Volume 6 Number 4 April 1979 Nucleic Acids Research

DNA replication in Physarum polycephalum: electron microscopic and autoradiographic analysis of replicating DNA from defined stages of the S-period

Steinar Funderud, Rolf Andreassen and Finn Haugli

Institute of Medical Biology, University of Tromsø, 9001 Tromsø, Norway

Received 2 January 1979

ABSTRACT

Electron microscopic and autoradiographic analysis of replicating DNA from Physarum showed that replication occurs at a rate of 0.4 μm/min/per replicon and that replicons of size 10-15 μ occur in temporal clusters with an average of about 4 replicons per cluster. These results are compared with previous hydrodynamic measurements and with those obtained in other organisms.

INTRODUCTION

We have previously used alkaline sucrose sedimentation analysis, in conjunction with radioactive pulse labelling, inhibitor experiments and bromodeoxyuridine induced ultra violet photolysis, to analyze mechanism and topology of DNA replication in Physarum polycephalum.[1-6] In the present communication we extend this analysis with the techniques of electron microscopy and autoradiography of replicating DNA from defined stages of the DNA replication period of Physarum.

Based on alkaline sucrose gradient analysis of pulse labelled DNA Funderud et al. (1978)[6] suggested the following model for DNA synthesis in Physarum: DNA replication occurs bidirectionally within replicons of size 35-45 S ($1.1\text{-}2.2 \times 10^7$ daltons single strand DNA) which are arranged in temporal clusters of 3-6. These mature to larger size DNA molecules only in discrete steps made possible by the termination of replication within replicons and between clusters. Distinct single stranded maturation intermediates appearing in the sedimentation analysis includes a class of molecules around 70 S (6.4×10^7 daltons) which could result from the joining of finished replicon size

DNA within clusters. The largest DNA molecules observed by alkaline sucrose gradient sedimentation analysis, 130 S or 3 x 10^8 daltons, may be the maturation limit, but could equally well result from a sedimentation artefact of chromatide size DNA.[19] The present work was initiated in order to try to verify and extend some aspects of this model.

EXPERIMENTAL PROCEDURES

1. Strain and culture methods.

Strains and culture techniques were as described previously.[1-6]

2. Isolation of nuclei.

Microscopy of ethanol fixed smears of the plasmodium was used to determine the time point of metaphase in the synchronous plasmodium. The DNA synthetic period starts about 5 min thereafter at completion of anaphase-telophase, and the time in minutes counted from that point on, defines the temporal stage of the S-period. (There is no G1 phase in the nuclear cycle of Physarum plasmodia). For electron microscopy, nuclei were simply isolated at the desired time point. For autoradiography small pieces of plasmodia, supported by the Millipore membrane, were labelled by placing them on 50 µl droplets of semidefined medium containing ^{3}H-deoxythymidine (Amersham code TRK 343, specific activity 19 Ci/mmol) at radioactive concentration 1 µCi/µl. No pretreatment was used, and nuclei were isolated immediately following the pulse. Nuclear isolation medium contained 0.25 M sucrose; 0.1% Triton X100; 0.01 M Tris pH 7.5 and 0.015 M $MgCl_2$ and homogenization was in a Sorvall Omnimixer, position 3 for 20 seconds. This procedure and the following centrifugal isolation of nuclei closely follows that of Mohberg and Rusch (1971).[20]

3. Electron microscopy.

2 x 10^7 nuclei were suspended in 0.4 ml of 0.15 M sodium chloride; 15 mM sodium citrate (SSC) and 1.0 ml of buffer A (10 mM NaCl; 10 mM EDTA; 10 mM Tris pH 7.5). 100 µl of proteinase K (5 mg/ml; Merck) and 10 µl of 30% N-lauroyl-sarcosine were added. After incubation at 37°C for 2 hours, the lysate was

very gently mixed with a solution of 9.2 g CsCl in 6.2 ml 1/10 SSC (DSC). To ensure a satisfactory deproteinization of DNA the mixture was kept on a rocker platform for 2 hours. The CsCl gradients were run in SW 40 rotors on the Beckman preparative centrifuge at 24000 rpm for 60 hours at 22°C. Fractions were collected from the bottom with the aid of a peristaltic pump. 3-5 µl of the CsCl gradient fractions were diluted and spread for EM as described by Davis et al. (1971)[7], with 40% and 10% formamide in the hyperphase and hypophase, respectively. The surface film was picked up onto Celloidin films supported on copper grids. The samples were stained in phosphotungstic acid (1 mg/ml in 0.18 M H_2SO_4, 90% ethanol), rinsed in 90% ethanol and shadowed with Pt/Pd (80:20). Preparations were scanned using a Hitachi HU-12 electron microscope. Contour length measurements were made on 10 times enlarged negatives with a map measurer and converted to micrometer by calibration with phage λ DNA spread under identical conditions.

