% CO_2 in air and 100% humidity except for the short times required for manipulation. In those experiments in which BrdUrd was used, the cells were protected completely from light during incubations, and manipulations were performed in reduced natural light which, since it was filtered through window glass, contained

little ultraviolet light. Specific labeling protocols are described in the figure legends and tables.

DNA extraction

DNA was extracted from cells using the modified Marmur procedure of Britten et al. (1974). It was sheared to 4.5 μm by passage four times through a 27 gauge needle or to 0.3 μm by making the DNA solution 67% with respect to glycerol and treating it for 30 minutes (with interruptions to prevent heating) at setting 100 in a Virtis 23 homogenizer. The size of the sheared DNA was measured by sedimentation in neutral sucrose gradients using Phi X 174 replicative form I as a marker.

Equilibrium centrifugation of DNA

The sheared DNA was centrifuged to equilibrium in alkaline CsCl as described elsewhere (Hand, 1976). DNA fragments sheared to 4.5 μm were centrifuged for a minimum of 40 h, while those sheared to 0.3 μm were centrifuged for 84 h. Fractions were collected from the bottom of the gradients and assayed for density by refractometry and for radioactivity by liquid scintillation counting.

In experiments in which native DNA was centrifuged to equilibrium the DNA, sheared to 4.5 μm, was dissolved in a

CsCl solution in TNE buffer (0.05 M Tris, pH 8, 0.15 M NaCl, 0.05 M EDTA) of a density of 1.72 g/ml. The samples were centrifuged for 40-48 h in a fixed angle rotor (Beckman S40.2).

DNA fiber autoradiography

This was performed as described elsewhere (Hand and Tamm, 1973). A drop of cell suspension containing 2000 to 3000 ^{3}H-dThd-labeled cells in phosphate-buffered saline was placed on a glass microscope slide. The cells were lysed gently on the slide by the addition of a drop of 1% sodium dodecyl sulfate. The DNA released from the cells was spread linearly along the slide with a glass rod. After fixation of the DNA, the slides were coated with NTB-2 emulsion (Kodak) and exposed one month or 6 weeks before development. This is somewhat shorter than the usual 6 month exposure time, but the grain tracks above the labeled DNA fibers were sufficiently dense after the short exposure to permit analysis.

RESULTS

When cellular DNA is sequentially labeled with ^{3}H-dThd and BrdUrd, radioactive label will be in end-to-end association with density label on those chains on which elongation takes place via replication fork displacement during both pulses.

Other chains elongating only during the radioactive pulse will contain ^{3}H but no BrdUrd. If elongation is sequential and continuous on the replicating chains, then the fraction of each radioactive chain length containing BrdUrd will vary from close to zero (elongation taking place almost entirely during the ^{3}H-dThd pulse) to close to one (elongation taking place almost entirely within the BrdUrd pulse). In an equilibrium density gradient, the ^{3}H counts from these partially substituted chains would band on the heavy side of normal density DNA, giving rise to a distribution of ^{3}H with some counts in normal density region, representing chains containing ^{3}H alone, and a skew toward higher buoyant density representing chains with increasing amounts of BrdUrd substitution. Equations have been developed relating the rate of fork movement to the shear size of the DNA, the ^{3}H pulse time, and the fraction of radioactive DNA that is density substituted (Painter and Schaefer, 1969; Roti Roti and Painter, 1977).

Equilibrium centrifugation analysis of replicating DNA

In some of our initial experiments, we found that, especially with short pulses of tritium, the fraction of BrdUrd-substituted DNA was low. This resulted in calculated rates of fork movement that were higher than expected. The fraction is estimated by normalizing the peak fraction of ^{14}C-dThd-labeled DNA sample run in the same gradient with

the equivalent fraction of sequentially labeled DNA. With short radioactive pulses, the fraction of heavy DNA might be low because of 1) incomplete substitution of BrdUrd for dThd in the DNA and 2) poor separation of heavy DNA from normal density DNA in the gradient. We therefore modified the procedure by adding FdUrd to the labeling medium to increase the incorporation of exogenous nucleosides and by centrifuging the DNA under denaturing conditions to increase the separation of normal and heavy DNA. Sequential labeling of DNA with ^{3}H-dThd for 15 min and BrdUrd for 3 h in the presence of FdUrd with analysis by equilibrium centrifugation in CsCl under alkaline conditions resulted in gradients of the type shown in figure 1a. A distinct second peak of heavy DNA of density 1.81 g/ml was seen. Centrifugation under neutral conditions (fig. 1b, c) or omission of the FdUrd (fig. 1c. resulted in a continuous distribution of ^{3}H label in the gradient with a skew of radioactivity towards the heavy density region as obtained by other investigators under similar experimental conditions (Painter and Schaefer, 1969, 1971; Gautschi and Kern 1973).

We were puzzled by the presence of the heavy peak of DNA and performed a series of experiments to investigate it further. We asked first whether the presence of the second peak was dependent on the ^{3}H pulse time or on the shear size of the centrifuged DNA. These results are shown in figure 2. DNA labeled for 5 min with ^{3}H-dThd before the 3 h BrdUrd pulse,

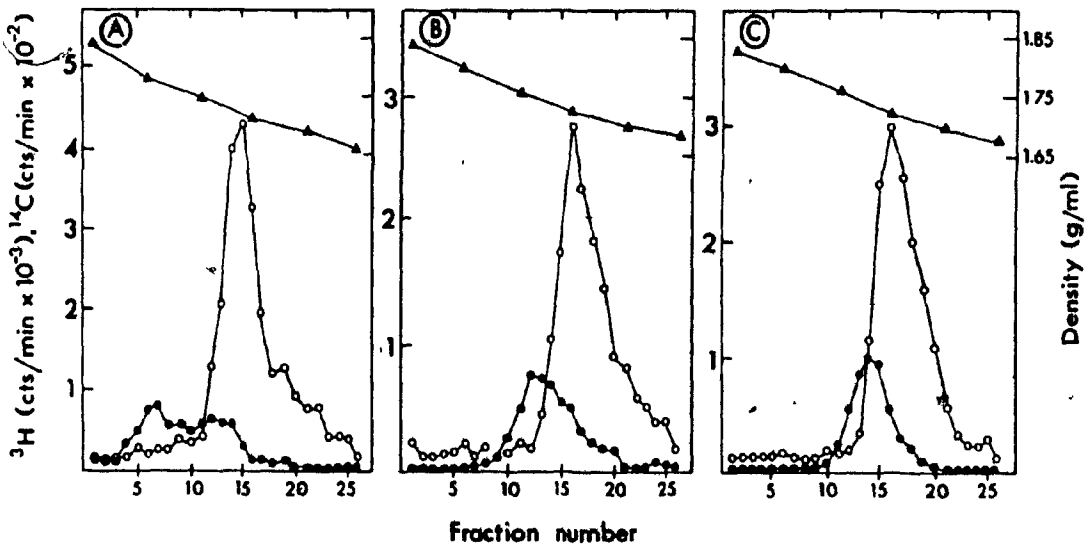

FIGURE 1

Alkaline and neutral equilibrium density gradients of DNA sequentially labeled with ^{3}H-dThd and BrdUrd. Logarithmically growing CV-1 cells were labeled overnight with ^{14}C-dThd (0.05 μCi/ml) to provide an internal normal-density marker. The following morning they were placed in normal medium for 3 h and then, in the presence of FdUrd (2×10^{-6} M), exposed for 15 min to ^{3}H-dThd (2.5×10^{-6} M, 27 μCi/ml). This was followed by a 3 h pulse with BrdUrd (2×10^{-6} M). After extraction, the DNA was sheared to 4.5 μm and centrifuged to equilibrium under alkaline (a) or neutral conditions (b,c). In (c), FdUrd was omitted from the protocol. (●), ^{3}H cts/min; (O), ^{14}C cts/min; (▲), density.

and then sheared to 4.5 μm showed a broad peak of radioactivity in the region of the gradient of density 1.81 g/ml and another peak banding with the normal density marker (fig. 2a). When the ^{3}H-dThd pulse was extended to 25 min, more tritium was associated with the normal density peak, but there was still a radioactive peak at density 1.81 g/ml (fig. 2b). Samples of the DNA used in the gradients in figure 2a, b, when sheared to 0.3 μm produced the gradient profiles seen in figure 2c, d. The peaks at density 1.81 g/ml were less evident, obscured in part by the increased diffusion of the highly sheared fragments during centrifugation, but nonetheless present.

DNA pulse-labeled with ^{3}H-dThd from 5 to 120 min prior to the long BrdUrd pulse consistently showed the heavy density peak when analyzed under these conditions, although the size of the peak was markedly decreased at pulse times longer than 30 min and was just barely evident after a pulse of 120 min (data not shown).

