DNA Replication in *Physarum polycephalum*: Electron Microscopic Analysis of Patterns of DNA Replication in the Presence of Cycloheximide

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ABSTRACT DNA from synchronously replicating nuclei of *Physarum polycephalum* was studied electron microscopically after 15, 30, 60, and 90 or 120 min of replication in the presence or absence of the protein synthesis inhibitor cycloheximide. The replication-loop size-distribution showed that replication fork progression is severely retarded in the presence of cycloheximide. Analysis of replication-loop frequency showed a similar pattern in control and cyclo-heximide-treated samples, with an increase from 15 to 30 and 60 min. This suggests, surprisingly, that initiations of new replicons either may not be inhibited by cycloheximide or, alternatively, that all initiations have already taken place at the very start of S-phase. The latter conclusion is favored in the light of previous results in our laboratory, discussed here.

We have previously suggested that the genome of *Physarum polycephalum* replicates as sets of replicons of size around 2–4 × 10^7 daltons (5–10 μm), occurring in temporal clusters of 3–4 (1). This model, arrived at by analysis of alkaline sucrose sedimentation patterns, was verified by electron microscope and autoradiographic analysis of replicating DNA (2). An earlier study on the effects of inhibition of protein synthesis with cycloheximide during S-phase suggested the following model for control of DNA-replication in Physarum (3): at least 10 temporal sets of replication units depend for their consecutive initiations on ongoing protein synthesis, while replicons once initiated are no longer protein-synthesis dependent. Evans et al. (5) concluded, however, that cycloheximide did not interfere with initiation of new replication units, while elongation of initiated progeny strands was inhibited. In our previous study on the effects of inhibition of protein synthesis on DNA replication (4), we found that the chain length increase of new DNA strands was slowed or prevented at three levels: (*a*) formation of Okazaki fragments, (*b*) joining of Okazaki fragments to replicon size DNA, and (*c*) maturation (joining) of replicon size DNA to high molecular weight DNA.

It is possible that the latter two effects could be a consequence of the first. This result, as well as that of Evans et al. (5), appears to be at variance with the results of Muldoon et al. (3).

In this communication we have analyzed DNA-replication in the presence and absence of the protein synthesis inhibitor, cycloheximide, using electron microscopy. The main purpose of this study was to shed light on the question of whether fork progression (replication rate) is unaffected or slowed or stopped in the presence of cycloheximide. We also hoped to be able to reveal whether new initiations can occur in the absence of protein synthesis.

MATERIALS AND METHODS

Strains and Culture Methods

Plasmodial strain TU291 was also used in all our previous studies on DNA replication in *Physarum polycephalum.* This strain is very closely related to the cycloheximide-resistant strain which we have used to demonstrate that the effects of cycloheximide on DNA replication are mediated exclusively through the effect on protein synthesis (4). The semi-defined medium used to maintain microplasmodia in liquid, shaken culture has been described (6).

15-ml cultures were grown at 25°C and shaken at 160 rpm in baffled 250-ml flasks. Synchronous surface plasmodia were grown on top of millipore membranes resting on a stainless-steel grid and fed the same medium. Mitotic stages were determined by microscopy of ethanol-fixed smears.

Cycloheximide Treatment

Millipore-supported pieces of plasmodia in defined mitotic stages were transferred to dishes containing cycloheximide at 50 μg/ml in semi-defined medium. While our previous studies with alkaline sucrose gradient used only 5 μg/ml of cycloheximide, we aimed for a higher dose in the present experiments to be absolutely certain that we had rapid and maximal inhibition. Inhibition of protein synthesis by cycloheximide is ~90% at 4 μg/ml (7) and better than 95% at 50 μg/ml as measured by incorporation of radioactive amino acids. The corresponding

