*Proc. Natl. Acad. Sci. USA*
Vol. 75, No. 12, pp. 6088–6092, December 1978
Cell Biology

# Actinomycin D inhibits initiation of DNA replication in mammalian cells

(replication origins/fluorodeoxyuridine/nascent double-stranded fragments)

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*Contributed by J. Herbert Taylor, September 18, 1978*

**ABSTRACT** **Mammalian (Chinese hamster ovary) cells were synchronized in the division cycle and blocked at the beginning of S phase with fluorodeoxyuridine. Traces of thymidine in the medium allowed cells to enter S phase and initiate DNA replication at some origins. For many hours after the traces of thymidine were depleted new sites for DNA replication accumulated in a small fraction of the DNA. However, these potential origins became active in bidirectional replication only when cells were released by adding [$^3$H]thymidine to the medium. Lysis at 37°C released most of the pulse-labeled DNA as linear double-stranded segments larger than Okazaki fragments and smaller than the unreplicated parental DNA. Release of the newly replicated DNA involves breakage of the template chains at or near each fork. The size of the fragments increased linearly with time of pulse labeling, but the efficiency of their release decreased. The excision could be prevented by lysis at 0°C. When cells were treated with actinomycin D for 3–5 min before release, the new origins failed to function, but chain growth continued from those sites at which initiation had taken place before depletion of thymidine. We interpret these results to indicate that initiation at origins requires an actinomycin D-sensitive step, presumably RNA transcription, while chain elongation, which involves the formation of Okazaki pieces, is relatively insensitive to actinomycin D during growth over long intervals.**

Some years ago it became evident that nascent DNA was more readily excised from chromosomes during cell lysis than were unreplicated parental segments (1–3). The excised segments are double-stranded parts of the newly replicated chromosomal regions and are distinctly larger than the more extensively studied and smaller single-stranded fragments (Okazaki pieces). As originally noted, it appears likely that the excision is effected by nucleases associated with the DNA at or near the growing forks in replication (1). Some more recent studies on the conditions of lysis that enhance or prevent release lend further support to the idea that the release is enzymatic (4). The best way yet known to reduce excision is to lyse the cells at 0°C instead of 37°C (5). This change in temperature has very little effect on the size of the unreplicated parental DNA found after lysis, but has a striking effect on the amount of the nascent DNA that is excised from the chromosomes—i.e., the number of breaks introduced into the template chains. High salt concentrations and denaturing agents for proteins, such as high concentrations of urea in the lysing solutions, reduced the breakage and excision, but apparently did not reach the sites of enzymatic action quickly enough to be completely effective (4). The enzymes involved are unknown, but one or more of the various nicking-closing activities named DNA topoisomerases by Wang and Liu (6) are presumably involved.

In the early reports on these excised segments we had the impression that the size did not change very much with pulse time after blocking with fluorodeoxyuridine (FdUrd), but as shown here and previously (3) it soon became evident that the fragment size increases as the chains grow and the "eye" loops extend from replication origins. This observation would indicate that excision occurs by breakage at or near the growing fork, in spite of the earlier suggestion that the fragments might represent forks (1). Some forks are found among the fragments (5), but most of the fragments are linear, double-stranded pieces with very little evidence that many of the single-stranded extensions at the ends are long enough to be seen regularly in electron micrographs of the surface spread DNA.

In the present report we present further evidence that: (*i*) the nascent double-stranded fragments released during cell lysis increase in size at approximately a linear growth rate when highly synchronized cells blocked at the beginning of S phase are released by a pulse label with [$^3$H]thymidine ([$^3$H]dThd); (*ii*) the temperature during lysis affects the efficiency of fragment release and the production of nicks in the newly replicated chains of released fragments; and (*iii*) actinomycin D inhibits or delays the initiation from potential origins otherwise ready to begin replication, but allows DNA chain elongation from origins at which initiation has already taken place.

