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Termination Point of Replication of Colicin E1 Plasmid DNA in Cell Extracts*

(precursor/segregation/restriction endonuclease *Eco*R1/NMN)

YOSHIMASA SAKAKIBARA† AND JUN-ICHI TOMIZAWA‡§

†National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo, Japan, and ‡National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT Closed-circular monomeric molecules were one of the major products of replication of colicin E1 plasmid DNA in cell extracts. However, when the plasmid DNA synthesized in the reaction mixture was labeled for 3 min after 27 min of incubation, most of the label was found in open-circular molecules. The open-circular molecules were converted to closed-circular molecules upon further incubation. The newly replicated open-circular molecules had a nick or small gap in their newly synthesized strand. The interruption was located at approximately 20% of the molecular length from the single site of cleavage by restriction endonuclease *Eco*R1 and at or very close to the origin of replication. The addition of 10 mM nicotinamide mononucleotide instead of nicotinamide adenine dinucleotide to extracts did not significantly affect the kinetics of the colicin E1 plasmid DNA synthesis. However, in the presence of nicotinamide mononucleotide the formation of completely replicated closed-circular molecules was suppressed and, instead, open-circular molecules accumulated with an interruption in the newly synthesized strand at the termination point of replication, which was located at or very close to the origin of replication.

A soluble system that is capable of replicating colicin E1 plasmid (Col E1) DNA was described in previous papers (1, 2). In that system, closed-circular molecules of Col E1 DNA can complete a round of semiconservative replication. The replication initiates in a fixed region and proceeds unidirectionally (3). A round of replication is completed with the formation of closed-circular monomers (1, 3). In this report we describe formation of closed-circular monomers from open-circular monomers that have a nick or small gap in a unique region of their newly synthesized strand. The effect of nicotinamide mononucleotide (NMN), which is known to inhibit the action of *Escherichia coli* DNA ligase [Poly(deoxyribonucleotide):poly(deoxyribonucleotide) ligase (AMP-forming, NMN-forming), EC 6.5.1.2] (4), on Col E1 DNA synthesis was also examined.

MATERIALS AND METHODS

Most of the materials used have been described in a previous paper (1). NMN was obtained from Sigma Chemical Co. and poly(U,G) from Miles Laboratories. Cell extracts were prepared from *E. coli* YS10 (Col E1) according to the methods described previously (1).

Assay of DNA Synthesis. The methods described in the previous paper (1) were followed. The standard reaction mixture (300 μl) contained 7.5 mM $MgCl_2$, 25 μM each of the four deoxynucleoside triphosphates (dNTPs), 200 μM each of the four ribonucleoside triphosphates, 2 mM nicotinamide adenine dinucleotide (NAD), 25 mM potassium phosphate buffer (pH 7.4), 67 mM KCl, and 100 μl of the extract. The specific activity of [α-^{32}P]dTTP was approximately 1 Ci/mmol to label DNA for 5 or 30 min and approximately 10 Ci/mmol to label DNA for 3 min. The mixture was incubated at 30°. In the density labeling experiments, the extract was incubated in the standard reaction mixture, except that 50 μM each of BrdUTP, dCTP, dGTP, and [α-^{32}P]dATP (approximately 1 Ci/mmol) were added instead of the four dNTPs.