Since the heavy peak was present when the DNA was sheared to a size of 0.3 μm, this suggested that it resulted from interspersion of density and radioactive label in replicating DNA at intervals of less than 0.3 μm. This appeared to be the case since, as shown in table 1, the fraction of radioactive DNA of heavy density was the same at shear sizes of 4.5 μm and 0.3 μm. In this experiment, DNA was labeled with ^{3}H-dThd from 5 to 30 min before the density pulse and replicate samples

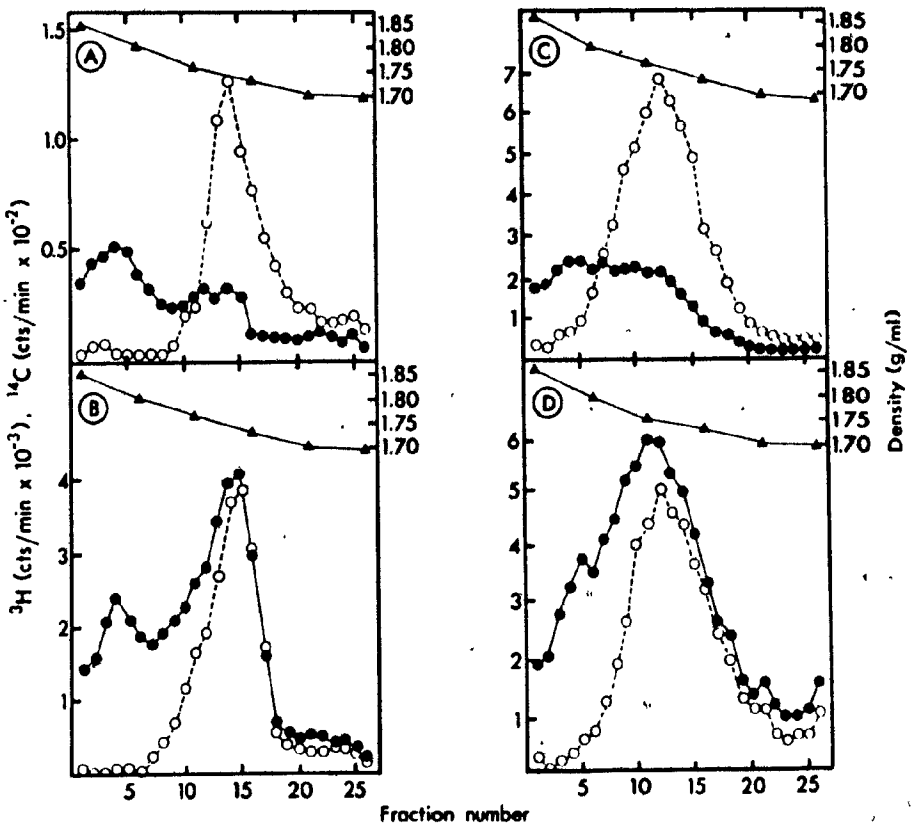

FIGURE 2

Alkaline equilibrium density gradients of DNA sequentially labeled with ^{3}H-dThd and BrdUrd. Logarithmically growing CV-1 cells were labeled overnight with ^{14}C-dThd (0.05 µCi/ml) to provide an internal normal-density DNA marker. The following morning they were placed in normal medium for 3 H and then exposed to ^{3}H-dThd (2.5 X 10^{-6} M, 27 µCi/ml) and FdUrd (2 X 10^{-6} M) for 5 min (a,c) or 25 min (b,d) followed by 3 h of treatment with BrdUrd (2 X 10^{-6} M). After extraction and shear to 4.5 µm (a,b) or 0.3 µm (c,d), the DNA was centrifuged to equilibrium in alkaline CsCl. (●), ^{3}H cts/min; (○), ^{14}C cts/min; (▲), density.

were sheared to 4.5 μm or 0.3 μm before centrifugation. If the heavy peak resulted from end-to-end association of the 2 tracers in stretches of DNA longer than 0.3 μm, then shear to the smaller size should have caused a marked reduction in the fraction of counts in this peak. At ^{3}H pulse times from 10 to 30 min, the fractions of heavy DNA are similar at both shear sizes.

The results obtained with the 5 min pulse are anomalous. This is probably an artifact of the normalization procedure. This consistently underestimates the true fraction of heavy DNA (Painter and Schaefer, 1969) and the underestimation is magnified at high values of heavy DNA and when the DNA is highly dispersed. Both values at 5 min are too low, but the degree of underestimation is greater in the sample sheared to 0.3 μm, accounting the difference between them.

Kinetics of nucleoside incorporation

One possible explanation for the interspersion of ^{3}H-dThd and BrdUrd in short stretches of DNA under the experimental conditions in figure 2 is the mixing of the 2 tracers in the precursor pool prior to incorporation into DNA. This could be caused by slow equilibration of the intracellular pools with the cell culture medium and by the preferential incorporation of dThd over BrdUrd or both. To investigate these possibilities, we performed a series of experiments aimed at examining the incorporation of exogenous nucleosides into DNA.

TABLE 1

Radioactive DNA of heavy density following sequential labeling with ^{3}H-dThd and BrdUrd

^{3}H pulse-time (min)	Fraction of heavy DNA[1] 4.5 μm	0.3 μm
5	.79	.59
10	.54	.52
15	.40	.43
20	.41	.42
25	.45	.36
30	.40	.41

[1]DNA was prepared according to the protocol in figure 2. Samples were sheared to 4.5 μm or 0.3 μm before centrifugation. The fraction of heavy DNA was estimated using the normalization procedure of Painter and Schaefer (1969). Each number represents the average of 2 or more gradients.

In the presence of FdUrd, ^{3}H-dThd is incorporated into DNA in a linear fashion at concentrations of inhibitor and isotope used in the sequential labeling protocol in figure 2. Linear incorporation was maintained for at least 60 min (fig. 3a) and the lag period from addition of isotope to linear incorporation was less than 1 min (fig. 3b).

We next looked at ^{3}H incorporation into DNA when the sequential labeling protocol in the experiment in figure 2 was duplicated exactly. In this experiment, cells were labeled in the presence of FdUrd with ^{3}H-dThd for 15 min and then with BrdUrd for 3 h, and incorporation of ^{3}H into DNA was measured. The results are shown in figure 3c, where the 0 time is the point at which BrdUrd was substituted for ^{3}H-dThd in the medium. Incorporation of radioactivity into DNA increased for at least 10 min after it had been removed from the medium. This indicated there was a slow transition from incorporation of ^{3}H-dThd to BrdUrd under these experimental conditions, which could explain the interspersion of the 2 tracers in the DNA.

The experiment in figure 3d confirmed this. Here the labeling protocol in figure 2 was followed and the radioactivity in the acid-soluble pool was measured. The amount of ^{3}H-dThd in the pool decreased by one-half within 5 min, but significant amounts of radioactivity remained in the pool through 30 min after the switch to BrdUrd. The possibility of preferential incorporation of dThd was examined in the