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 OCTOBER 1982 323-331
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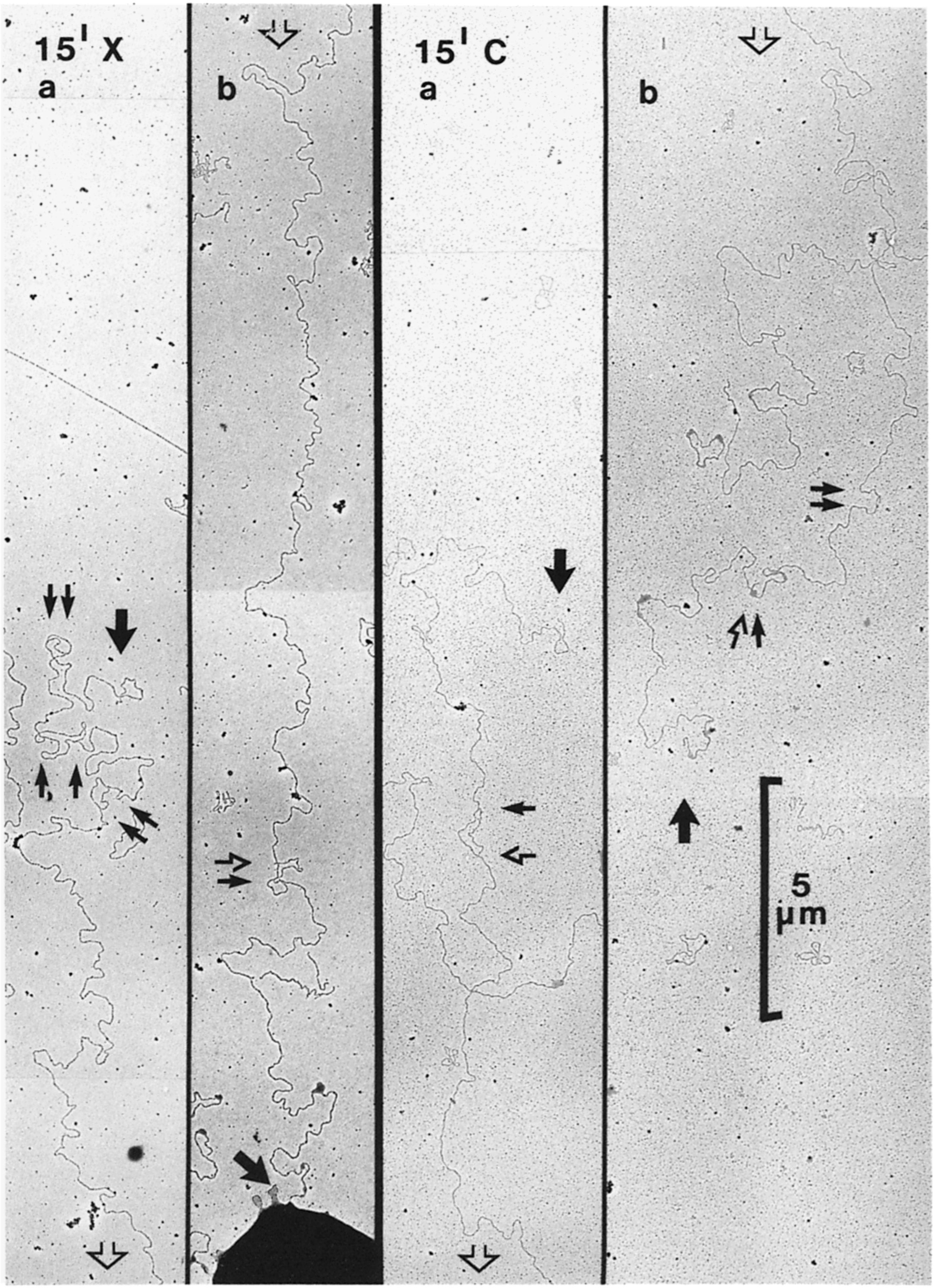
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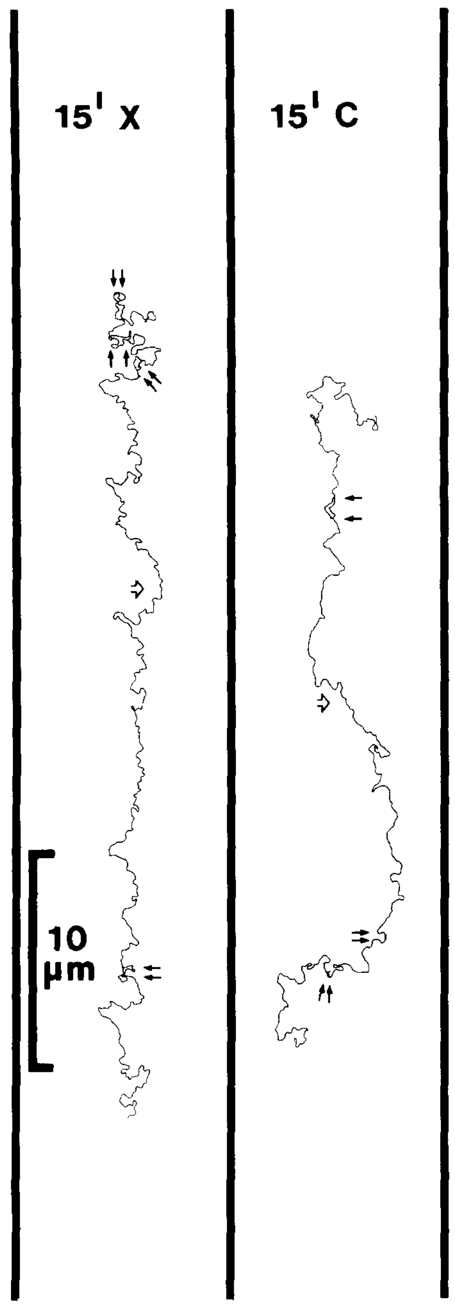


FIGURE 1 (*A*) Actual photograph. (*B*) Line drawing of molecule. One synchronous plasmodium of *Physarum* was cut in two in metaphase, and the two halves were transferred to medium with and without cycloheximide. Fifteen minutes into S-phase (which immediately follows telophase), nuclei were isolated and DNA was prepared for electron microscopy (EM) as described. *15' X*, 15 min into S-phase with cycloheximide. *15' C*, 15 min into S-phase without cycloheximide. Symbols: ⇅, junction of scissor-cut micrograph; ↓↓, replication loop forks; and ↓, end of molecules.

inhibition of DNA synthesis as measured by incorporation of radioactive precursors into the acid-insoluble fraction varies with the kind of precursor used, because of metabolism and pool expansions. It has been shown that exogenously added ^{14}C-labeled thymidine is rapidly broken down and released as CO$_2$ by *Physarum* (8) while deoxyadenosin is not catabolized (9). Furthermore, it has been shown that cycloheximide causes a dTTP pool expansion while not changing the dATP pool appreciably (10, 11). Both contribute to a seemingly greater inhibition of DNA synthesis when monitored by use of radioactive thymidine than when deoxyadenosin is used. Thus, in our hands 4 µg/ml of cycloheximide after 5 min of treatment gives >95% inhibition of DNA synthesis when [^{3}H] deoxythymidine is used as label, and ~75% inhibition when [^{3}H]deoxyadenosin is used to label DNA. At 50 µg/ml of cycloheximide, the corresponding values are 99% and 85%, respectively, but these numbers do not take into account contributions from endogenous pool alteration, nor do they differentiate between nucleolar, nuclear, and mitochondrial contributions to total DNA synthesis.

