

DNA REPLICATION IN MAMMALIAN CELLS

Altered Patterns of Initiation during Inhibition of Protein Synthesis

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ABSTRACT

The effects of inhibition of protein synthesis by the antibiotics cycloheximide and puromycin on the initiation of DNA replication in mouse L cells were studied. Cellular DNA was pulse labeled with [³H]thymidine of high, then of low specific activity and prepared for fiber autoradiography. Autoradiograms containing multiple (up to four) replication units were analyzed. In control cells, the proportion of replication units that initiated during a 10-min, high specific activity pulse was approximately equal to the proportion initiating immediately before the pulse. The addition of cycloheximide or puromycin at the start of the pulse inhibited the frequency of initiation in that there was a decrease by up to one-third of units initiating during the pulse relative to controls. Replication direction was also altered. Addition of the antibiotics 2 h before the pulse reduced the proportion of bidirectional units observed from 0.98 to 0.70. Antibiotic treatment for 2 h also decreased initiation synchrony in that the proportion of multiunit autoradiograms on which neighboring units showed similar replication patterns (indicating temporally coordinated initiation) was reduced by one-half. These observations indicate that inhibition of protein synthesis alters the normal pattern of DNA initiation.

DNA replication in eukaryotic cells occurs on multiple units arranged in tandem on the chromosome (1, 2, 16, 20, 24, 25) through a mechanism that involves initiation at the centers of individual units and subsequent bidirectional daughter chain elongation. Initiation and fork progression are the two processes determining the overall rate of DNA replication on a chromosome. The factors regulating these processes are poorly understood. Continued cellular protein synthesis is necessary to sustain replication (12, 13, 19, 21, 22, 27, 30, 31). Recent evidence indicates that inhibition of protein synthesis slows replication fork progression (6, 9, 29), although not all investigators have found this to be so (14). There is also conflicting evidence as

to whether initiation of replication is decreased (3, 4, 8, 14) or unaffected (5, 6, 29) when protein synthesis is cut off.

Initiation itself involves several factors that may be measured. These include the frequency of initiation events, the degree of synchrony of events on clusters of units (7, 10, 16, 17, 25), and whether an individual initiation event results in uni- or bidirectional replication (2, 9, 16–18, 28).

The present study investigates the effect of inhibition of protein synthesis on these factors. The technique of DNA fiber autoradiography was used to measure alterations in the normal initiation pattern produced by treatment of mammalian cells with cycloheximide or puromycin, two antibiotics

whose primary action appears to be inhibition of protein synthesis (reviewed in references 23 and 26).

MATERIALS AND METHODS

Cell Line

The continuous line of mouse fibroblasts, L-929, was used in this study. Numerous aliquots of cells had been stored in liquid nitrogen at two or three subculturings beyond that of the cells originally received in Dr. I. Tamm's laboratory at The Rockefeller University from Dr. E. Kilbourne. The cells, when recovered from liquid nitrogen, were maintained in monolayer cultures in Eagle's Minimal Essential Medium (MEM) (Grand Island Biological Co., Grand Island, N.Y.) containing 5% fetal calf serum (FCS), gentamicin 50 $\mu\text{g}/\text{ml}$, amphotericin B 0.25 $\mu\text{g}/\text{ml}$, and tylocine 60 $\mu\text{g}/\text{ml}$. Twice a week, confluent monolayers in 75-cm² plastic flasks (Falcon Plastics, Div. of B.-D. Laboratories, Los Angeles, Calif.) were diluted 10-fold and transferred to new culture flasks. The line was maintained for 30–40 such subculturings before being discarded and another aliquot was taken from liquid nitrogen storage and maintained for a similar number of subculturings. For individual experiments, cells were seeded into 60-mm Petri dishes (Falcon Plastics) at a density of 300,000 cells per dish in fresh medium identical to the maintenance medium, but without added tylocine. The experiments were performed 16–24 h after seeding, when the cells were in logarithmic growth. All experiments were performed in a walk-in warm room at 37°C.

Techniques of Pulse Labeling and DNA

Fiber Autoradiography

These procedures were essentially as described elsewhere (9, 10). [³H]Thymidine, 50–60 Ci/mmol, 1 mCi/ml, was supplied by New England Nuclear, Boston, Mass. At the appropriate time, the cell monolayers were treated with fluorodeoxyuridine (2×10^{-6} M) for 30 min to exhaust the endogenous thymidine nucleotide pools. The cells were then pulse labeled according to one of two protocols (Table I). In the first, [³H]thymidine was used at 50 Ci/mmol, 5×10^{-6} M for 10 min (the hot pulse), and then at 5 Ci/mmol, 5.5×10^{-6} M for 120 min (the warm pulse). In the second, the hot pulse was applied for 30 min and the warm pulse for 30 min. The amounts of radioactivity and the concentrations of thymidine were the same. With both protocols, fluorodeoxyuridine was left in the medium during the labeling period to increase the incorporation of the exogenous [³H]thymidine. At the conclusion of the pulse, the cells were washed, detached from the Petri dish, and processed for DNA fiber autoradiography. This involved gentle lysis and spreading of the released DNA on glass microscope slides before coating with nuclear track emulsion. Up to 10

separate slides were prepared from each sample cell monolayer.

Scoring of Autoradiograms

The preparations were examined by light microscopy. Microscope slides were scanned horizontally at 1-mm intervals for appropriate autoradiograms. Those autoradiograms containing two to four replication units were identified at a magnification of 125–200 and no more than one per microscope field or 25 per microscope slide was chosen. This increased the chance of the autoradiograms coming from a large number of different cells and the results being representative of all S-phase cells. The autoradiograms were then scored for the number and types of replication units they contained (pre- and postpulse or uni- and bidirectional, see Results) for determinations of frequency and synchrony of initiation and direction of replication. In individual experiments, slides were prepared from two replicate cultures for each control and experimental point.