Sedimentation Analysis. Sucrose solutions were made up in 0.15 M NaCl–0.015 M Na_3citrate containing 5 mM EDTA or in 0.3 N NaOH–1.0 M NaCl containing 5 mM EDTA. Samples of 0.1–0.2 ml were layered on 4.7 ml of 5–20% sucrose gradient with an 0.3-ml underlayer of CsCl-saturated 20% sucrose and centrifuged at 45,000 rpm for 150 min at 10° in neutral or for 300 min at 5° in alkaline gradients in a Beckman SW50.1 rotor. For analysis by CsCl density gradient centrifugation, CsCl was added to 3 ml of a sample to give a density of 1.72 g/cm^3. Centrifugation was performed at 36,000 rpm for 60 hr at 20° in a Beckman Type 40 rotor. For analysis by CsCl density gradient centrifugation in the presence of ethidium bromide, a solution that contained 8 ml of a sample, 7.5 g of CsCl and 8 mg of ethidium bromide was centrifuged at 36,000 rpm for 48 hr at 20° in a Beckman Type 40 rotor. Fractions were collected from the bottom of the tube. Elimination of ethidium bromide from the DNA sample was performed by shaking with an equal volume of isopropanol stored over CsCl-saturated water followed by dialysis. The reference DNA labeled with [^{3}H]thymidine was prepared as described previously (1).

Treatment with Restriction Endonuclease EcoR1. *Eco*R1 was supplied by Drs. D. Nathans, T. J. Kelly, Jr., and Dr. K. Matsubara. Col E1 DNA was treated with the enzyme as described previously (3).

Separation of the Strands of Col E1 DNA. Approximately 1 μg of open-circular and/or linear Col E1 DNA was heated at 93° for 4 min with 20 μg of poly(U,G) in 0.5 ml of 50 mM Tris·HCl buffer (pH 8.5) containing 15 mM NaCl, 1.5 mM Na_3citrate, 10 mM EDTA, and 0.25% sodium lauroyl sarkosinate and then the mixture was rapidly cooled, followed by addition of 2.5 ml of chilled water. After adjustment of the density to 1.74 g/cm^3 with CsCl, the mixture was centrifuged

Abbreviation: Col E1, colicin E1 plasmid.

* This paper is the fourth of a series. The third is ref. 3.

§ To whom reprint requests should be addressed.

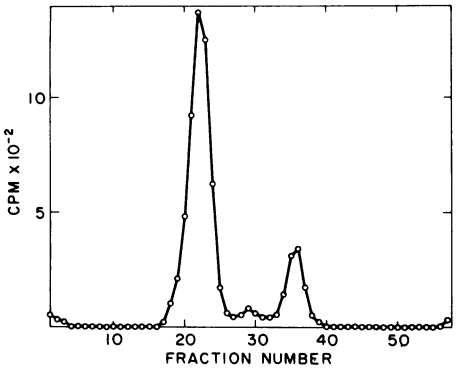

FIG. 1. CsCl-ethidium bromide density gradient centrifugation analysis of Col E1 DNA synthesized in a cell extract. The extract was incubated for 30 min with [α-^{32}P]dTTP in the standard assay conditions. The DNA was extracted and centrifuged in a CsCl density gradient containing ethidium bromide. Trichloroacetic acid-insoluble radioactivity of a portion of each fraction was measured. In this and the following figures, the radioactivity counts have been multiplied by the indicated factors to give the numbers on the ordinates.

at 36,000 rpm for 60 hr at 20° in a Beckman SW50.1 rotor. Fractions were collected from the bottom of the tube.

RESULTS

Synthesis of open-circular molecules carrying an interruption in the newly synthesized strand

The major species of Col E1 DNA synthesized in the extracts are completely replicated molecules and a class of replicative intermediates containing newly synthesized 6S DNA (1). When Col E1 DNA synthesized during a 30-min incubation was analyzed by CsCl–ethidium bromide density gradient centrifugation, the DNA formed two major bands (Fig. 1). The heavier density band consisted of completely replicated closed-circular DNA and the intermediates containing 6S DNA. The former accounted for approximately 65% and the latter approximately 20% of the total amount of labeled DNA (data not shown). The DNA in the lighter density band consisted almost exclusively of open-circular monomeric molecules (Fig. 2a) of which approximately 80% of the newly synthesized strands were linear and had the same sedimentation rate as the unit single strand of Col E1 DNA (Fig. 2b). The small peak between these two bands (Fig. 1) contained catenanes (interlocked rings) of a closed-circular molecule and an open-circular one.

