PROPERTIES OF A DNA LIGASE MUTANT OF ESCHERICHIA COLI.

II. INTERMEDIATES IN DNA REPLICATION

Crellin Pauling and Lyla Hamm

Department of Life Sciences
University of California
Riverside, California 92502

Received October 23, 1969

Summary

A fast-renaturing component has been recovered from DNA replicated under restrictive conditons in a DNA ligase-deficient mutant of Escherichia coli. This component exists as an intermediate during DNA replication, and may represent the forked molecule proposed in the model of Kornberg.

Introduction

The replication of bacterial DNA occurs in a semi-conservative mode[1], and is generally considered to be initiated at a unique site, and to proceed in a linear manner along the length of the chromosome[2]. However, the detailed mechanism of chain elongation remains poorly understood. Recent evidence[3,4,5,6,7,8] has suggested that chain elongation proceeds in a discontinuous manner, in that the DNA strands are synthesized as fragments that are subsequently joined together through the action of the enzyme DNA ligase[9,10]. A model has been proposed[11,12] in which DNA replication proceeds for some distance in the 5' to 3' direction along one template strand, and then switches to the complementary template strand, and continues in the 5' to 3' direction. Thus the newly-replicated fragment of DNA exists for a short period as a fork; the fork is subsequently cleaved by an endonuclease. This communication reports in vivo evidence in support of such a forked molecule as an intermediate in DNA replication.

Materials and Methods

Escherichia coli ts-7[13], a temperature-sensitive, radiation-sensitive derivative of E. coli TAU-bar[14], was cultured aerobically in a glucose-salts

medium with appropriate supplements as previously described[15]. Media changes were accomplished by centrifugation.

E. coli ts-7 cells were grown at 25°C to a density of 2 x 10^8 cells/ml. The cells were harvested and resuspended in 1/5 vol growth medium lacking thymine and prewarmed to 40°C. Following 1 min growth in the fresh medium, 5 mc H^3-thymine (New England Nuclear, specific activity 18.2 c/mM) was added, and growth continued for 7.5 min. Growth was terminated by the addition of ice-cold NET[16] buffer supplemented with 10^{-3} M KCN. The cells were harvested and lysates were prepared as previously described[8]. The lysate was layered directly onto 5 to 20 percent linear sucrose density gradients, and sedimented for 6 hr at 27,000 rpm in an SW27 rotor of a Beckman L2-50 ultracentrifuge. Fractions were collected from the bottom of the centrifuge tube.

Pooled fractions selected from the sucrose density gradient were denatured by heating to 100°C for 10 min followed by rapid cooling. The denatured DNA so obtained was applied to a hydroxylapatite (Calbiochem) column, and eluted with a 0.03 M to 0.35 M linear gradient of phosphate buffer, pH 6.8[17].

Pooled fractions selected from the first hydroxylapatite columns were again denatured by boiling, and again fractionated by hydroxylapatite column chromatography.

Radioisotope assays of acid-precipitable material were performed as previously described[18].

Results

The profiles obtained by sedimentation of pulse-labeled DNA through neutral and alkaline sucrose density gradients are presented in Figure 1. Under neutral conditions the isotope incorporated during a 7.5 min pulse sediments in two major fractions. The majority of the isotope sediments rapidly; this is the pattern observed for the sedimentation of random-labeled DNA under these conditions. A second fraction, however, is slowly-sedimenting; this fraction represents the most newly-synthesized DNA, as it

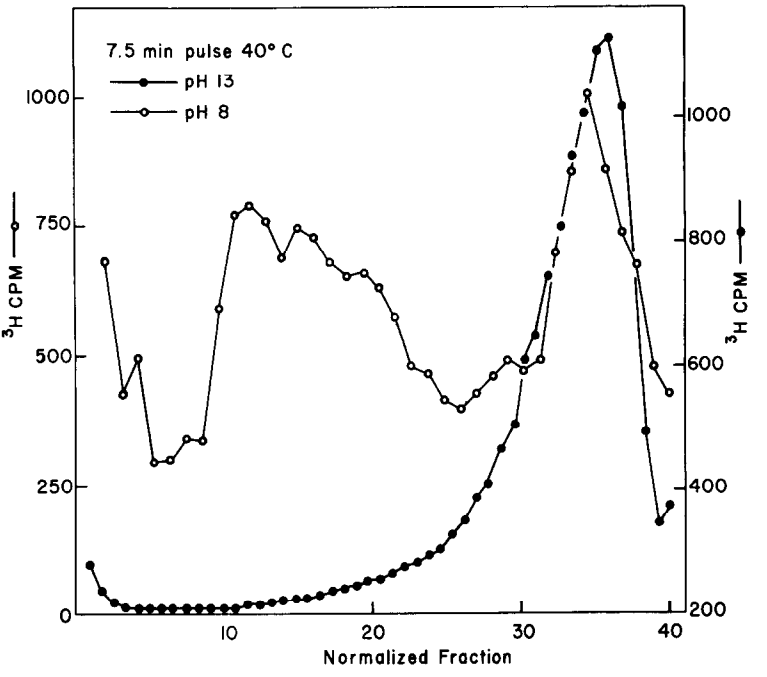

Figure 1. Sedimentation of pulse-labeled DNA. A culture of ts-7 was grown, pulse labeled, and a lysate prepared as described (see Methods). Fractions of the lysate were sedimented through neutral (open circles) and alkaline (closed circles) sucrose density gradients.

