Proc. Nat. Acad. Sci. USA
Vol. 71, No. 8, pp. 3189–3193, August 1974

Replication of the *Escherichia coli* Chromosome with a Soluble Enzyme System

(spermidine/folded DNA/DNA polymerase I/DNA ligase)

THOMAS KORNBERG, ARTHUR LOCKWOOD, AND A. WORCEL

Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08540

Communicated by Arthur B. Pardee, May 6, 1974

ABSTRACT **Semi-conservative DNA synthesis is observed when the isolated, folded *E. coli* chromosome is supplemented with a DNA-free, soluble enzyme fraction, the four deoxynucleoside 5′-triphosphates, ATP, and Mg^{++}. The DNA synthesized *in vitro* remains associated with the folded chromosome during sedimentation through neutral sucrose, but is released as small DNA fragments in alkali. Sealing of these replicative intermediates to the chromosome requires the presence of both *E. coli* DNA polymerase I (EC 2.7.7.7) and DNA ligase (EC 6.5.1.2).**

Study of the replication of *Escherichia coli* DNA has been complicated both by the need for coordinated interplay of many enzymes and by the fragile nature of the template. For these reasons *E. coli* DNA replication has not been successfully reconstructed in solution. DNA synthesis observed in cell-free extracts (1–7) represents catalytic actions of the DNA polymerases (1, 5, 7–10), but lacks many of the important features of replication. Moses and Richardson (11) showed that *E. coli* cells rendered permeable by toluene to low-molecular-weight precursors are able to replicate their DNA; however, the cells remained impermeable to proteins and thus to fractionation and dissection of the enzymatic steps of replication. Schaller *et al.* (12) demonstrated that replication could proceed in cells lysed on cellophane discs; however, this system appears to require the tight complex of macromolecular components of the bacterial cell, rendering its fractionation extremely difficult.

We were encouraged to attempt the replication of DNA in a soluble system by two recent advances. First, a special high-speed supernatant from gently lysed cells (13, 14) has been shown to contain the active gene products (14, 15) defined genetically to be essential for *E. coli* DNA synthesis (the *dnaA*, *B*, *C*, *E*, and *G* proteins) (16) as well as other replication proteins not yet defined by genetic lesions (17). Second, a method has been developed whereby *E. coli* spheroplasts yield a nonviscous preparation of intact, folded, and supercoiled chromosomes (18, 19).

These isolated folded chromosomes behave like compact particles, and sediment either as free, folded chromosomes at 1300–2200 S or at 3000–4000 S in a membrane-attached form (20, 21). The physical properties of these extracted particles vary with the cell's physiological state: replicating chromosomes isolated at 10°–15° in 1 M NaCl remain associated with the bacterial envelope, whereas nonreplicating chromosomes are released membrane-free (20). The size distribution of the chromosomes also reflects their growth during replication (19).

The exploitation of these techniques has allowed us to demonstrate and characterize the unique DNA synthesis that occurs when the folded chromosomes are combined with a DNA-free, soluble enzyme system. As reported below, this DNA synthetic reaction contains many of the features embodied in *in vivo* chromosome replication.

Abbreviations: Pol I, DNA polymerase I; EDTA, ethylenediaminetetraacetate; BrdUTP, bromodeoxyuridine 5′-triphosphate.

MATERIALS AND METHODS

Sources were as follows: radioactive nucleotides [^{3}H]dTTP (17.1 Ci/mmole) and [^{3}H]dATP (13.4 Ci/mmole) from New England Nuclear Corp.; deoxybromouridine 5′-triphosphate (BrdUTP) from Terra Marine Research; other unlabeled nucleotides, lysozyme, spermidine·HCl, Brij-58, and deoxycholate from Sigma; Angio-Conray (sodium iothalamate) from Malinckrodt; phage M13 [^{14}C]DNA a gift from Dr. T. Henry; DNA polymerase I (DNA nucleotidyl transferase, EC 2.7.7.7, 3 mg/ml, 18,000 units/mg) a gift from Dr. D. Brutlag; and *E. coli* DNA ligase [Polynucleotide synthetase (NAD^+), EC 6.5.1.2, 1.5 mg/ml, 12,500 units/mg] a gift from Dr. P. Modrich. *E. coli* strain H560 (*polA 1*$^-$, *endo 1*$^-$, *thy*$^-$) was used for the preparation of the cell-free extracts and chromosomes.