4. Autoradiography

Isolated nuclei were resuspended in nuclear isolation medium minus Triton X 100 at concentration of 10^5 - 10^6 nuclei per ml. 5 µl of this nuclear suspension was put on a gelatinized microscope slide together with 5 µl of lysis solution (containing 2% Sarkosyl in 0.01 M Tris pH 8.0 and 0.01 M EDTA). After gentle mixing, 5 minutes was allowed before the lysate was spread on the slide with one stroke of a flame-polished glass rod. The slides were dried in a vertical position and the spread lysate fixed and washed at 0°C by dipping for 5 min in 96% ethanol, dried and then dipped 2 x 5 min in 0.25 M PCA and finally rinsed 2 x 5 min in 96% ethanol. After drying the slides were dipped for 1 second in Kodak L4 liquid emulsion, prepared by mixing 15 g of emulsion and 30 ml of 2% glycerol at 42°C. After drying, the slides were stored light and airtight at 4°C until time of development (ca. 6 months). Developing was in Kodak liquid developer D19 for 5 min at 22°C, stopped in 1% acetic acid for 1 min and fixed in acid fixer for 5 min. Finally the slides were rinsed and dried and autoradiograms screened with a 10 x achromat in light field. Photographs were

taken through a 40 x Zeiss planachromate with phase optics to increase contrast, using the Zeiss photomicroscope and Agfa Ortho 25 film.

RESULTS AND DISCUSSION

1. Electron microscopy.

DNA from nuclei isolated at 0, 5, 15, 25, 40, 55, 85 and 115 minutes into the S-phase was prepared. Figure 1 shows typical molecules from 5, 25 and 55 minutes and Figure 2 similarly shows molecules from 85 and 115 minutes. Figures 3 and 4 sums up the analysis of a larger number of such molecules, giving the center-center distance of replication loops where two or more are found in tandem (Figure 3) and the replication loop size at the different time points (Figure 4). From this analysis we would like to make the following points.

a. Replicon size.

On the assumption that replication loops arranged in tandem represent replication units or replicons, the center-center distances are taken to be equivalent to the origin-origin distances in neighbouring replicons (this presupposes bidirectional replication at equal rates, as shown by Funderud et al. (1978).[4] These center-center distances should be reliable measures of origin-origin distances, and thus of average replicon size, up to such times when joining of neighbouring replicons may occur. This, according to previous estimates from alkaline sucrose gradient analysis, will not occur to any appreciable extent until 45-60 minutes into the S-phase. Based on such considerations, Figure 3 reveals a slightly bimodal size distribution of replicons with a peak around 8 ± 2 μm and another size class at around 16 ± 2 μm which contains somewhat fewer individuals. It is, however, quite possible that the distribution is really unimodal around 8-10 μm, and that the somewhat larger distances, particularly conspicous at earlier times and at 55 minutes, results from interspersal of a few later initiating replicons (Hori and Lark 1973)[8] and from replicon fusion, respectively. The main conclusion from these considerations is that most replicons in Physarum occur with an origin-origin distance of

