Microbubbles in Replicating Nuclear Deoxyribonucleic Acid from *Physarum polycephalum*

By David A. F. GILLESPIE and Norman HARDMAN

Department of Biochemistry, Marischal College, University of Aberdeen, Aberdeen AB9 1AS, Scotland, U.K.

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Clusters of microbubbles, representing probable sites of newly initiated DNA synthesis, were identified in nuclear DNA from *Physarum polycephalum* by using the electron microscope. Their presence is associated specifically with S-phase. Each microbubble corresponds in size to a replicating segment of DNA about 100–5000 nucleotide residues in length. The DNA structures containing microbubbles are metastable, and revert to native DNA in the presence of moderate concentrations of formamide used to prepare samples for electron microscopy. It is suggested that each cluster of microbubbles may correspond to a unit of replication (a replicon) in *Physarum* DNA.

The duplication of eukaryotic chromosomes is, of necessity, a highly organized process (Hand, 1978). It is now well established that the replication of eukaryotic DNA occurs in an ordered manner, by phased initiation of numerous replication units, termed replicons, whose existence is revealed by radioactive pulse-labelling and DNA-fibre radioautography (Huberman & Riggs, 1968). In structural terms, a replicon is considered to consist of a segment of DNA containing a discrete origin of replication, from which DNA synthesis starts and proceeds bidirectionally. The boundaries of the replication unit are defined by the fusion of converging replication forks from adjacent replicons. Structures of this sort have been reported in a wide range of eukaryotic organisms, and they extend over segments of DNA 10–100 μm in length (Edenberg & Huberman, 1975). Replicons can thus be considered to be intermediate structural units for DNA replication, since their size is several orders of magnitude larger than the primary intermediate in DNA synthesis, the Okazaki fragment, but much smaller than the size of an individual eukaryotic chromosome.

The low resolution of DNA-fibre radioautography precludes an analysis of the fine structure of the replicon, or of the events that might lead to controlled initiation of DNA synthesis in the replication unit. Electron microscopy, which is capable of far greater resolution than fibre radioautography, has also been applied to the study of replicating DNA (Kriegstein & Hogness, 1973; Newlon *et al.*, 1974). In general, the results from both techniques are consistent. However, studies on replicating DNA in embryos from *Drosophila virilis* (Zakian, 1976) and the sea urchin (Baldari *et al.*, 1978; Kurek *et al.*, 1979) have revealed that, in addition to replicon-sized replication bubbles, smaller 'microbubbles' are

present with properties that suggest that a single replicon may be composed of a cluster of smaller replication units, each of which initiates DNA synthesis simultaneously.

Physarum polycephalum has proved to be a particularly interesting and useful system in which to investigate how the process of DNA replication is co-ordinated during S-phase (Funderud *et al.*, 1978), primarily because *Physarum* macroplasmodia exhibit natural mitotic synchrony. Replicons 10–15 μm in length have been identified and characterized in *Physarum* DNA in S-phase (Funderud *et al.*, 1979) by using both the techniques described above. In the present work, we have examined replicating DNA from *Physarum* using the electron microscope, and have been able to detect similar replicating structures to those described by Funderud *et al.* (1979). In addition, however, we also detect a novel type of DNA structure. These new structures contain microbubbles, in many respects similar to those observed in replicating DNA in other systems (Zakian, 1976). They are seen much more frequently in *Physarum* DNA in S-phase than other replicating structures, but in contrast with these other structures they appear to be metastable and disappear after incubation of S-phase DNA for a short period under partly denaturing conditions. We believe that these structures provide evidence that DNA synthesis in *Physarum* may be initiated simultaneously at several points within the same replicon.