Preparation of Cell-Free Extracts. Growth of cells and preparation of DNA-free extracts were essentially as described by Wickner *et al.* (17). Experiments described here utilized the 0–40% ammonium sulfate fraction prepared according to a minor modification (W. Wickner, personal communication) of the method of W. Wickner *et al.* (17).

Preparation of Folded Chromosomes. Folded chromosomes were prepared by a modification of the method of Stonington and Pettijohn (18). Cells were grown in 160 ml of M9 minimal medium supplemented with 40 μg/ml of thymine to OD_{600} = 0.500 at 37°, harvested by centrifugation at 4°, and resuspended at 0° in 0.5 ml of a solution containing 0.01 M Tris·HCl, pH 8.2, 0.1 M NaCl, and 20% sucrose. Incubation with lysozyme [0.1 ml of a solution containing 4 mg/ml of lysozyme, 0.12 M Tris·HCl, pH 8.2, and 0.05 M ethylenediaminetetraacetate (EDTA)] for 40 sec on ice was followed by the addition of 0.5 ml of a solution containing 1% Brij-58, 0.4% deoxycholate, 0.01 M EDTA, and 10 mM spermidine·HCl. Cells were lysed at 10° for 3 min. The suspension of lysed cells (about 1.2 ml) was subjected to centrifugation for 17 min at 9,000 × *g* and 4° through a 12–60% sucrose gradient (4.4 ml) which contained 0.01 M Tris·HCl, pH 8.2, 1 mM 2-

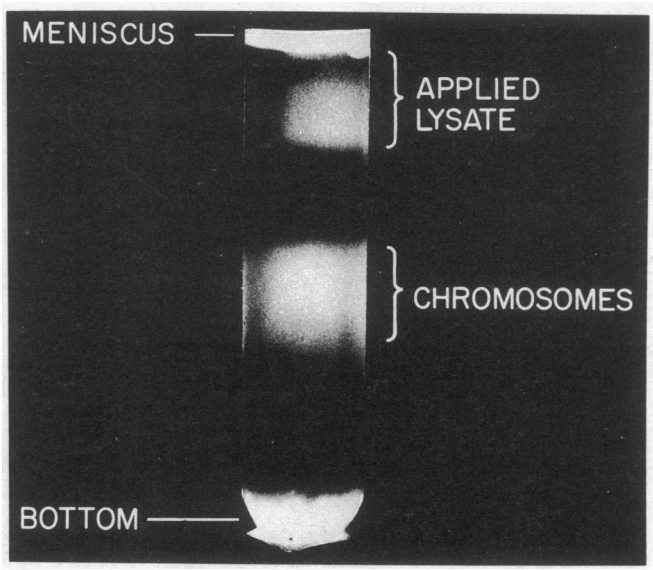

FIG. 1. Sucrose gradient centrifugation of folded chromosomes. Folded chromosomes were prepared as described under *Materials and Methods*, and illuminated with a narrow beam directed from the top of the tube. The band of chromosomes, as well as the meniscus, bottom of the tube, and volume of lysate applied are clearly visible from their light scattering.

mercaptoethanol, 1 mM EDTA, and 5 mM $MgCl_2$. The white, opalescent band at the center of the tube (see Fig. 1) was carefully removed with a wide-bore pipet and was assayed immediately for DNA synthesis. Storage for periods greater than one-half hour led to rapid loss in the ability of the folded chromosomes to support DNA synthesis. ^{14}C labeled chromosomes were prepared as described above except that the M9 medium contained 5 μg/ml of thymine and 10 μCi of [^{14}C]-thymine (New England Nuclear Corp.).