RESULTS

The Inhibition of Leucine and Thymidine

Uptake by Cycloheximide or Puromycin in L Cells

As a preliminary experiment, the inhibition of incorporation of [³H]leucine and [³H]thymidine into L cells by cycloheximide or puromycin under the conditions of culture used was determined.

Logarithmically growing L cells were treated with either cycloheximide at 50 $\mu\text{g}/\text{ml}$ (1.8×10^{-4} M) or puromycin at 200 $\mu\text{g}/\text{ml}$ (4.2×10^{-4} M). The incorporation of [³H]leucine and [³H]thymidine was determined in replicate cultures at various times after addition of either antibiotic. The results are shown in Fig. 1.

Both cycloheximide and puromycin inhibit 90% of the uptake of [³H]leucine into acid-precipitable material immediately after addition. The incorporation of [³H]thymidine into acid-precipitable material is also inhibited by both antibiotics, although the inhibition occurs somewhat more slowly and is not maximal until 15 min. The time course of inhibition is similar to that obtained with the same cell line under slightly different culture conditions (3, 9), but the degree of inhibition is greater than seen previously (9).

[³H]Thymidine incorporation may not be an accurate indicator of the level of DNA synthesis (reviewed in reference 11). However, both cycloheximide and puromycin do produce inhibition of DNA replication (5, 6, 29). The characteristics of this were analyzed in subsequent experiments.

TABLE I
Labeling Protocols for Initiation Experiments

Experiment	[³ H]Thymidine pulse*		Time of addition of antibiotics†	[³ H]Thymidine incorporation % of control‡	
	Hot	Warm		Cycloheximide treated	Puromycin treated
	<i>min</i>	<i>min</i>	<i>min</i>		
1	10	120	0	40.4	26.0
2	10	120	0	44.2	20.1
3	30	30	-120	27.4	19.4
4	30	30	-120	20.4	10.3

* Monolayers of L-929 cells were labeled with [³H]thymidine as described in Materials and Methods for the lengths of time indicated.

† Antibiotics, either cycloheximide (1.8×10^{-4} M) or puromycin (4.2×10^{-4} M), were added to monolayers at the indicated times in relation to the beginning of the hot pulse.

‡ Determined in replicate monolayers pulse labeled with [³H]thymidine (0.5 μ Ci/ml) for the same length of time as the hot pulse. Acid-precipitable radioactivity was measured by liquid scintillation counting.

The same doses were used, and the resulting modification in the pattern of DNA replication was analyzed by DNA fiber autoradiography. A summary of the antibiotic treatment protocols used in these experiments and the inhibition of [³H]thymidine incorporation in treated cells is shown in Table I.

Replication Patterns in Control Cells

Cellular DNA was labeled *in vivo* with [³H]thymidine as indicated in Materials and Methods and the cells were processed for fiber autoradiography. The autoradiograms showed linear tracks of silver grains of high or low density, reflecting DNA replication during the hot or the warm pulse, respectively. Replication of individual units (16) produced patterns of three types. Units which began replication before the hot pulse showed a short clear area (presumably indicating DNA replicated before the beginning of the pulse) flanked by two linear grain tracks of heavy density proceeding directly to tracks of lighter density (Fig. 2 *a*). These are prepulse bidirectional initiation units; the high-density tracks representing DNA replicated during the hot pulse, and the low-density tracks representing that replicated during the warm pulse. Units which began replication after the pulse showed a central track of high grain density, flanked on either end by tracks of lower grain density (Fig. 2 *b*). These are postpulse bidirectional initiation units. Less frequently, units in which replication was clearly unidirectional

were observed (Fig. 2 *c*). In these, it could not be determined whether initiation took place before or after the beginning of the hot pulse.

These replicating units were arrayed in clusters (7, 14, 16). In control cells, these clusters most frequently exhibited like units, all prepulse (Fig. 3 *a* and *d*) or all postpulse (Fig. 3 *b* and *e*), indicating synchrony of initiation (7, 10, 14, 15). The few unidirectional units seen were almost always observed singly in association with the more common bidirectional units (Fig. 3 *c*). A low frequency of unidirectional units in mammalian cells has been observed previously (9, 17). Whether these represent true unidirectional replication occurring as an alternative to the more common bidirectional mode is not known. They could be artifacts resulting from a chance linear association which occurred during preparation of the autoradiograph of a broken bidirectional unit on one fiber with an unbroken bidirectional unit on another fiber.

Replication Patterns in Antibiotic-Treated Cells

DNA from antibiotic-treated cells showed the same three basic patterns of replicating units. DNA from cycloheximide-treated cells (Fig. 4) showed replicating units organized in clusters. In general, hot-pulse track lengths were shorter even immediately after addition of the antibiotic (Fig. 4 *b*), reflecting a decreased rate of replication fork