Labeling with Radioactive Isotopes

For the purpose of isolation of DNA for electron microscopy, plasmodia were prelabeled with [^{3}H]deoxyadenosin by growing for ~24 h on semi-defined medium to which was added 1 µCi/ml of [^{3}H]deoxyadenosin (New England Nuclear, Boston, MA; NET-123). This allowed easy identification of DNA in a purifying CsCl gradient centrifugation.

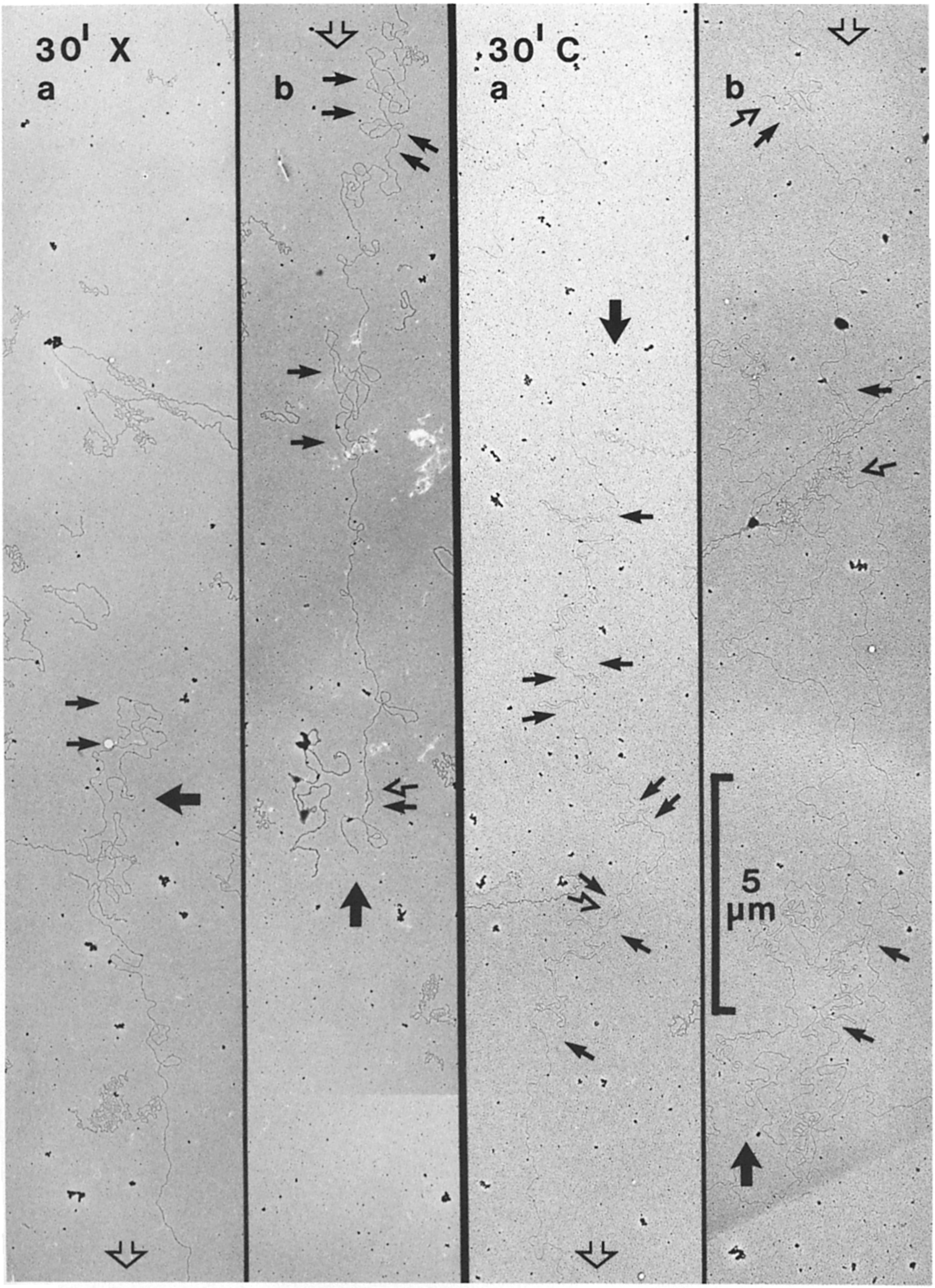
Nuclear Isolation and Preparation of DNA