Preparation of Hybrid Density DNA. Cells (100 ml) were grown with 10 μCi of [^3H]thymine (New England Nuclear Corp.) to OD_{600} = 0.250, filtered, resuspended in M9 medium containing 20 μg/ml of 5-bromodeoxyuridine instead of thymine, and incubated for an additional hour with aeration, in the dark at 37°. Cells were concentrated 20-fold and the DNA extracted with Sarkosyl and protease as previously described (22).

Conditions of the Standard Assay. DNA synthesis was measured in a reaction mixture (0.05 ml) that contained, in order of mixing, 50 mM Tris·HCl, pH 7.5; 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM ATP, 100 μM each of CTP, UTP, and GTP, 20 μM each of dCTP, dATP, and dGTP, 20 μM [^3H]dTTP (4400 cpm/pmole), 4 mM spermidine·HCl, 10 μl of folded chromosomes, and 28.4 μg of enzyme fraction. Incubation was at 30°. Acid-insoluble radioactivity was determined as previously described (5). Incubation mixtures lacking extract served as controls and generally incorporated less than 1.5 pmoles of nucleotide.

RESULTS

Preparation of the Template. We have modified the procedure of Stonington and Pettijohn (18) to permit the isolation of the folded chromosome in low salt and at high chromosome concentration. Folded chromosomes were prepared in the presence of spermidine and Mg^{++} which provide sufficient counterion strength and eliminate the problem of aggregation

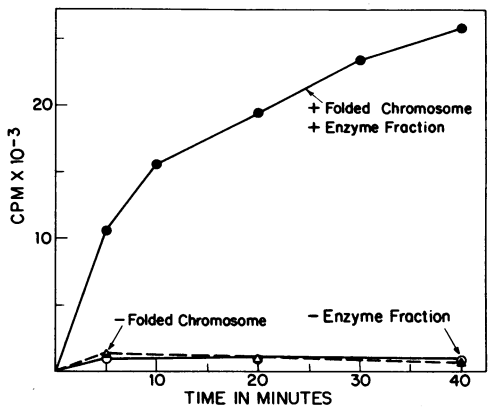

FIG. 2. Kinetics of *in vitro* DNA synthesis. Incubations were as described under *Materials and Methods* except that enzyme fraction or folded chromosomes were omitted where indicated.

at high chromosome concentration. Sedimentation of these spermidine lysates in neutral sucrose containing 5 mM Mg^{++} yields a single peak of material absorbing at 260 nm which has more than 90% of the acid-precipitable DNA from the lysate, an A_{260}/A_{280} ratio of about 1.5, and a sedimentation value of approximately 4000 S. This chromosome suspension contains approximately 2×10^{10} chromosomes per ml (about 100 μg/ml); it is relatively shear resistant, permitting manipulation by pipet. Polyacrylamide gel electrophoretic analysis of the protein components (Burgi and Worcel, unpublished) indicates that these DNA particles are analogous to the membrane-attached chromosomes prepared in 1 M NaCl (20). The membrane-attached, folded chromosomes are discrete particles approximately 1 μm in diameter as seen by phase contrast light microscopy; due to their large size they are visually apparent by Tyndall light scattering. A typical preparation seen by indirect lighting is shown in Fig. 1.

TABLE 1. *Properties of DNA synthesis*

Reactants	% Activity
Complete*	100
Complete − extract (+ heated extract)†	18
Complete − template (+ heated template)†	<1
Complete − $MgCl_2$ + EDTA (4 mM)	<1
Complete − 2-mercaptoethanol + NEM (10 mM)	<1
Complete − dATP, dCTP, dGTP	<1
Complete − CTP, GTP, UTP	107
Complete − ATP	17
Complete + DNase (100 μg/ml)‡	<1
Complete + NaCl (0.1 M)	16
Complete + KCl (0.1 M)	20
Complete − Extract + Pol I (1.4 units)	<1
Complete + Pol I	87

NEM, *N*-ethylmaleimide; Pol I, DNA polymerase I.

