EVIDENCE FOR THE ATTACHMENT OF RNA TO PULSE-LABELED DNA IN THE SLIME MOLD, PHYSARUM POLYCEPHALUM

M.Anwar Waqar and Joel A. Huberman

Department of Biology, Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Received January 17,1973

SUMMARY

When Physarum polycephalum is pulse-labeled for up to 20 minutes with ^{3}H-thymidine and the shortest labeled DNA strands are partially purified by sedimentation through a neutral aqueous sucrose gradient and then through a formamide-sucrose gradient, these short strands band in Cs_2SO_4 isopycnic density gradients at a density greater than that of bulk single-stranded DNA. Their density is brought partially or nearly completely back to that of single-stranded DNA by hydrolysis with pancreatic RNase A or alkali, respectively. Therefore the dense material attached to the short pulse-labeled DNA strands consists at least partially of RNA.

Okazaki and co-workers (1-6) have done much to clarify the mechanism of DNA replication in Escherichia coli and certain other prokaryotes. They have demonstrated that a radioactive DNA precursor (^{3}H-thymidine) is first incorporated into relatively short DNA strands (about 1000 nucleotides long, 2) and that the radioactivity is subsequently found in the much longer DNA strands characteristic of fully replicated DNA (1-3). Recently Sugino, Hirose and Okazaki have shown that some of the shorter pulse-labeled DNA strands contain covalently linked RNA (6).

Because RNA polymerases can initiate synthesis of new strands in vitro (7) while DNA polymerases have not yet shown evidence of such an ability, an attractive possibility for the role of the RNA detected by Sugino, Hirose and Okazaki (6) is that of primer for the synthesis of new DNA strands. Kornberg and co-workers (8-10) have demonstrated a need for RNA priming during the in vitro and in vivo conversion of phage M13 or ϕX174 single-stranded DNA molecules to double-stranded replicating forms.

There are several suggestions that RNA may also serve a priming function in eukaryotic DNA replication. The reverse transcriptase of eukaryotic tumor viruses initiates DNA synthesis with an RNA primer (11). Keller (12) and Chang and Bollum (13) have demonstrated the capability of mammalian DNA polymerases to use RNA as a primer in vitro, and Keller has pointed out that the abundant RNase H (14) of eukaryotic cells might serve to hydrolyze an RNA primer during DNA synthesis (12). Very recently, Sato et al (15) have obtain-

ed evidence for attachment of RNA to nascent DNA in Ehrlich ascites tumor cells

We have chosen to use the slime mold, Physarum polycephalum, as a test system in our investigation of the role of RNA synthesis during DNA synthesis in eukaryotic organisms. Physarum can grow as a syncytial plasmodium which can reach a diameter of several centimeters and contain over 10^8 nuclei within a single cytoplasm (12). These nuclei go through mitosis and DNA replication in synchrony. Such natural synchrony is a big advantage in studies of DNA replication.

In this paper we report our finding that a portion of the nascent DNA of Physarum polycephalum is, like that of E. coli (6) and Ehrlich ascites tumor cells (15), attached to RNA.

MATERIALS AND METHODS

Physarum polycephalum, CLM1 strain, was generously provided by C.E. Holt.

Methyl-^{3}H-thymidine (56 Ci/m mole) and ^{14}C-thymidine (51.6mCi/m mole) were obtained from New England Nuclear Corp.

Pancreatic RNaseA was purchased from Worthington Biochemical Corp. and was used after heating for 10 min at 90°C in 10 mM EDTA, 0.1M NaCl, PH 7.0.

^{14}C-labeled HeLa cell ribosomal RNA was a gift of Martin Hirsh and ^{14}C-labeled E. coli tRNA was a gift of Dennis Lynch.

Physarum microplasmodia were grown in shaker culture medium and macroplasmodia were formed by fusion of microplasmodia and then grown on filter papers supported above culture medium by glass beads, as described by Holt and Gurney (17). Other replacement or alterations of chemicals in the medium was as described by Horwitz & Holt (18). The macroplasmodia were uniformly labeled with ^{14}C-thymidine (0.3μCi/15 ml medium) for 32 to 38 hours after fusion. Pulse labeling was carried out during the third postfusion S phase. 5-fluorodeoxyuridine was added to a final concentration of 3μg/ml for 5 minutes before labeling with ^{3}H-thymidine. For pulse labeling with ^{3}H-thymidine, the plasmodium on the filter paper was blotted on another filter paper and then cut into sections which were floated on drops (1 ml) of culture medium containing ^{3}H-thymidine (150μCi/ml) and 5-fluorodeoxyuridine (3μg/ml) at 20°C. The pulses were terminated and the plasmodia were lysed by modification of the method of Sonenshein & Holt (19). The plasmodia were immediately scraped with a blunt scalpel into 1.5 ml ice-cold 0.1M EDTA, 0.1M tris, PH 8.1. An equal volume of 15% (v/v) Sarkosyl NL30 (Geigy Chemicals) was added. The lysates, in test tubes, were rotated for 45 minutes at 60 r.p.m., around a circle of 20 cm diameter in a vertical plane, in the cold room at 2-4°C.