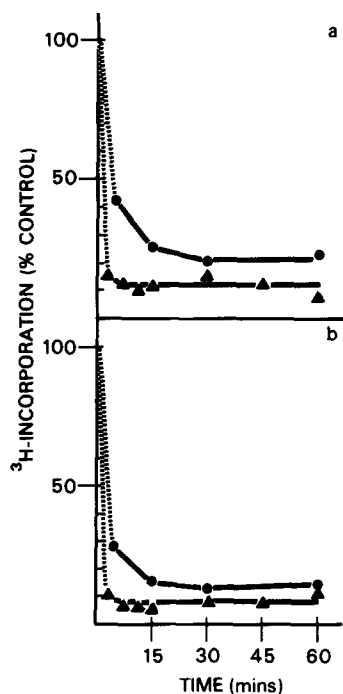


FIGURE 1 Inhibition of incorporation of [^3H]thymidine and [^3H]leucine into acid-precipitable material by cycloheximide or puromycin. Replicate 60-mm Petri dishes of monolayers of L cells in logarithmic growth received antibiotic as a 100 times-concentrated solution in minimal essential medium (MEM) or MEM alone at time 0. Those cultures to be labeled with [^3H]leucine had been in MEM containing 0.1 the normal amount of leucine for 30 min before time 0. At various times after addition of antibiotic, control and experimental cells were pulse labeled for 5 min with [^3H]thymidine ($0.5 \mu\text{Ci/ml}$) or [^3H]leucine ($1.0 \mu\text{Ci/ml}$). At the completion of the pulse, the monolayers were washed and scraped, the acid-precipitable material was collected after treatment with 5% trichloroacetic acid, and incorporation of isotope was determined by liquid scintillation counting. The plotted times represent the midpoint of each pulse. Each point represents the average of four replicate plates. Data are plotted as the percent of counts incorporated as compared to the concomitantly pulsed control. (a) Cycloheximide, 1.8×10^{-4} M. (b) Puromycin 4.2×10^{-4} M., ●, [^3H]thymidine incorporation; ▲, [^3H]leucine incorporation.

progression (5, 6, 9, 29). This was more evident when cycloheximide was added 2 h before the pulse. Under these conditions the warm-pulse grain tracks were less frequently continuous between adjacent hot-pulse tracks (Fig. 4 c-f). The autoradiograms from cells to which cycloheximide had been added at the time of the hot pulse more

often were clusters of prepulse units (Fig. 4 a), although some clusters of postpulse units were present (Fig. 4 b). When cycloheximide was added 2 h before the pulse, clusters frequently showed mixtures of pre- and postpulse units, i.e. they contained unlike units (Fig. 4 c, the three units indicated on the right) although clusters of like units were seen (Fig. 4 f). There was a higher incidence of unidirectional units, some of which appeared in tandem (Fig. 4 d).

DNA autoradiograms from puromycin-treated cells (Fig. 5) were similar to those from cycloheximide-treated cells. Clusters of prepulse units predominated when puromycin was added at the time of the pulse (Fig. 5 a). When puromycin was added 2 h before the pulse, clusters more frequently were composed of unlike units (Fig. 5 b), although those with like units were still evident (Fig. 5 f). Unidirectional units were also more frequent (Fig. 5 c) and occurred in tandem (Fig. 5 d, e).

During the initial examinations of autoradiograms from antibiotic-treated cells, it appeared as if the spatial intervals between initiation sites was reduced (Fig. 4 b, e, f; Fig. 5 c, f). However, in well-spread isolated fibers of replicating DNA containing multiple units (where the observer could be reasonably sure the units were on one fiber even if they were not linked by a warm grain track), the initiation intervals from antibiotic-treated cells were not significantly different in length from the controls. Initiation intervals much shorter than the mean occurred with reasonable frequency in control cells (10), and these shorter intervals were probably more frequently recognized and therefore selected in antibiotic-treated cells because of slowed fork progression. The use of multiple-unit autoradiograms minimizes many of the problems of selection and only such autoradiograms were used for the remainder of the analyses in this report.

Alterations in the Frequency of DNA Initiation Produced by Cycloheximide or Puromycin

The observation that there appeared to be fewer autoradiograms containing postpulse units from cells to which the antibiotics had been added at the time of the pulse suggested that cycloheximide and puromycin inhibited initiation of replication. The overall ratio of postpulse to prepulse units in a control preparation is close to unity (10). If an agent that inhibits initiation is added to cells at the

time of the hot pulse, it should reduce this ratio. Using this rationale, the effects of cycloheximide and puromycin on initiation of DNA replication were assessed in the autoradiographic slides from experiments 1 and 2 in Table I. Autoradiograms containing two to four bidirectional units were scored and the number of pre- and postpulse initiation units (Fig. 2 *a* and *b*) determined. The results (Table II *a*) showed fewer postpulse initiations in antibiotic-treated cells compared to controls. The degree of inhibition of initiation was determined by comparing the ratios of postpulse to prepulse units in control and treated cells. Both cycloheximide and puromycin appear to produce a modest but significant reduction in the frequency of DNA initiation events within 10 min of their addition to cells.

A similar analysis was carried out on slides from experiments 3 and 4 in Table I. This protocol utilized a 2-h pretreatment with antibiotics. Here it was felt that the proportion of postpulse units in antibiotic-treated cells should be similar to controls, since the antibiotic should inhibit initiation equally before and after the start of the pulse. The data (Table II *b*) support this, in that there is no significant difference in the proportion of postpulse units between control DNA and DNA from cells pretreated for 2 h with antibiotics. Having established this, I was able to use these same autoradiographic preparations from experiments 3 and 4 to investigate the direction of replication and the synchrony of initiation in antibiotic-treated cells; the advantage of this protocol being that the proportion of postpulse units was not a variable.