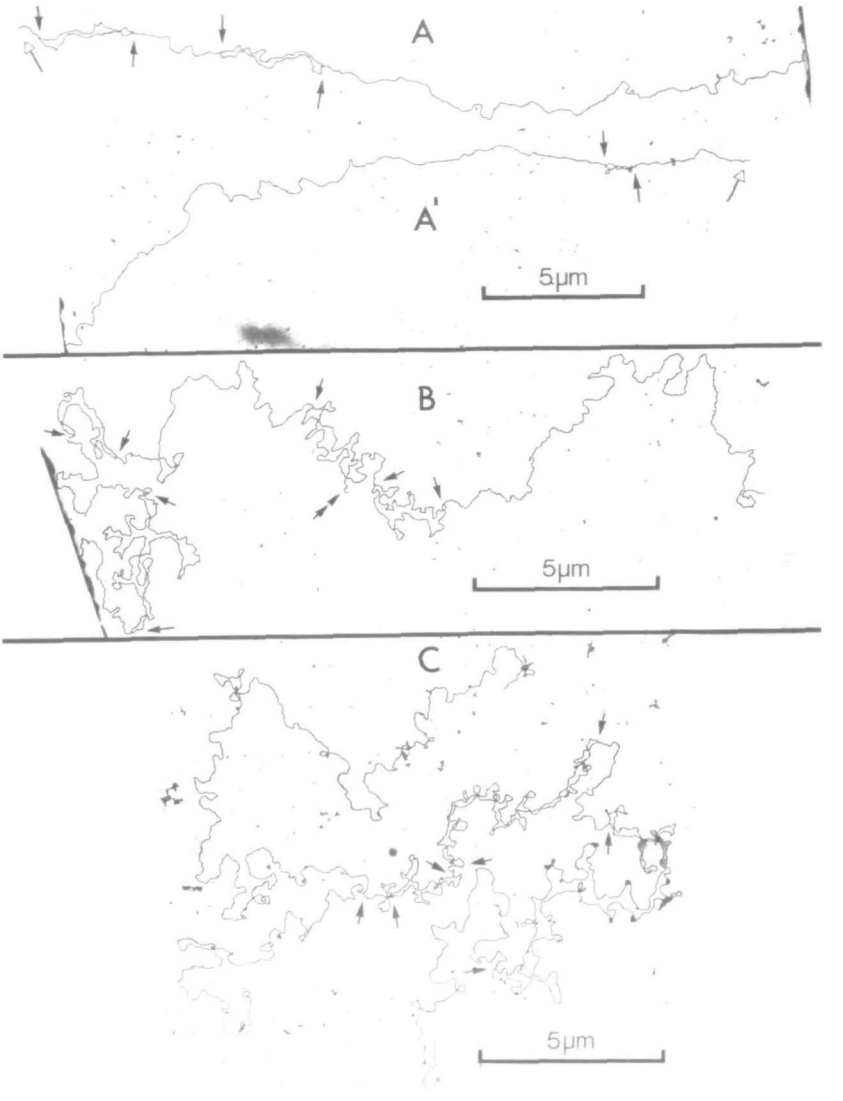


Figure 1. Electron micrographs of replicating DNA molecules from early S-phase. Nuclei and DNA was prepared and spread as described in the text. Molecules of interest have been traced with black ink. (Regular contrast can be seen in occasional DNA molecules. Black arrows: replication forks. In frame A: open arrows point to where molecule was cut to allow space for presentation. Double black arrow: place where one end of replication loop was broken at fork. Bars = 5 μm.
A and A': one molecule isolated at time 5 minutes.
B: one molecule isolated at time 25 minutes.
C: one molecule isolated at time 55 minutes.

