

**MECHANISM OF DNA CHAIN GROWTH, IV. DIRECTION OF
SYNTHESIS OF T4 SHORT DNA CHAINS AS REVEALED
BY EXONUCLEOLYTIC DEGRADATION***

BY TUNEKO OKAZAKI AND REIJI OKAZAKI

INSTITUTE OF MOLECULAR BIOLOGY, FACULTY OF SCIENCE, NAGOYA UNIVERSITY, NAGOYA, JAPAN

Communicated by Arthur Kornberg, October 8, 1969

Abstract.—T4 nascent short chains labeled at their growing ends with H³-thymidine and uniformly with C¹⁴-thymidine were prepared, separated into complementary strands, and degraded by *E. coli* exonuclease I in the 3' to 5' direction or by *B. subtilis* nuclease in the 5' to 3' direction. The kinetics of release of H³ and C¹⁴ labels by both enzymes was consistent with the conclusion that the H³ label is at the 3' end of the nascent short chains of both strands and that the short chains are products of discontinuous synthesis in the 5' to 3' direction along the two template strands.

Experiments reported in previous papers¹⁻⁵ from this laboratory revealed the following: (1) The most recently replicated portion of the bacterial and T4 phage chromosome can be isolated after denaturation as short DNA chains with a sedimentation coefficient of about 10S.^{1, 2, 4} (2) Temporary inhibition of polynucleotide ligase, an enzyme for the covalent joining of the DNA chains, results in a marked accumulation of the nascent short chains.^{3, 4} (3) The T4 nascent short chains found under the normal steady-state conditions as well as those accumulated upon temporary inhibition of polynucleotide ligase anneal equally to the separated complementary phage DNA strands.⁵ These results and similar observations made in other laboratories⁶⁻¹³ support the idea that the two strands of DNA are fashioned in a discontinuous manner, whereby short segments of chromosomal DNA are synthesized in the 5' to 3' direction and are subsequently joined by ligase action. An alternative possibility remained that two DNA strands are synthesized by a continuous mechanism in the 5' to 3' and 3' to 5' direction, respectively, but selective nicks are introduced *in vivo* in the newly replicated portion, these nicks being sealed subsequently by ligase.

To decide between these possibilities it is crucial to determine the direction of synthesis of the short DNA chains. In this paper we present evidence that the T4 nascent short DNA chains of both strands are products of synthesis in the 5' to 3' direction. Thus, the above alternative mechanism for the generation of the short chains involving the continuous synthesis and nicking of both strands is highly unlikely.

Materials and Methods.—H³-thymidine and C¹⁴-thymidine were purchased from New England Nuclear, γ -P³²-ATP was prepared according to the method of Glynn and Chappell,¹⁴ H³-dTTP as described by Okazaki and Kornberg,¹⁵ and calf thymus DNA was prepared according to the procedure of Kay, Simmons, and Dounce.¹⁶ The following enzymes were used: *E. coli* exonuclease I¹⁷ (crystalline preparation generously provided by Dr. I. R. Lehman), exonuclease III (Fraction VI, kindly provided by Dr. C. C. Richardson or prepared according to the technique of Richardson and Kornberg¹⁸), *B. subtilis* nuclease (Fraction I-A, described previously¹⁹), micrococcal nuclease²⁰ (a gift from

Dr. A. Ohsaka), *B. subtilis* DNA polymerase (Fraction IV described by Okazaki and Kornberg²¹), and polynucleotide kinase (Fraction VI, prepared according to the method of Richardson²²). Other materials were described previously.⁵