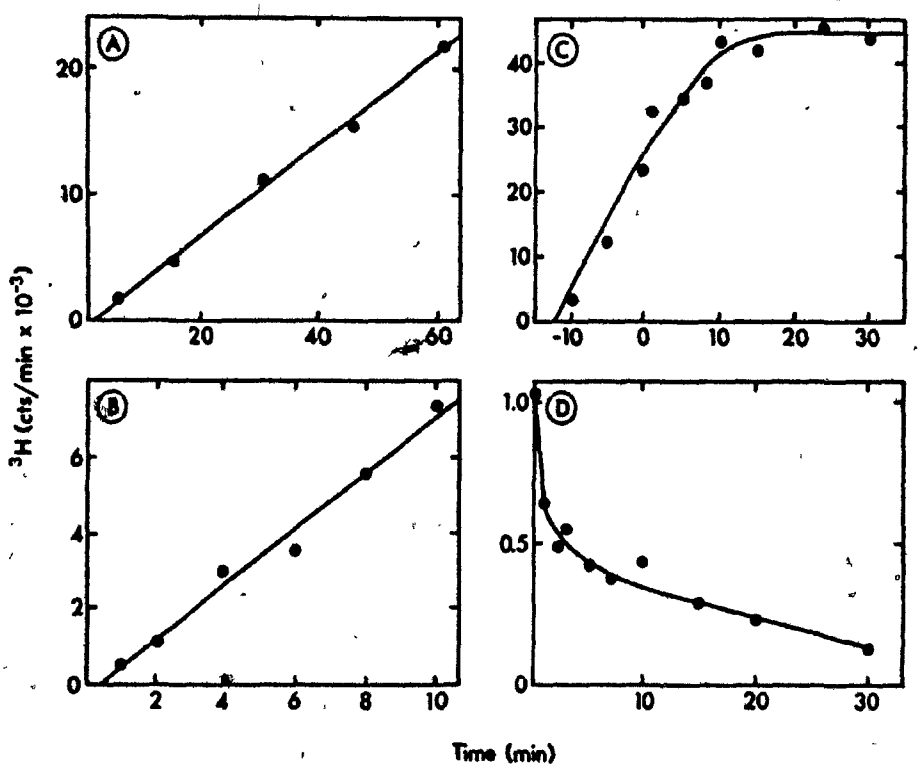

FIGURE 3

Incorporation of ^{3}H-dThd into CV-1 cells under conditions of the sequential ^{3}H-dThd to BrdUrd labeling protocol. Logarithmically growing cells were treated with ^{3}H-dThd (2 X 10^{-6} M, 10 µCi/ml) and FdUrd (2 X 10^{-6} M) for the times indicated, (a,b), or were treated with ^{3}H-dThd (2 X 10^{-6} M, 10 µCi/ml) and FdUrd (2 X 10^{-6} M) for 15 min prior to treatment with BrdUrd and FdUrd, (c,d) which was then continued for the duration of the experiment. In (a,b,c), the DNA precipated by addition of 5% TCA plus 0.5% sodium pyrophosphate. In (d), the acid-soluble radioactivity was extracted from the cellular pools by treatment with 5% TCA at 4^o for 10 min. Each point is the average of 3 replicate samples.

experiment in table 2. Here, known concentrations of ^{3}H-BrdUrd and ^{14}C-dThd were mixed in the labeling medium and incorporation of the 2 isotopes into the acid-soluble pool and into DNA was measured. The results showed that there was preferential incorporation of ^{14}C-dThd into the acid-soluble pool and into DNA. This preferential incorporation was not altered by the presence of FdUrd in the labeling medium.

Incorporation kinetics when density label preceded radioactive label

Accurate measurements of rate of fork displacement using this hydrodynamic method depends upon the sharp transition from radioactive to density label in the replicating DNA. We thought we could take advantage of the preferential incorporation of dThd over BrdUrd by reversing the sequence of the 2 labels. By administering the density label first, the switch to ^{3}H-dThd label might result in a sharp transition and minimize the length of DNA with both tracers interspersed.

When, in the presence of FdUrd, BrdUrd treatment preceded labeling with ^{3}H-dThd, the incorporation of radioactivity into DNA was linear over 60 min (fig. 4a) and the lag period before incorporation was less than 1 min (fig. 4b).

In the experiment in figure 4c, ^{3}H-BrdUrd was administered for 3 h and chased with unlabeled dThd. The amount of BrdUrd

TABLE 2

^{3}H-BrdUrd and ^{14}C-dThd incorporation into CV-1 cells

Sample	$^{3}H:^{14}C$	
	with FdUrd	without FdUrd
Culture medium	2.290	2.428
Acid-soluble pool	1.213	1.283
TCA-precipitable DNA	.466	.534
KOH-digested DNA	.809	.903

Cells were treated for 15 min with medium containing ^{3}H-BrdUrd (2×10^{-6} M, 2.5 µCi/ml) and ^{14}C-dThd (2×10^{-6} M, 0.5 µCi/ml in the presence or absence of FdUrd (2×10^{-6} M). The ratio of $^{3}H:^{14}C$ in the culture medium was measured by spotting acidified samples on glass fiber filters and determining the radioactivity on the dried filters by liquid scintillation counting. The acid-soluble pools were extracted with TCA at 4° for 10 min and samples of the extract then spotted on filters. The DNA samples were precipitated with TCA and the precipitates counted directly or further treated with 0.3 N KOH at 37° for 16 h, acidified again, and counted.

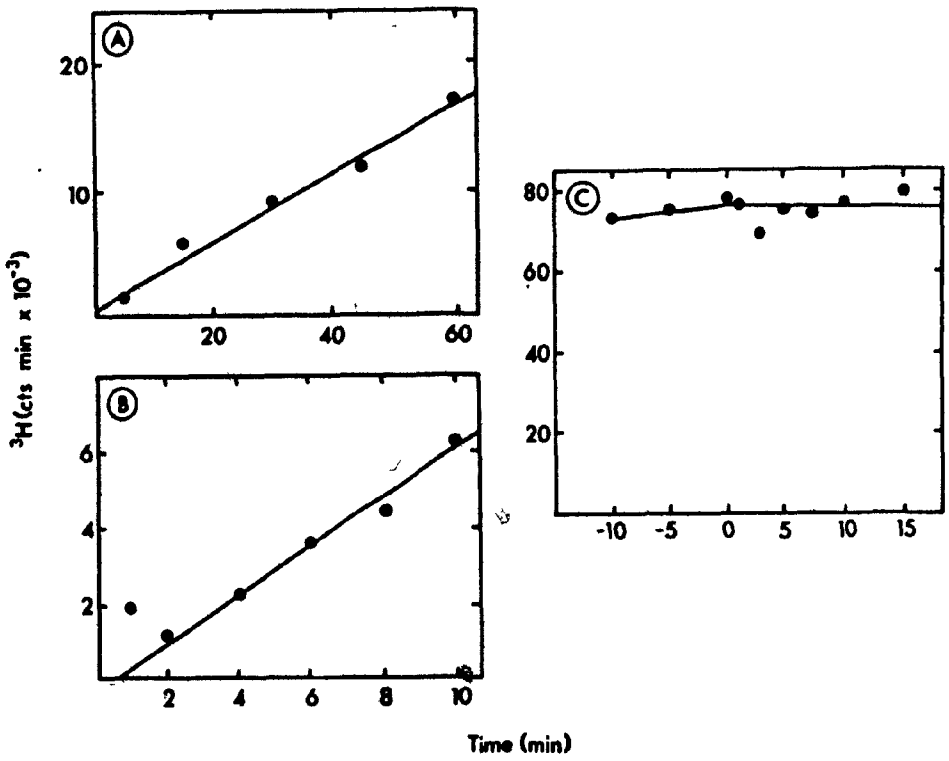

FIGURE 4

Incorporation of exogenous nucleosides under conditions of the sequential BrdUrd to ^{3}HTd (reverse) protocol. Logarithmically growing cells were treated with BrdUrd (2 X 10^{-6} M) and FdUrd (2 X 10^{-6} M) for 3 h prior to treatment with ^{3}H-dThd (2 x 10^{-6} M, 10 µCi/ml) and FdUrd (2 X 10^{-6} M) for the times indicated (a,b), or were treated with ^{3}H-BrdUrd (2 X 10^{-6} M, 20 µCi/ml) and FdUrd (2 X 10^{-6} M) prior to a chase with unlabeled dThd (2 X 10^{-6} M) in presence of FdUrd (2 X 10^{-6} M) for 2 h (c). The DNA was acid-precipitated and the radioactivity measured. Each point represents 3 replicate samples.

in DNA remained constant following the transition to dThd label.

These results suggested that by reversing the sequence of labeling, a sharp transition from density to radioactive DNA could be achieved.

Equilibrium centrifugation analysis of DNA labeled sequentially with BrdUrd and ^{3}H-dThd (reverse protocol).

When DNA labeled with this reverse protocol was analyzed by centrifugation in alkaline CsCl, we obtained the results shown in figure 5. The ^{3}H-labeled DNA banded in a continuous distribution with a peak buoyant density slightly heavier than unsubstituted DNA. This result was obtained with ^{3}H pulses of 5 min and 25 min as illustrated, and in many other gradients with ^{3}H pulse times form 5 to 30 min. Shearing the DNA from 4.5 μm to 0.3 μm before centrifugation resulted in a wider band of the same configuration and a reduced fraction of DNA banding at heavy density (table3). Intermixing of density and radioactive tracers in the precursor pool was not completely eliminated by the reverse labeling protocol, since the highly sheared fragments still showed displacement toward heavier than normal density. This was most marked with the shortest ^{3}H labeling times.