* Incubations were performed under standard reaction conditions for 5 min at 30°. 100% activity represents the incorporation of 7.2 pmoles of nucleotide.

† An aliquot of extract or folded chromosome was preincubated in the standard reaction mixture for 5 min at 70°.

‡ DNase was added after the 5-min incubation and treatment proceeded for 10 min at 30°.

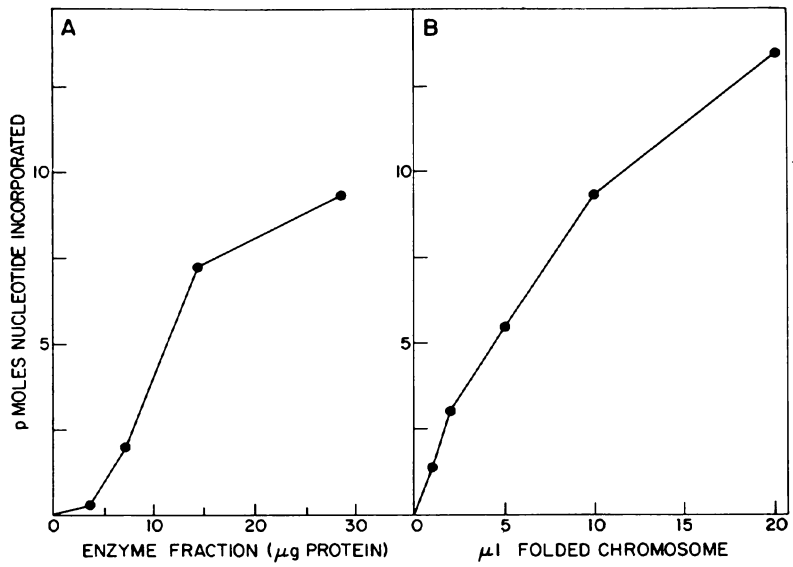

FIG. 3. Effect of cell-free extract and folded chromosome on the initial reaction rate. Incubations were for 5 min at 30° as described under *Materials and Methods* except that extract and folded chromosome quantities were varied as indicated.

Properties of the Reaction. Folded chromosomes did not catalyze the synthesis of DNA. However, when supplemented with a soluble enzyme fraction prepared from gently lysed cells, the chromosomes supported a rapid burst of synthesis (Fig. 2). Incorporation of deoxynucleotides, which proceeded for approximately one-half hour, required the enzyme fraction and chromosome template (Fig. 2), both of which are thermolabile (Table 1). The lability of the template preparation may be due to instability of the DNA structure or a protein component or both.

The rate of synthesis was proportional to additions of the enzyme fraction and template (Fig. 3). Requirements for synthesis, performed with saturating amounts of extract, are summarized in Table 1. All four deoxynucleoside 5′-triphosphates were required, with an apparent K_m of approximately 12 μM. The product of the reaction was sensitive to DNase. Maximal activity required Mg^{++} and 2-mercaptoethanol; omission of Mg^{++} and addition of EDTA, or replacement of 2-mercaptoethanol by *N*-ethylmaleimide abolished incorporation. Ionic strength increased by addition of NaCl or KCl to 0.1 M reduced incorporation more than 5-fold. ATP was required for maximal activity; omission of the other three ribonucleoside triphosphates was without effect.

Spermidine (4 mM) had no effect on either the rate or extent of DNA synthesis, but the folded chromosome preparation may have retained sufficient amounts of spermidine added in the lysis procedure. In the absence of added spermidine the chromosomes unfold and the incubation mixture becomes viscous. Thus, maintenance of the folded state depends on the presence of added spermidine. As shown in Fig. 4, incubation with the enzyme fraction under the standard assay conditions (4 mM spermidine) does not affect the sedimentation behavior of the folded chromosomes.