Preparation of C¹⁴-T7 DNA labeled with H³ at the 3' end: C¹⁴-labeled T7 DNA was prepared by the procedure described by Richardson, Inman, and Kornberg²³ for the preparation of H³-labeled T7 DNA except that 5-fluorodeoxyuridine was added to 5×10^{-5} M at the time of phage infection and C¹⁴-thymidine (6 μ Ci/ μ mole) was added to 2×10^{-5} M at 4 min after infection. The specific activity of the DNA obtained was 1.1×10^5 cpm/ μ mole.²⁴ The 3'-end of the C¹⁴-T7 DNA was labeled with H³-thymidylate by the "limited reaction"²⁵ of *B. subtilis* DNA polymerase. The reaction mixture (1 ml) contained 67 mM Tris-HCl (pH 8.0), 6.7 mM MgCl₂, 1 mM β -mercaptoethanol, 0.4 mM C¹⁴-T7 DNA, 2 μ M H³-dTTP (2.7×10^8 cpm/ μ mole), and 12 μ g of enzyme. After incubation at 37° for 1 hr, NaCl was added to 0.2 M, and the mixture heated at 70° for 10 min and dialyzed extensively against 0.5 M KCl and finally against 10 mM Tris-HCl (pH 7.6) containing 50 mM KCl. The H³-labeled terminal portion of the doubly labeled DNA, estimated from the H³ to C¹⁴ ratio, corresponded to 0.018% of the molecule.

Doubly labeled T7 DNA with a larger H³-labeled region at the 3' end was prepared by limited digestion of the C¹⁴-T7 DNA with *E. coli* exonuclease III followed by partial "repair"²³ with *B. subtilis* DNA polymerase. The reaction mixture (0.75 ml) for the digestion with exonuclease III contained 67 mM Tris-HCl (pH 7.8), 6.7 mM MgCl₂, 10 mM β -mercaptoethanol, 0.4 mM C¹⁴-T7 DNA, and 2 μ g of enzyme, and was incubated at 37°. An additional 2 μ g of enzyme and 10 mM β -mercaptoethanol were added at 90 and 130 min, respectively. After 160 min of incubation, 22% of the radioactivity was rendered acid soluble. NaCl was added to 0.2 M and the mixture was heated at 65° for 10 min and dialyzed successively against 0.5 M KCl and 10 mM Tris-HCl (pH 7.6) containing 50 mM KCl. The reaction mixture (3.75 ml) for repair contained: 67 mM Tris-HCl (pH 8.0), 6.7 mM MgCl₂, 1 mM β -mercaptoethanol, 1 μ M H³-dTTP (2.7×10^8 cpm/ μ mole), 1.35 μ M each of dATP, dTTP, dGTP, and dCTP, 40 μ M C¹⁴-T7 DNA degraded 22% by exonuclease III and 9 μ g of *B. subtilis* DNA polymerase. A synthesis of 0.8% was attained during incubation at 20° for 250 min. NaCl was added to 0.2 M and the mixture was heated at 70° for 10 min, dialyzed extensively against 0.5 M KCl, and finally obtained in 10 mM Tris-HCl (pH 7.6) containing 50 mM KCl.

Preparation of DNA labeled with H³ at the 3' end and with P³² at the 5' end: Calf thymus DNA was degraded by micrococcal nuclease to 2% acid solubility. The reaction mixture (2.1 ml) contained: 45 mM glycine-KOH (pH 9.2), 10 mM CaCl₂, 0.5 mg of bovine serum albumin, 0.9 mM calf thymus DNA, and 5 μ g of micrococcal nuclease. Incubation was carried out at 37° for 10 min. The mixture was dialyzed successively against 1 M NaCl and 10 mM Tris-HCl (pH 7.5) containing 50 mM KCl. The terminal 3' phosphate was removed from this DNA with *E. coli* exonuclease III. The reaction mixture (2 ml) containing 62 mM Tris-HCl (pH 7.4), 8 mM MgCl₂, 12 mM β -mercaptoethanol, 0.45 mM micrococcal nuclease-treated DNA, and 0.12 μ g of exonuclease III was incubated at 37° for 30 min. NaCl was added to 0.2 M, and the mixture was heated at 70° for 10 min and dialyzed successively against 10 mM NaCl and 50 mM Tris-HCl (pH 8.0). H³-thymidylate was then added to the 3'-hydroxyl end of the DNA by the limited reaction of *B. subtilis* DNA polymerase. The reaction mixture (2.7 ml), containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM β -mercaptoethanol, 2 μ M H³-dTTP (7.8×10^8 cpm/ μ mole), 0.3 mM 3'5'-hydroxyl terminated DNA, and 15 μ g of *B. subtilis* DNA polymerase, was incubated at 37° for 40 min. NaCl was added to 0.2 M and the mixture was heated at 70° for 10 min and dialyzed successively against 0.5 M NaCl, 50 mM NaCl, and 0.1 M Tris-HCl (pH 7.5). This material was then used as a phosphate acceptor in the polynucleotide kinase reaction. The reaction mixture (3 ml), containing 80 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 17 mM β -mercaptoethanol, 83 μ M γ -P³²-ATP (2.1×10^8 cpm/ μ mole), 0.25 mM DNA labeled with H³ at the 3' end, and 2.8 μ g of polynucleotide kinase, was incubated at 37° for 60 min. The mixture was treated with phenol and the doubly labeled DNA was purified by filtration through a Sephadex G50 column