The lighter band in the CsCl–ethidium bromide gradient of the preparation continuously labeled for 30 min contained

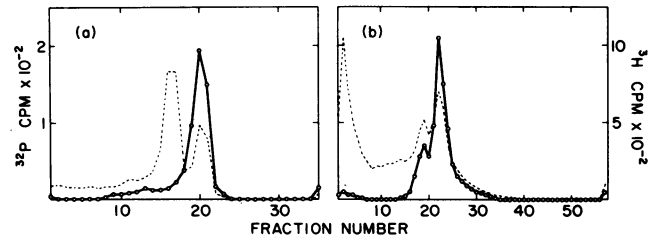

FIG. 2. Sedimentation analysis of the DNA in the lighter density band in the CsCl–ethidium bromide density gradient shown in Fig. 1. The DNA in fractions 35 and 36 of Fig. 1 was analyzed by neutral (a) and alkaline (b) sucrose gradient centrifugation. The reference Col E1 [^{3}H]DNA (- - - -) represents from the left, double-stranded closed- and open-circular forms in the neutral sucrose gradient and single-stranded circular and linear forms in the alkaline sucrose gradient (O——O): [^{32}P]-DNA.

approximately 15% of the incorporated radioactivity (Fig. 1). In contrast, in the DNA labeled for 5 min from 25 min and for 3 min from 27 min after incubation, the DNA in the lighter band accounted for approximately 50 and 70% of the total labeled DNA, respectively (Fig. 3a and b). The DNA in the lighter band in Fig. 3b consisted almost exclusively of open-circular molecules which contained a newly synthesized linear strand of unit length (Fig. 4). A portion of the reaction mixture, in which DNA was labeled for 3 min from 27 min after incubation, was diluted with an excess of dNTPs and the standard reaction mixture which had been preincubated in the presence of rifampicin and nonradioactive dNTPs. In the DNA prepared after incubation for an additional 30 min, most of the label was found in closed-circular molecules (Fig. 3C). These results show that the interruption was present in the newly synthesized strand of the freshly formed molecules and that these open-circular molecules were precursors of the closed-circular molecules.

Location of the interruption in the newly synthesized strand

The location of the interruption in the newly synthesized strand of the open-circular molecules was studied using restriction endonuclease *Eco*R1, which introduces one unique double-strand break into the Col E1 DNA molecule (3, 5). The open-circular molecules labeled in the newly synthesized linear strand were treated with the endonuclease and analyzed by alkaline sucrose gradient centrifugation. As shown in Fig. 5, cleavage of the open-circular molecules resulted in the formation of two well-defined labeled single-strand fragments.

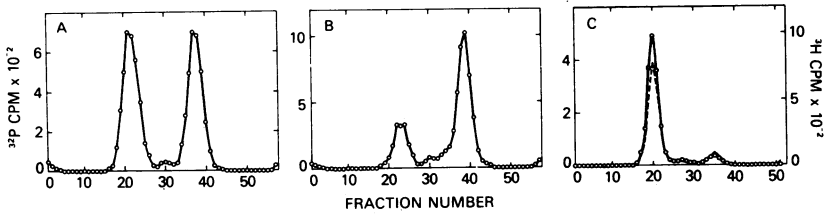

FIG. 3. CsCl–ethidium bromide density gradient centrifugation analysis of Col E1 DNA labeled during short periods. [α-^{32}P]dTTP was added to the standard reaction mixture at 25 (A) and 27 (B) min after incubation and the labeling was terminated at 30 min. Twenty microliters of (B) were diluted with 25 μl of 2.5 mM unlabeled dNTPs and 500 μl of the standard reaction mixture which had been preincubated for 10 min with 10 μg/ml of rifampicin in the absence of labeled dTTP. The diluted mixture was further incubated for 30 min (C). The DNA was extracted and analyzed by CsCl–ethidium bromide density gradient centrifugation. (O——O): [^{32}P]DNA; (- - -): reference [^{3}H]DNA.