## MATERIALS AND METHODS

**Cell Cultures.** Chinese hamster ovary (CHO) cells, provided to us by L. R. Gurley of the Los Alamos Scientific Laboratory, have been maintained in our laboratory at 37°C in supplemented Ham's F-10 medium for several years (7). To obtain a parasynchronous culture, CHO cells in a subconfluent roller vessel were deprived of the essential amino acids isoleucine and glutamine for 24 hr. Approximately 17 hr after restoration of these amino acids, mitotic cells were collected for 1–2 hr by increasing the rate of roller vessel rotation to prevent settling and reattachment of dividing cells (7, 8). Usually about $10^6$ recently divided cells were distributed to each 25-cm$^2$ polystyrene flask in an experiment and allowed to settle and reattach for 1–2 hr. Total time of collection and attachment to flasks was always 3 hr. At 3 hr after mitosis, cultures were rinsed twice with isotonic Tris [tris(hydroxymethyl)aminomethane] buffer and overlaid with Ham's medium containing 10 $\mu$M FdUrd and no thymidine. Without thymidine supplied by the medium and with synthesis *de novo* inhibited by FdUrd, cells accumulate at the G1/S interface with very limited synthesis of DNA for a short time, followed by what appears to be a complete block of synthesis for many hours (3).

**Pulse Labeling and Lysis.** Cultures were incubated in the presence of 10 $\mu$M FdUrd for 7 hr prior to release by supplying [$^3$H]dThd in fresh Ham's medium (25 $\mu$Ci/ml; 50 Ci/mmol) as a pulse label for various times (1 Ci = $3.7 \times 10^{10}$ becquerels). Pulse labeling of cells in culture was terminated by rinsing with

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either isotonic Tris buffer or Ham's medium and lysing the cells at 0° or 37°C, usually in 2 ml of a nondenaturing solution containing 0.1 M $NaHCO_3$, 0.05 M $Na_2$ EDTA, and 0.3% Sarkosyl (ICN-K & K Laboratories), pH 10.8 (standard lysing solution). Cellular dissolution was always rapid as observed in the light microscope.

**Velocity Sedimentation.** Self-generating NaI velocity sedimentation gradients (9) were used to make estimates of molecular weight and to determine the efficiency of liberation of the nascent DNA fragments. Centrifugation was performed at 20°C in a Beckman L2-65B preparative ultracentrifuge in the SW 41 rotor at a speed of 20,000 rpm. Fractions of 0.27 ml were collected from the gradients and precipitated by mixing with a solution containing 5% trichloroacetic acid, 0.01% bovine serum albumin, 0.01% calf thymus DNA, and 0.02% sodium azide on the surface of a glass fiber filter, Type A-E (Gelman Instrument Co.). Filters were subsequently washed with 0.1 M sodium bisulphite to prevent discoloration by the oxidation of NaI, and water was removed by successive washes of 70% and 95% (vol/vol) ethanol. Filters were then oven-dried and radioactivity was determined by methods previously described (7).

**Treatment with Actinomycin D.** The antibiotic actinomycin D was applied to cell cultures in Ham's medium at a concentration of 5 μg/ml by using one of two treatment methods. After the double synchronization procedure described above, 5 μg of actinomycin D per ml was applied to cells while they were in medium containing FdUrd, for various times immediately prior to pulse labeling with [$^3$H]dThd. The other method of treating CHO cells with actinomycin D consisted of a similar cell synchronization procedure followed by a 2-min pulse label with [$^3$H]dThd. After 2 min the labeled medium was quickly exchanged for medium containing [$^3$H]dThd and 5 μg of actinomycin D per ml, and DNA replication was allowed to continue for 0, 2, 6, 10, 14, or 22 min. In both methods, pulse labeling was terminated after a quick rinse with isotonic buffer by cell lysis at 37°C using our standard lysing solution.