Nuclei for isolation of DNA for electron microscopy were isolated in 250 mM sucrose, 10 mM Tris, pH 8.5, 15 mM MgCl$_2$, 0.1% Triton X-100, essentially as described (12). DNA for electron microscopy was prepared by suspending 2 × 10^7 nuclei in 0.4 ml of 0.15 M NaCl, 0.015 M trisodium citrate and adding: 0.1 ml proteinase K (Worthington Biochemical Corp., Freehold, NJ) at 5 mg/ml in buffer A; 1.0 ml buffer A (10 mM Tris pH 7.4; 10 mM EDTA; 10 mM NaCl); 10 µl 30% (wt/vol) sarkosyl. The nuclei were digested for 2 h at 37°C, and the digest was then mixed with 9.2 g of CsCl dissolved in 6.2 ml of 1/10 standard saline-citrate (0.15 M NaCl, 0.015 M sodium citrate). Mixing was completed in polycarbonate ultracentrifugation tubes by very gentle rocking for 2 h at 26°C. Centrifugation was done for 60 h at 24,000 rpm in SW40 rotor (Beckman ultracentrifuge; Beckman Instruments, Fullerton, CA). After fractionation of the gradients into 24 fractions of ~400 µl each, the DNA was located by measuring the concentration of radioactivity, and the peak DNA fractions were used directly in spreading for electron microscopy. The hypophase, 10 mM tris (pH 8), 1 mM EDTA, and 10% formamide, was placed in a 10-cm plastic petri dish. The hyperphase contained the DNA at a concentration 0.5–1.0 µg/ml and cytochrome *c* in 40% (vol/vol) formamide. 30 µl of the hyperphase was spread by letting it run down a clean glass slide onto the hypophase. DNA was picked up on carbon-coated formvar film on a copper grid within 30 s of spreading and was stained for 30 s in phosphotungstic acid (1 mg/ml in 90% ethanol, 1% sulfuric acid). Dehydration for 30 s in 90% ethanol was followed by rotary shadowing with Pt/Pd alloy 80/20 and carbon-coating.

RESULTS AND DISCUSSION

In the present work the duration of the nuclear cycle of the synchronous plasmodia is ~9 h. The total S-phase as judged by gross incorporation of radioactive precursors ends in ~150 m after initiation, which occurs in telophase of mitosis. However, a joining of DNA molecules made during the last S-phase and a low level chromosomal DNA synthesis is going on all through the following G2 phase (1).

Figures 1, 2, 3 and 5 which display replicating molecules at 15, 30, 60, 90, and 120 min after initiation thus show replicating structures after roughly 0.1, 0.2, 0.4, 0.6, and 0.8 of the total major part of the S-phase, in the absence (*C*) and presence (*X*) of cycloheximide. (*A*-figures show actual photographs of molecules while B-figures show line-drawings of the same molecules). Fig. 4 shows a diagrammatic representation of populations of such molecules, and Fig. 6 sums up all of the data in the form of histograms. It can be seen that replication loops from control, untreated samples are significantly larger than loops from treated plasmodia. This difference in size is discernible even at 15 min and becomes more striking with increasing times of incubation into S-phase. It should be noted that one might have expected the difference in replication-loop size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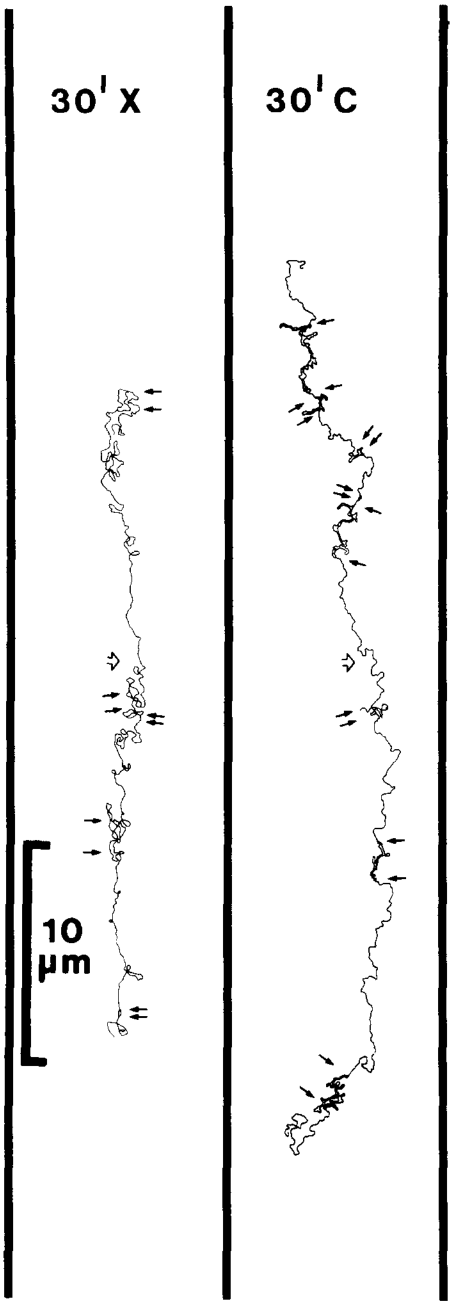
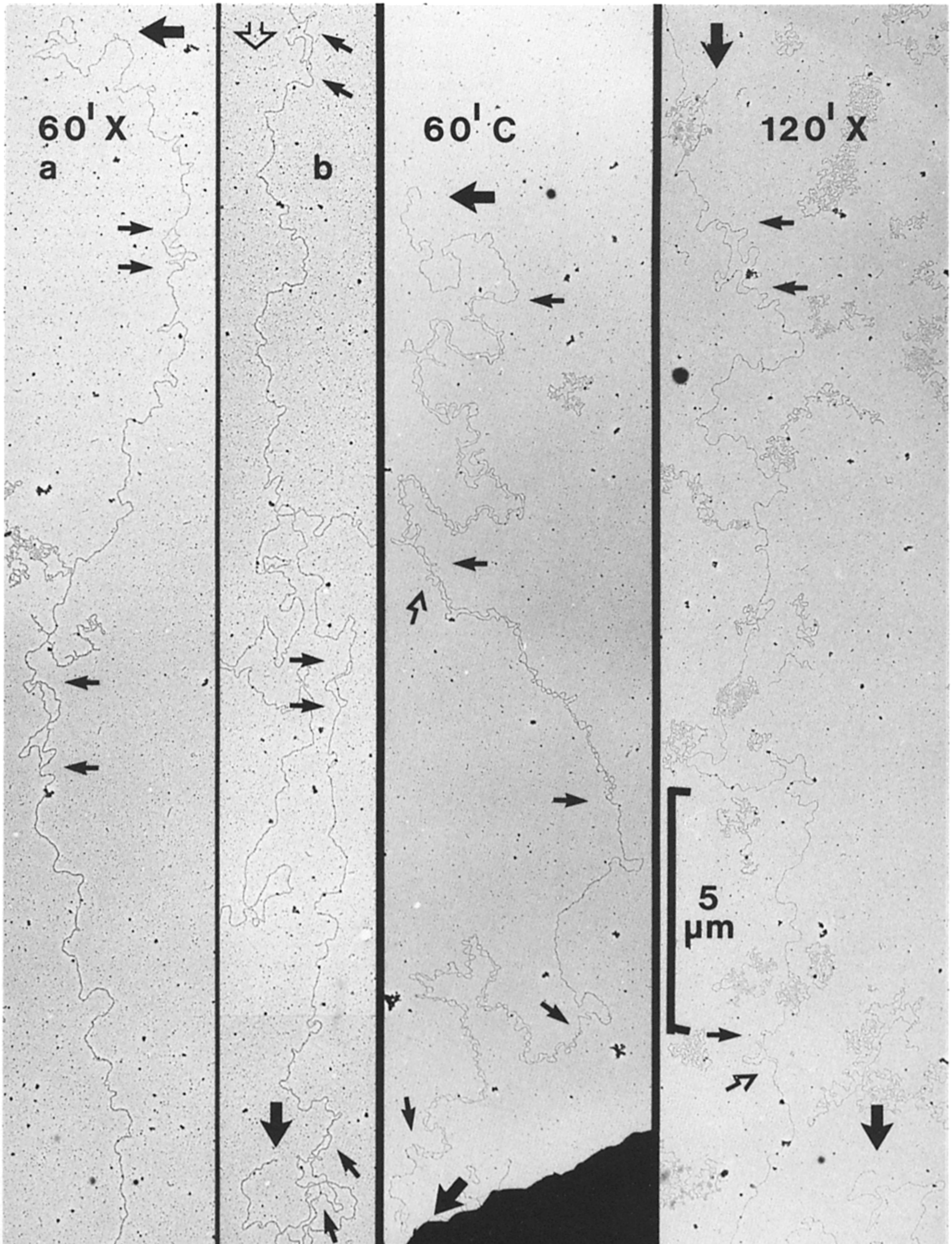