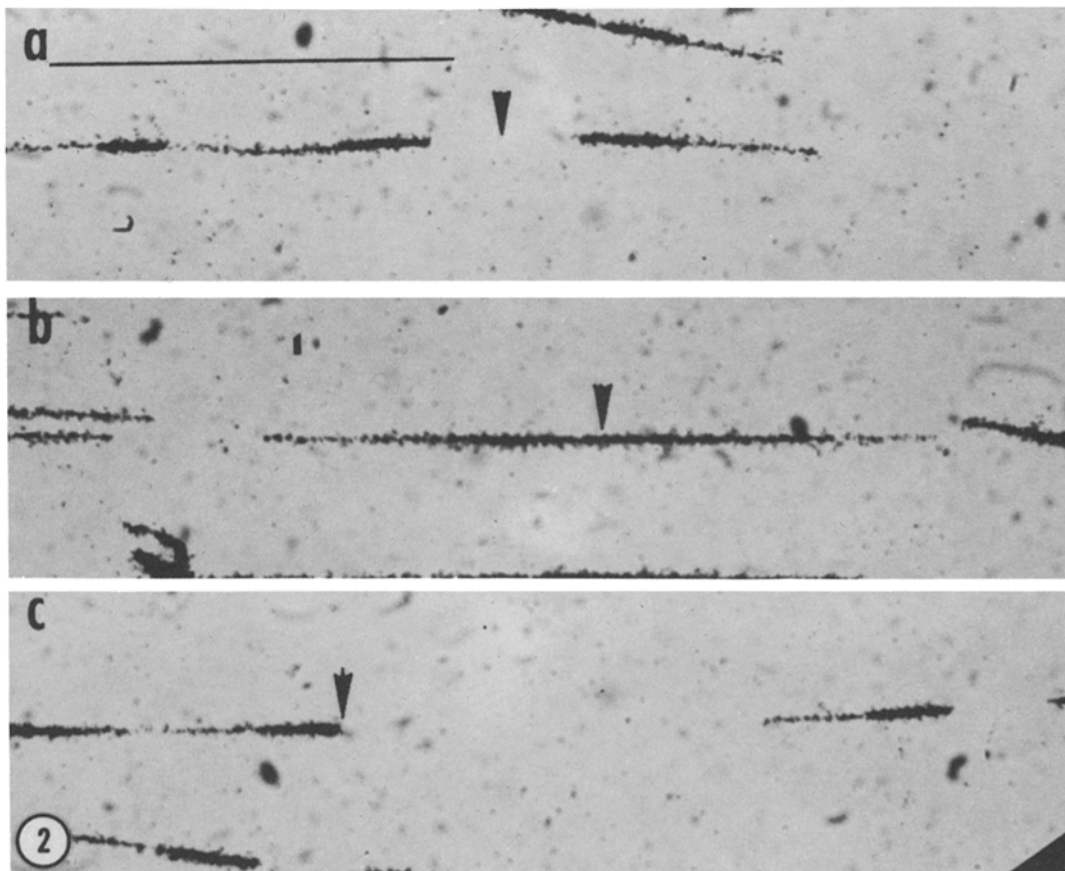


FIGURE 2 DNA autoradiograms of replication units. These are from DNA labeled with a 30-min hot followed by a 30-min warm pulse. The arrowheads indicate presumed initiation points. (a) Prepulse bidirectional initiation unit. (b) Postpulse bidirectional initiation unit. (c) Unit with unidirectional replication. The bar represents 50 μ m. All micrographs are at a magnification of 1,100.