(1.2×22 cm). The DNA was denatured in 0.1 *N* NaOH and sedimented through an alkaline sucrose gradient in a SW 25.1 rotor for 41 hr at 22,500 rpm and 15°. The radioactivities showed a peak at 5.6*S*. The peak fraction (5–6*S*) was neutralized with KH_2PO_4 , concentrated, and dialyzed against 50 mM Tris-HCl (pH 7.7). The final product had ratios of H^3 -thymidylate to total nucleotides of 1:3000 and P^{32} -phosphate to total nucleotides (or phosphate) of 1:380.

Preparation of H^3 - and C^{14} -pulse-labeled replicative T4 DNA: Two 180-ml cultures of *E. coli* B grown in a glucose salt medium (Medium A described previously²) to a titer of 10^9 cells/ml were infected with T4D (MOI = 10), incubated for 70 min at 20° with shaking and transferred to beakers in a 8° bath. After 10 min of stirring at 8°, the cultures were pulse labeled. To one culture, H^3 -thymidine (19.2 mCi/ μmole) was added to 5×10^{-7} *M* and the labeling terminated after 0.1 min by pouring onto crushed ice and 20 mM KCN. To the other culture, C^{14} -thymidine (54 $\mu\text{Ci}/\mu\text{mole}$) was added to 1.2×10^{-6} *M* and the labeling terminated after 2.5 min. The labeled cells were collected by centrifugation, the DNA extracted by the Thomas procedure,²⁶ and concentrated by pressure dialysis with a collodion membrane as described previously.²

Separation of the labeled nascent short DNA chains into components hybridizable with each strand of the T4 phage DNA: The W and C strands of T4 phage DNA were isolated as described previously.⁵ Thirty membrane filters (25-mm disks) loaded with the W strand and the same number of membrane filters loaded with the C strand were prepared by immobilizing 2 μg of the isolated strand to each disk by the method of Denhardt.²⁷ The two types of membrane filters (W and C) were placed alternately and horizontally in a vial containing 8 ml of solution of the labeled short chains in $3 \times \text{SSC}$ containing 0.02% Ficoll, polyvinylpyrrolidone, and bovine serum albumin, and incubated for 12 hr at 65°. The membrane filters were washed three times in 500 ml of SSC, and the labeled short DNA chains annealed to each phage DNA strand were eluted by dipping each type of the membrane filters in 5 ml of 0.1 *N* NaOH containing 10 mM EDTA at room temperature for 10 min.

Assay of nucleolytic degradation of radioactive DNA: The reaction was stopped by chilling the incubation mixture and adding 50% cold trichloroacetic acid to a final concentration of 6%. Thirty microliters of 1% bovine serum albumin were added at 0° and 5 min later the sample was centrifuged. The precipitate was suspended in 300 μl of 5% trichloroacetic acid, heated at 90° for 30 min, and centrifuged. The radioactivity in the cold acid-soluble and in hot acid-soluble fractions was measured in a Tri-Carb liquid scintillation spectrometer to obtain the percentage of the radioactive material rendered cold acid soluble.

Results and Discussion.—The basic plan of the present study is to determine the direction of synthesis of DNA chains by labeling the growing ends of the nascent short DNA chains and by identifying the labeled end by stepwise degradation from the 3' or 5' end by specific exonucleases. The usefulness of *E. coli* exonuclease I and *B. subtilis* nuclease for this purpose was demonstrated by model experiments which compared the rate of release of H^3 and C^{14} from C^{14} -labeled T7 DNA whose 3' end was labeled with H^3 by the DNA polymerase reaction. As is evident from Figure 1, *E. coli* exonuclease I degrades single-stranded DNA in a stepwise manner beginning from the 3' end, whereas *B. subtilis* nuclease initiates an exonucleolytic attack at the 5' end of the single-stranded DNA. This was also indicated by the kinetics of the release of H^3 and P^{32} from DNA chains whose 3' and 5' ends were labeled with H^3 and P^{32} by the DNA polymerase and polynucleotide kinase reactions, respectively (Fig. 2).