FIGURE 2 (*A*) Actual photograph. (*B*) Line drawing of molecule. Culture treated as described in legend to Fig. 1. Nuclei isolated 30 min into S-phase, and DNA prepared for EM. *30' X*, 30 min into S-phase with cycloheximide. *30' C*, 30 min into S-phase without cycloheximide. Symbols: ⇓, junction of scissor-cut micrograph; ↓↓, replication loop forks; ↓, strand broken at fork; ⬇, end of molecule.

between controls and cycloheximide-treated samples at the latest times to be considerably greater than was actually observed. The reason for this is probably that the very large replication loops expected at later times break more easily during preparation than the smaller ones and thus tend to get underrepresented. In addition, it can be seen that although the majority of replication loops in cycloheximide-treated plasmodia stay small, at around 1–2 μm and 3–4 μm, a few appear to grow slightly during the 1.5-h incubation. The reason for this is not known, and at the present time, we can only assume either that this may be due to enough of the necessary proteins being present to allow some replication or that there might be some low level protein synthesis going on in the presence of the cycloheximide. Thus this electron microscope investigation of replication-loop growth with and without ongoing protein synthesis shows that ongoing DNA-replication is dependent on simultaneous protein synthesis. This conclusion is contrary to the widely accepted conclusion of Muldoon et al. (3).

With regard to this difference the following comments seem appropriate. At the time Muldoon and co-workers performed their study, two important facts about the use of thymidine to monitor DNA replication in *Physarum* were not known. First, exogenously supplied thymidine is extensively catabolized (8) and thus will label both protein and RNA to a large extent (Haugli, unpublished observations). Second, addition of cycloheximide more than doubles the endogenous pool of dTTP, thus effectively lowering the specific activity of exogenously added ^{3}H thymidine [10, 11]. Both facts contribute to a great uncertainty about the data given by Muldoon and co-workers. Since those authors only measured accumulated radioactivity in total acid-insoluble fraction, it is not possible to know how much of the label was in fact in DNA, and how much in RNA and protein. The increase in the accumulated acid-insoluble radioactivity that was observed after the addition of 10 μg/ml of cycloheximide may, therefore, not reflect DNA synthesis at all, or only to a limited, unknown, extent. Thus, their conclusion that initiated replicons do finish replication in the absence of protein synthesis is not well supported by their observations. On the other hand, the present work is independent of radioactive precursors and thus avoids the problems of catabolism and pool-sizes. We therefore believe that we can state with confidence that ongoing DNA replication is, in fact, severely inhibited when protein synthesis is prevented.