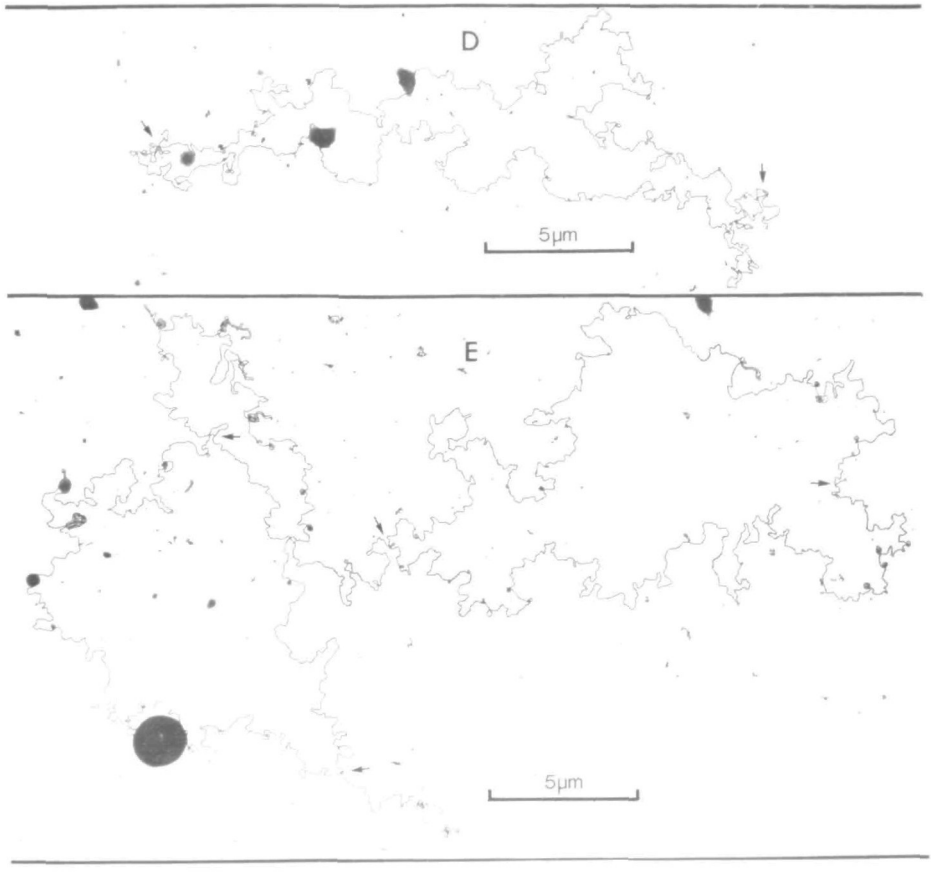


Figure 2. Electron micrographs of replicating DNA molecules from late S-phase. Nuclei and DNA prepared as described in the text. As in Figure 1, DNA molecules of interest have been traced with black ink, for clarity of presentation. Black arrows: points of replication forks. Bars = 5 μm.
D: one molecule isolated at 85 minutes.
E: one molecule isolated at 115 minutes.

roughly 8-10 μm, which must also be the average replicon size. 10 μm corresponds to roughly 10^7 daltons of single strand DNA or 35 S, while 20 μm would correspond to 2×10^7 daltons or roughly 45 S. This is the very range that was estimated for replicon size from the sucrose gradient analysis.[6] As mention-

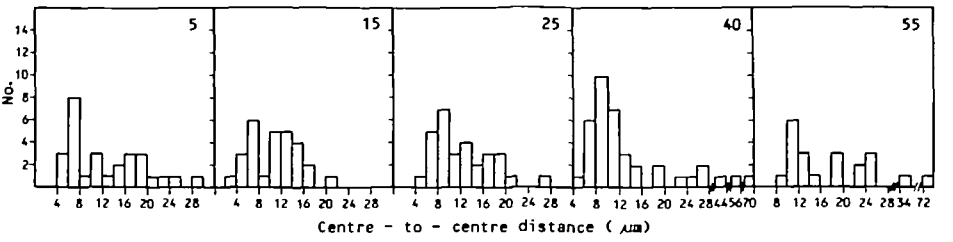


Figure 3. Center-Center distance of replication loops. The center-center distance of replication loops found in tandem, was measured. This center-center distance should be a measure of origin-origin distance of clustered replicons. The measurements for timepoints 5, 15, 25, 40 and 55 minutes into S-phase have been measured in μm and plotted as histograms in this figure.

ed above, the upper range, based on center-center distances, may be overestimated if within a temporal tandem cluster of replicons there is an occasional late-initiating unit. Similarly, the upper range estimated from the sedimentation studies, 20 μ, could be an overestimation due to replicon fusion.

b. Rate and replication loop size.

Figure 4 shows size distribution of replication loops at different time points in the S-phase. One difficulty with this analysis is the technical bias against large loops, since these are easily broken during preparation. While the earlier time points are likely to give a fair representation of the size distribution, there is some danger at 55 and 85 minutes, possibly even at 40 minutes, that larger molecules (20-70 μm) may be under represented. Another difficulty of analysis results from the broadening of the pattern with time. This is a natural consequence of having several temporal sets of initiation, resulting in newly initiated, semi-finished and essentially finished replicons, as well as higher order fusion products, from time 15 min - 25 min on. Attempting to select the molecules representing the first temporal set, as the largest major class of loops at the various time points, we find a size of 2.5 μm at 5 min, 6.75 μm at 15 min and 10.0 μm at 25 min. This gives a rate of total replicon expansion of 0.4 μm per min, or 0.2 μm per min per fork, or 2 x 10^5 daltons per fork per min. This