The experiments shown in Figure 1 suggest that the size of the terminal label should be made very small to obtain unequivocal results. To facilitate restrictive labeling of the growing end of the nascent chains it is necessary to slow down

FIG. 1.—Exonucleolytic degradation of C^{14} -T7 DNA labeled with H^3 at the 3' end. C^{14} -T7 DNA with a H^3 -labeled 3'-terminal region corresponding, on the average, to 0.018% or 0.8% of the whole molecule was prepared by the limited reaction and the repair reaction of *B. subtilis* DNA polymerase, respectively (see *Materials and Methods*). The DNA was used after heat denaturation at 100° for 4 min. (A) Digestion with exonuclease I of doubly labeled DNA prepared by the limited reaction. Reaction mixtures (70 μ l), containing 70 mM glycine-KOH (pH 9.2), 7 mM $MgCl_2$, 0.7 mM β -mercaptoethanol, 57 μ M DNA, and 0.05 μ g of enzyme, were incubated at 37° for various times (1–180 min). (B) Digestion with exonuclease I of doubly labeled DNA prepared by the repair reaction. Reaction mixtures (50 μ l), containing 70 mM glycine-KOH (pH 9.2), 7 mM $MgCl_2$, 0.7 mM β -mercaptoethanol, 20 μ M DNA, and 0.025 μ g of enzyme, were incubated at 37° for various times (2–40 min). (C) Digestion with *B. subtilis* nuclease of doubly labeled DNA prepared by the limited reaction. Reaction mixtures (50 μ l), containing 50 mM Tris-HCl (pH 8.8), 40 μ M DNA and 0.08 μ g of enzyme, were incubated at 37° for various times (5–80 min). (D) Digestion with *B. subtilis* nuclease of doubly labeled DNA prepared by the repair reaction. Reaction mixtures (50 μ l), containing 50 mM Tris-HCl (pH 8.8), 20 μ M DNA, and 0.11 μ g of enzyme, were incubated at 37° for various times (4–80 min).

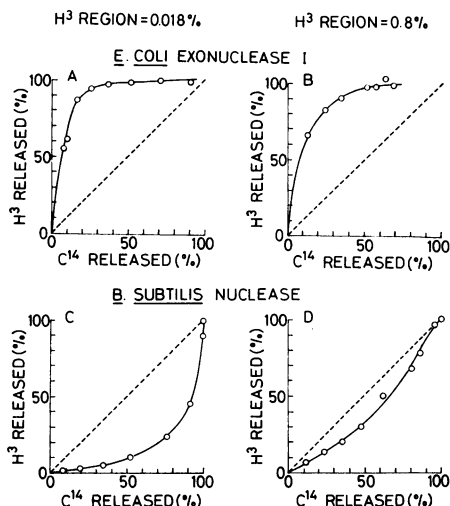
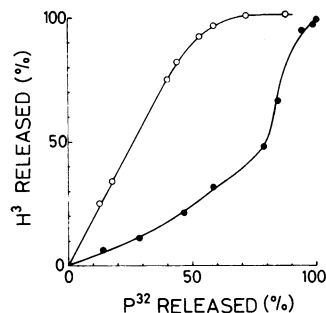


FIG. 2.—Exonucleolytic degradation of DNA labeled with H^3 at the 3' end and with P^{32} at the 5' end. The doubly labeled DNA was prepared as described in *Materials and Methods*. (—○—), Digestion with *E. coli* exonuclease I. Reaction mixtures (50 μ l), containing 70 mM glycine-KOH (pH 9.2), 7 mM $MgCl_2$, 0.7 mM β -mercaptoethanol, 80 μ M H^3P^{32} -DNA, and 0.15 μ g of enzyme were incubated at 37° for various times (3–120 min). (—●—), Digestion with *B. subtilis* nuclease. Reaction mixtures (50 μ l), containing 50 mM Tris-HCl (pH 8.8), 80 μ M H^3P^{32} -DNA, and 0.05 μ g of enzyme were incubated at 37° for various times (3–120 min).