## RESULTS

**Double-Stranded DNA Fragments Excised from Chromosomes during Lysis of Cells Synchronized at Beginning of S Phase Result from Cleavage at or near Replication Forks.** Replicate cultures of CHO cells were prepared from cells collected at division and blocked until 10 hr from division (7 hr in 10 μM FdUrd). Individual flasks of cells were then released from the block by the addition of [$^3$H]dThd for 2, 4, 8, 12, 16, or 24 min, rinsed rapidly with Tris buffer, and then lysed in our standard lysing solution. Fig. 1 demonstrates the NaI gradient profiles of DNA from lysates prepared in this manner. Three distinct size classes of DNA can be seen banded in the gradients. Examination of the entire series reveals that the smaller classes of DNA, corresponding in size to the familiar Okazaki pieces, remain relatively constant regardless of pulse length. It is possible, however, that some of the labeled material seen near the top of these gradients may be the result of some nonspecific association of [$^3$H]dThd with proteins, including those liberated from the cellular membranes. The amount of labeled material banding in this region is somewhat dependent on the specific activity of the [$^3$H]dThd in the labeling medium and the number and method of rinses prior to cell lysis. The amount of labeled material does, therefore, remain fairly constant in any one experimental series but may vary from one experimental series to another. The largest particles of DNA in these lysates band near the botton of the gradient and represent what we call parental-sized DNA (greater than 300 × $10^6$ daltons). The parental-sized particles are for the most part unlabeled and

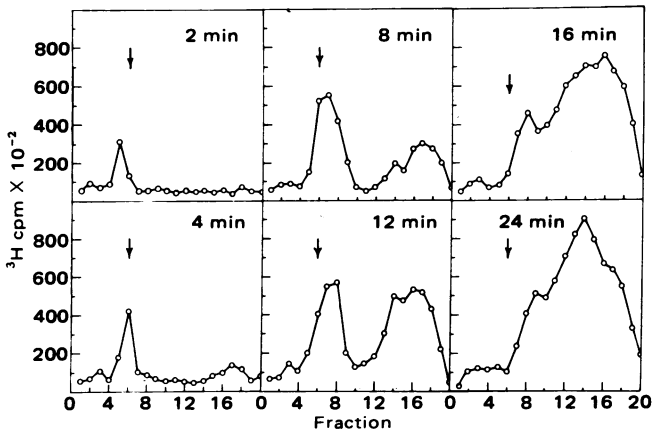


FIG. 1. Growth of nascent fragments with duration of pulse label. CHO cells were blocked for 10 hr after mitosis and released by pulse labeling with [$^3$H]dThd for 2, 4, 8, 12, 16, or 24 min. Pulse labeling was terminated by lysing cells with our standard lysing solution, and lysates were layered on 3 M NaI gradients. Centrifugation was for 5 hr at 20,000 rpm ($\omega^2 t$ was $8 \times 10^{10}$ rad$^2$ sec$^{-1}$). The markers (arrows) represent the position of the peak fraction of a sheared $^{14}$C-labeled CHO DNA. Sedimentation is from left to right in all figures.

therefore not detected at the shortest pulse times (Fig. 1, 2-min pulse). While remaining relatively constant in size, these large particles showed a more or less linear increase in incorporated radioactive label from the 2-min to the 8-min pulse. At 12 min a rather large increase in label associated with this fraction may be seen and an increased rate of incorporation was maintained at longer pulses up to the 24-min one.

The intermediate-sized classes of DNA represent the nascent, double-stranded fragments released from the replication complex, presumably during cell lysis. The time course series illustrated in Fig. 1 demonstrates that, with increasing lengths of pulse labeling, the nascent fragments show a progressive increase in size. These results can be observed most easily by comparing the sedimentation of the fragments to the sedimentation of a marker DNA, which in these experiments is aliquants of a single sample of sheared [$^{14}$C]dThd-labeled CHO cell DNA prepared for this purpose and added to each tube. Pulse-labeling, times of 2 min through 24 min produced nascent fragments that increased in size at what appears to be a fairly linear rate of growth. Pulse times longer than 24 min were generally uniformative because the fragments no longer sedimented as a band discreet from the larger labeled parental-sized DNA. Along with the increase in fragment size there is, as previously mentioned, a concomitant increase in labeled DNA associated with the parental-sized particles, but it is apparent that the efficiency of nascent fragment release brought about by our standard cell lysis procedures is inversely proportional to the duration of the pulse label.