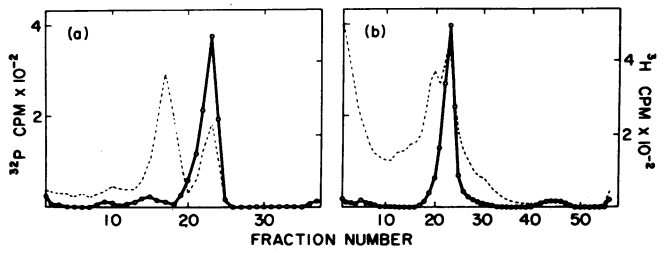

FIG. 4. Sedimentation analysis of the DNA in the lighter density band of the CsCl–ethidium bromide density gradient. The DNA in fractions 38 and 39 of Fig. 3B was pooled and analyzed by neutral (a) and alkaline (b) sucrose gradient centrifugation. (O——O): [^{32}P]DNA; (– – –): reference [^{3}H]DNA.

The mass of the smaller and larger fragments formed after the cleavage is calculated (6) to be approximately 20 and 80% of the mass of a unit single strand of Col E1 DNA molecule, respectively. The result indicates that most of the newly synthesized strands had an interruption at approximately 20% of the molecular length from the endonuclease-sensitive site.

Accumulation of newly synthesized open-circular molecules in the presence of NMN

The rate of Col E1 DNA synthesis was not affected by the addition of 2.5 or 10 mM of NMN instead of NAD, as shown in Fig. 6. These concentrations of NMN are sufficient to completely inhibit the action of *E. coli* DNA ligase in purified preparations (4) as well as in cell lysates (7). Circular Col E1 DNA molecules with a few nicks that were made by pancreatic DNase I (deoxyribonucleate 5′-oligonucleotidohydrolase, EC 3.1.4.5) were efficiently converted to the closed-circular form in the reaction mixture; this conversion was almost completely inhibited by 10 mM NMN (data not shown). Although the rate of Col E1 DNA synthesis was not

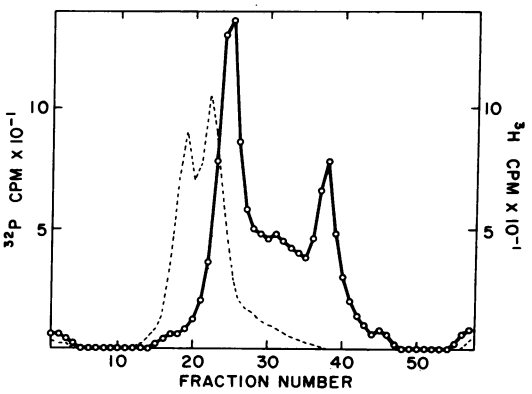

FIG. 5. Characterization of the cleavage products of the newly synthesized open-circular molecules by restriction endonuclease *Eco*R1. The open-circular molecules in the DNA labeled for 3 min after 27 min of incubation were purified by a CsCl–ethidium bromide density gradient centrifugation and then by a neutral sucrose gradient centrifugation as described in Figs. 3 and 4. The DNA was dialyzed against 50 mM Tris·HCl (pH 7.5) containing 50 mM NaCl and 1 mM EDTA. After addition of $MgCl_2$ to give a final concentration of 6 mM, the DNA was converted to the linear structure by treating with *Eco*R1 at 37° (3) and analyzed with ^{3}H-labeled open-circular reference DNA (– – –) by an alkaline sucrose gradient centrifugation. The underlayer of CsCl–sucrose solution was omitted. (O——O): [^{32}P]DNA.