The fractions of heavy DNA were used to estimate the rate of replication fork movement. These results are shown in the last column of table 3. The calculated rate appears to

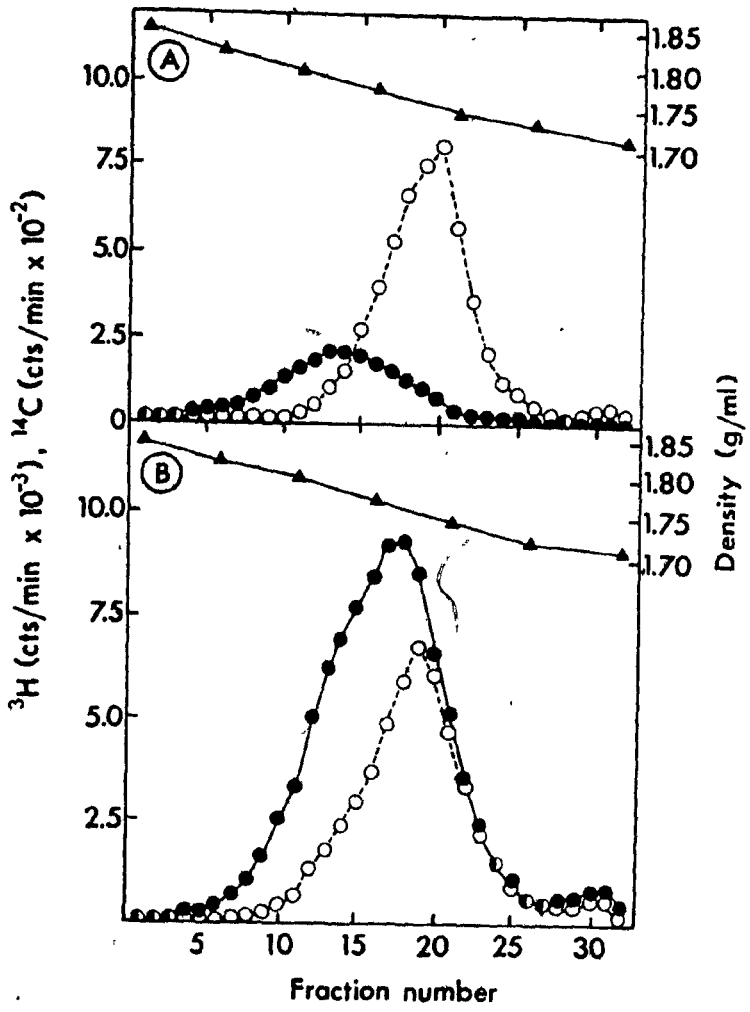

FIGURE 5

Alkaline equilibrium density gradients of DNA sequentially labeled with BrdUrd and ^{3}H-dThd. The experiment is identical to the one in figure 2 except the sequence of the BrdUrd and ^{3}H-dThd pulses was reversed. ^{3}H pulses were for 5 min (a) or 25 min (b). DNA was sheared to 4.5 μm. (●), ^{3}H cts/min; (○), ^{14}C cts/min; (▲), density.

TABLE 3

Calculated rates of replication fork movement from sequential labeling with BrdUrd and ^{3}H-dThd

^{3}H pulse-time (min)	Fraction of heavy DNA[1] 4.5 μm	0.3 μm	Rate[2] (μm/min)
5	.77	.56	-
10	.61	.23	.59
15	.53	.21	.47
20	.46	.15	.36
25	.37	.13	.38
30	.35	.10	.30

[1]DNA was prepared according to the protocol in figure 5. Samples were sheared to 4.5 μm or 0.3 μm before centrifugation. The fraction of heavy DNA was estimated using the normalization procedure of Painter and Schaefer (1969). Each number represents the average of 2 or more gradients.

[2]Calculated using the equation, $R = B/2Ft$, where R is the rate of fork movement, B is the shear size of the DNA, F is the fraction of heavy DNA, and t is the time of the ^{3}H pulse. F is corrected by subtraction of the value at 0.3 μm from that at 4.5 μm. This method of calculation follows that of Painter and Schaefer (1969). The modification introduced by Roti Roti and Painter (1977) change the results by less than 5%. The values of F at 5 min are anomalous and none of the equations in Painter and Schaefer (1969) or Roti Roti and Painter (1977) applies.

decrease as the ^{3}H pulse is increased from 5 to 30 min. The short pulses (up to 15 min) yield too high a rate because of the underestimation of the fraction of heavy DNA is greater at high values.

DNA fiber autoradiography

Fiber autoradiography has been used as another method to measure rate of replication fork movement. It was of interest to compare it to the equilibrium centrifugation method.

As shown in figure 6, fiber autoradiograms prepared from the DNA labeled for sequential 30 min periods with high-and low-specific-activity ^{3}H-dThd showed patterns similar to those seen in other mammalian cells. To determine the rates of replication fork movement, we measured lengths of the thick portions of tracks on replication units that had initiated synthesis before the beginning of the high-specific-activity pulse. The results are shown in figure 7a. These tracks showed a linear increase with increasing pulse time, with a slope of 0.56 μm/min, corresponding to the rate of fork movement.

The size of replication units may also be measured by fiber autoradiography. Knowledge of unit size is important in studies of fork movement. If a long pulse time is chosen so that many forks complete replication within their units

FIGURE 6

Autoradiograms of DNA fibers from CV-1 cells. Logarithmically growing cells were labeled in the presence of FdUrd (2×10^{-6} M) with ^{3}H-dThd of high specific activity (50 Ci/mmole, 5×10^{-6} M) for 10 min (a) or 30 min (b) and then for an additional 30 min with ^{3}H-dThd of low specific activity (5 Ci/mmole, 5.5×10^{-5} M). The DNA was prepared for autoradiography and the autoradiograms were developed after 6 weeks exposure. The large arrowheads in (a) and (b) mark the ends of fibers longer than 250 µm. The small arrowheads at x indicate the length of a high-grain-density track used to measure rate of replication fork movement; the small arrowheads at y, the distance between 2 initiation points used to measure the size of a replication unit. The bar at upper left is 100 µm. Both micrographs are at a magnification of 410.

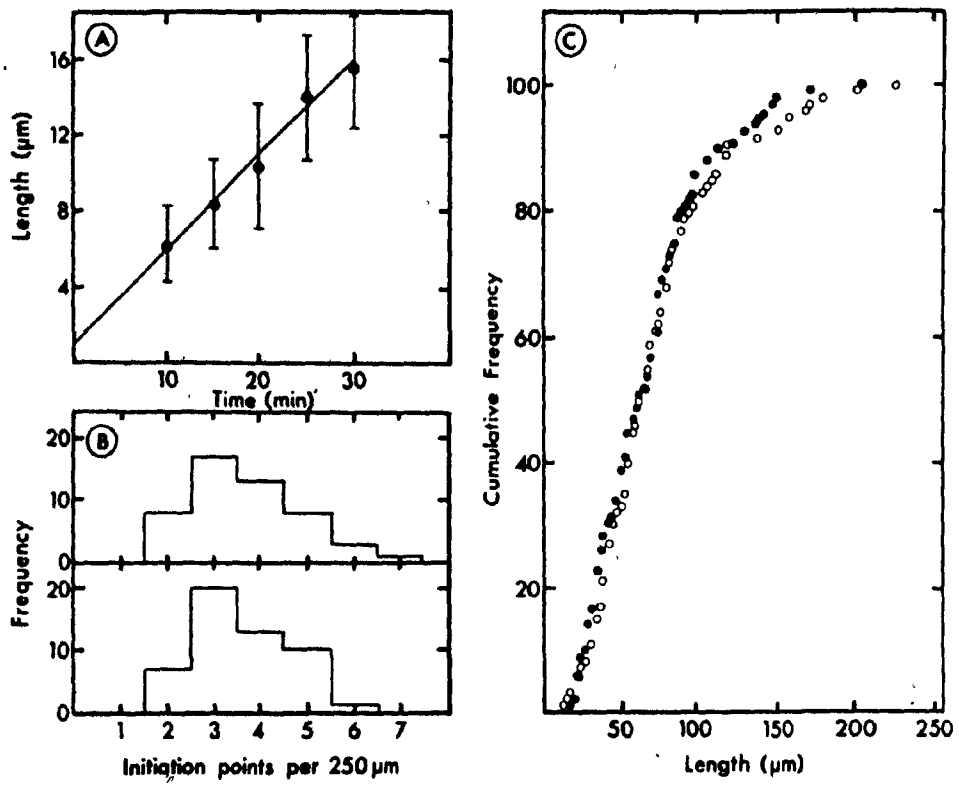

FIGURE 7

Replication fork movement and unit size by fiber autoradiography. (a) Lengths of high-grain-density tracks (fig. 6, x) at high specific-activity pulse times form 10 to 30 minutes. The error bars indicate the standard deviations of 50 measurements. The line was fitted by regression analysis using the method of least squares, $r^2 = 0.98$. (b) Initiation points on 250 μm fibers (fig. 6,y) pulsed with high-specific-activity ^{3}H-dThd for 10 min (upper panel) or 30 min (lower panel). (c) Lengths between initiation points on 250 μm fibers. (●), 10 min pulse with high-specific-activity ^{3}H-dThd; (O), 30 min pulse with high-specific-activity ^{3}H-dThd.

before the end of the pulse, then the rate of fork movement may be underestimated.