**Release of Nascent Fragments Is Temperature Dependent and Can Be Prevented by Lysis at 0°C.** When CHO cells were blocked until 10 hr after division with FdUrd, pulse-labeled for 4 min, and lysed at 37°C in standard lysing solution, the majority of radioactivity was associated with intermediate-sized DNA (nascent fragments) in NaI velocity gradients (Fig. 2*A*). A marked change occurred when CHO cells were handled exactly as above except that the rinse and lysis took place at 0°C. Sedimentation of the DNA from these lysates as double strands at pH 9.5 is illustrated in Fig. 2*C*. As expected on the basis of previous studies, the liberation of the nascent DNA from the large parental DNA was almost toally inhibited by rinsing and lysing the cells at 0°C. The DNA from these lysates was also sedimented through NaI gradients as single strands at pH 11.5 (Fig. 2*D*). In the gradient profile of the 0°C lysate under de-

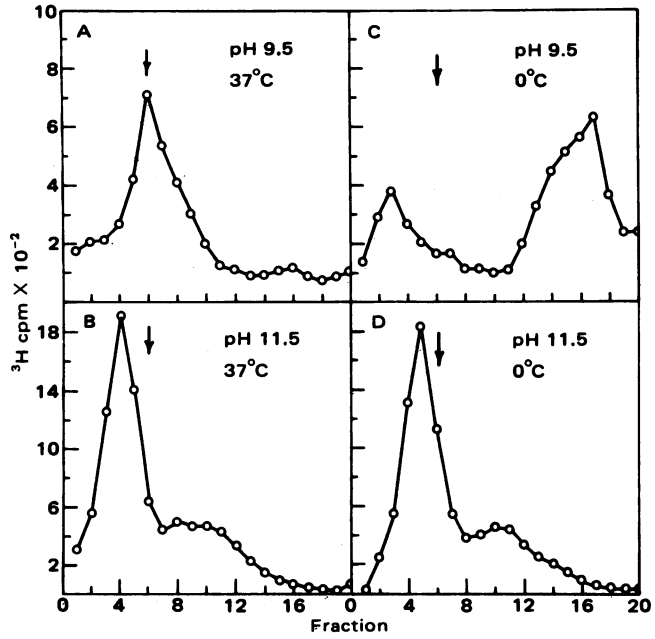


FIG. 2. Standard lysis of CHO cells at 37°C and 0°C. CHO cells were blocked with 10 μM FdUrd for 10 hr after mitosis and pulse-labeled 4 min with [$^3$H]dThd. Pulse labeling was terminated by rinsing cells with isotonic Tris buffer and lysing with standard solution (pH 10.8) at either 37°C or 0°C. Lysates were analyzed by centrifuging for 5 hr at 20,000 rpm ($\omega^2 t$ was $8 \times 10^{10}$ rad$^2$ sec$^{-1}$) in either nondenaturing (pH 9.5) or denaturing (pH 11.5) gradients.

naturing conditions, the nascent DNA sedimented with a peak in fraction 5. However, when the nascent fragments, which were released from the replicating DNA by our standard cell lysis at 37°C, were centrifuged though similar alkaline NaI gradients, the labeled single strands sedimented with a peak in fraction 4 (Fig. 2 *B* and *D*). This one-fraction difference in sedimentation represents an approximate 2-fold difference in molecular weight of the newly synthesized single chains of DNA—i.e., from $3 \times 10^6$ to about $6 \times 10^6$ daltons. These results suggest that, during liberation of the nascent DNA as intermediate-sized fragments, breaks are introduced near the middle of the newly synthesized DNA strand simultaneously with the introduction of the breaks in the template strand and perhaps the new chain near each replicating fork. Assuming that most CHO cell DNA replication is bidirectional, the middle of the newly made DNA strand corresponds to the center of the replicon in which it is synthesized. The breaks in the nascent strands, therefore, may be localized near the site of origin for DNA replication.