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does not accumulate with time, and upon a chase with unlabeled thymine the isotope is transferred to the rapidly-sedimenting fraction. Under alkaline conditions all of the isotope incorporated during a 7.5 min pulse is recovered as slowly-sedimenting material. We interpret these data as indicating that in this strain, ts-7, H^3-thymine incorporated during pulse-labeling at the restrictive temperature is recovered only as Okazaki fragments of newly-replicated DNA[3,4,5,6,7]. A complete discussion of these sedimentation experiments will be presented elsewhere.

Rapidly-sedimenting material from fractions 11 through 17 of the pH 8 sucrose gradient were pooled, heat denatured, and chromatographed through a hydroxylapatite column as described above. The elution profile is presented in Figure 2 (top). The majority of the counts elute as single-stranded DNA[17]; 6 percent of the counts elute in a tail at the region where double-stranded DNA elutes.

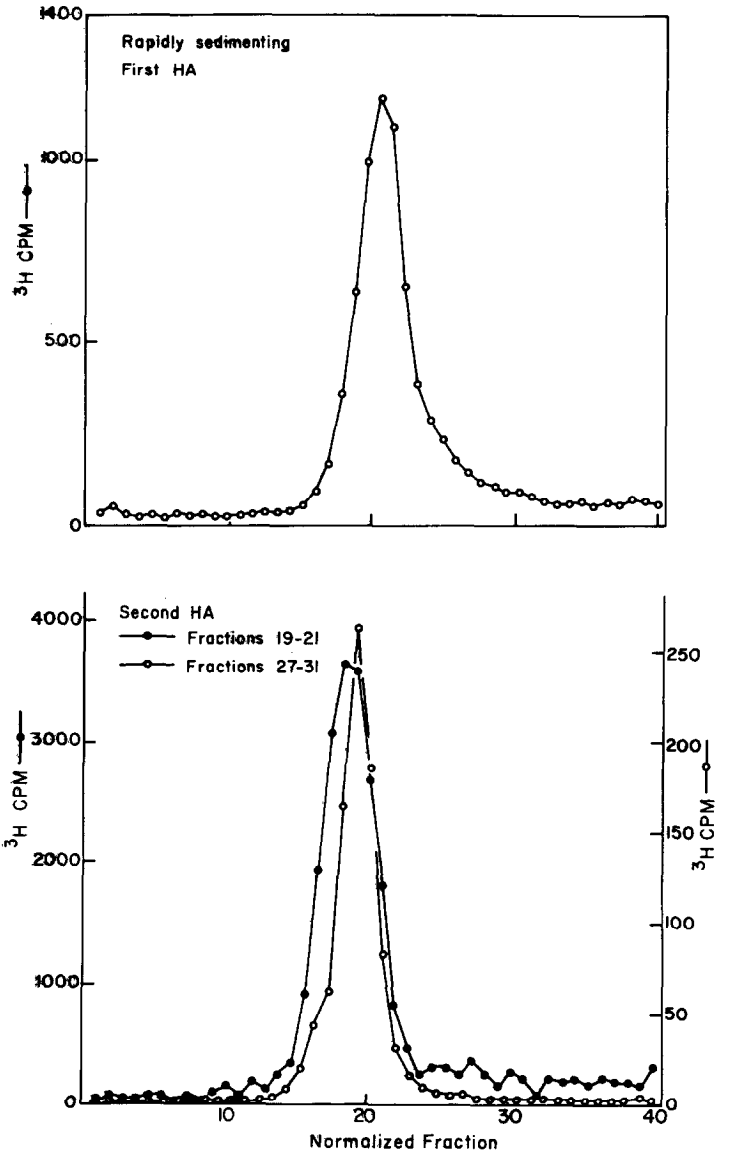

Figure 2. Hydroxylapatite column chromatography of rapidly-sedimenting DNA. See text for details.

Fractions 19 through 21, representing single-stranded DNA, and fractions 27 through 31, representing double-stranded DNA, were pooled, denatured, and chromatographed again. The results are presented in Figure 2 (bottom). The material that eluted from the first hydroxylapatite column as single-stranded

DNA again elutes as single-stranded DNA (closed circles), and the small amount of material that eluted from the first hydroxylapatite column as double-stranded DNA elutes from the second hydroxylapatite column as single-stranded DNA as well (open circles).