Unit size was measured by a modification of the method of Martin and Oppenheim (1977). Autoradiograms of DNA fibers of minimal length 250 μm were scored for the number of initiation points. Figure 7b shows the modal number was 3 initiation points from fibers labeled with high-specific-activity ^{3}H-dThd for 10 and 30 minutes. The lengths between initiation points of replication units of 250 μm fibers was also measured. The lengths reflect the size of replication units (Huberman and Riggs, 1968). The cumulative frequency distribution of lengths from DNA labeled for 10 and 30 minutes with ^{3}H-dThd of high specific activity are shown in Figure 7c. The distributions are similar and the medians of both are 62 μm.

DISCUSSION

Accuracy with the hydrodynamic method of measuring rate of fork movement depends on a sharp transition from radioactive to density label in the replicating DNA. A factor that could interfere with this is slow turnover of exogenous label in the precursor pool, which could be caused by preferential uptake of ^{3}H-dThd over BrdUrd if the radioactive tracer precedes the density tracer. We have shown that this can occur. The effect of preferential uptake of dThd can be

eliminated by administering this compound after the BrdUrd. With this reversal of the sequence of density and radioactive label, we were able to achieve rapid substitution of dThd for BrdUrd so that end-to-end association of the two tracers on the newly replicated chains was obtained.

The calculated rates decreased with increasing time of the ^{3}H-dThd pulse. This has been observed previously when 2 different pulse times have been used in the same set of experiments (Painter and Schaefer, 1969; Martin and Oppenheim, 1977). The method overestimates rate at short pulse times. This is caused by underestimation of the fraction of heavy DNA when this fraction is very high. It would seem best to choose ^{3}H pulse times that give heavy DNA fractions that are less than 0.5. Errors in measurement of the fraction can also be minimized by increasing the separation between heavy and normal DNA in the gradients. This can be done by use of FdUrd to increase exogenous nucleoside incorporation and by use of alkaline centrifugation conditions which increase the separation of heavy and normal density DNA.

There may also be an underestimation of rate at long ^{3}H pulse times if a significant proportion of replication forks complete elongation before the end of the pulse. Autoradiography showed that the average size of replication units in the CV-1 cell line is 62 μm. With bidirectional replication, the average time that each replication fork is in operation is

about one hour (assuming a rate of fork movement of about 0.5 μm/min). However some 20% of units are less than half this average size, and therefore could complete synthesis in 30 min or even less. With the longest pulse time used in these experiments - 30 min - there could be some underestimation of the rate.

We believe the most accurate estimates are those from the 20 and 25 min pulses in Table 3. This is because with these pulse times, the fraction of heavy DNA is small enough to be accurately measured and almost all replication forks are in operation for the duration of the pulse.

We have also demonstrated a preferential incorporation of dThd over BrdUrd. This has been reported in uninfected bacterial cells (Hackett and Hanawalt, 1966), in phage-infected bacterial cells (Le Pecq and Baldwin, 1968) and in certain animal cell systems such as developing chick red blood cells (Weintraub et al., 1972). However, in other animal cell systems, the two tracers have been incorporated equally well in whole cells (Myers and Feinendegan, 1975; O'Brien and Stellwagen, 1977) and in vitro using a lysed cell systen (Gautschi et al., 1978). If this hydrodynamic method is to be used to measure rate of fork movement, we believe that incorporation of both tracers must be measured and the labeling protocol modified according to the results. If there is no preferential incorporation, the sequence dThd to BrdUrd may be used. If dThd is preferentially incorporated, then the reverse sequence of labeling must be used.

Finally, it is worth commenting on the higher rates found using fiber autoradiography. The thick portion of the grain track used in the measurement gradually tapers to the thinner portion, therefore transition from high-to low-specific-activity labeling may not be sharp. The measurements encompass the entire tapered region of the track, and this results in overestimation. Whatever error is introduced, it is systematic, since the calculated rate was independent of pulse time.

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4. DNA REPLICATION DURING A SERUM INDUCED S PHASE IN PRIMATE CV-1 CELLS

SUMMARY We have investigated components of DNA replication in a serum-induced S phase of primate CV-1 cells. Using DNA fiber autoradiography, we found a relative decrease in the frequency of initiation events in mid-S compared to early and late S phase. The other components of DNA replication measured by autoradiography - synchrony of initiation events, size of replication units, incidence of bidirectional replication, and the rate of replication fork movement - remained constant through S phase. When fork movement was measured by density gradient analysis of BrdUrd - and ^{3}H thymidine-substituted DNA, it was also found to remain constant. These results show that most components of DNA replication are invariable through a serum-induced S phase. The changes in initiation frequency support the view that it may be critical in the regulation of ongoing replication.

Proliferating eukaryotic cells replicate their DNA in a defined period in interphase, the DNA synthetic (S) phase [1]. Cells can also be in a nonproliferating state in which there is no cell division for prolonged periods [2]. A cell reaches a decision during G_1 whether to initiatite S phase and continue to mitosis or to stop proliferation. Many factors can influence the cell's decision; less than optimal growth conditions generally favor entry into a quiescent state.

Once a cell enters S phase, the entire genome is replicated. The eukaryotic genome is composed of many thousands of tandemly arranged replication units, each of which contains a central origin for the initiation of DNA synthesis [3, reviewed in 4-6]. From these origins, replication forks proceed bidirectionally toward the outlying termini of the units where the newly replicated daughter chains fuse with the chains synthesized on adjacent units to form the large molecules characteristic of chromosomal DNA. The factors regulating the rate of DNA synthesis through the S phase are largely unknown.

Evidence has been presented to show that, in diploid and pseudodiploid cells synchronized by mitotic selection, the overall rate of DNA synthesis varies through the S phase with three maxima present in early, mid, and late S phase [7,8]. These studies depended on thymidine incorporation as a measure of DNA synthesis, and no attempt was made to analyze the individual components that make up the overall rate of synthesis, such as speed of replication fork movement or frequency of initiation events.

Several studies have reported on the rate of replication fork movement as the cells progress through S. Painter and Schaefer [9] showed that rate increased from early to mid S phase. They used HeLa cells synchronized by mitotic selection or by pharmacologically induced alignment at the G_1-S interface, and measured rate by equilibrium centrifugation of DNA sequentially labeled with [^{3}H] thymidine and BrdUrd. Housman and Huberman [10] also concluded that rate increased in CHO cells synchronized by colchicine block followed by mitotic selection. Rate was measured in their experiments by DNA fiber autoradiography. More recently, Kapp and Painter [11] have reported that, following synchronization by mitotic selection, the increase in rate of fork movements does not occur in all cell lines. They compared 2 hydrodynamic methods for measuring rate, one a modification of the equilibrium centrifugation procedure used earlier [9], and the other a procedure that measures the frequency of light-induced breaks in replicating DNA substituted with BrdUrd [12]. Similar results were obtained by both methods. HeLa cells showed the characteristic increase, while CHO cells showed a uniform rate through the S phase. Human diploid fibroblasts showed a periodic variation in rate that correlated with changes in thymidine incorporation into DNA. The reason for the difference in the results of this study and earlier ones is unknown.

Other aspects of DNA replication, such as the frequency of initiation and the degree of synchrony of initiation events have not yet been examined in synchronized cells.

In the experiments reported here, we have examined several components of DNA replication in synchronized cells. We have used stimulation of quiescent cells by fresh medium and serum to induce S phase, and the techniques of fiber autoradiography and equilibrium centrifugation to examine DNA synthesis. The results show that, under our experimental conditions, there is a relative decrease in the frequency of initiation events in mid S phase cells compared to early and late S phase cells. Other aspects of DNA replication including the rate of replication fork movement remain constant through the S phase.

MATERIALS AND METHODS

Cells

CV-1 cells, a continuous line of African Green Monkey cells (a gift from Dr. E. Gershey, The Rockefeller Univ., N.Y.), were grown in Eagle's Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 50 µg/ml of Gentamicin, 0.25 µg/ml of Amphotericin B, and 60 µg/ml of Tylocine.

Thymidine Incorporation into DNA of Cells Stimulated to Proliferate by Addition of Fresh Medium and Serum

Incorporation into acid-precipitable material. CV-1 cells were seeded into 60 mm plastic cells culture dishes at a density of 5×10^4 cells/cm^2 in MEM plus 2% FCS. At 96 hours after seeding, the medium was removed from half of the dishes and replaced by fresh MEM plus 10% FCS. The other dishes were left as serum-depleted controls.