Methods

Growth of *Physarum* cultures

Physarum polycephalum LU648 $\times$ LU688 (Cooke & Dee, 1975) was used in this work. Microplasmodia were grown as described previously (Hardman *et al.*,

1979a). In order to trace nuclear DNA through the isolation procedures, DNA was labelled in microplasmidia in the presence of $2\mu\text{Ci}$ of [*methyl*- ^3H]-thymidine (The Radiochemical Centre, Amersham, Bucks., U.K.)/ml of culture. For macroplasmoidal cultures, microplasmidia in exponential growth were harvested by centrifugation for 2–3 min at 50g, washed with sterile distilled water and resuspended in sterile water (1:1, v/v). A $300\mu\text{l}$ portion of this suspension was plated on to a 50mm-diam. Sartorius nitrocellulose filter ($0.45\mu\text{m}$ pore size). Plasmoidal fusion was allowed to take place by incubation in the dark for 1 h at 26°C , after which the culture was grown at the same temperature on the semi-defined medium used to grow microplasmidia (Hardman & Jack, 1977). Under these conditions the time between subsequent synchronous mitoses was 8 h. S-phase commenced 5–10 min after mitosis and was essentially complete at 2–3 h after mitosis.

Isolation of nuclei and extraction of DNA

The following methods were developed from the original work of Mohberg & Rusch (1971). Macroplasmidia were scraped from the filter used for growth and each stored at -20°C after freezing in acetone/ CO_2 . When required, a plasmodium was thawed, washed in 10 ml of homogenization buffer (0.25M-sucrose, 10mM- CaCl_2 , 0.25M- NaCl , 0.1% Triton X-100, 10mM-Tris/HCl, pH8.0) at 4°C . Homogenization was carried out by passage of the suspension ten times through a 21-gauge syringe needle. The homogenate was centrifuged at 4°C for 5 min at 50g, and the supernatant was then centrifuged at 4°C for 10 min at 1000g. The nuclear pellet obtained was washed three times in homogenization buffer at 4°C and recovered each time by centrifugation, as described above.

The washed nuclear pellet was resuspended in 0.8 ml of nuclear lysis buffer (0.25M- NaCl , 10mM-EDTA, 50mM-Tris/HCl, pH8.0) containing $50\mu\text{g}$ of ribonuclease A (Sigma; pre-boiled to inactivate deoxyribonuclease)/ml. Sarkosyl NL (Ciba-Geigy, U.K.) was added to a final concentration of 1% (v/v) and then incubation was carried out at 37°C for 30 min. Then $200\mu\text{g}$ of proteinase K (Sigma)/ml was added and incubation continued at 37°C for 4 h. Nuclear lysate (1 ml) was layered over a 3 ml CsCl step gradient consisting of equal volumes of two CsCl solutions in lysis buffer (refractive indices 1.405 and 1.395 respectively) in a 5 ml Beckman polyallomer centrifuge tube. Lysates were centrifuged in a SW50.1 rotor at 20°C for 3 h at 40000 rev./min. DNA was recovered from the interphase between the CsCl steps, and its concentration determined spectrophotometrically, assuming that $1\mu\text{g}$ of DNA/ml has $A_{260} = 0.02$.

Electron microscopy

Samples were prepared by diluting DNA directly from CsCl solution. Under these conditions CsCl did not appear to affect adversely DNA-sample preparation for electron microscopy, or the quality of micrographs. Microscopy was performed by using a Philips EM400 microscope, by a procedure described previously (Davis *et al.*, 1971; Hardman *et al.*, 1979a,b) with formamide hyperphase and hypophase. The hyperphase contained 40% (v/v) formamide, $30\mu\text{g}$ of cytochrome *c*/ml, 0.18M- NaCl , 10mM-disodium EDTA, 100mM-Tris/HCl, pH8.5. The hypophase contained a freshly prepared solution of 20% (v/v) formamide in 10mM-Tris/HCl, pH8.5. Immediately before spreading, DNA was diluted from the CsCl solution used for its preparation (1:100, v/v) into hyperphase solution, to give a final concentration of about 0.05M-CsCl and 0.5–1.2 μg of DNA/ml. The time that DNA was present in the hyperphase solution (Table 1) was measured immediately after DNA addition to the time required for monolayer formation on spreading. The minimum time for this operation was 20 s.

Specimens were rotary shadowed by using Pt/Pd, and the mass/unit length of duplexes and single-stranded DNA estimated as described previously (Hardman *et al.*, 1979b).