The frequency of replication loops was calculated from the total number of DNA fragments observed (including those without replication loops) and the calculated average length of these DNA molecules as measured in electron micrographs. These calculations are based on the assumption that a random field of DNA observed on the grid after spreading is representative of the total population of DNA fragments obtained from the genome. The frequency thus obtained, gives the average distance between replication loop centers as a function of time and will only be meaningful as a measure of replicon origins and initiations as long as neighboring replicons have not started to fuse. Extensive replicon fusion occurs from around 60 min (1). Another limitation, with regard to the question of initiation in presence of cycloheximide, is that replication loops can only be seen after they reach a minimal size which in the present work approaches 500 Å. Thus, an initiated area covering 10–30 nucleotide pairs would be <100 Å long and would hardly be discernible until fork progression has enlarged the loop. Since this study shows that fork progression is severely retarded by cycloheximide, an initiation might not be immediately detectable in the presence of cyclo-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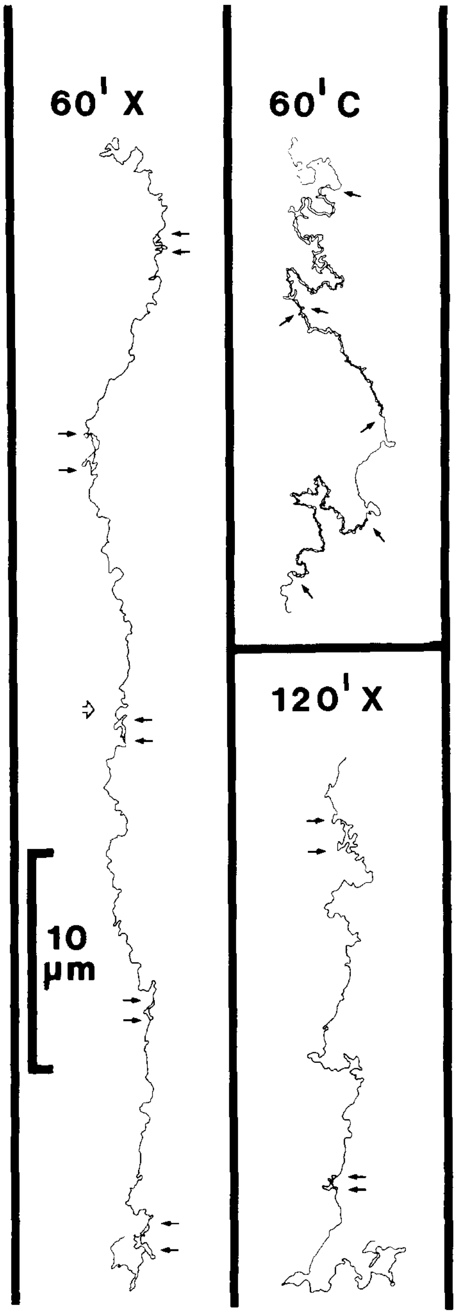


FIGURE 3 (*A*) Actual photograph. (*B*) Line drawing of molecule. Culture treated as described in legend to Fig. 1. Nuclei isolated 60 min into S-phase, and DNA prepared for EM. In addition, a molecule treated with cycloheximide for 120 min is included in this figure, since the size of the corresponding untreated molecules at 90 min shown in Fig. 5 did not allow presentation in the same figure. *60' X*, 60 min into S-phase with cycloheximide. *60' C*, 60 min into S-phase without cycloheximide. *120' X*, 120 min into S-phase with cycloheximide (see Fig. 5 for comparison with untreated late-S-phase molecules). Symbols: ⇓, junction of scissor-cut micrographs; ↓↓, replication loop forks; ↓, strand broken at fork; ↓, end of molecule.

heximide. With these uncertainties in mind, we find an expected frequency increase in the control from an average of one replication loop every 175 µm at 15 min into S-phase, to one loop every 86 µm at 30 min, and one loop every 45 µm at 40 min. Control data for 60 min cannot be calculated due to lack of registered DNA fragments without replication loops. Data for later points are difficult to interpret since replicon fusion will occur, and so they have not been included. In the cycloheximide-treated samples, the frequencies of replication loops follow the control closely, giving an average replication-loop center-center distance of 148 µm at 15 min, 66 µm at 30 min, and 39 µm at 60 min.

Thus, the unexpected finding is that, although the growth rate of replicons is clearly much slower in cycloheximide-treated cultures, there does not appear to be any significant difference in the frequence of newly initiated replicons compared to controls. This result should be considered in the light of results obtained previously in our laboratory (13). Here we studied the frequency of initiation at time zero (start) in S-phase. Start of S-phase is around anaphase-telophase of mitosis, since *Physarum* lacks a G1-phase. The technique was UV-scission of bromodeoxyuridine-labeled sites, followed by molecular weight estimations on alkaline sucrose gradients. In these experiments we invariably found that UV-sensitive sites, presumably defining sites of initiation, were separated by 1.1–2.2 × 10^7-daltons single-strand weight of DNA or ~7–12 µm. We suggested that this could only mean that all replication units of the *Physarum* genome were initiated at one single time point. This would be at master initiation of the S-phase. However, among the initiated replicons, only selected groups actually proceeded in DNA replication at given time points, giving rise to sets of early and later replicating units, all of