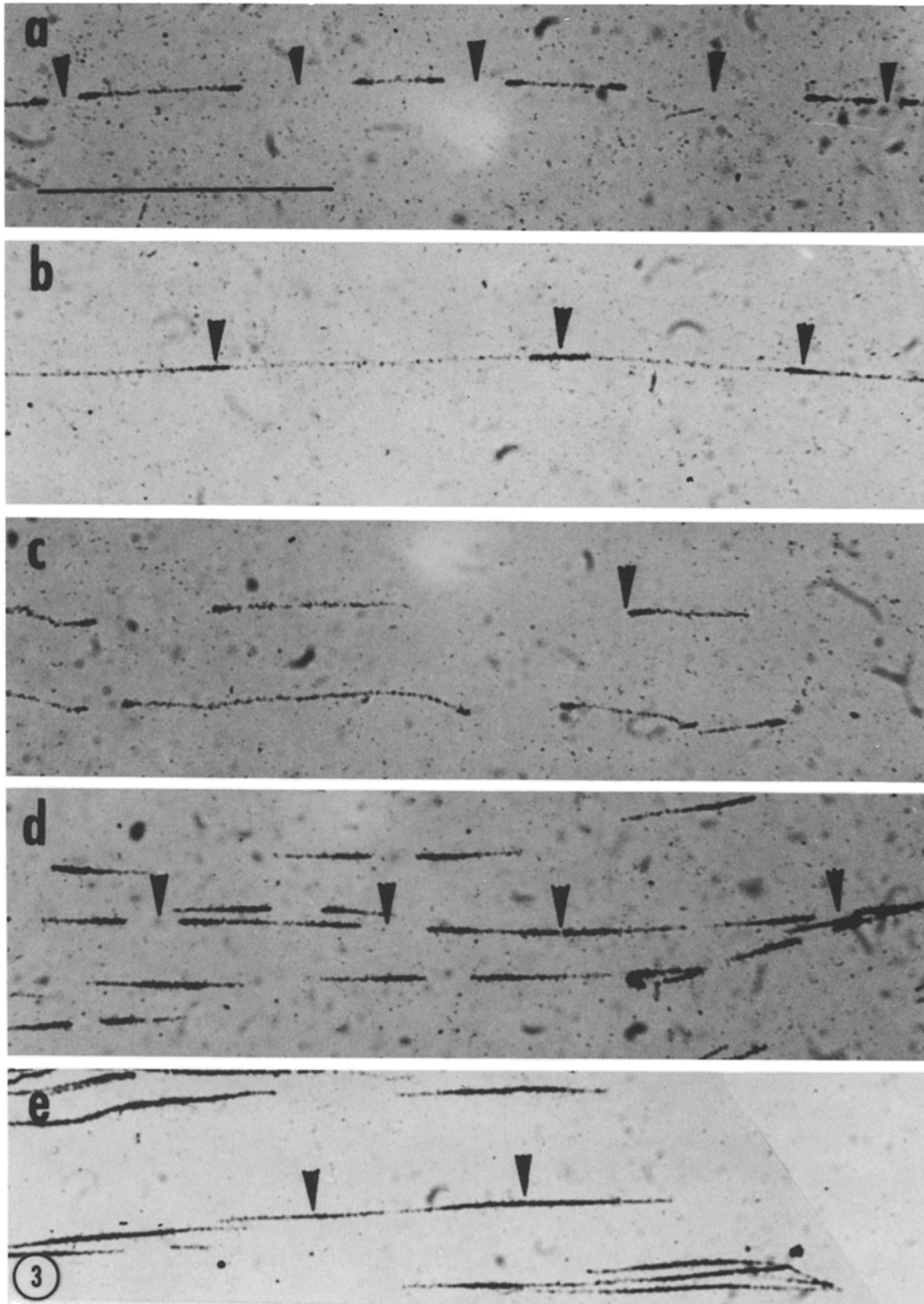


FIGURE 3 Multiple-unit DNA autoradiograms from control cells. In (a-c) the autoradiograms are from experiments 1 and 2 in Table I; (d) and (e) they are from experiments 3 and 4. The arrowheads indicate presumed initiation points. (a) Cluster of five prepulse units, 10-min hot pulse. (b) Cluster of three postpulse units, 10-min hot pulse. (c) Unidirectional unit within a replicating DNA fiber, 10-min hot pulse. (d) Cluster of four prepulse units, 30-min hot pulse. (e) Cluster of two postpulse units, 30-min hot pulse. The bar represents 100 μm . All micrographs are at a magnification of 450.