Characterization of the Product. The DNA synthesized *in vitro* has been characterized by zonal sedimentation in neutral and in alkaline sucrose. The reaction product remains associated with the folded chromosomes under conditions of neutral sedimentation (Fig. 4), but when denatured, it sediments slowly, as small DNA fragments (Figs. 5 and 6). The DNA fragments are approximately 6–13 S after a 1-min incubation and grow to a size of 20–50 S after 10 min of incubation.

Under conditions of the standard assay the DNA fragments do not join to the high-molecular-weight DNA. Since both the extract and folded chromosomes were prepared from a mutant deficient in DNA polymerase I (Pol I), and since both fractions have been purified from a substantial portion of the

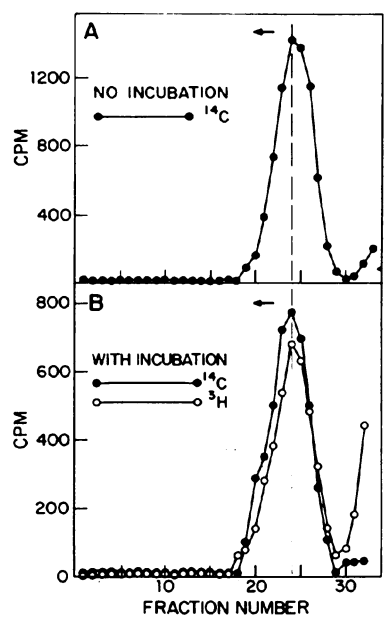

FIG. 4. Neutral sucrose gradient reruns of folded chromosomes. Folded chromosomes labeled with [^{14}C]thymine were isolated as described under *Materials and Methods*. After mixing with the standard 50 μl of reaction mixture, samples with (*Panel B*), or without (*Panel A*) incubation for 10 min at 30° were applied to a 5.2-ml 12–30% sucrose gradient which contained 0.01 M Tris·HCl, pH 8.2, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM $MgCl_2$, and 1 M NaCl. Centrifugation was for 20 min at 9000 × *g* and 4°. Seven-drop fractions were collected on GF/A filters and acid-insoluble radioactivity was determined. Sedimentation is from *right* to *left* as indicated by the *arrows*. In the bottom panel, ^{3}H cpm represents *in vitro* synthesized DNA.