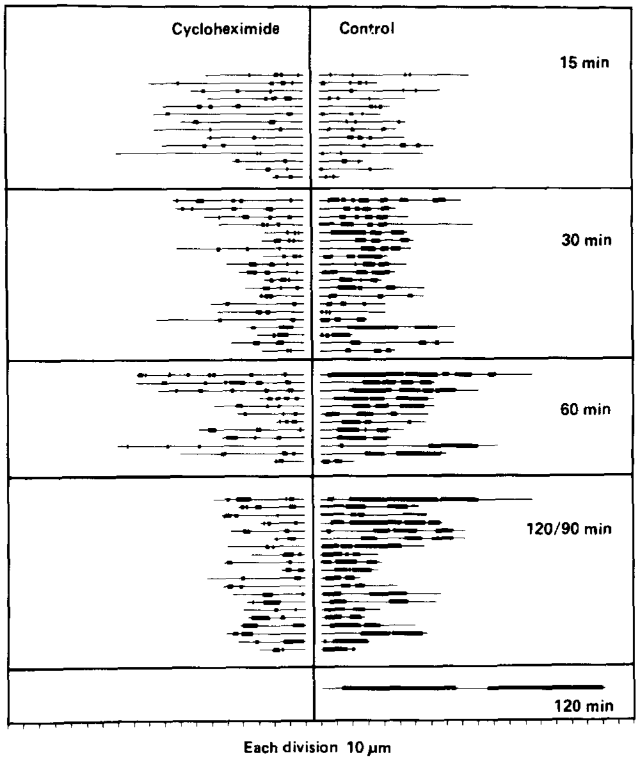
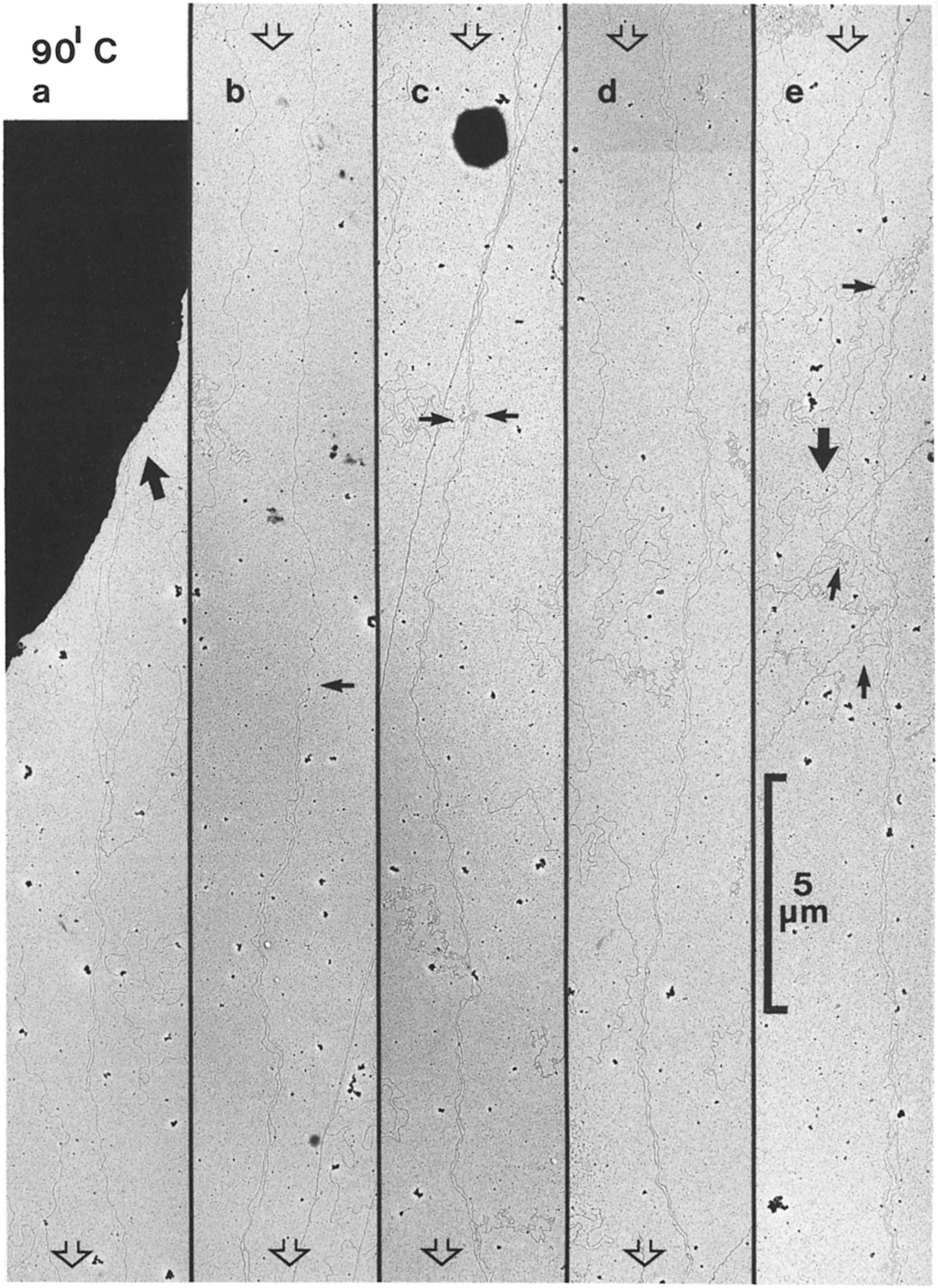