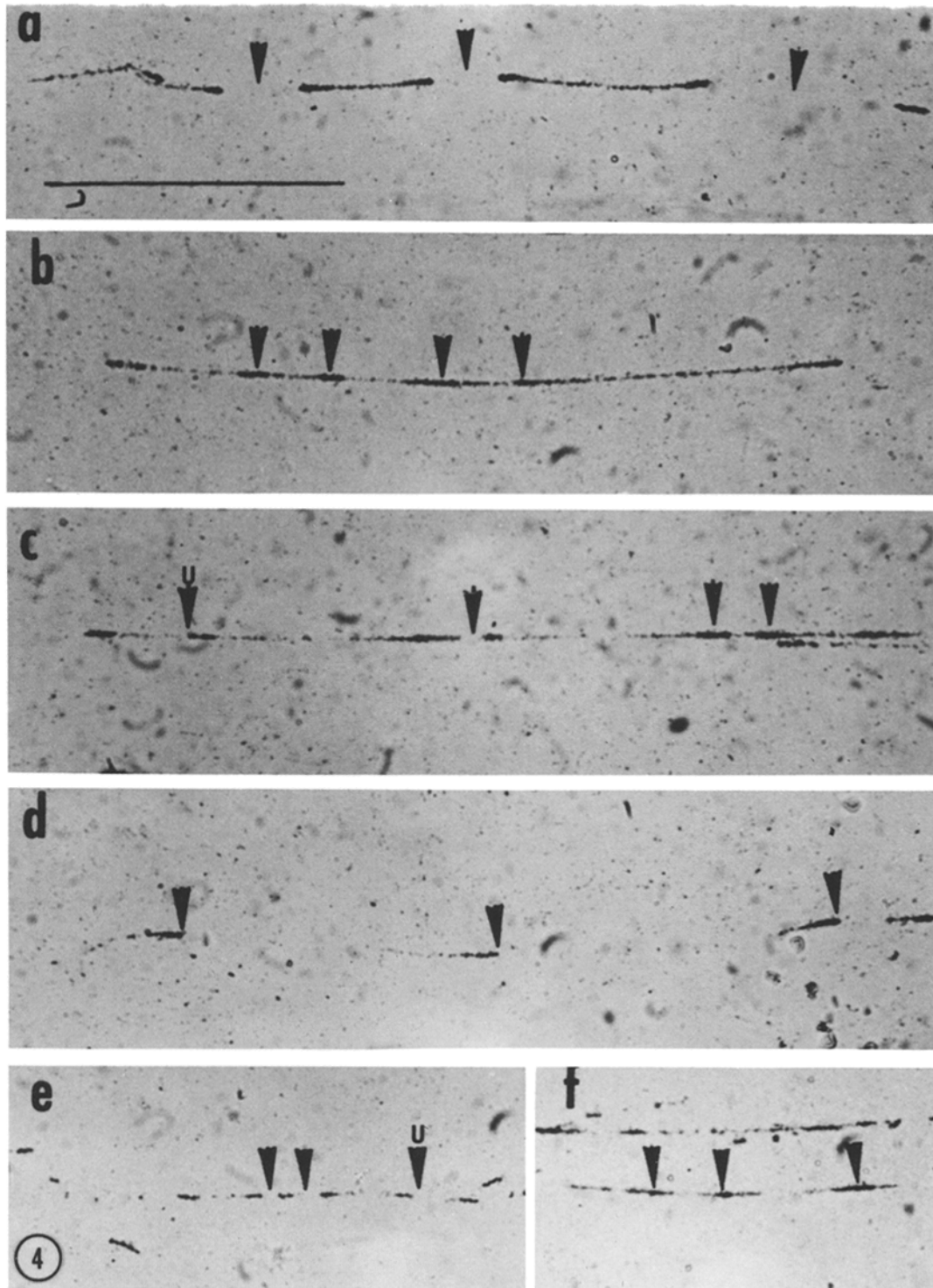
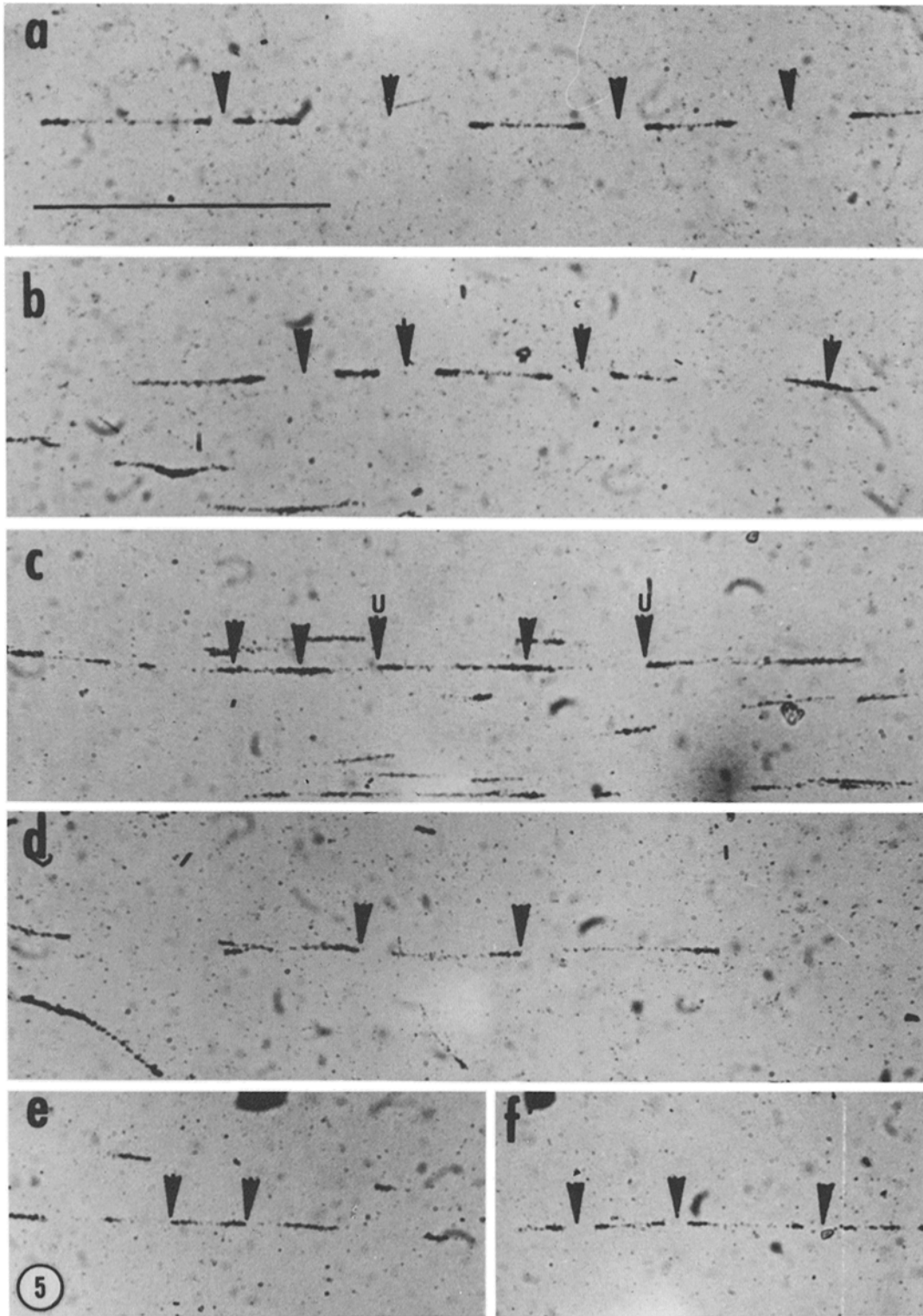


FIGURE 4 Multiunit autoradiograms from cycloheximide-treated cells. In (a) and (b) the autoradiograms are from experiments 1 and 2 in Table I; c–f, they are from experiments 3 and 4. The arrowheads indicate presumed initiation points. (a) Cluster of three prepulse units, cycloheximide added at the start of the 10-min hot pulse. (b) Cluster of four postpulse units, cycloheximide added at the start of the 10-min hot pulse. (c) Cluster of four units: from left to right, unidirectional (arrowhead *u*), prepulse bidirectional, and two postpulse bidirectional units, cycloheximide added 2 h before the start of a 30-min hot pulse. (d) Cluster of three unidirectional units, cycloheximide added 2 h before the start of a 30-min hot pulse. (e) Cluster of three units: from left to right, two bidirectional prepulse and a unidirectional unit (arrowhead *u*) cycloheximide added 2 h before the start of a 30-min hot pulse. (f) Cluster of three postpulse units, cycloheximide added 2 h before the start of a 30-min hot pulse. The bar represents 100 μm . All micrographs are at a magnification of 450.



Unidirectional vs. Bidirectional Fork Progression in Antibiotic-Treated Cells

Since the characteristic of uni- or bidirectional replication might be determined by factors governing initiation, the apparent decrease in bidirectional units in antibiotic-treated cells was also quantitated using the preparations from experiments 3 and 4 in Table I. Autoradiograms containing two to four units were scored and the number of units showing bidirectional as opposed to unidirectional replication was determined. This protocol, which used a 2-h pretreatment period with antibiotic, was chosen because there is no way of determining whether a unidirectional unit initiated before or after the beginning of the hot pulse. Almost all units in controls showed bidirectional replication. This proportion was decreased signifi-

cantly in the antibiotic-treated cells (Table III). Thus, bidirectional DNA replication appears to be decreased in cells in which protein synthesis is inhibited. Examples of autoradiograms with more than one unidirectional unit from antibiotic-treated cells are shown in Fig. 4 *d* and Fig. 5 *d, e*.