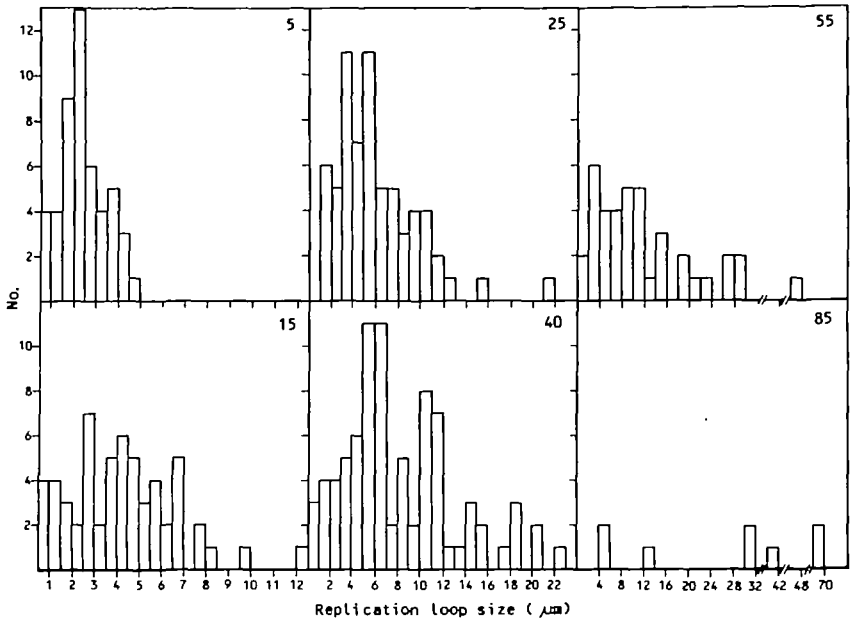


Figure 4. Replication loop size. The fork-fork distance within a replication loop was measured for a number of loops found at the different time points into S-phase, indicated in each frame.

corresponds well with the estimate of 3-5 x 10^5 daltons per min per replicon obtained from the sedimentation analysis.[4,6]

c. Clustering and replicon topology.

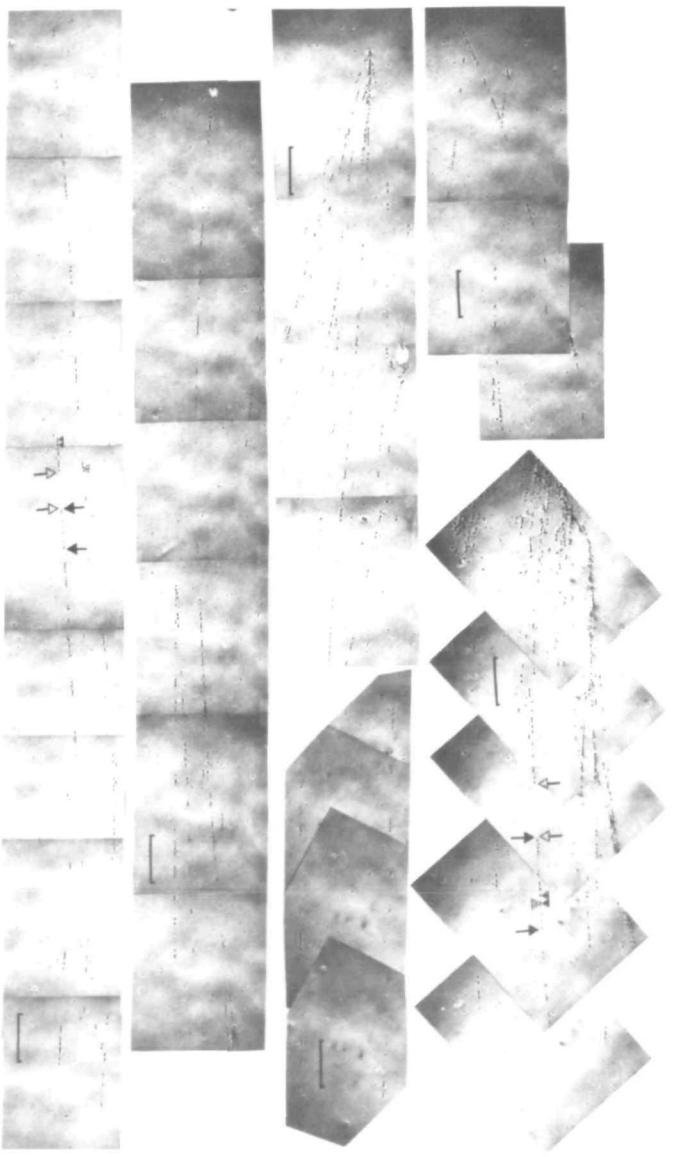
The molecules shown in Figure 1 and Figure 2 show clustering of replication loops, indicating that such clustering occurs at least some of the time, but due to the danger of technical bias against large and replicating molecules we did not make a quantitative analysis of the extent of clustering from EM analysis. For this purpose we chose to attempt autoradiographic analysis.