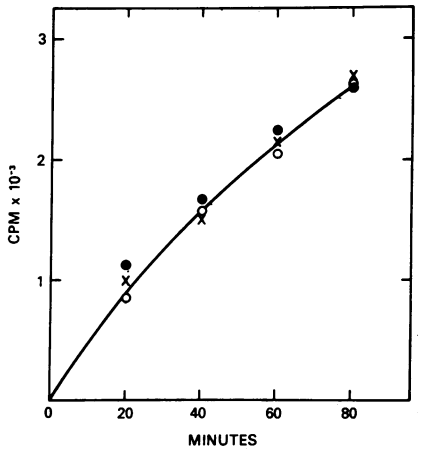

FIG. 6. Kinetics of Col E1 DNA synthesis in the presence of NMN. The extract was incubated in the standard assay conditions with [^{32}P]dTTP (250 cpm/pmol) (O) and in the presence of 2.5 (●) and 10 mM (×) NMN instead of NAD. Fifty microliters of each was withdrawn at the times indicated.

significantly affected by NMN, approximately 50 and 80% of the DNA labeled during 30 min of incubation in the presence of 2.5 and 10 mM NMN, respectively, was open-circular (Fig. 7A and C). This is in contrast to the formation of completely replicated closed-circular molecules as the major product in the absence of NMN (Fig. 1 and ref. 1). The labeled DNA that banded near the position of closed-circular DNA in Fig. 7C consisted mostly of the closed-circular replicative intermediates.

The open-circular molecules contained a newly synthesized linear strand having the same sedimentation rate as the unit single strand of Col E1 DNA (Fig. 7B and D). The position of the nick or gap in these open-circular molecules was determined by alkaline sucrose gradient centrifugation after treatment with endonuclease *Eco*R1. The sedimentation pattern of the treated molecules was almost the same (data not shown) as that obtained with the similarly treated open-

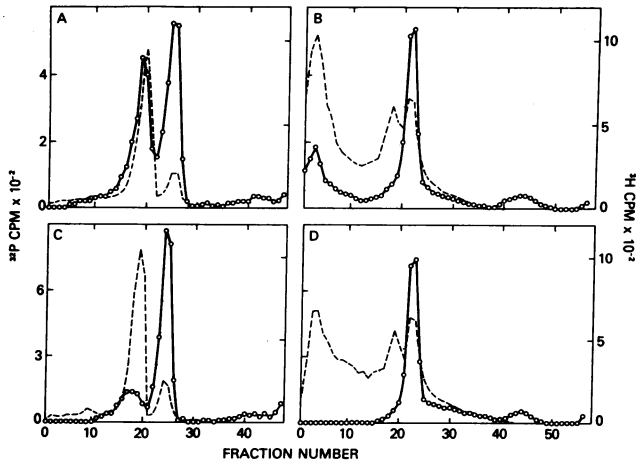

FIG. 7. Accumulation of open-circular molecules in the presence of NMN. The extract was incubated for 30 min in the standard assay conditions, except that 2.5 (A and B) and 10 mM (C and D) NMN was added instead of NAD. The DNA was extracted and analyzed by neutral (A and C) and alkaline (B and D) sucrose gradient centrifugation. (O——O): [^{32}P]DNA; (– – –): reference [^{3}H]DNA.