**Initiation from Potential Origins Otherwise Ready for DNA Replication is Inhibited in Presence of Actinomycin D.** Actinomycin D is a highly toxic chromopeptide antibiotic that binds to DNA and thereby interferes with functions such as RNA transcription. Strong binding occurs by intercalation of the chromophore moiety into the base pairs of double-helical DNA but specifically does not occur with double-helical RNA (10, 11). DNA-directed RNA synthesis is approximately 100 times more sensitive to actinomycin D binding than the replication of the DNA itself. Erickson (12) observed that, when CHO cells were blocked with FdUrd and treated 15 min prior to pulse labeling with 5 μg of actinomycin D per ml, DNA replication was reduced to about 15% in short pulse times as measured by incorporation of labeled dThd. By using essentially the same procedure, replicate cultures of CHO cells were blocked until 10 hr after mitosis, treated for 15 min with 5 μg of actinomycin D per ml, and pulsed for 2, 4, 8, 12, 16, or 24 min. Pulse labeling was terminated by our standard lysis, and lysates were layered on 3 M NaI gradients for analysis. Fig. 3 illustrates the results of such an experiment. As expected for short pulse times, very small amounts of labeled DNA are detectable. In fact, virtually no nascent fragments can be seen for any pulse labeling time up to 24 min. For example, in sedimentation of lysates from 4-min pulses, nascent fragments should be coincident with the marker DNA. No such peak is visible. Examination of the entire series reveals a progressive increase in the [$^3$H]dThd incorporated into parental-sized DNA as the pulse time was increased. This increase in labeled parental DNA was somewhat surprising, because the absence of nascent fragments in the lysates indicated that there were very few newly activated replicons. It appeared that either DNA replication was being initiated at new sites and was proceeding without release of nascent fragments at lysis or the replication observed was not associated with sites at which synthesis had been initiated at the beginning of the pulse. Therefore, an experiment was designed to test the ability of actinomycin D to stop *de novo* initiation of DNA replication and to determine what time period is required for actinomycin D to enter CHO cells and exert an effect.

Replicate cultures of CHO cells were again prepared from cells collected at division and blocked until 10 hr after division with FdUrd. The cells were then treated with 5 μg of actinomycin D per ml for 15, 10, 5, 3, 1, or 0 min prior to pulse labeling for 4 min. Cells were lysed in standard lysing solution and lysates were analyzed by NaI sedimentation as shown in Fig. 4. For actinomycin D pretreatment times of 10 or 15 min, there was no detectable peak of intermediate-sized nascent DNA fragments. This result we interpret as complete inhibition of active origins for replication. A 5-min pretreatment with actinomycin D is the shortest of the intervals in which actinomycin D entered the CHO cells and essentially inhibited the initiation at new origins. The experiment indicates to us that the nascent DNA fragments produced during lysis provide a good indication of the number of new origins for DNA replication which are functioning and that such initiation events are effectively blocked by actinomycin D.