2. Autoradiography

As discussed above, electron microscopy could only reveal that temporal clusters of replicons do exist, but could not reveal the extent of such clustering. We therefore made autora-

diograms of DNA pulse labelled for 5 minutes at 3 time points during S-phase. Here, the emphasis of analysis is placed on the pulse given at 5 minutes into the S-phase, since at this time the first temporal set of replicons, exclusively, are expected to have initiated. This gives a clearer picture for analysis. In addition, pulses given at 60 minutes and 150 minutes have been considered. Examples of patterns observed at 5 minutes are shown in figures 5 and 6. Since previous estimates of replication rate is 0.2 μm per minute per fork (see discussion of EM data above, and Funderud et al. (1978)[4,6]), any two forks within a bidirectional replicon should not have moved more than 5-10 minutes, or 2-4 μm away from each other. Thus, the grain pattern from one replicon must be expected to occur in very short doublets or singlets (newly initiated), and this is used as basis for selection of patterns corresponding to individual replicons. The following parameters have been measured on a number of patterns, and average values as well as range, is discussed below.

i. Cluster size, defined as range of clustered grains, as shown in figure 5 and 6. ii. Intercluster distance, defined as range without grains occurring within a linear pattern, as shown in figures 5 and 6. iii. Center-center distances between doublets or singlets of grains within a cluster, judged to represent single replicons, and yielding the presumed origin-origin distance between neighbouring replicons, as shown in figures 5 and 6. iv. The distance between grains in doublets was also measured, as a rough estimate of replication rate. v. Finally, the number of replicons within a cluster was estimated by counting doublets or singlets occurring within a cluster. This analysis for the 5 min pulse experiment yielded an average cluster size of 42.2 μm, with a range of 23-83 μm. One particular difficulty with this measurement, as well as with the analysis of intercluster distance, was to estimate the cut off point when clusters appeared in tandem, as they often appear to do. The uncertainty of this measurement is compounded by the suggestion of Hori and Lark (1973)[8] and Funderud et al. (1978)[6] that temporal clusters may contain an occasional late replicating unit. Thus, the result of 42 μm



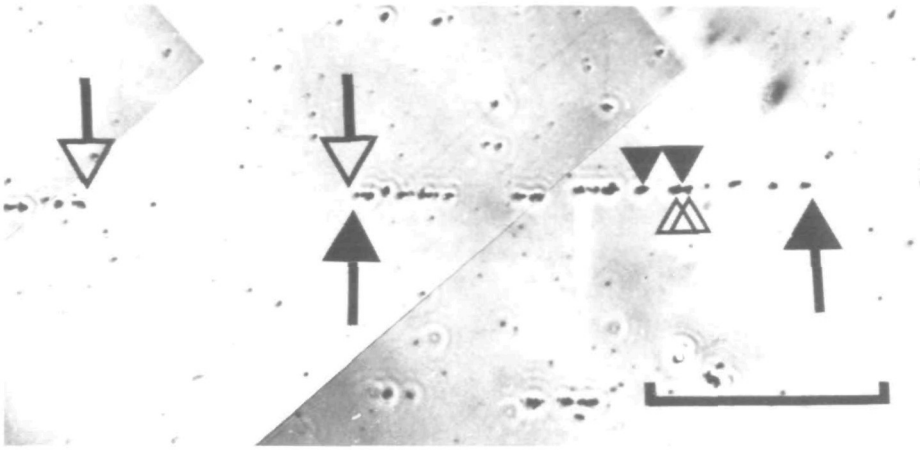


Figure 6. Autoradiograms of replicating DNA from beginning of S-phase. Enlarged details of Figure 5.

for a cluster size must be regarded as an absolute minimum. Indeed, identical measurements at 60 min give an average cluster size of about 90 µm with a range of 40-120 µm. The intercluster distance at 5 min appears to be an average of 44 µm with a range of 20-140 µm. Actually, the clusters of the temporally first replicons may themselves be clustered to some degree. The low intercluster distance of 44 µm may reflect

Figure 5. Autoradiograms of replicating DNA from beginning of S-phase. Synchronous plasmodia were pulse-labelled for 5 min at 5 min into S-phase, nuclei isolated and prepared for autoradiography as described in the text. Exposure: 5 months. Bars correspond to 50 µm in length.