At various times, quadruplicate dishes were pulse-labeled with [^{3}H]-thymidine (1.0 µCi/ml) for 30 min, washed, and the cells removed from the dishes by scraping. One half of each sample was used for protein determination by the Lowry method [13] using albumin as the standard. The other half was used to determine trichloroacetic-acid-insoluble radioactivity by liquid scintillation counting.

Per cent of cells in S phase. CV-1 cells were seeded into 60 mm cell culture dishes each containing one sterile 22 mm glass coverslip and grown as described above. At various

time points, duplicate dishes were labeled for 30 min with [^{3}H]thymidine (1.0 μCI/ml). Following the pulse, the dishes, were washed and the cells on the coverslips were prepared for whole cell autoradiography. After exposure for 7 to 10 days, the preparations were developed, stained, and examined with a light microscope. Consecutive fields were inspected until 250 cells on each of the coverslips had been scored for the presence of silver grains over the nucleus.

Fiber Autoradiography for the Measurement of DNA Replication

CV-1 cells, seeded into 35 mm plastic cell culture dishes, were brought to quiescence and then stimulated to proliferate as described above. Cells in triplicate dishes were labeled with [^{3}H]thymidine and processed for DNA fiber autoradiography as described previously [14] after growth in fresh medium for 11, 15.5 or 20 hours. In brief, the cells were labeled at the indicated times for sequential 30 min periods with [^{3}H] thymidine of high specific activity (50 Ci/mmol, 500 μCi/ml) and low specific activity (5 Ci/mmol, 500 μCi/ml). Following washing, they were lysed gently with 1% sodium dodecyl sulfate and the released DNA fibers extended and spread on glass microscope slides. The preparations were fixed and coated with NTB-2 emulsion. After exposure and development, the autoradiograms were examined by light microscopy. The methods used for scoring and analyzing the fiber autoradiograms for initiation frequency, replication unit size, rate of fork movement, bi-

directional replication, and initiation synchrony have been described [15].

Determination of Rate Replication Fork Movement by Equilibrium Centrifugation of Denatured DNA

Density labeling. CV-1 cells in 250 ml plastic cell culture flasks were brought to quiescence and stimulated to proliferate as described above. From 19.5 hours after seeding to the addition of fresh medium, they were labeled with [^{14}C]thymidine (0.05 µCi/ml). Density labeling was performed as described previously [16]. Three hours before the time points, BrdUrd (2×10^{-6}M), and FdUrd (2×10^{-6}M) were added to the cells which were then protected from light. At each time point (11, 15.5 and 20 h after addition of fresh medium) the medium was replaced by labeling medium ([^{3}H]thymidine, 10 µCi/ml, 2×10^{-6}M, and FdUrd, 2×10^{-6}M, in MEM plus 10% FCS). After a pulse of 30 minutes the cells were washed and the DNA extracted.

Extraction of DNA. A modification of the phenol-chloroform method of Marmur was used [17]. The extracted DNA was sheared to a molecular weight of 8×10^{6} by passage 4 times through a 27 gauge needle.

Equilibrium centrifugation of denatured DNA. A sample of extracted DNA containing 5000 ^{14}C cpm was brought to a final volume of 6.2 ml in a solution of 0.002 M NaCl, 0.1 N NaOH, 0.002 M EDTA, 0.1% Sarkosyl plus 7 g CsCl. This was centrifuged

to equilibrium for 48 to 72 h in a 40.2 fixed angle rotor (Beckman Instruments). Fractions of 0.25 ml were collected from the bottom of the gradient and analyzed for density and radioactivity.

RESULTS

Induction of Cellular DNA Synthesis by the Addition of Fresh Medium and Serum

In our initial experiments, we determined the time course of DNA synthesis in CV-1 cells brought to quiescence by low serum and then stimulated to proliferate by fresh medium and serum. The results are shown in Fig. 1. There is a steady decline in thymidine incorporation into acid-precipitable material through 96 h in low serum. In the unstimulated controls, the low incorporation is maintained for the rest of the experiment. In the cells that received fresh medium with 10% FCS, there is a sharp rise in the rate of tritiated thymidine incorporation that is evident by 9 h and peaks at 15.5 h after medium change. This represents a ten-fold stimulation in the rate of incorporation compared to controls. The duration of this DNA synthetic phase is 11 h.

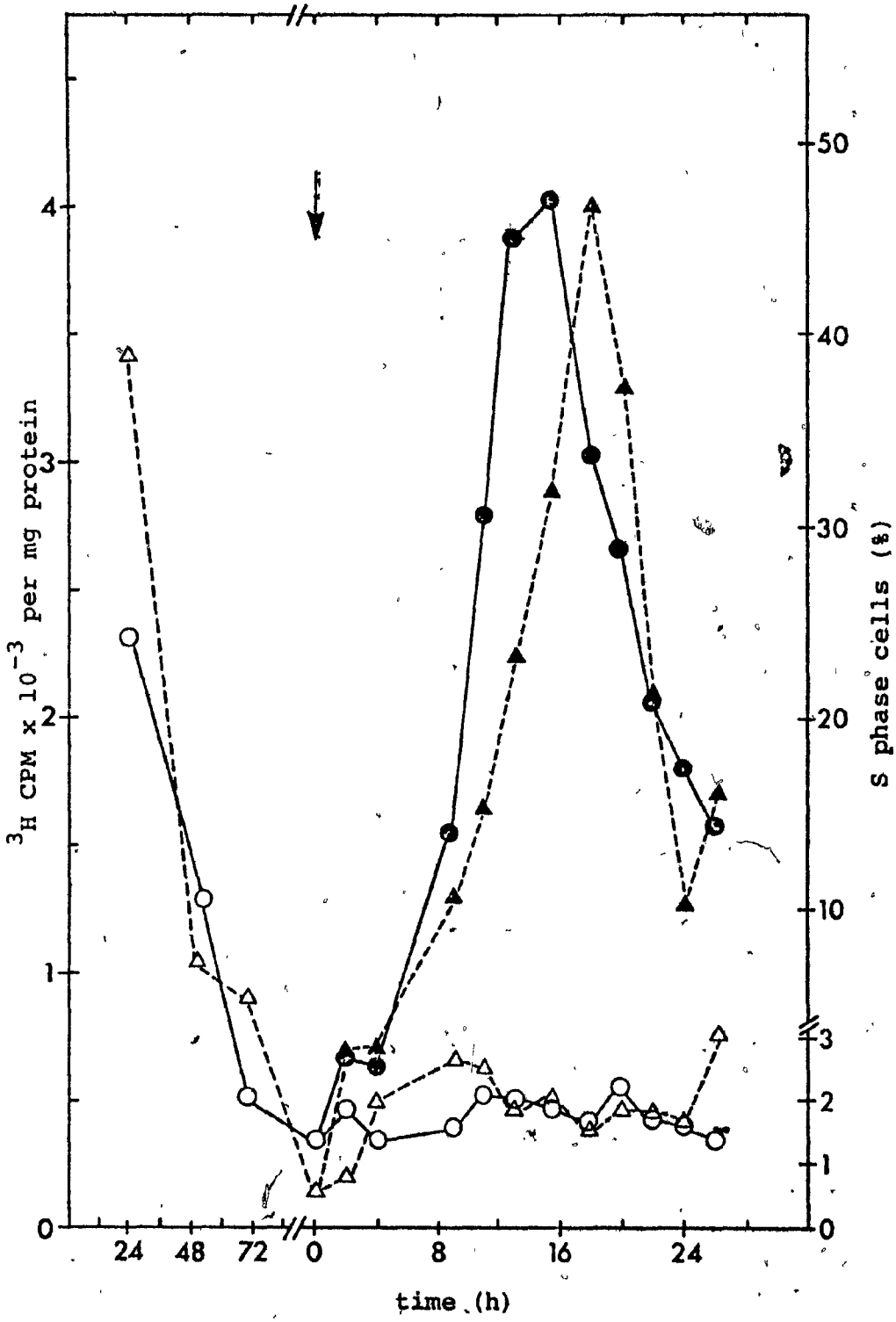
Whole cell autoradiography following pulse-labeling with [^{3}H]thymidine also revealed a synchronous wave of DNA synthesis in stimulated cells. Following addition of fresh medium, the proportion of labeled nuclei increased from 1% to 47% by 18 h.

FIGURE 1

Abscissa: time (h); ordinate: (left) ^{3}H CPM X 10^{-3} per mg protein (right) S phase cells (%).

DNA synthesis in CV-1 cells brought to quiescence in low serum and then stimulated to proliferate by the addition of fresh medium and serum. Acid precipitable incorporation (● , ○) and percent of cells in S phase (▲ , △) was measured following pulses with [^{3}H]thymidine. Open symbols indicate cells in MEM plus 2% FCS, closed symbol, cells to which fresh MEM plus 10% FCS were added 96 h after plating (arrow).