Changes in Synchrony of Initiation Produced by Inhibition of Protein Synthesis

The more frequent appearance in antibiotic-treated cells of clusters containing both pre- and postpulse units suggested that the normal degree of synchrony of initiation on clusters might be decreased by inhibition of protein synthesis.

Initiation of DNA replication is to an extent synchronized in control cells. Thus, adjacent units

TABLE II
Postpulse DNA Initiation in Antibiotic-Treated Cells*

Sample	Number of units scored	Postpulse units, proportion	Postpulse initiation† % of control
<i>(a)</i> No pretreatment with antibiotic, 10-min hot pulse			
Control	550	0.49	—
Cycloheximide-treated	550	0.39 ($P < 0.001$)§	66
Puromycin-treated	550	0.43 ($P < 0.05$)§	78
<i>(b)</i> 2-h pretreatment with antibiotic, 30-min hot pulse			
Control	550	0.49	—
Cycloheximide-treated	550	0.49 ($P > 0.25$)§	100
Puromycin-treated	550	0.50 ($P > 0.25$)§	100

* Antibiotics were added to cells at the time of a 10-min hot pulse with [³H]thymidine (*a*) or 2 h before the start of a 30-min hot pulse (*b*), and DNA fiber autoradiographs were prepared from the labeled cells. Multiunit (100 two-unit, 50 three-unit and 50 four-unit) autoradiograms containing bidirectional units were scored from each sample and the proportion of these units that initiated after the start of the hot pulse was determined.

† Determined from the ratios of postpulse to prepulse units in each sample.

§ *t*-Test, one-tailed.

FIGURE 5 Multiunit autoradiograms from puromycin-treated cells. In (*a*), the autoradiogram is from experiment 1 in Table I; in (*b*–*f*) they are from experiments 3 and 4. The arrowheads indicate presumed initiation points. (*a*) Cluster of four prepulse units, puromycin added at the start of a 10-min hot pulse. (*b*) Cluster of four units: from left to right, three prepulse and a postpulse unit, puromycin added 2 h before the start of a 30-min hot pulse. (*c*) Cluster of five units: from left to right, two bidirectional postpulse, one unidirectional (arrowhead *u*), one bidirectional postpulse, one unidirectional unit (arrowhead *u*), puromycin added 2 h before the start of a 30-min hot pulse. (*d*) Cluster of two unidirectional units, puromycin added 2 h before the start of a 30-min hot pulse. (*e*) Cluster of two unidirectional units (fork progression in opposite directions), puromycin added at the start of a 30-min hot pulse. (*f*) Cluster of three prepulse units, puromycin added at the start of a 30-min hot pulse. This micrograph was damaged by a crease in processing for the final print. The bar represents 100 μm. All micrographs are at a magnification of 450.

TABLE III
Bidirectional DNA Replication in
Antibiotic-Treated Cells*

Sample	Number of units scored	Bidirectional units, proportion
Control	201	0.98
Cycloheximide-treated	186	0.71 ($P < 0.001$)‡
Puromycin-treated	152	0.70 ($P < 0.001$)‡

* Antibiotics were added to cells 2 h before the start of a 30-min hot pulse with [³H]thymidine and DNA fiber autoradiographs prepared from the labeled cells. 50 multiunit autoradiographs from each sample were scored and the proportion of bidirectional units in these autoradiographs was determined.

‡ *t*-Test, one-tailed.

on multiunit autoradiographs are more frequently of the same type, either prepulse (Fig. 3 *a*) or postpulse (Fig. 3 *b*), than would be expected if initiation had occurred randomly (7). Whether this synchrony could be altered by inhibition of protein synthesis was investigated in the present study. Using preparations from experiments 3 and 4 in Table I, multiple-unit autoradiographs containing two to four bidirectional units were scored for the proportions which contained units of all the same type (either all prepulse or all postpulse). These are the observed proportions. The expected proportions of autoradiographs containing only like units, assuming random initiation, were calculated from the proportions of individual pre- and postpulse units in each sample using the binomial equation. The data are presented in Table IV.

TABLE IV
Synchrony of DNA Initiation in Antibiotic-Treated Cells*

Autoradiograms	Proportion with like units‡		Synchrony§ % of control
	Observed	Expected	
Two-unit			
Control	0.82	0.50	—
Cycloheximide-treated	0.66 ($P < 0.005$)	0.50	50
Puromycin-treated	0.64 ($P < 0.003$)	0.50	44
Three-unit			
Control	0.72	0.25	—
Cycloheximide-treated	0.52 ($P < 0.025$)	0.25	57
Puromycin-treated	0.56 ($P < 0.05$)	0.25	66
Four-unit			
Control	0.70	0.13	—
Cycloheximide-treated	0.38 ($P < 0.001$)	0.13	44
Puromycin-treated	0.42 ($P < 0.005$)	0.13	51

* Antibiotics were added to cells 2 h before the start of a 30-min hot pulse with [³H]thymidine and DNA fiber autoradiographs prepared from the labeled cells. Multiunit (100 two-unit, 50 three-unit, and 50 four-unit) autoradiographs containing bidirectional units were scored from each sample and the numbers of pre- and postpulse units on each autoradiogram were recorded.