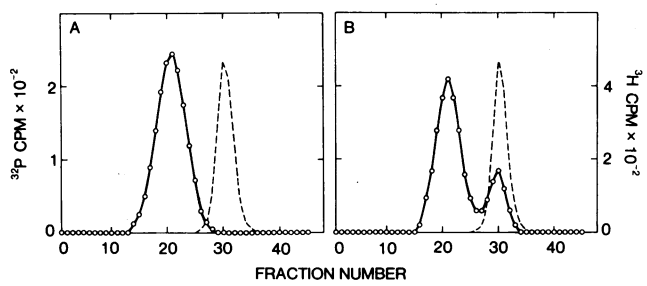

FIG. 8. Equilibrium CsCl density gradient centrifugation of BrdU-labeled Col E1 DNA. The extract was incubated for 30 min in the standard reaction mixture, except that 50 μM each of BrdUTP, dCTP, dGTP, and [α-^{32}P]dATP were added instead of the four dNTPs. The DNA was purified by neutral sucrose gradient centrifugation after heating at 90° for 2 min to eliminate the 6S DNA synthesized (1). The DNA was centrifuged in a CsCl density gradient (A). The extract was similarly incubated in the medium with 10 mM NMN instead of NAD. The DNA was centrifuged without further purification so that the molecules containing 6S DNA formed a separate peak (1, 2) (B). (O——O): [^{32}P]DNA; (- - -) reference [^{3}H]DNA.

circular molecules which were precursors of the closed-circular molecules (Fig. 5). In the presence or absence of NMN, approximately 80% of the parental molecules did not participate in replication during 30 min of incubation (compare ref. 1) and approximately 15% of labeled parental DNA was found in open-circular forms after the incubation with NMN for 30 min (data not shown). In other words, interruption of strands in unreplicated molecules was relatively infrequent compared to that found in the monomeric molecules synthesized in the presence of NMN. Since replication was not preferentially reinitiated in molecules that had completed a round of replication (1), the high incidence of interruption in the newly formed molecules in the presence of NMN probably does not relate to further replication of closed-circular molecules that had completed replication in the presence of NMN. From these results it can be concluded that NMN blocks the completion of replication by inhibiting the closure of a nick or gap that is located at or near the origin of replication.

When the DNA was labeled with BrdUMP in the presence of 10 mM NMN, the major product showed the same density

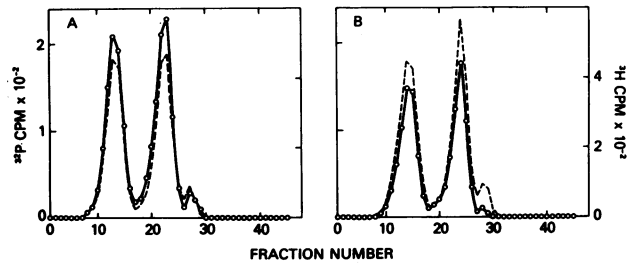

FIG. 9. Separation of the strands of the newly synthesized circular molecules. The closed-circular molecules synthesized during 30 min in the standard reaction mixture were purified as described in the legend of Fig. 8 and treated with *Eco*R1 to convert them to the linear form. The open-circular molecules accumulated during 30-min incubation on the addition of 10 mM NMN were purified by neutral sucrose gradient centrifugation. ^{3}H-labeled open-circular reference Col E1 DNA was added (- - -) to the ^{32}P-labeled linear (A) and open-circular molecules (B). DNA was denatured, annealed with poly(U,G), and then analyzed by CsCl density gradient centrifugation. (O——O): [^{32}P]DNA.

as that of the closed-circular molecules synthesized in the standard assay conditions (Fig. 8), which consist of a hybrid of a newly synthesized heavy strand and a parental light strand (1). The major band in Fig. 8B consisted of the open-circular monomeric molecules and the minor band the replicative intermediate containing newly synthesized 6S DNA. These results confirm the conclusion that the open-circular molecules that accumulated in the presence of NMN have almost completed a round of replication but contain an interruption in the newly synthesized strand.

The strands newly synthesized in the presence of NMN were annealed with poly(U,G) and analyzed by CsCl density gradient centrifugation. The result showed that the newly synthesized DNA contained equal amounts of the "light" and "heavy" strands, as did that synthesized in the presence of NAD (Fig. 9). Thus DNA synthesis continues on both strands of Col E1 DNA even in the presence of NMN, and both of the newly synthesized strands have an interruption.