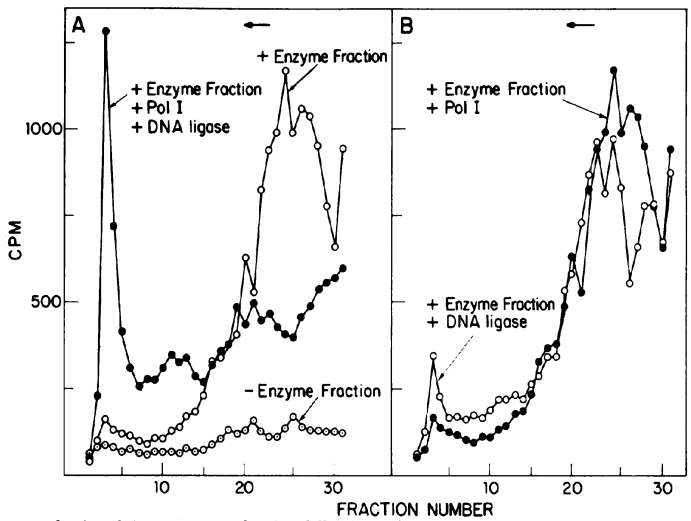

FIG. 5. Alkaline sedimentation analysis of *in vitro* synthesized DNA: effect of DNA polymerase I plus DNA ligase. Incubations were carried out in a total volume of 50 μl as described under *Materials and Methods* for 4 min, except that Pol I (1.4 units) or DNA ligase (1.2 units plus 0.5 mM NAD) were included where indicated. Fifty microliters of 0.1 M EDTA was added following incubation; after 10 min at 0° an equal volume of 0.5 M NaOH was added and the mixture was kept at room temperature. Samples were applied to 4.8 ml of alkaline 5–20% sucrose gradients (containing 0.7 M NaCl, 0.3 M NaOH, and 1 mM EDTA) layered over 0.3-ml Angio-Conray cushions. Centrifugation was for 9 hr at 26,000 rpm at 10° in a Spinco SW 50.1 rotor.

cellular protein, the effect of the addition of Pol I and DNA ligase was examined. Pol I alone failed to use the folded chromosomes as template, nor did its addition to the complete system affect either the rate (see Table 1) or extent of the *in vitro* incorporation (with or without DNA ligase). However, both Pol I and ligase were essential for the sealing of the small DNA fragments. Early incorporation into high-molecular-weight single-stranded DNA was observed with ligase acton (Fig. 6*A*) whereas only slow-sedimenting DNA accumulated when ligase action was inhibited by NMN (Fig. 6*B*).

In order to test for semi-conservative DNA synthesis, folded chromosomes were labeled with [^{14}C]thymidine *in vivo* and DNA was synthesized *in vitro* with [^{3}H]dATP plus BrdUTP density label. The product DNA was sheared to a uniform molecular weight of approximately 3×10^6 and sedimented to equilibrium in CsCl at neutral pH (Fig. 7). All of the product (^{3}H-label) appeared at a position coincident with a hybrid density marker. Upon denaturation, the [^{3}H]DNA banded at the density of completely heavy single-stranded DNA. Since no new DNA of intermediate density was seen in either gradient, we conclude that the product of the *in vitro* reaction extends for long stretches (relative to 3×10^6 daltons), and that the DNA synthesized is made in a normal semiconservative manner.

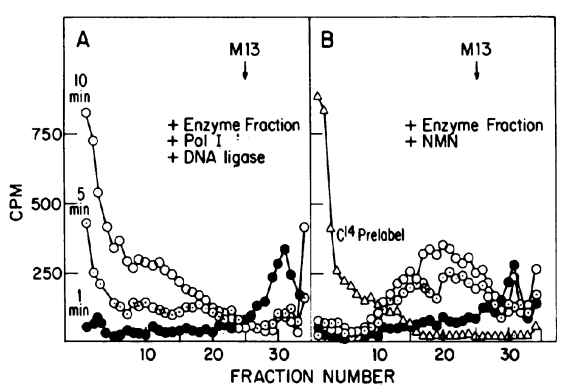

FIG. 6. Alkaline sucrose sedimentation analysis of *in vitro* synthesized DNA; kinetics in the presence or absence of DNA polymerase I and DNA ligase. Incubations (●, 1 min; ⊙, 5 min; O, 10 min) were as described under *Materials and Methods* except that Pol I (1.4 units), DNA ligase (1.2 units plus 0.5 mM DPN) or NMN (250 μg/ml) were included as indicated. Folded chromosomes were prelabeled *in vivo* with [^{14}C]thymine. Conditions of centrifugation were as described in Fig. 5 except that the alkaline gradient (5.0 ml) was layered over 0.15 ml of a 50% (v/v) Angio-Conray–70% sucrose mixture, covered with 0.1 ml of 40% sucrose. The sedimentation of phage M13 DNA is indicated by *arrows*.

DISCUSSION

The ability of cells rendered permeable to the low-molecular-weight precursors (11, 23) and of lysed cells concentrated on cellophane discs (12) to replicate their DNA demonstrated that this complex synthetic process can survive cell death and lysis. It has previously been shown that DNA-free extracts can catalyze the efficient initiation and replication of the single-stranded DNAs of the coliphages φX174 and M13 (13–15). The results presented here indicate that an *E. coli* replication apparatus that synthesizes DNA on double-stranded templates can also be reassembled in soluble form *in vitro*.