‡ These are autoradiographs in which all replication units showed the same pattern, i.e. all prepulse or all postpulse. Observed values were determined as indicated in the footnote above. Expected values were calculated from the equation for the binomial distribution $(p + q)^n = 1$, where p = proportion of prepulse units in the sample, q = proportion of postpulse units in the sample, and n = number of units per autoradiogram (two, three, or four) The sum of p^n and q^n is the expected proportion of autoradiographs containing only like units.

§ Synchrony is defined arbitrarily as the difference between expected and observed proportions for each sample of like autoradiographs. $(\text{Synchrony}_{\text{treated}}/\text{Synchrony}_{\text{control}}) \times 100$ gives the values in this column.

|| The P values in parentheses show the significance of the difference between these values and the control autoradiographs with the same number of units (*t* test, one-tailed). In addition, all observed values are significantly different from expected ($P < 0.001$, except for row 5 where $P < 0.025$ and row 8 where $P < 0.05$; chi-square for goodness to fit).

In control cells, there is a higher proportion than expected of multiunit autoradiograms containing like units, confirming that synchrony of initiation occurs on these subchromosomal clusters (7). In DNA from cycloheximide- and puromycin-treated cells, the observed proportions of autoradiograms with like units are significantly decreased compared to the control values (compare observed two-unit control with observed two-unit cycloheximide-treated, or observed three-unit control with observed three-unit cycloheximide-treated, etc.). The observed values in the antibiotic-treated cells remain significantly different from the expected values. The degree of synchrony in antibiotic-treated cells is roughly half that observed in control cells. A complete analysis of the data, comparing the observed and expected frequencies for each of the terms of the binomial equations expanded to the second, third, and fourth powers for two-unit, three-unit, and four-unit autoradiograms respectively, showed the same result: observed frequencies deviated significantly from those expected for random initiation, and the deviations were less marked in antibiotic-treated cells. Thus, inhibition of protein synthesis is associated with a decay in the synchrony of DNA initiation events, although initiation is not completely randomized.

Multiple-unit autoradiograms from experiments 1 and 2 in Table I were analyzed for synchrony in the same fashion. The observed proportions of autoradiograms with like units were decreased in antibiotic-treated cells compared to controls, but the differences were not statistically significant. The change in synchrony produced by inhibition of protein synthesis therefore seems to be a function of time, with a significant decrease occurring 2 h after, but not immediately after addition of the antibiotics.

The slower rate of fork progression in antibiotic-treated cells may result in selection of autoradiograms from these cells having shorter initiation intervals than is usually observed in untreated cells. These clusters might have initiation patterns less synchronized than those with longer intervals. If replication on this subset of clusters with short intervals was more resistant to inhibition of protein synthesis, then this might explain the reduced synchrony of initiation seen when protein synthesis was inhibited. To investigate this, initiation synchrony was determined in control cells, comparing autoradiograms with short initiation intervals ($< 25 \mu\text{m}$) to those with long intervals ($> 25 \mu\text{m}$).

There was no difference in the degree of synchrony between the two groups. This supports the concept that the reduction in synchrony observed in antibiotic-treated cells results from inhibition of protein synthesis.

DISCUSSION

The data in this report define three alterations produced by inhibition of protein synthesis on the pattern of initiation on replication units of mammalian DNA. In the presence of cycloheximide or puromycin, (a) fewer units initiate replication, (b) those that do initiate show bidirectional replication less frequently, and (c) active units in clusters show a lesser degree of synchrony of initiation.

The lower proportion of replication units initiating in the presence of cycloheximide or puromycin may be interpreted as a decrease in the frequency of initiation when protein synthesis is inhibited. The data here document only a modest decrease, but this occurs within the first 10 min after addition of the inhibiting agents. The decreased proportion of bidirectional units observed after 2 h of treatment with the antibiotics might also be a reflection of decreased frequency of initiation if units with unidirectional replication resulted from initiation events in which formation of one of the two forks was inhibited by the antibiotics.

That inhibition of protein synthesis would decrease the frequency of eukaryotic DNA initiation had been suggested in several previous studies (3, 4, 8, 9, 14). In these studies, the overall decrease in DNA synthesis resulting from inhibition of protein synthesis could not be accounted for by the observed retardation in the rate of replication fork progression and it was postulated that initiation had been inhibited.

The interpretation that inhibition of protein synthesis decreases initiation is not necessarily in conflict with the findings of Weintraub and Holtzer (29) or Gautschi (5). These investigators interpreted their data as showing that inhibition of protein synthesis inhibited fork progression alone. In both studies, the investigators, using sedimentation techniques, asked whether fork progression or initiation was inhibited. The experiments could not detect decreases in both, especially if inhibition of fork progression predominated. Fiber autoradiography, the technique used in this report, allows an independent analysis of both variables. However, analyses using sedimentation techniques (5, 29) or autoradiography (9) indicate that a very prominent early effect of inhibition of protein synthesis on

DNA replication is retardation of fork progression.

The present experiments do not exclude the possibility that the unidirectional units observed in the presence of antibiotics may represent a subset of units which normally contain only one fork and are resistant to inhibition of protein synthesis. That clusters of unidirectional units were observed in inhibited cells and not in controls suggests that unidirectional units arise from modifications of bidirectional units induced by the inhibitors. However, further experiments are needed to prove this.

The concept that initiation occurs synchronously on subchromosomal clusters of active units (7, 10, 16, 17) has been strengthened by the present observation that clusters up to four units in length exhibit initiation patterns indicating nearly simultaneous initiation. This synchrony is decreased by inhibition of protein synthesis. Available data on mammalian DNA replication do not allow a simple explanation for this. However, it is not unreasonable to postulate that the orderly replication of the mammalian chromosome requires continued synthesis of both structural and functional proteins. Absence of protein synthesis would result in a breakdown of the regulation of this process and those initiation events that do occur might be more likely to occur randomly.

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