↓ ↓ defines "cluster size" (range of grains occurring in tandem)

▽ ▽ defines "inter-cluster distance" (range without grains in a linear pattern).

▼ ▼ defines "origin-origin distance between replicons" (center-center distance between doublets or singlets of grains within a cluster).

▽ ▽ defines "rate" (distance between grains in doublets).

Symbols have been marked only on a few representative examples of each type to facilitate interpretation. Actual measurements were done in enlargements about 10 x the size of this figure.

this possibility rather than the true average separation of clusters over the entire genome. If we assume a chromatide length of 2300 μm and 10 sets of temporally isochronous replicons[6], then the average intercluster separation should be closer to 200 μm. The intercluster distance found at 60 min is about 200 μm. Center-center, or origin-origin distance, within clusters, should yield the average size of replicons. At 5 min the average value has been calculated to be 9.1 μm with a range of 3.7 - 13.4 μm. At 60 min the corresponding values are 14.5 and 3.7-40.3 μm respectively. These values are in good agreement with the electron microscopic observations in the present work, and by previous hydrodynamic measurements[4-6]. The rate of replication can only be very roughly estimated by measuring the grain-to-grain distances within doublets. At 5 min the average value is about 2 μm for a 5 min pulse giving a rate of 0.4 μm per min which is in good agreement with rates estimated from EM here, and sedimentation studies previously done.[6] Finally, the approximate number of replicons within a temporal cluster at 5 min was estimated by measuring number of doublets and singlets judged to be newly initiated replicons. The average value obtained was 4.1 with a range of 2-8. Here, patterns of only one grain doublet, which also occured, was not included. Measurements at 150 min have been largely excluded from this discussion since the patterns at this time, which is approaching the end of S-phase, are very diffuse and clear clusters or linear patterns were rarely observed. This, too, is in agreement with what one would expect, since at this time only scattered terminal synthesis can be expected to occur.

FINAL DISCUSSION

The present electron microscopic analysis suggests that the replicons of Physarum polycephalum have a size of around 10 μm, while autoradiographic analysis suggests a size of 9 μm and a somewhat larger size of 14 μm when tested later in S-phase. EM suggests a total rate of replication at 0.4 μm per min per replicon, or 0.2 μm per min per replication fork. Previous es-

timates from our hands based on alkaline sucrose gradient sedimentation analysis, gave the rate of size increase of replicons as 3-5 x 10^5 daltons per minute per replicon.[4-6] Taking into account the proven bidirectional synthesis[4] this gives a fork rate of approximately 2 x 10^5 daltons or 0.2 μm per minute, in good agreement with the present estimate from EM analysis. The estimates from the hydrodynamic measurements suggested a replicon size of 35-45 S, corresponding to 1.1-2.2 x 10^7 daltons or 11-22 μm. This is a little higher than the present result, yet within the same range. In the following we have compared these results with those from a selection of other organisms. In Saccharomyces, Newlon et al. (1974)[9] found two size classes of replication loops by electron microscopy, one at 15-20 μm and the other at 30-35 μm. They argued against the possibility that the larger size class resulted from fusion of the smaller ones.

In Drosophila, Blumenthal, Kriegstein and Hogness (1973)[10] by autoradiography found an average replicon size of 13.6 μm while Hand and Tamm (1974)[11] using autoradiographic analysis of bovine cells found a replicon size of around 17 μm. Huberman and Riggs (1968)[12] however, in their classic autoradiographic study of Chinese Hamster cells reported a range of 5-180 μm for replicon size. They did, however, suggest that these results could include overestimations. In general, a replicon size of 10 μm (9-14 μm) as reported in the present work, is in reasonable agreement with data from a number of other eucaryotes as discussed above, and reviewed by Edenberg and Huberman (1975).[13]

The present results from electron microscopic analysis suggest a rate of 0.2 μm per min per fork, on the assumption that replication is bidirectional.[4] Hand and Tamm (1973)[11] in mouse L cells estimated a fork rate of 0.3-0.35 μm per min; Housman and Huberman (1975)[14] in CHO cells found an initial rate of 0.2 μm, increasing to 0.6 μm per min at late times in S-phase. Stimac et al. (1977)[15], working with mouse, CHO and HeLa cells estimated fork progression to be 0.25 - 0.41 μm per min. All these workers used autoradiography, while Planck and Mueller (1977)[16] who used alkaline sucrose gradient analysis of UV photolysed BUdR substituted nascent DNA, suggested a rate of

0.15 - 0.43 μm per min.