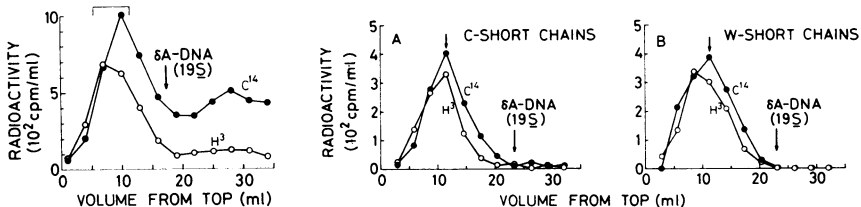


the reaction rate. This can be achieved by lowering the temperature. However, it is also essential that under the conditions used, DNA synthesis proceeds normally although at a reduced rate. After a search for such conditions, the labeling at 8° of T4-infected *E. coli* B was found to be satisfactory. When the temperature was lowered from 20° to 8° at 70 minutes after infection at 20° , phage DNA synthesis continued at a reduced but constant rate. Upon addition of radioactive thymidine, the label incorporated first appeared in the short DNA chains and was subsequently transferred to large chains. Furthermore, nascent short chains labeled at 8° for various times (0.1–2.5 minutes) annealed equally to the separated W and C strands of the phage DNA. Thus, the mode of replication after the temperature shift-down to 8° during the period of active phage DNA synthesis appears to be the same as that at 20° or higher temperatures.^{2, 4, 5}

To prepare the T4 nascent short DNA chains labeled at the growing ends with

H^3 and those labeled uniformly with C^{14} , one culture of phage-infected cells was pulse labeled for 0.1 minute with H^3 -thymidine at 8° and another culture with C^{14} -thymidine for 2.5 minutes under the same conditions. After DNA extraction, the H^3 - and C^{14} -labeled preparations were mixed and the short DNA chains were isolated by alkaline sucrose gradient sedimentation (Fig. 3).²⁸ The labeled short chains were separated into complementary components by annealing to and elution from membrane filters loaded with the W or C strand of phage DNA. Of the H^3 - and C^{14} -short chains, 21 and 26 per cent, respectively, were recovered from the membranes loaded with the W strand, and 20 and 21 per cent of the H^3 - and C^{14} -short chains, respectively, were recovered from the membranes loaded with the C strand. These separated complementary components of the short chains (C and W short chains) were further subjected to alkaline sucrose gradient sedimentation to obtain populations of the H^3 - and C^{14} -labeled C and W short chains which are relatively homogeneous in size (Fig. 4).

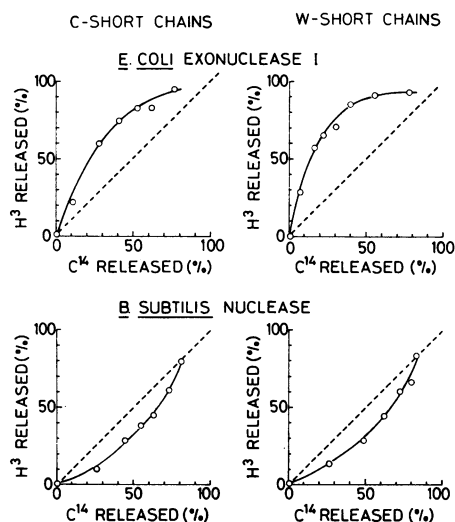
The C and W short chains recovered from the peak fractions having a sedimentation coefficient of about 9 to 10S (the fourth fraction from the top of the gradient shown in Figs. 4A, B) were degraded by *E. coli* exonuclease I or by *B. subtilis* nuclease, and the extent of release of H^3 compared with that of C^{14} at various times during digestion. The results in Figure 5 show that with both C and W short chains, the release of H^3 preceded the release of C^{14} when the DNA was degraded by *E. coli* exonuclease I, whereas the release of C^{14} preceded the release of H^3 when *B. subtilis* nuclease was used. Similar results were obtained with the C and W short chains sedimented at a slower rate (the third fraction from the top of the gradients in Figs. 4A, B) and with preparations of the short chains which were not separated into complementary strands.