Slowly-sedimenting material from fractions 33 through 36 of the pH 8 sucrose gradient were pooled, heat-denatured, and chromatographed through

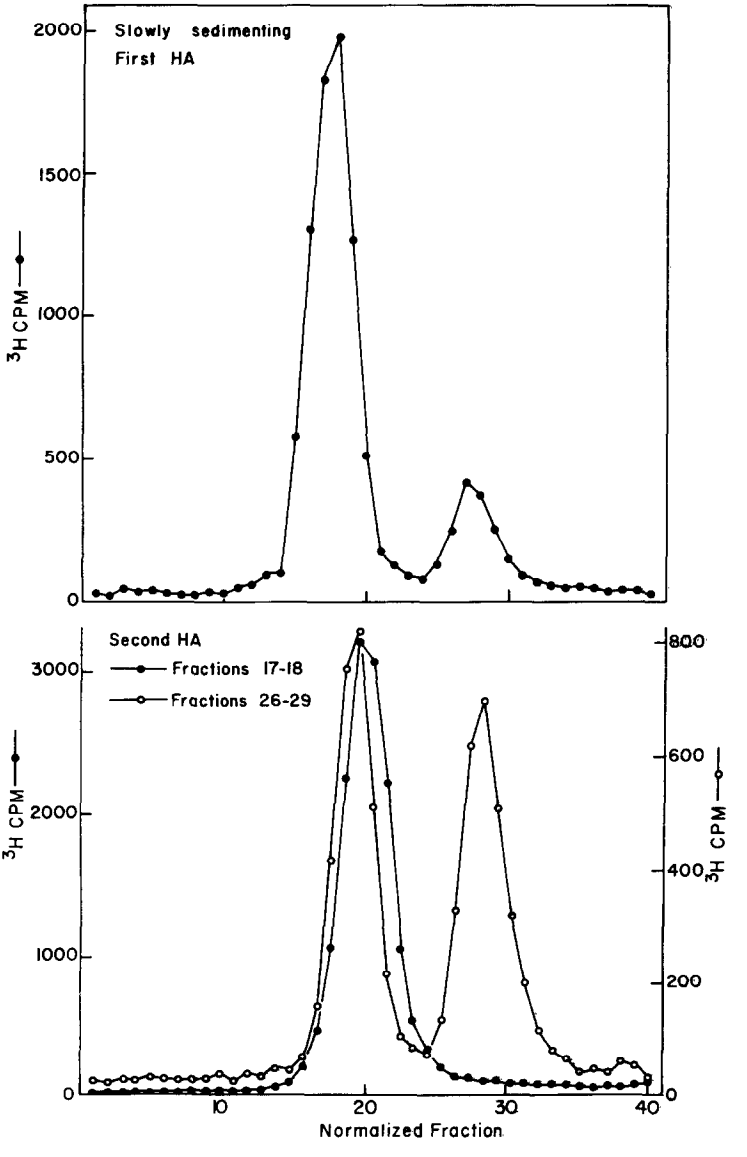

Figure 3. Hydroxylapatite column chromatography of slowly-sedimenting DNA. See text for details.

hydroxylapatite. The elution pattern is presented in Figure 3 (top). In this case 17 percent of the counts elute as double-stranded DNA, and the remaining elute as single-stranded DNA. Fractions 17 and 18, representing single-stranded DNA, and fractions 26 through 29, representing double-stranded DNA, were pooled, denatured, and chromatographed again; the elution patterns are presented in Figure 3 (bottom). The material that eluted from the first hydroxylapatite column as single-stranded DNA again elutes as single-stranded DNA (closed circles); however, the material that eluted as double-stranded DNA now elutes half as single-stranded, and half remains as double-stranded DNA (open circles).

Discussion

We have obtained evidence, to be presented elsewhere, that DNA replicated at the restrictive temperature in a DNA ligase-deficient mutation of *E. coli* can be recovered in a unique state, as the most newly-replicated DNA is recovered as slowly-sedimenting material under non-denaturing conditions. We have presented evidence in this paper that a significant fraction of this slowly-sedimenting material elutes from hydroxylapatite columns as double-stranded DNA after repeated heat-denaturation. We interpret this observation as evidence that this fraction of newly-replicated DNA is in a form that rapidly renatures following heat-denaturation. We have not detected fast-renaturing material from the rapidly-sedimenting fraction.

We interpret these results as evidence that newly-replicated DNA exists temporarily in a form that is fast-renaturing following heat-denaturation. One possible conclusion is that this fast-renaturing material represents the forked molecule postulated as an intermediate in DNA replication by Guild[11] and by Kornberg[12]. Evidence of a similar fast-renaturing intermediate in DNA replication of *Bacillus subtilis* has been obtained by R. Burger (personal communication).

Acknowledgments

We acknowledge the technical assistance of Laura Ann Douglas. This research is supported by U. S. P. H. S. grant AI-07798.

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