FIGURE 1


^{3}H CPM x 10^{-3} per mg protein
S phase cells (%)
time (h)
0
1
2
3
4
10
20
30
40
50
24
48
72
0
8
16
24

The duration of the synthetic phase was 9 h. Unstimulated controls showed 1 to 3% of the cells labeled.

The expected overlap of peaks by the two methods of measuring DNA synthesis is not seen. The onset and peak of DNA synthesis is 2 h later when measured by autoradiography. A similar lack of coordination of peaks of activity of acid precipitable counts and percentage of labeled nuclei has been observed in WI-38 cells synchronized by mitotic selection [8,18]. Aside from this, the cells are well-synchronized, although slightly less than half of them enter S phase. On the other hand, less than 3% of the unstimulated controls synthesize DNA and this low background is of advantage in the fiber autoradiography studies described below. We chose time points, at 11, 15.5 and 20 h following medium change as representative of proliferating cells in early, mid and late portions of the DNA synthetic period for subsequent studies.

DNA Fiber Autoradiography

Fiber autoradiography was used to examine DNA replication in these cells. At each of the time points in S, we measured the relative frequency of initiation events, the incidence of bidirectional replication, the synchrony of initiation events, the size of replication units, and the rate of replication fork movement.

In order to make these measurements, we used a step-down labeling protocol, that is, a 30 min pulse with [^{3}H] thymidine

of high specific activity followed immediately by a 30 min pulse with [^{3}H]thymidine of low specific activity. The protocol yields two types of autoradiograms. Replication units that initiate before the high specific activity pulse have a pattern in which a small gap is flanked by two stretches of heavy grain density, followed by lower grain density tails. Since initiation took place prior to the pulse, these are prepulse autoradiograms. Units that initiate during the high-specific-activity pulse give patterns in which a continuous stretch of heavy grain density is flanked by two low grain density tails. These are postpulse autoradiograms. The 2 types of patterns are easily recognized by light microscopy and are used to make the measurements of DNA replication.

Relative frequency of initiation. The ratio of prepulse to postpulse autoradiograms reflects the relative numbers of units initiating before and during the pulse of high specific activity. With constant pulse time, a change in the ratio measures a change in the frequency of initiation. We measured this ratio in preparations from early, mid and late S phase cells (Table 1). The results show an increase in the ratio in mid S DNA, indicating that there are relatively fewer units initiating then, compared to early and late S.

Proportion of bidirectional replication units. DNA replication is bidirectional from a central initiation point [3] but there is always a measurable incidence of unidirectional replication. This incidence increases when DNA synthesis is

TABLE 1

Relative initiation frequency in synchronized cells through s phase

Time after serum addition (h)	Initiation frequency (prepulse: postpulse patterns)[a]	Sample size
11.0	35:65	386
15.5	49:51	360
20.0	39:61	478

[a]Random microscopic fields from the autoradiographs were scored for the number of prepulse and postpulse patterns. The ratio of these is used as a relative indication of initiation frequency. The increase in the ratio observed at 15.5 h compared to the other 2 times points indicates relatively fewer postpulse units and therefore that fewer units initiated during the pulse at mid S.

inhibited by cycloheximide [15]. In the present experiments, a high proportion of bidirectional replication units was found at all 3 time points (Table 2). Thus, the factors that induce simultaneous initiation of 2 replication forks from a common origin continue throughout the serum-induced S phase.

Synchrony of initiation events. Adjacent units initiate synthesis synchronously [4,6]. With fiber autoradiography, this synchrony is demonstrated by adjacent units on a DNA fiber with the same pattern, that is, both prepulse or both postpulse. If initiation were random rather than synchronous, then the proportions of 2-unit autoradiograms showing like or unlike patterns would be determined by the binomal distribution for any given ratio of prepulse to postpulse patterns in a preparation. A significant deviation from the expected proportions in the direction of an increase in units with like patterns indicates synchrony.

The results of observations on 2-unit autoradiograms are shown in Table 3. There is no significant difference in the synchrony of initiation in early, mid and later portions of a serum-induced S phase, and the usual high degree of synchrony is maintained thoughout.

Replication unit size. To determine the size of replication units, the center-to center distances between adjacent replication units were measured. The cumulative frequency distributions are shown in Fig. 2. The median values for the early, mid and late S measurements are similar. There are

TABLE 2

Bidirectional DNA Replication

Time after Serum addition (h)	Bidirectional units proportion[a]	Sample size
11.0	.94	307
15.5	.93	222
20.0	.93	345

[a]Random microscopic fields were scored for the numbers of bidirectional and unidirectional patterns on replication units. Unidirectional patterns are those with a high density track flanked on one end by a low density track, and on the other end by a gap adjacent to another low density track.

TABLE 3

Synchrony of initiation events on 2-unit autoradiograms

Time after serum addition(h)	Proportion of autoradiograms with like units[a] observed	expected	Sample Size
11.0	.83	.56	82
15.5	.78	.54	81
20.0	.85	.56	73

[a]Autoradiograms containing 2 units were scored as to whether the units had the same patterns, either both prepulse or both postpulse, or whether they had different patterns, one prepulse and the other postpulse. This gave the observed proportions. Expected proportions were calculated using the binomial equation $(p + q)^2 = 1$, where p = proportion of prepulse units in the sample and q = proportion of post pulse units. The observed values are all significantly different from those expected ($P < 0.005$, X^2 for goodness of fit).

slightly fewer large units (>50 μm) in late S cells, but on the whole, there is little difference in the size of units active at different times in S. The median values for unit size are 30 to 35 μm. Given a rate of replication fork movement of about 0.33 μm/min (see below, Table 4) and that almost all units have bidirectional replication, then the median time a unit functions is 45 min.

Rate of replication fork movement. The heavily labeled portions of prepulse autoradiograms were measured to determine the rate (Table 4). There is no significant difference through the S phase in these cells.

Determination of Rate of Fork Movement by Equilibrium Centrifugation of Denatured DNA

An equilibrium centrifugation method for the measurement of the rate of fork progression has been described by Painter and Schaefer [19]. In this procedure, a short radioactive label preceeds a long density labeling period. The DNA is extracted, sheared and centrifuged to equilibrium in CsCl. The proportion of radioactive DNA that is greater than normal density in the CsCl gradient is inversely proportional to the rate of fork movement. We felt it would be of value to compare this method with the autoradiographic method for measuring fork movement in the same cell line. Two modifications were introduced: 1) the sequence of radioactive and density labeling was reversed so that sufficient time for density labeling was available at all 3 time points, and 2) equili-

FIGURE 2

Abscissa. replication unit size (μm); ordinate: cumulative frequency (%).

Cumulative frequency distribution of replication unit size through S phase. Center-to-center distances on 2-unit autoradiograms were measured. (●), 11 h after serum addition, median = 30 μm, $\bar{X}$ ± S.D. = 40 ± 22.3 μm, sample size (N) = 96; (○), 15.5 h after serum addition, median = 35 μm, $\bar{X}$ ± S.D. = 41 ± 20.7 μm, N = 97; (▲), 20 h after serum addition, median = 30 μm, $\bar{X}$ ± S.D. = 37 ± 18.1 μm, N = 83.

