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Biochemistry

Structure of the ColE1 DNA molecule before segregation to daughter molecules

(ColE1 plasmid/DNA replication/catenane)

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ABSTRACT **The segregation of daughter DNA molecules at the end stage of replication of plasmid ColE1 was examined. When circular ColE1 DNA replicates in a cell extract at a high KCl concentration (140 mM), a unique class of molecules accumulates. When the molecule is cleaved by a restriction enzyme that cuts the ColE1 DNA at a single site, an X-shaped molecule in which two linear components are held together around the origin of DNA replication is made. For a large fraction of these molecules, the 5′ end of the leading strand remains at the origin and the 3′ end of the strand is about 30 nucleotides upstream of the origin. The 3′ end of the lagging strand is located at the *terH* site (17 nucleotides upstream of the origin) and the 5′ end of the strand is a few hundred nucleotides upstream of the *terH* site. Thus the parental strands of the molecule intertwine with each other only once. When the KCl concentration is lowered to 70 mM, practically all of these molecules are converted to daughter circular monomers or to catenanes consisting of two singly interlocked circular units.**

At the last stage of replication of a circular DNA molecule, the template strands intertwine only in a short unreplicated region where they are held together by base pairing. If the template strands in the unreplicated region are separated by unpairing of the bases before removal of the strand intertwining, a catenane that is composed of two circular units must be formed. Thereafter the catenane could be converted to two circular daughter molecules. On the other hand, if intertwining of the template strands is removed before their separation by unpairing of the bases, two daughter circular molecules must be formed without passing through formation of a catenane. Separation of the parental strands before release from intertwining has been suggested by accumulation of catenanes under conditions where type II topoisomerase activity is supposed to be suppressed (1–6). On the other hand, in some other systems (7–8), including replication of ColE1 plasmid DNA in extracts of *Escherichia coli*, a catenane is not an obligatory intermediate in formation of daughter molecules. The process of formation of a catenane or daughter circular molecules at the final stage of replication is named segregation. Segregation in various replication systems has been reviewed (1, 7, 9).

Here, we examine the process of segregation of ColE1 DNA in some detail. Replication of closed circular (CC) ColE1 DNA begins by formation of the precursor of the RNA primer for DNA synthesis by the host RNA polymerase. The precursor is cleaved by RNase H to form primer for synthesis of the leading strand, which initiates from a unique site called the origin, catalyzed by DNA polymerase I (10). Then, synthesis of the lagging strand by a replisome initiates at a certain region in the replication loop (11, 12) and terminates at the *terH* site (13). Subsequently, semiconservative synthesis of the DNA proceeds unidirectionally (14) by replisomes (12, 13). The replication completes by formation of two CC monomers or a molecule called a CC-CC catenane, which is composed of two interlocked monomeric CC units (7). An open circular (OC) monomer and an OC-OC catenane are respective precursors. Under the standard replication conditions, CC monomers predominate in the products. Two OC molecules and an OC-OC catenane must be made by processing of a molecule with a short base-paired unreplicated region, called a late intermediate (LI). In this paper, we report formation of LI molecules in which the parental strands intertwine with each other only once.

MATERIALS AND METHODS

Bacteria and Plasmid. *Escherichia coli* YS1 *endI* (15) was used for preparation of cell extracts, MK398*recB21* (16) for preparation of fraction II (17) of a cell extract, and HB101 (18) for transformation by and preparation of plasmid DNA. Plasmid pSD71 is a derivative of pSD1 (19). It has the *inc1* and *inc2* mutations and does not have the *cer* site.

***In Vitro* DNA Synthesis.** Bacterial extract (20) was adjusted to pH 7.0 with 1 M KOH, divided into 100-μl aliquots, frozen, and stored at −70°C. Supercoiled monomeric pSD71 DNA (50 μg/ml) was incubated at 30°C in reaction mixtures containing 25 mM potassium phosphate buffer (pH 7.5), 7.5 mM $MgCl_2$, 25 μM dNTPs, 200 μM rNTPs, [α-^{32}P]dCTP (20 Ci/mmol), the cell extract (45% by volume), and various concentrations of KCl. After incubation, radioactivity in trichloroacetic acid-insoluble material was determined. To analyze the properties of the products, the reaction was stopped by adding EDTA (10 mM) and SDS (0.1%). After treatment with proteinase K (200 μg/ml) at 37°C for 1 hr, ^{32}P-labeled products were purified by 2-propanol precipitation after phenol extraction.

Sedimentation and Gel Electrophoretic Analyses. For CsCl/ethidium bromide equilibrium centrifugation, samples were centrifuged at 50,000 rpm for 14 hr at 15°C in a Beckman VTi 65 rotor. For neutral sucrose gradient centrifugation, samples were layered on a 5-ml gradient of 5–20% (wt/vol) sucrose in 0.1 M NaCl/10 mM Tris·HCl, pH 7.5/5 mM EDTA and centrifuged at 45,000 rpm for 5 hr at 9°C in a Beckman SW 50.1 rotor. Neutral agarose gel electrophoresis was carried out in 89 mM Tris base/89 mM boric acid/2 mM EDTA for analysis of products and in 40 mM Tris acetate, pH 8.0/2 mM EDTA for isolation of products. After gel electrophoresis, DNA in gel slices were recovered by using glassmilk (Geneclean, Bio 101, La Jolla, CA). Alkaline agarose gel electro-

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Abbreviations: CC, closed circular; OC, open circular; LI, late intermediate.