Lysates were centrifuged through neutral sucrose density gradients (33ml of 5-20% sucrose in 2 mM EDTA, 5mM potassium phosphate, PH 7.5) in polyallomer tubes for 2.5 hours at 5°C and 20,000 r.p.m. in the Spinco SW 27 rotor. 1.2 ml fractions were collected from the top of the tube. 0.1 ml aliquots were dried onto filter paper discs (#895-E, Schleicher & Schuell, Inc., Keene, N.H.), followed by a 1 hour wash in cold 10% TCA and 5 one hour washes in cold 5% TCA and finally by 2 five minute washes with 95% ethanol. The filter discs were then dried and counted in a scintillation counter. Pellets were resuspended in 1.2 ml of 99% formamide and counted as above.

Selected fractions (indicated in figures) from the neutral sucrose gradients were pooled, brought to 90% formamide, heated at 37°C for 30 minutes (6), concentrated by evaporation under vacuum, and then centrifuged through 0-15% sucrose gradients in 99% formamide at 25°C and 25,000 r.p.m.

for 20 hours in the SW 27 rotor. 1.2 ml fractions were collected and 0.1 ml aliquots were taken onto filter discs which were washed and counted as above. Again selected fractions were pooled, dialyzed at 0-4°C against 10mM Tris, 1mM EDTA, PH7.4, for 18 hours, and then centrifuged in Cs_2SO_4 equilibrium density gradients (6). Cs_2SO_4 gradients were centrifuged in a Beckman SW 50L rotor for 48 hours at 36,000 r.p.m. and 15°C. Ten-drop fractions were collected directly onto filter discs through the needle by puncturing at the bottom of the tube. Drying, washing of the discs with TCA and counting were as described above.

Pancreatic RNase A digestion was carried out at 37°C for 3 hours in 1.0M NaCl, 0.1M EDTA,pH 7.0,at a RNase A concentration of 1 mg/ml.

The KOH digestion was in 0.3M KOH at 37°C for 18 hours. KOH was removed by dialysis against the appropriate buffer before running the Cs_2SO_4 equilibrium density gradients.

RESULTS

For preparation of short strand intermediates in DNA replication, we sedimented pulse-labeled cell lysates through two different kinds of sucrose gradients. The lysates were first analyzed on neutral aqueous sucrose gradients with the results shown in Figure 1. Bulk DNA (labeled for several generations with ^{14}C-thymidine) sediments both into a pellet at the bottom of the tube and in a broad peak near the top of the tube. The proportion of material in the top peak was roughly 50% for plasmodia in S phase (as in Fig.1). When cells in S phase are pulse-labeled with ^{3}H-thymidine for varying lengths of time (Fig.1), the ^{3}H label is always found both in the pellet and in the top fractions of neutral sucrose gradients. The proportion of ^{3}H label in the top fractions is invariably somewhat less than the proportion of ^{14}C in the top fractions as might be expected from the fact that the ^{14}C label in the top fractions includes some mitochondrial and nuclear satellite DNA, which replicate in G2 as well as S phase (19).

Note that the ^{3}H label at the top of the neutral sucrose gradients sediments more slowly than the ^{14}C label after a short pulse (30 seconds-Fig.1a), implying that this ^{3}H label is in separate structures from the bulk DNA.It is this slowly-sedimenting ^{3}H-labeled DNA which, as we shall describe below, contains attached RNA.

Fractions containing the ^{3}H label at the top of the neutral sucrose gradients (indicated in Fig. 1) were pooled, denatured by incubation in 90% formamide at 37°C for 30 minutes, and then sedimented through 0-15% sucrose gradients in 99% formamide. Under these conditions DNA is totally denatured (20) so that sedimentation should be a true measure of single strand length for linear strands. In addition, since alkali is not used, RNA is not hydrolyzed.