DISCUSSION

The complete replication of Col E1 DNA in cell extracts results in the formation of closed-circular monomeric molecules (Fig. 1 and 3C). When the DNA was labeled for 3 min before termination of the incubation at 30 min, most of the labeled products were the open-circular monomeric molecules carrying a nick or small gap in the newly synthesized strands (Fig. 3B and 4). The open-circular molecules were converted to the closed-circular form upon further incubation (Fig. 3C). These results indicate that the interruption is present in the newly formed molecules and that the open-circular molecules are precursors of the completely replicated closed-circular molecules. Replication proceeds semiconservatively on both strands and the two daughter molecules segregate from each other before completion of replication of the closed-circular molecules.

Electron microscopic observation of Col E1 DNA molecules replicated *in vitro* shows that one of the branch points (defined as the left branch point) of replicating loops is located at a position approximately 17% of the molecular length (to the right) from the endonuclease-*Eco*R1-sensitive site. This location is independent of the extent to which the right branch point has proceeded (3). These results indicate that the DNA replication initiates at a fixed region and proceeds unidirectionally. If the rightward replication continues to complete a round of replication, the termination point of replication is expected to be located at the origin of replication. In agreement with this expectation, most of the freshly formed open-circular molecules that have almost completed a round of replication have an interruption in the newly synthesized strand at approximately 20% of the molecular length from the endonuclease-*Eco*R1-sensitive site (Fig. 5). These results support the idea that the termination point of replication in most of the molecules is located at or very close to the left branch point which, in turn, is located at or very close to the origin of replication. The presence of linear fragments between the well-defined peaks of the smaller and larger fragments in the alkaline sucrose gradient after the cleavage of the open-circular molecules by the endonuclease (Fig. 5) may be due to random nicking of the circular molecules during incubation and DNA preparation. However, the possibility that a minor fraction of the molecules terminate replication at various distances from the origin of replication is not ruled out.

Since newly replicated DNA transiently accumulates as open-circular molecules (Fig. 3), the final sealing process must be a rate-limiting step in replication. Some other *in vitro* systems for DNA replication are similarly limited. Thus in the cellophane disc system, which converts single-stranded bacteriophage ϕX174 DNA to the double-stranded form, the conversion of the newly-formed circular molecules to the closed-circular form is rate-limiting (7), while in the soluble system for bacteriophage M13 DNA replication this step is totally defective (8). The complementary strand of M13 DNA synthesized in the soluble system has been found to have a gap, corresponding to several percent of the genome length, at a unique region (9). Another parallel case exists in the *in vivo* replication of simian virus 40 DNA, where replication has been shown to initiate in a fixed region and to proceed bidirectionally to a termination point opposite the origin (10). In this system as well, open-circular molecules with an interruption in the newly synthesized strand at the termination point were observed (11).

The addition of NMN to the reaction mixture instead of NAD did not significantly affect the kinetics of Col E1 DNA synthesis (Fig. 6), but led to accumulation of open-circular molecules which had almost completed a round of replication (Fig. 7). The replication proceeds on both strands of Col E1 DNA (Figs. 8 and 9) and both of the newly synthesized strands have an interruption at a unique region where replication terminates. These results show that NMN selectively blocks the final sealing process of the freshly formed open-circular molecules but does not significantly interfere with the propagation of Col E1 DNA chains, which occurs by a discontinuous mechanism (Y. Sakakibara and J. Tomizawa, manuscript in preparation). Since the conversion of open-circular molecules formed by treatment with DNase I to the closed-circular form in cell extracts was inhibited by NMN, if *E. coli* ligase is involved in the joining process during the discontinuous replication, it must be in some way protected from the inhibitory action of NMN. Alternatively the joining process might be carried out by another ligase, the action of which is not inhibited by NMN. The final sealing process is prevented by NMN, which inhibits either the ligation itself or a reaction that immediately precedes the ligation. The final sealing process may not be simply the last ligation process in discontinuous replication, since the process occurs after segregation of daughter molecules and is a rate-limiting and NMN-sensitive step, in contrast to the propagation process.

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