We were, however, still uncertain about the origin of the labeled DNA which was seen in spite of a 15-min pretreatment

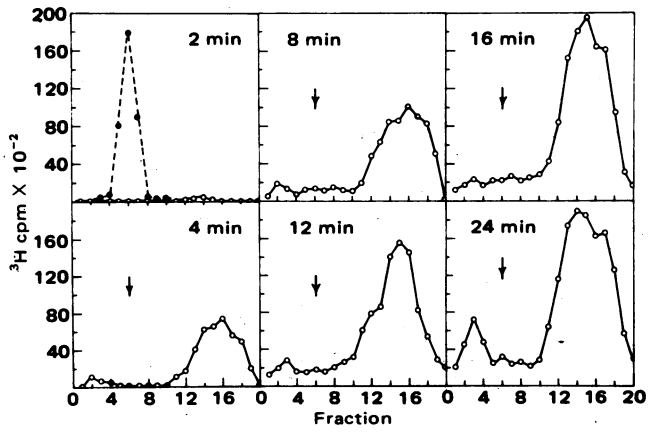


FIG. 3. Effect of 15-min treatment with actinomycin D prior to pulse labeling of CHO cells. Cells were blocked for 10 hr after mitosis with 10 μM FdUrd followed by addition of 5 μg of actinomycin D per ml for 15 min. Cells in the presence of actinomycin D were pulse labeled for 2, 4, 8, 12, 16, or 24 min with [$^3$H]dThd and lysed in our standard lysing solution. Lysates were layered on 3 M NaI gradients and centrifuged for 5 hr at 20,000 rpm ($\omega^2 t = 8 \times 10^{10}$ rad$^2$ sec$^{-1}$). The marker DNA ([$^{14}$C]dThd-labeled) is shown in the 2-min frame by ●s and by arrows in all other figures; pulse-labeled DNA is shown by Os.

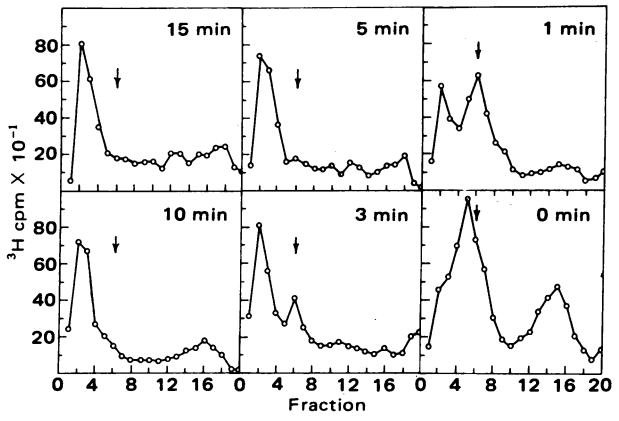


FIG. 4. Variations in time of actinomycin D treatment of CHO cells prior to pulse labeling. CHO cells were blocked for 10 hr after mitosis with 10 μM FdUrd and subsequently treated for 15, 10, 5, 3, 1, or 0 min prior to pulse labeling. Pulse labeling was with [$^3$H]dThd for 4 min followed by cell lysis in standard lysing buffer. Lysates were layered on 3 M NaI gradients and centrifuged 5 hr at 20,000 rpm ($\omega^2 t = 8 \times 10^{10}$ rad$^2$ sec$^{-1}$).