Again, we conclude that the present and previous results on Physarum regarding the rate of fork progression are reasonable values also when compared with other organisms. The final and important question we want to discuss regards replicon topology and the degree of clustering. Previous results regarding size maturation, as measured by sedimentation in alkaline sucrose gradients, were interpreted by us to imply a degree of temporal clustering of replicons.[6] Specifically, these results suggested that 3-6 replicons of size 1.1 - 2.2 x 10^7 daltons (ss DNA) were initiated in a temporal cluster, finally being joined to a 6.6 x 10^7 daltons species of DNA as a specific intermediate. The present results from electron microscopy as well as autoradiography suggests that the common size of replicons may be closer to the lower limit, at 10 μm (10^7 d) single strand equivalent. Electron microscopy furthermore (see figure 1 and figure 2) show that temporal clustering do occur, and autoradiography gave convincing evidence that a high degree of topological order and temporal clustering occur in Physarum. Specifically the AR data show that an average of 4 replicons (range 2-8) of average size 10-14 μm occurs in temporal clusters and that these clusters again show some degree of clustering, appearantly being interspersed with later replicating region of size 44 μm. Furthermore, occasional nonlabeled regions within appearant replicon cluster, allow for the possibility of rare later initiating replicon within a temporal cluster that could explain delayed size maturation, such as found by Funderud et al. (1978)[6] and proposed by Hori and Lark (1974).[8] The present cluster size of 40-50 μm, as estimated from the AR studies, are lower than the previous estimate of around 60-70 μm based on alkaline sucrose gradient sedimentation. The reason for this is not known.

The evidence for temporal clustering in other organisms come mostly from AR studies. Huberman and Riggs (1968)[12] observed patterns that indicated a degree of clustering and Hori and Lark (1973)[8] found 4-5 replicons per cluster, in hamster cells. Convincing evidence for the occurrence of physical clustering of groups of isochronously initiating replicons have also been

provided by Hand (1975)[17], Kowalski and Cheevers (1976)[18] and a number of other workers. (For review, see Edenberg and Huberman, 1975).[13]

REFERENCES

1. Funderud, S. and Haugli, F. (1975) Nucleic Acids Research 2, 1381-1930.
2. Funderud, S. and Haugli, F. (1977) Nucleic Acids Research 4, 405-413.
3. Funderud, S. and Haugli, F. (1977) Biochem. Biophys. Res. Comm. 74, 941-948.
4. Funderud, S., Andreassen, R. and Haugli, F. (1978) Nuclei Acids Research 5, 713-721.
5. Funderud, S., Andreassen, R. and Haugli, F. (1978) Nuclei Acids Research 5, 3303-3313.
6. Funderud, S., Andreassen, R. and Haugli, F. (1978) Cell, Des. 1978.
7. Davis, R.W., Simon, M. and Davidson, N. (1971). Methods in Enzymology 21, 413-428.
8. Hori, T. and Lark, K.G. (1973) J. Mol. Biol. 77, 391-404.
9. Newlon, C.S., Petes, T.D., Hereford, L.V. and Fangman, W.L. (1974) Nature, 247, 32-35.
10. Blumenthal, A.B., Kriegstein, H.J. and Hogness, D.S. (1973) Cold Spring Harbor Symp. Quant. Biol. 38, 205-223.
11. Hand, R. and Tamm, I. (1974) J. Mol. Biol. 82, 175-183.
12. Huberman, J.A. and Riggs, A.D. (1968) J. Mol. Biol. 32, 327-341.
13. Edenberg, H.J. and Huberman, J.A. (1975) Ann. Rev. Genet. 9, 245-284.
14. Housman, D. and Huberman, J.A. (1975) J. Mol. Biol. 94, 173-181.
15. Stimac, E., Housman, D. and Huberman, J.A. (1977) J. Mol. Biol. 115, 485-511.
16. Planck, S.R. and Mueller, G.C. (1977) Biochemistry, 16, 1808-1813.
17. Hand, R. (1975) J. Cell Biol. 64, 89-97.
18. Kowalski, J. and Cheevers, W.P. (1976) J. Mol. Biol. 104, 603-615.
19. Zimm, B.H. and Schumaker, V.N. (1976) Biophys. Chem. 5, 265-270.
20. Mohberg, J. and Rusch, H.P. (1971) Exptl. Cell. Res. 66, 305-316.