The data of Figure 2 show that the ^{14}C-labeled DNA pooled from the top of the neutral sucrose gradients consists of single strands which sediment heterogeneously, with two peaks near the top of the formamide-sucrose grad-

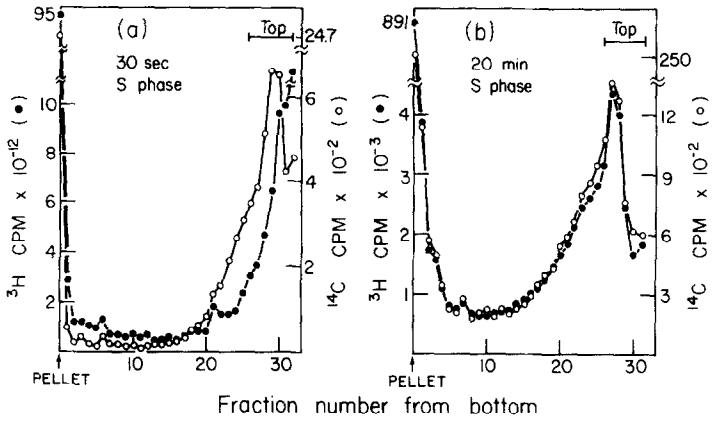

Fig. 1. Sedimentation of pulse-labeled Physarum DNA through neutral sucrose gradients. Pulse-labeling, lysis, and sedimentation were as described in Materials and Methods.
The ^{3}H-thymidine pulse was in early S phase for (a) 30 seconds, and (b) 20 minutes. The indicated top fractions were separately pooled for further analysis The recovery of ^{14}C label was about 100%, and the recovery of ^{3}H label was about 99%.

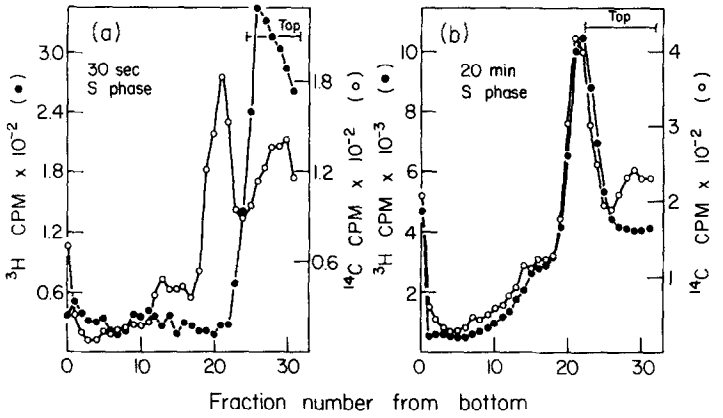

Fig. 2. Sedimentation of pooled top fractions from Fig. 1 through formamide-sucrose gradients. The procedure is described in Materials and Methods. The ^{3}H-thymidine pulse was in early S phase for (a) 30 seconds and (b) 20 minutes. The indicated top fractions were pooled for analysis in isopycnic gradients. The recovery of ^{14}C label was about 92%, and the recovery of ^{3}H label was about 100%.

ient. On the other hand, the ^{3}H-labeled DNA from a 30 second pulse (Fig. 2a) sediments entirely as very short strands, at about the same position as the slower of the ^{14}C peaks. When the pulse time is extended to 20 minutes (Fig.2b) the ^{3}H-labeled DNA sediments more rapidly, at approximately the position of the faster ^{14}C peak. Preliminary characterization of these formamide-sucrose gradients shows that tRNA sediments with the same speed as the peak of ^{3}H-DNA pulse-labeled for 30 seconds.