The reconstructed *E. coli* system mimics *in vivo* DNA synthesis: it catalyzes semi-conservative DNA replication (24) by a mechanism involving the synthesis and joining of short DNA fragments (25); the DNA synthesis utilizes deoxynucleoside 5′-triphosphate substrates, and is ATP-dependent (3, 11, 12, 23). Unlike the replication system of Schaller *et al.* (12), it is sensitive to 0.1 M salt.

Synthesis proceeds for about 30 min with an initial rate equivalent to about 5% of the *in vivo* rate; approximately 1% of the genome is replicated within 30-min *in vitro*. These calculations are based on the assumption that all of the chromosomes are active; thus, the rate and extent of synthesis on individual chromosomes may be considerably higher. Autoradiographic measurements are needed to determine the degree of heterogeneity in the population.

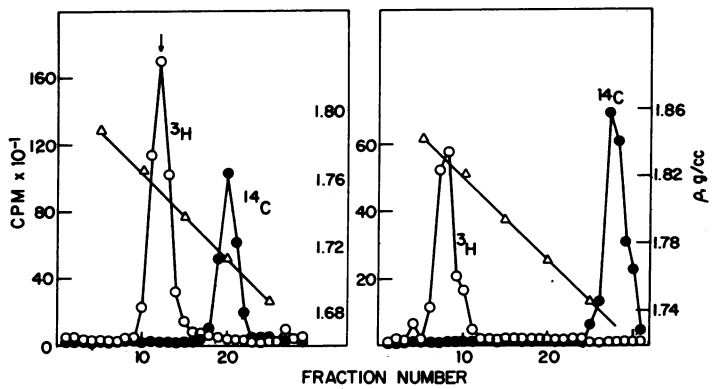

FIG. 7. Buoyant density sedimentation analysis of *in vitro* synthesized DNA in neutral CsCl. *Left,* native DNA; *right,* denatured DNA. Incubations were for 20 min as described under *Materials and Methods* except that 5 μCi of [³H]dATP was included and 40 μM BrdUTP replaced the [³H]dTTP. Template was prelabeled with [¹⁴C]thymine. Samples were diluted into 1 ml of 0.01 M Tris·HCl, pH 8.2, 50 mM EDTA, after incubation. DNA was denatured by incubation for 10 min at 30° in 0.1 M NaOH and the solution was neutralized with 0.1 ml of 1 M HCl and 0.1 ml of 1 M Tris·HCl, pH 8.2. The mass of each DNA sample was adjusted to 2 g with water, and CsCl (2.65 g to the native sample and 2.88 g to the denatured sample) was added. Each sample was sheared for 10 min in a Virtis homogenizer at maximum speed and then subjected to centrifugation for 60–65 hr at 30,000 rpm and 20°. Six-drop fractions were collected. The *arrow* indicates the position of the hybrid DNA marker.

Evidence that the folded chromosomes are supercoiled has indicated that they remain largely unperturbed by their isolation (19). The failure of Pol I to use the folded chromosome as a template testifies to the absence of nicks or gaps in the duplex DNA. The fact that the catalytic action of Pol I is apparently involved solely in the joining of "Okazaki" fragments (Table 1, Figs. 5 and 6) and that DNA nucleases present in the extract fail to attack the folded chromosomes (Fig. 4) suggests that these enzymes retain their *in vivo* specificity toward the chromosome.

We do not know whether the compact state of the folded chromosome is important for its *in vitro* replication. The visible unfolding of the chromosomes during incubation in the absence of added spermidine does not appear to affect synthesis; however, the increase in viscosity observed may reflect the complete relaxation of a small proportion of the folded chromosomes or the unfolding of several discrete loops (19), neither of which need affect the regions of replication. On the other hand, the shut-off of synthesis may be due to structural decay in the region of the replication fork that is undectable by sedimentation analysis (Fig. 4). An understanding of the role of DNA tertiary structure in replication must await more information about the nature of the replication fork and the properties of the enzymes active in this region.