FIGURE 4 Scale-drawing of DNA molecules from cultures treated and not treated with cycloheximide. Time periods given for each horizontal set of data give the time from start of synchronous S-phase until isolation of nuclei. Thick bars, extension of replication loops.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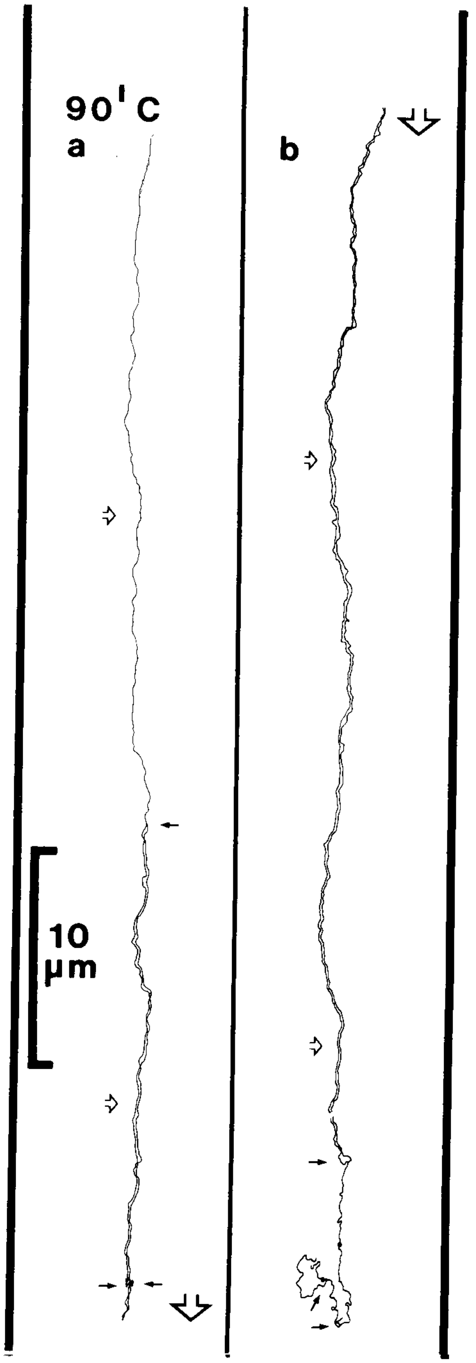

FIGURE 5 (*A*) Actual photograph. (*B*) Line drawing of molecule. Cultures treated as described in legend to Fig. 1. Nuclei isolated 90 min into S-phase, and DNA prepared for EM. *90' C*, 90 min into S-phase without cycloheximide. Corresponding cycloheximide-treated molecule at 120 min is presented in Fig. 3, for space reasons. Symbols: ⇓, junction of scissor-cut micrographs; ↓↓, replication loop-fork; ↓, end of molecule.

which, however, were initiated simultaneously, at the start of S-phase (13). If this is in fact so, the question of whether initiations can occur in the presence of cycloheximide must be rephrased as whether fork progression can proceed in the presence of cycloheximide. While the present results show that fork progression is severely retarded in the presence of cycloheximide, the data on frequency of replication loops suggest that fork progression, at least in the early stages of loop expansion, is great enough to allow visualization with the present technique. Furthermore, the frequency data suggest

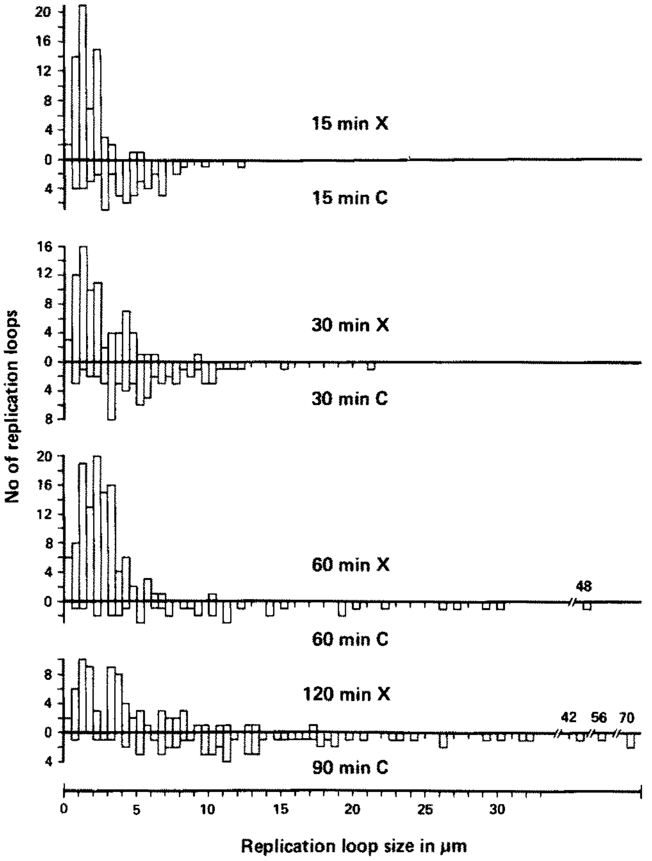

FIGURE 6 Histogram showing number of replication loops of a given size at various time points into S-phase. Bars above base line, cycloheximide treated. Bars below base line, untreated controls.

either that replicon initiation can occur in the presence of cycloheximide or that initiations have all occurred at the very beginning of S-phase. In view of the results discussed above (13), we favor the latter explanation.

Received for publication 15 March 1982, and in revised form 10 June 1982.

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