Results and Discussion

Conditions were devised in the present study to minimize the loss of nascent DNA fragments from the parental DNA template. These included the use of moderately high NaCl concentrations during nuclear isolation, lysis and DNA extraction, ensuring that DNA was not exposed to low ionic strengths, conditions that are believed to promote branch migration and consequent loss of nascent chains from replicating structures (Wanka *et al.*, 1977). Similar precautions were taken in the preparation of DNA for electron microscopy. It was considered necessary to use formamide solutions to prepare specimens for electron microscopy because of the additional structural information afforded by the application of this technique (Davis *et al.*, 1971), compared with the aqueous spreading method, in which single-stranded DNA segments form collapsed structures.

The DNA structures observed were classified according to whether they contained replication bubbles or other replicating structures. The results of a series of experiments are shown in Table 1. About 7–10% of the DNA molecules isolated from nuclei about 15 min into S-phase contained putative replicating regions. Of these, approx. 10–15% were structures with properties similar to those described previously in S-phase *Physarum* DNA by Funderud *et al.* (1979). These structures have been described

EXPLANATION OF PLATE I

Electron micrographs of S-phase Physarum nuclear DNA

Samples were prepared from DNA isolated from macroplasmodia 20 min after the third mitosis after plating (Table 1). Specimens were exposed for 20 s to 40% hyperphase solution during preparation for electron microscopy. Microbubbles containing duplex segments are shown (D), as are single-stranded microbubbles (S) and single-stranded tails (T). Bars indicate a length of 0.5 μ m.

Table 1. *Electron microscopy of Physarum DNA isolated at various stages during the mitotic cycle*

Interphase DNA and S-phase DNA were respectively prepared 3 h before, and 20 min after, the third mitosis which followed plating of macroplasmidia (see the Methods section). Mitosis was defined by microscopic examination of nuclei in samples of macroplasmidia. DNA was spread for observation in the electron microscope as indicated in the text. The times shown in the second column are those for which DNA was exposed to hyperphase solution during sample preparation. Conditions were as described in the text, except for one sample in which the formamide concentration was raised and the NaCl concentration lowered in the hyperphase. Values in parentheses refer to the percentage of the total scored molecules in a given sample.

Experimental sample	Conditions in hyperphase solution	No. of structures scored	Total no. of replicating structures	No. of micro-bubble-containing structures
S-phase	20s, 40% formamide	292	28 (9.6%)	24 (8.2%)
Interphase	20s, 40% formamide	400	None	None
Metaphase	20s, 40% formamide	400	None	None
S-phase	10min, 40% formamide	174	2 (1.2%)	None
S-phase	20min, 50% formamide/0.09 M-NaCl	200	1 (0.5%)	None

fully in that previous report, and so are not discussed further here. However, in contrast with that previous work, most of the putative replicating structures observed here were found to contain clusters of much smaller replication bubbles (Plate 1), similar to replicating DNA from certain other systems (Zakian, 1976; Baldari *et al.*, 1978; Kurek *et al.*, 1979). These novel structures in *Physarum* DNA are associated specifically with DNA isolated from S-phase nuclei, as shown in Table 1. DNA molecules prepared from interphase nuclei 3–4 h before mitosis, and from nuclei isolated at mitosis just before the onset of DNA replication, were devoid of these structural features.

During the early part of this study we encountered some difficulty in detecting structures containing microbubbles reproducibly. It transpired that this was due to the instability of these structures in the presence of formamide, as illustrated in Table 1. We were able to shorten the time of exposure of DNA to the formamide hyperphase to 20s, the time taken to add DNA to the hyperphase solution and form the monolayer. Under these conditions the number of microbubble-containing structures was reproducible. The structures are stable at least up to 44 h in CsCl solution, but are completely eliminated after 10 min in the hyperphase solution (Table 1). In contrast with structures containing microbubbles, the large replicating structures with replication bubbles and branches similar to those described by Funderud *et al.* (1979) were stable under all these conditions. This may offer a possible explanation for the ability of Funderud *et al.* (1979) to observe the latter structures in their preparations, but not the former structures, since they may have been unaware of the sensitivity of microbubble-containing structures to exposure to formamide. A considerable proportion of the microbubbles observed in structures after 20s in hyperphase solution contained entirely single-

stranded DNA. Further bubbles contained partly single-stranded regions and tails, and others entirely duplex segments of DNA (Plate 1). This suggests that possibly in some replicating regions nascent DNA chains may be removed from the parental template even under mildly denaturing conditions, well below the 'melting' temperature of native DNA. We cannot totally exclude branch migration, and consequent extrusion of nascent DNA chains from microbubbles, as a possible additional contributory explanation for the instability of microbubbles in the hyperphase solution. In any event, promotion of the removal of nascent DNA fragments by either mechanism could account readily for the properties of microbubble-containing structures. Some of these observations may also help to explain the differing degrees of success with which various authors have been able to detect microbubbles in replicating eukaryotic DNA (Baldari *et al.*, 1978; Kurek *et al.*, 1979).