The template used in all our studies was the membrane-attached form of the chromosome (20). The membrane-free chromosome is relatively unstable and we have not been able to isolate it (in the absence of 1 M NaCl) at the high DNA concentrations required for our assays. Therefore, the role, if any, of the bacterial membrane in DNA replication cannot yet be ascertained.

Schaller *et al.* (12) have shown that replication *in vitro* reflects its *in vivo* sensitivity to nalidixic acid, mitomycin C, ultraviolet light and *dna* temperature-sensitive mutants (16). We have observed a 4-fold inhibition of *in vitro* DNA synthesis by nalidixic acid (40 μg/ml). Experiments to assess the role of the *dna* gene products are now indicated.

We gratefully acknowledge Dr. W. Wickner's generous assistance with both advice and material. This research has been supported by grants from the National Science Foundation and the American Cancer Society.

1. Kornberg, A. (1969) *Science* **163,** 1410–1418.
2. Ganesan, A. T. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 1296–1300.
3. Smith, D. W., Schaller, H. & Bonhoeffer, F. (1970) *Nature* **226,** 711–713.
4. Knippers, R. & Stratling, W. (1970) *Nature* **226,** 713–717.
5. Kornberg, T. & Gefter, M. L. (1970) *Biochem. Biophys. Res. Commun.* **40,** 1348–1355.
6. Okazaki, R., Sugimoto, K., Okazaki, T., Imae, Y. & Sugino, A. (1970) *Nature* **228,** 223–226.
7. Knippers, R. (1970) *Nature* **228,** 1050–1053.
8. Moses, R. E. & Richardson, C. C. (1970) *Biochem. Biophys. Res. Commun.* **41,** 1557–1564.
9. Wickner, R. B., Ginsberg, B., Berkower, I. & Hurwitz, J. (1972) *J. Biol. Chem.* **247,** 489–497.
10. Kornberg, T. & Gefter, M. L. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 761–764.
11. Moses, R. E. & Richardson, C. C. (1970) *Proc. Nat. Acad. Sci. USA* **67,** 674–681.
12. Schaller, H., Otto, B., Nusslein, V., Huf, I., Herrmann, R. & Bonhoeffer, F. (1972) *J. Mol. Biol.* **63,** 183–200.
13. Wickner, W., Brutlag, D., Schekman, R. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 965–969.
14. Schekman, R., Wickner, W., Westergaard, O., Brutlag, D., Geider, K., Bertsch, L. & Kornberg, A. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 2691–2695.
15. Wickner, R. B., Wright, M., Wickner, S. & Hurwitz, J. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 3233–3237.
16. Wechsler, J. & Gross, J. (1971) *Mol. Gen. Genet.* **113,** 273–284.
17. Wickner, W., Schekman, R., Geider, K. & Kornberg, A. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 1764–1767.
18. Stonington, G. O. & Pettijohn, D. E. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 6–9.
19. Worcel, A. & Burgi, E. (1972) *J. Mol. Biol.* **71,** 127–147.
20. Worcel, A. & Burgi, E. (1974) *J. Mol. Biol.* **82,** 91–105.
21. Delius, H. & Worcel, A. (1974) *J. Mol. Biol.* **82,** 107–109.
22. Worcel, A. (1970) *J. Mol. Biol.* **52,** 371–386.
23. Vosberg, H. P. & Hoffmann-Berling, H. (1971) *J. Mol. Biol.* **58,** 739–753.
24. Meselson, M. & Stahl, F. W. (1958) *Proc. Nat. Acad. Sci. USA* **44,** 671–682.
25. Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., Kairuma, R., Sugino, A. & Iwatsuki, N. (1968) *Cold Spring Harb. Symp. Quant. Biol.* **33,** 129–142.