FIGURE 2

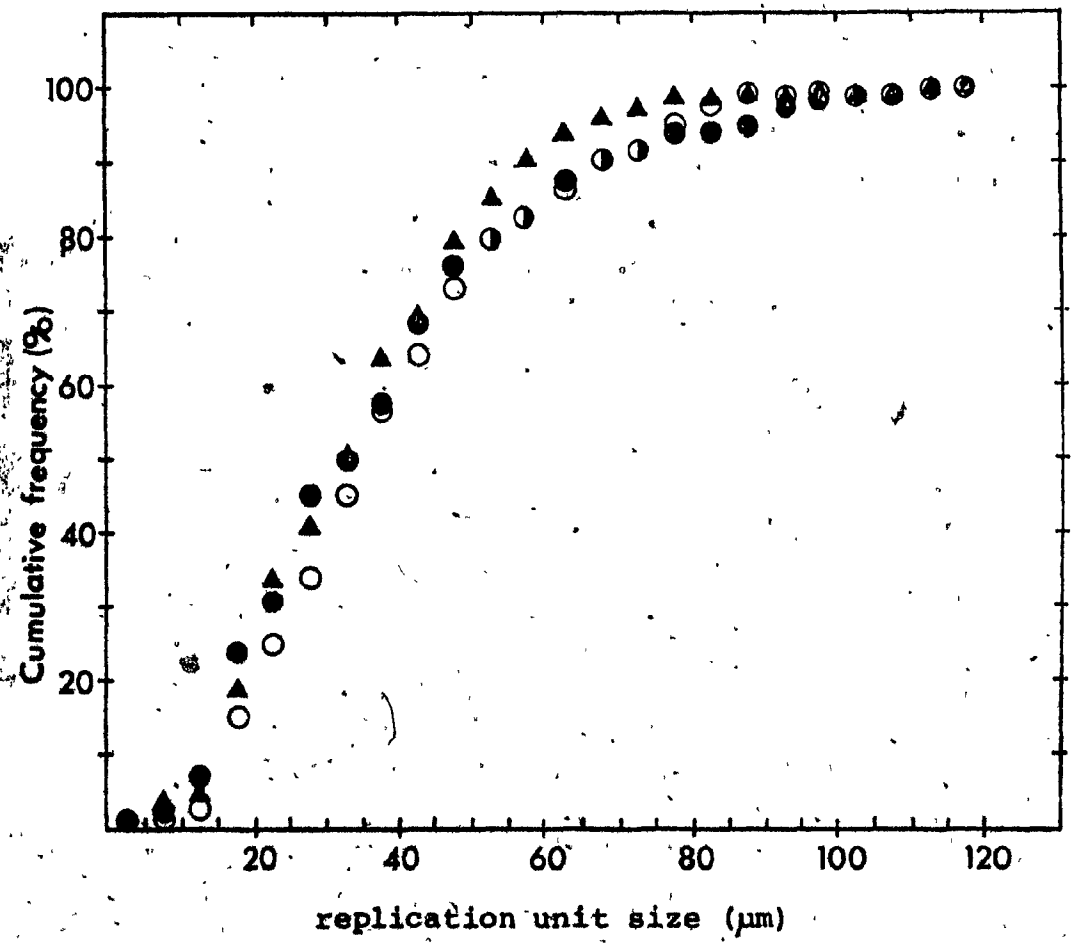

Cumulative frequency (%)
100
80
60
40
20
20
40
60
80
100
120
replication unit size (μm)

TABLE 4

Rate of DNA replication fork movement by autoradiography

Time after serum addition (h)	Rate[a] (μm/min, X ± SD)	Sample size
11.0	.34 ± .113	166
15.5	.34 ± .091	232
20.0	.32 ± .115	219

[a]The high density tracks of prepulse autoradiograms were measured, and the mean lengths divided by the duration of the pulse of high specific activity (30 min).

TABLE 5

Rate of DNA replication fork movement by equilibrium centrifugation[a]

Time after serum addition (h)	F ($\bar{X}$ ± SD)	Rate (μm/min)
11.0	.51 ± .069	.15
15.5	.46 ± .042	.16
20.0	.56 ± .090	.13

[a]DNA was labeled with bromodeoxyuridine for 3 h and with [^{3}H]-thymidine for 30 min according to the protocol in Materials and Methods. The time after serum addition is the beginning of the radioactive pulse. Following equilibrium centrifugation, the fraction (F) of DNA heavier than normal density was measured. The rate of fork movement was calculated using the formula $R = \frac{B}{2Ft}$ where R = rate of fork movement, L = length of the DNA chains synthesized during the ^{3}H pulse, t = time of ^{3}H pulse, B = shear size of centrifuged DNA, and F = fraction of heavy DNA [19]. The values of F represent the means of 5 gradients from 3 separate experimehts.

brium centrifugation was carried out under alkaline conditions to increase the separation of normal density and heavy DNA.

In agreement with the findings from fiber autoradiography, there is no change in the rate of fork movement as the cells move through S phase (Table 5). A notable difference, however, is that average rates calculated by this method are less than those found by autoradiography.

DISCUSSION

We have measured several aspects of DNA replication through the S phase in cells synchronized by medium repletion. We find a relative decrease in the frequency of initiation events in mid S phase cells, compared to those in early and late S. The other measurements - incidence of bidirectional replication, synchrony of initiation events, size of replication units, and rate of replication fork movement - remain constant through the S phase in the CV-1 cell line with the method of synchronization used.

The frequency of initiation decreases as DNA synthesis peaks in mid S phase. This is in no way inconsistent, since the overall rate of DNA synthesis reflects the number of operating replication units. Half the units in these cells operate for 45 min or longer and nearly 20% for more than 90 min. The peak of initiation therefore could be more than 45 min before the peak in overall DNA synthesis. The relative

increase in initiation in late S phase without a rise in overall synthesis might reflect the slightly higher proportion of small replication units active towards the end of S. At any given rate of fork movement, small units are functional for less time than large units. Therefore, fewer units are operating simultaeously and the overall rate of DNA synthesis is less. Small units have been found in satellite DNA [20], and satellite is frequently late-replicating. In particular, the alpha component satellite of CV-1 cells is known to replicate towards the end of S [2].

The constancy of the other measures of DNA replication argues in favor of the view that regulation of overall synthesis is attributable to changes in initiation frequency. This is consistent with the findings in several invertebrates in which there are wide fluctuations in the rate of synthesis in cells of the same organism [22-24]. In each case studied, the changes in the rate of synthesis have been caused by changes in initiation frequency.

The high degree of initiation synchrony and bidirectional replication through the S phase suggest that strict organization of replication is maintained. There is probably little "filling-in" of unreplicated regions late in the S phase. Synchronous initiation of clusters of replication units has been found using a variety of techniques [6] and our findings add further evidence to the concept that regulation within these clusters is stringent [25].

The rate of replication fork movement remained constant through S phase in our experiments. These results differ somewhat from those reported by other investigators [9,10]. This cannot be attributed to the methods used to measure the rate, since the present experiments yielded a constant rate with both fiber autradiography and equilibrium centrifugation, and 2 different hydrodynamic methods gave consistent results in the same cell lines in the study by Kapp and Painter [11]. One possible explanation, offered by Kapp and Painter [11], is that the increase in rate is found in heteroploid lines such as HeLa, but not in diploid or pseudodiploid cells such as human skin fibroblasts or CHO cells. The CV-1 line used in the present experiments is heteroploid, but it behaves like many diploid cell strains in that it can be brought to the quiescent state by medium depletion and stimulated to proliferate by addition of fresh medium and serum. The cells may be functionally similar to the diploid cells from which they were derived, at least insofar as the control of rate of fork movement is concerned.

The findings of Housman and Huberman [10] are also consistent with the view that rate is constant through the greater part of S phase. In their studies with the pseudodiploid CHO cell line, the most reliable estimate of rate showed it to be low only at the earlies point in S, 2.3 h after mitotic selection. By 4.3 h after mitosis (still early in S), the rate had nearly tripled, but then it was constant through the

remainder of S. In the present experiments, the earliest point at which measurements were taken was about 2 h past the G_1-S interface. The constant rate should be established by this time.

The fiber autoradiography technique yielded a higher estimate of rate of fork movement than the equilibrium centrifugation method. This has also been found in other studies in which the 2 methods have been compared [16,26]. The most probable explanation for this is the opposite effects that thymidine triphosphate pool turnover has on the calculations. With fiber autoradiography, the transition from high to low specific activity of the radioactive thymidine results in transition from heavy to light density tracks. The longer the pool turnover, the more gradual the transition and the more likely the length of the heavy track will be overestimated. With the equilibrium centrifugation method, intermixing of [^{3}H]thymidine and BrdUrd in the pool during the switch from one label to the other results in interspersion of the 2 in the DNA. The calculation of rate depends on the fraction of DNA with density and radioactive label in end-to-end association. Heavy DNA from interspersion of the labels rather than end-to-end association will lower the calculated apparent rate, since rate is inversely proportional to the fraction of heavy DNA. Painter and Schaefer[19] describe a procedure that corrects for the interspersion, but this correction is incomplete when the rate of fork movement is low as in the

present experiments. The true rate of replication fork movement probably lies somewhere between the values estimated by these 2 methods.

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5. CONCLUSIONS

These studies have shown that the reversal of the sequence of density and radioactive labeling period can yield the required sharp transition needed for accurate measurement of the rate of DNA chain growth by the hydrodymnamic method.

This modified hydrodynamic method was then used to measure the rate of chain growth in serum induced CV-1 cells. The rate of DNA chain growth, the frequency of initiation events, the size of replication units, the synchrony of initiation and the incidence of bidirectional replication were measured using DNA fiber autoradiography. These parameters were examined through the S phase in cells synchronized by the addition of serum to serum-depleted cultures. There is a relative decrease in the frequency of initiation events seen in mid S phase cells when contrasted to early and late S phase cells. The other parameters of DNA replication do not change during the serum induced S phase.