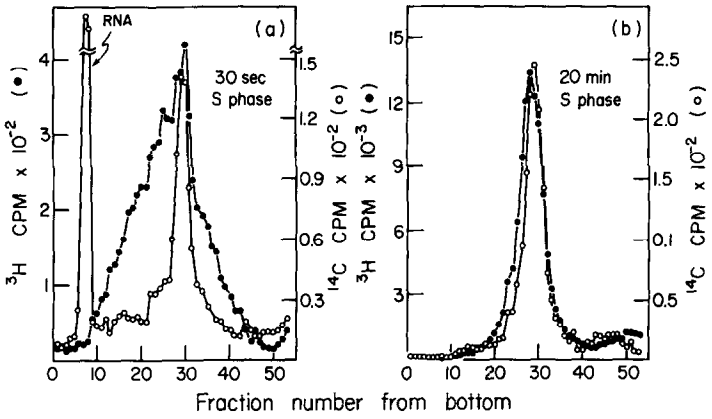

Fig. 3. Isopycnic centrifugation of slowly-sedimenting pulse-labeled DNA chains. The indicated fractions from the formamide-sucrose gradients shown in Fig. 2 were pooled, dialyzed to remove formamide, and centrifuged in Cs_2SO_4 gradients as in Materials and Methods. In (a) marker ^{14}C-labeled HeLa cell ribosomal RNA was added. The ^{14}C marker for the density of single-stranded DNA is ^{14}C overnight-labeled Physarum DNA (denatured by formamide). (a) 30 second pulse, from Fig. 2a, (b) 20 minute pulse, from Fig. 2b. The recovery of ^{14}C label was about 100% and the recovery of ^{3}H label was about 88%.

In order to test for possible attached RNA, the slowly sedimenting material from the formamide-sucrose gradients (fractions indicated in Fig.2) was pooled, dialyzed, and then centrifuged in isopycnic Cs_2SO_4 gradients along with added ^{14}C-labeled HeLa cell ribosomal RNA and ^{14}C-labeled single-stranded Physarum DNA. As shown in Fig. 3, the density of a portion of the ^{3}H-thymidine labeled DNA was greater than that of the marker DNA, the difference in density being much more pronounced for the material labeled for 30 seconds (Fig. 3a) than for the material labeled for 20 minutes (Fig. 3b). This density difference could be partially abolished by pretreatment of the ^{3}H-thymidine labeled DNA strands with agents capable of hydrolyzing RNA. As shown in Fig. 4a and b, alkaline hydrolysis resulted in nearly complete loss of detectable density difference. On the other hand, treatment with pancreatic RNase A (Fig. 4c) caused only partial loss of density difference under conditions where the marker ribosomal RNA was completely degraded.

These facts suggest that the material causing the density shift includes RNA. If it is only RNA, one can calculate from the position of the ^{3}H label between the RNA and DNA markers in Fig. 3a. that some of these short chains contain more RNA than DNA!

DISCUSSION

Our results show that a portion of the DNA of Physarum polycephalum which is pulse-labeled with ^{3}H-thymidine contains attached material which gives this DNA a higher buoyant density than bulk single-stranded DNA. The

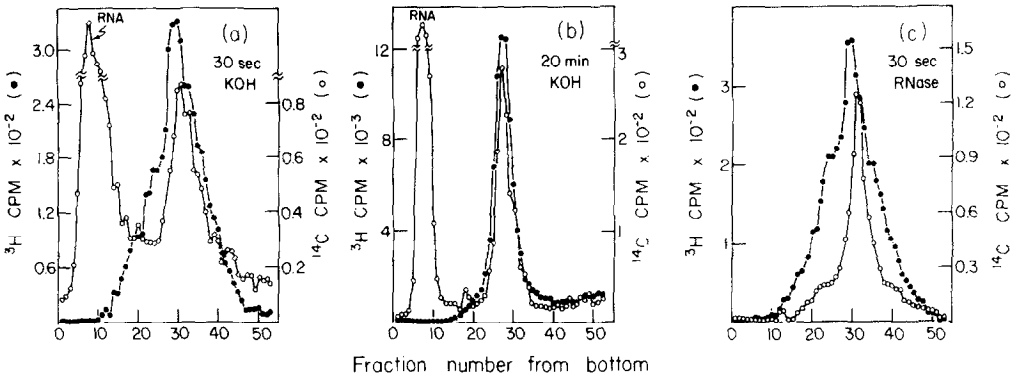

Fig. 4. Effect of hydrolysis by KOH or RNase A on the density of pulse-labeled DNA chains. Hydrolyses were carried out as described in Materials and Methods. Conditions for Cs_2SO_4 centrifugation were like those of Fig. 3. (a) 30 second pulse from Fig. 2a, hydrolysis by KOH; (b) 20 minute pulse from Fig. 2b, hydrolysis by KOH, (c) 30 second pulse from Fig. 2a, hydrolysis by RNase A. ^{14}C labeled HeLa ribosomal RNA was added to (a) and (b) after hydrolysis, but was added to (c) before hydrolysis.

attached material is almost completely sensitive to alkaline hydrolysis and is partially sensitive to hydrolysis by pancreatic RNase A. Therefore the material is very likely either to consist entirely of RNA or to depend on RNA for its attachment to the pulse-labeled DNA. The fact that the putative RNA is only partially sensitive to RNase A might be explained by high purine content, by configurational restrictions, or by the presence of an RNA-DNA double-stranded hybrid. The last possiblity seems unlikely because the ^{3}H-labeled material was first denatured in formamide.