The microbubbles in structures similar to those shown in Plate 1 varied in length from about 100 to 5000 nucleotide residues, and were arranged in clusters of 2–37 bubbles, with an average of 11 bubbles/cluster. The distances between adjacent microbubbles ranged from about 200 to 40000 nucleotides, longer lengths reflecting the separation of the nearest bubbles in separate clusters. The average separation of the microbubble clusters was seemingly bimodal, with peaks around 15000 and 25000 nucleotides. Occasional distances of up to 40000 nucleotides were observed. The length of DNA occupied by a single cluster of microbubbles was 16000 nucleotide pairs, on average, so that the separation of the centres of adjacent clusters is in the range 31000–56000 nucleotides, or 10–18.2 μ m. These estimates provide a similar range of values to those obtained by Funderud *et al.* (1979) for the centre-to-centre distance of the much larger replication loops

observed in *Physarum* DNA at the same stage in S-phase. This is consistent with our belief that structures containing clusters of microbubbles may represent replicons in the early stages of replication, in which initiation of DNA synthesis has occurred at about the same time at several distinct points in close proximity along the DNA. The high frequency with which these structures are found compared with other putative replicating structures, under appropriate conditions, suggests that multiple initiation of DNA synthesis within a replicon may be a general feature of the mechanism of DNA replication in *Physarum*, as in certain other species. These findings raise the possibility of quite a different interpretation of the nature of the mechanism of DNA chain elongation in a replicon in *Physarum*. It will be of interest, for example, to consider the factors that may potentiate multiple initiation of DNA synthesis within a single replication unit, and to investigate whether DNA sequences themselves, or some other structural feature of chromatin, are directly involved.

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References

- Baldari, C. T., Amaldi, F. & Buongiorno-Nardelli, M. (1978) *Cell* **15**, 1095–1107
- Cooke, D. J. & Dee, J. (1975) *Genet. Res.* **15**, 35–41
- Davis, R., Simon, M. & Davidson, N. (1971) *Methods Enzymol.* **21**, 413–428
- Edenberg, H. J. & Huberman, J. A. (1975) *Annu. Rev. Genet.* **9**, 245–284
- Funderud, S., Andreassen, R. & Haugli, F. (1978) *Cell* **15**, 1519–1526
- Funderud, S., Andreassen, R. & Haugli, F. (1979) *Nucleic Acids Res.* **6**, 1417–1431
- Hand, R. (1978) *Cell* **15**, 317–325
- Hardman, N. & Jack, P. L. (1977) *Eur. J. Biochem.* **74**, 275–283
- Hardman, N., Jack, P. L., Brown, A. J. P. & McLachlan, A. (1979a) *Eur. J. Biochem.* **94**, 179–187
- Hardman, N., Jack, P. L., Brown, A. J. P. & McLachlan, A. (1979b) *Biochim. Biophys. Acta* **562**, 365–376
- Huberman, J. A. & Riggs, A. D. (1968) *J. Mol. Biol.* **32**, 327–341
- Kriegstein, H. J. & Hogness, D. S. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 135–139
- Kurek, M. P., Billig, D. & Stambrook, P. (1979) *J. Cell Biol.* **81**, 698–703
- Mohberg, J. & Rusch, H. P. (1971) *Exp. Cell Res.* **66**, 305–316
- Newlon, C. S., Petes, T. D., Hereford, L. V. & Fangman, W. L. (1974) *Nature (London)* **247**, 32–35
- Wanka, F., Brouns, P. M. G. M. E., Aelen, J. M. A. & Eygen-Stein, J. (1977) *Nucleic Acids Res.* **4**, 2083–2097
- Zakian, V. A. (1976) *J. Mol. Biol.* **108**, 305–331