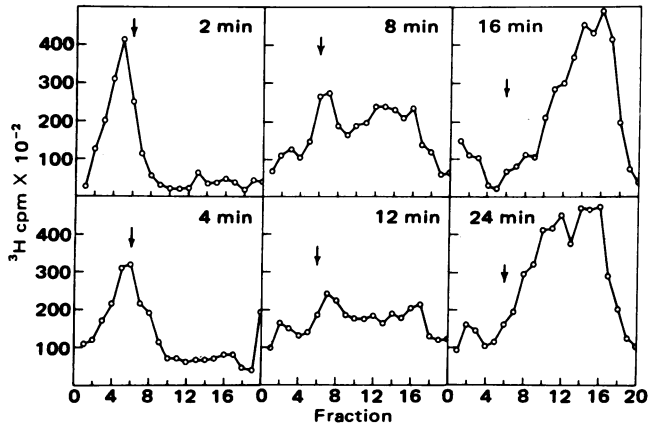


FIG. 5. Growth of nascent DNA fragments in the presence of actinomycin D. CHO cells were blocked for 10 hr after mitosis with 10 μM FdUrd. Release from the block was by [$^3$H]dThd for 2 min immediately followed by the addition of 5 μg of actinomycin D per ml. Pulse labeling was allowed to continue in the presence of actinomycin D for 0, 2, 6, 10, 14, or 22 min and terminated by the addition of our standard lysing solution. Lysates were analyzed on NaI gradients ($\omega^2 t = 8 \times 10^{10}$ rad$^2$ sec$^{-1}$).

with 5 μg of actinomycin D per ml (Fig. 3). An experiment was, therefore, designed to determine if the incorporation of [$^3$H]-dThd in the presence of the antibiotic was the result of the elongation of DNA chains from origins already established at pulse time or possibly the result of some other type of DNA synthesis such as repair replication. Replicate cultures of CHO cells were collected, blocked in FdUrd until 10 hr, and pulse-labeled for 2 min. After the 2-min pulse time, the labeled medium was quickly exchanged for conditioned medium containing both [$^3$H]dThd and 5 μg of actinomycin D per ml. Replication was allowed to continue for 0, 2, 10, 14, or 22 min. Cells were lysed at the proper time by the addition of standard lysing buffer and lysates were analyzed on NaI gradients. This experiment was designed to allow initiation of DNA replication at potential origins (otherwise prepared during the block but without thymidylate for chain growth) before the actinomycin D could affect them. However, most of the newly made chain extensions would have to occur in the presence of the drug. The pulse times with and without actinomycin D were equivalent to the pulse-labeling times employed in the measurements shown in Fig. 1. The estimates of molecular weights of the nascent DNA fragments synthesized in the presence of actinomycin D and liberated by standard cell lysis should be a fairly accurate measure of elongation rates occurring in spite of the antibiotic binding to the DNA. Fig. 5 illustrates the results of this experiment. The nascent fragments, representing new starts of replication, are clearly visable for all pulse times except the 24-min total treatment time. The fragments can be seen to become progressively larger with increasing duration of pulses. The parental-sized DNA again shows increasing amounts of incorporated label with longer pulse times. The results indicate that elongation of nascent chains that can be released during lysis occurs more or less normally even in the presence of 5 μg of actinomycin D per ml, if initiation is allowed to proceed before the actinomycin D has produced its effects.

## DISCUSSION

The data presented here, along with previous reports, indicate that FdUrd is an effective inhibitor of DNA replication *in vivo*. Replication stops because of the lack of thymidine triphosphate (dTTP). Because thymidine cannot be completely excluded from the complex medium used for growing mammalian cells in culture, a small amount of DNA replication occurs, which soon depletes all usable thymidine. In cells released from a $G_0$ block in the presence of FdUrd, S phase is reached and chain growth is initiated from many potential origins in early replicating DNA (13), but as soon as the residual thymidine is depleted, initiation at new origins stops. The use of dialyzed serum, because serum is the principal source of residual thymidine in the medium, reduced the number of initiations.