(Left) FIG. 3.—Alkaline sucrose gradient sedimentation of H^3 - and C^{14} -pulse-labeled T4 DNA. T4-infected cells pulse-labeled with H^3 -thymidine for 0.1 min at 8° and those labeled with C^{14} -thymidine for 2.5 min at 8° were prepared and DNA was extracted from these cells as described in *Materials and Methods*. H^3 - and C^{14} -labeled DNA were combined (total volume: 30 ml), denatured in 0.2 N NaOH containing 5 mM EDTA and subjected to alkaline sucrose gradient sedimentation. The 5-ml samples were layered together with δA DNA (internal marker²) over 30 ml of a 5 to 20% linear alkaline sucrose gradients containing 0.1 N NaOH, 0.9 M NaCl, and 1 mM EDTA and centrifuged in a SW 27 rotor for 19 hr at 25,000 rpm and 4° . The 3-ml fractions were collected. Bracketed fractions were combined and used for isolation of the short chains complementary to each phage DNA strand.

(Right) FIG. 4.—Alkaline sucrose gradient sedimentation of the complementary components of H^3 - and C^{14} -labeled short chains. The labeled nascent short chains complementary to each strand of T4 phage DNA (C and W short chains) were isolated as described in *Materials and Methods*. The 5-ml samples were layered together with δA DNA over 32 ml of a 5 to 20% linear alkaline sucrose gradient containing 0.1 N NaOH, 0.9 M NaCl, and 1 mM EDTA and centrifuged in the SW 27 rotor for 17 hr at 27,000 rpm and 4° ; 3-ml fractions were collected.

FIG. 5.—Degradation of the H^3 - and C^{14} -labeled C and W nascent short chains by *E. coli* exonuclease I and *B. subtilis* nuclease. The labeled short chains recovered after alkaline sucrose gradient sedimentation in the fractions indicated by arrows in Fig. 4 were used following neutralization with KH_2PO_4 and successive dialysis against: 0.5 M NaCl containing 20 mM Tris-HCl (pH 8.2) and 2 mM EDTA; 20 mM Tris-HCl (pH 8.2) containing 2 mM EDTA; and 20 mM Tris-HCl (pH 8.2). Digestion with *E. coli* exonuclease I was carried out at 37° for various times (3–80 min) in reaction mixtures (240 μ l) containing 40 mM glycine-KOH (pH 9.2), 4 mM $MgCl_2$, 0.6 mM β -mercaptoethanol, 200 μ l of the solution of the labeled C or W nascent short chains, and 0.05 μ g of enzyme. Digestion with *B. subtilis* nuclease was carried out at 37° for various times (5–80 min) in reaction mixtures (230 μ l) containing 40 mM Tris-HCl (pH 8.9), 3 mM $CaCl_2$, 200 μ l of the solution of the labeled C or W nascent short chains, and 0.15 μ g of enzyme.



It may be concluded from these results that the H^3 label was at the 3' ends of both the C and W short chains indicating that the direction of synthesis of both types of the short chains is 5' to 3'. The results are in accordance with the two-strand discontinuous replication mechanism, but are not compatible with the contention that the short chains are produced by selective *in vivo* (or *in vitro*) nicking in the nascent regions of two daughter strands that are synthesized by a continuous mechanism.²⁹ If the latter were the case, the short chains of either one of the strands should have been labeled with H^3 at the 5' end.

One possible mechanism for discontinuous replication involves the formation of hairpin structures along the two template strands in the fork region followed by a specific endonucleolytic cleavage of the hairpins at their apex.^{30, 31} The present study revealed that the nascent DNA labeled by pulse as short as six seconds at 8° was highly susceptible, after denaturation in alkali, to exonuclease I, which degrades only single-stranded DNA. Thus, the bulk of the initial products of discontinuous replication do not display a property of hairpins, which are not denatured irreversibly and hence insusceptible to exonuclease I. This would imply either that the hairpin structures are not formed or that they are extremely short lived.

We are grateful to Drs. Arthur Kornberg and Nicholas Cozzarelli for critical reading of the manuscript.

* This work was supported by the Research Fund of the Ministry of Education of Japan and by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

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