Further experiments are in progress in our laboratory to clarify the nature of the attached material and the means of its attachment to the pulse-labeled DNA. These experiments will help determine whether the attached RNA is acting as a primer for DNA synthesis. We are also investigating the relationship between the short DNA strands detected in a 30 second pulse (Fig. 2b) and the bulk replicated DNA. Preliminary results suggest that ^{3}H label is first found in short strands and then moves into the longer strands of bulk DNA, in a manner analogous to the findings of Okazaki and co-workers (1-3) for prokaryotes.

The sedimentation behavior of the bulk ^{14}C-labeled DNA in our experiments is puzzling. About half the bulk DNA sediments to the pellet in a neutral sucrose gradient (Fig. 1). Sedimentation analysis of this pelleted material in formamide-sucrose gradients shows that most of the strands are long enough to sediment into the bottom half of gradients such as those in Fig.2 (Waqar and Huberman, unpublished results). Yet the other half of the

bulk DNA sediments very slowly in neutral sucrose gradients (Fig.1) and shows much shorter single strands when analyzed in formamide-sucrose gradients (Fig.2). The reason for such a large difference in size between these two halves of the bulk DNA is not clear and is the subject of further investigation.

ACKNOWLEDGEMENTS

This work could not have been carried out without the advice, assistance and facilities of C.E. Holt. We are also grateful to David Botstein, Henry Horwitz, David Housman and Robert Minkoff for discussions. The work was supported by grants from the National Science Foundation and the National Institutes of Health.

REFERENCES

1. Sakabe,K. & Okazaki, R., Biochim. Biophys. Acta, 129,651 (1966).
2. Okazaki, R.,Okazaki, T.,Sakabe, K., Sugimoto, K., Kainuma, R., Sugino,A., & Iwatsuki, N., Cold Spring Harbor Symp. Quant. Biol., 33, 129 (1968).
3. Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., & Sugino, A., Proc. Natl. Acad. Sci. USA, 59, 598 (1968).
4. Okazaki, T. & Okazaki, R., Proc. Natl. Acad. Sci. USA, 64, 1242 (1969).
5. Sugino, A. & Okazaki. R., J.Mol. Biol., 64, 61 (1972).
6. Sugino, A., Hirose, S., & Okazaki, R., Proc. Natl. Acad. Sci. USA, 69, 1863 (1972).
7. Maitra, U. & Hurwitz, J., Proc. Natl. Acad. Sci. USA, 54, 815 (1965).
8. Brutlag, D., Schekman, R., & Kornberg, A., Proc. Natl. Acad. Sci. USA, 68, 2826 (1971).
9. Wickner, W., Brutlag, D., Schekman, R., & Kornberg, A., Proc. Natl. Acad. Sci. USA, 69 , 965 (1972).
10. Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L.L. & Kornberg, A., Proc. Natl. Acad. Sci. USA, 69, 2691(1972).
11. Verma, I.M., Meuth, N.L., Bromfeld, E., Mauly, K.F., & Baltimore, D., Nature New Biol., 233, 131 (1971).
12. Keller, W., Proc. Natl. Acad. Sci. USA 69, 1560 (1972).
13. Chang, L.M.S. & Bollum, F.J., Biochem. Biophys. Res. Commun. 46, 1354 (1972).
14. Hausen, D. & Stein,H., Eur. J. Biochem. 14, 278 (1970).
15. Sato, S., Ariake, S., Saito, M., & Sugimura, T., Biochem. Biophys. Res. Commun. 49, 827 (1972).
16. Rusch, H.P. in "Advances in Cell Biology", Vol 1.,ed. D.M. Prescott, L. Goldstein, E.McConkey, pp. 297-327, Appleton-Century-Crofts, New York (1970).
17. Holt, C.E. & Gurney, E.G., J. Cell Biol. 40, 484 (1969).
18. Horwitz, H.B. & Holt, C.E., J. Cell Biol. 49, 546 (1971).
19. Sonenshein, G.E.& Holt, C.E., Biochem. Biophys. Res. Commun. 33, 361 (1968).
20. Marmur, J. & Ts'o, P.O.P., Biochim. Biophys. Acta, 51, 32 (1961).