In the absence of thymidine, potential origins in a small fraction of the DNA continue to be prepared for initiation (13) at nearly a linear rate for many hours (3). As soon as thymidine is available, provided by a pulse label for example, chain growth begins from origins where synthesis has already been initiated as well as those potential origins that have accumulated during the block with FdUrd. Our interpretation of the data is that in the presence of actinomycin D (given at least 5 min before the pulse) such potential origins, which would otherwise support initiation, fail to do so. Only those origins at which chain growth has been initiated before the pulse continue replication. In addition, only those chains at the newly initiated origins are excised efficiently by the nucleases at lysis. Therefore, we obtain very few, if any, double-stranded segments even when the lysis is carried out at 37°C in actinomycin D-treated cells. There is, however, a significant amount of thymidine incorporated into DNA that remains attached to the larger fragments of chromosomal DNA in lysates. Because the amount increases nearly linearly with time, and otherwise behaves like newly replicated DNA, it is unlikely that a significant part of the incorporation is due to repair synthesis. The conclusion is that actinomycin D effectively inhibits initiation at origins, but has little effect on chain growth over thousands of nucleotide pairs if initiation has already occurred. The simplest explanation is that an RNA transcript is required for initiation in mammalian cells, but that whatever process causes initiation of Okazaki pieces is relatively insensitive to actinomycin D. Another conclusion is that the transcript or primer for initiation is unstable or cannot form without dTTP. Otherwise origins would be prepared for chain growth during the FdUrd block. This process does not appear to occur. There is also evidence that an analog of thymidine, such as bromodeoxyuridine (BrdUrd), either delays (7) or effectively prevents initiation when it is the only substitute for thymidine available. Mixtures of thymidine and BrdUrd are, however, increasingly effective as the relative concentration of thymidine increases (T. J. Laughlin and J. H. Taylor, unpublished observations).

The release of the excised segments of newly replicated DNA is best observed after a rather long block of DNA replication induced by FdUrd. The highest percentages of labeled DNA released can be obtained during the first 10–12 min of a pulse-labeling period in highly synchronized cells held at the beginning of S phase. However, we have observed the release in unsynchronized cells after a 5-min FdUrd block. In such cells the percentage released is smaller than the almost complete release obtainable in the highly synchronized, FdUrd-treated cells. The percentage released decreased nonlinearly with the pulse time from about 70% in 2 min to only 5–10% after 30 min (5). Efficient release requires rapid lysis—i.e., high ratios of lysing solution to cell number and a quick rinse with isotonic Tris buffer to remove medium before lysis of cells attached to a substrate. Once cells are lysed and the DNA is in solution, excision does not occur even if nucleases are not completely inhibited. Excision appears to occur at the moment of rapid lysis of the cell.

The work reported was supported in part by Contract AT-(40-1)-2690 with the Division of Biomedical and Environmental Research, Energy Research and Development Administration; by Contract EY-78-S-05-5854 with the Division of Biomedical and Environmental Research, Department of Energy; and by The Florida State University. During part of this time A.G. was a predoctoral trainee in genetics under Grant 5 T01 GM 01316-08, National Institutes of Health.

1. Taylor, J. H. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1083–1087.
2. Taylor, J. H., Wu, M., Erickson, L. C. & Kurek, M. P. (1975) *Chromosoma* **53,** 75–189.
3. Kurek, M. P. & Taylor, J. H. (1977) *Exp. Cell Res.* **104,** 7–14.
4. Guy, A. L. (1977) Dissertation (Florida State University, Tallahassee, FL).
5. Taylor, J. H., Adams, A. G. & Kurek, M. P. (1973) *Chromosoma* **41,** 361–384.
6. Wang, J. C. & Liu, L. F. (1978) *in Molecular Genetics,* ed. Taylor, J. H. (Academic, New York), Part 3, in press.
7. Taylor, J. H., Myers, T. L. & Cunningham, H. L. (1971) *In Vitro* **6,** 309–321.
8. Ley, K. D. & Tobey, R. A. (1970) *J. Cell Biol.* **47,** 453–459.
9. Hozier, J. & Taylor, J. H. (1975) *J. Mol. Biol.* **93,** 181–201.
10. Goldberg, I. H. & Friedman, P. A. (1971) *Annu. Rev. Biochem.* **40,** 775–810.
11. Bloomfield, V. A., Crothers, D. M. & Tinoco, I. (1974) *Physical Chemistry of Nucleic Acids* (Harper & Row, New York), pp. 461–464.
12. Erickson, L. C. (1974) Dissertation (Florida State University, Tallahassee, FL).
13. Taylor, J. H. (1977) *Chromosoma* **62,** 291–300.