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phoresis was carried out in 40 mM NaOH/1 mM EDTA, and polyacrylamide gel electrophoresis in 8 M urea in the Tris borate buffer described above.

Hybridization of Labeled Strands. Labeled DNA fragments were mixed with an excess of single-stranded DNA from phage M13 in which either of the strands of pSD71, cleaved with *Eco*RI, was cloned. The mixture was heated at 95°C for 5 min and incubated at 65°C for 1 hr in the presence of 0.2 M NaCl. The hybridized fragments were isolated by agarose gel electrophoresis, denatured, and analyzed.

RESULTS

Conditions for Accumulation of LI Molecules. We reasoned that LI molecules are likely to have certain hydrodynamic properties similar to those of OC-OC catenanes. In preliminary experiments, we found that a significant accumulation of such molecules could be achieved by manipulating the concentration of KCl in the reaction mixture. We began *in vitro* synthesis of plasmid pSD71 DNA by incubation for 30 min at 25°C in the presence of 110 mM KCl. Most products were molecules carrying newly synthesized small DNA fragments (Fig. 1, step I). Rifampicin was added to inhibit initiation of DNA synthesis (10), and then KCl was increased to 140 mM. Because DNA synthesis was inefficient, it was enhanced by supplementation with fraction II (step II). During neutral gel electrophoresis, two major bands were made by the products of a 30-min incubation. One of these migrated at a rate similar to that of singly interlocked OC-OC catenanes, and the other at a rate similar to that of OC monomers. Because the unit-length linear (L) molecules predominated in the alkaline analysis, replication of most molecules was almost complete. The collapsed CC-CC and CC molecules were derived from corresponding relaxed CC molecules. Subsequent to step II, the mixture was diluted with a low-salt reaction mixture to reduce the final KCl concentration to about 70 mM. The concentration of dCTP was increased to 1 mM. During incubation for 20 min (step III), the total amount of labeled material was not significantly changed. Molecules that migrated at rates similar to those of CC-CC catenanes and CC monomers predominated in the products.

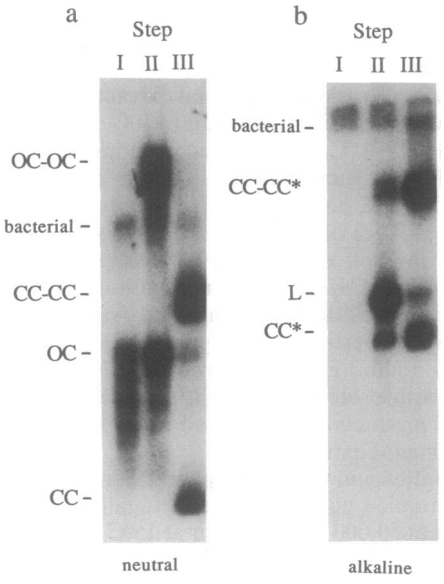

FIG. 1. DNA products of stepwise incubation. pSD71 DNA was incubated for 30 min at 25°C in a mixture containing YS1 extract, [α-^{32}P]dCTP, and 110 mM KCl (step I). An equal amount of reaction mixture containing rifampicin (20 μg/ml), 170 mM KCl, and fraction II (1/20th the volume of the extract) was added and incubation was continued for another 30 min (step II). Then 3 volumes of the mixture without additional KCl but with rifampicin (10 μg/ml) and 1 mM nonlabeled dCTP was added and incubation was continued for 20 min (step III). At the end of each step, products were analyzed by neutral (*a*) and alkaline (*b*) 1% agarose gel electrophoresis. The samples were appropriately diluted so that each sample contained the same amount of the mixture derived from step I. The migration positions of pSD71 species are indicated: CC and CC-CC are supercoiled molecules, and CC-CC and OC-OC catenanes are singly interlocked; L shows unit-length linear molecules, and CC* and CC-CC* are collapsed molecules. Most labeled products from step I ran off the gel under the alkaline condition.

The molecules formed after step II were fractionated by CsCl/ethidium bromide equilibrium centrifugation (Fig. 2*a*). The DNA in the lighter peak (Fractions 22–25), where OC and linear molecules banded, was subjected to neutral sucrose gradient centrifugation and fractionated (Fig. 2*b*). When aliquots of these fractions were examined by neutral agarose gel electrophoresis (Fig. 2*c*), the molecules in the faster sedimenting major peak (peak I) migrated similarly to or slightly faster than OC-OC catenanes in which most daughter units were interlocked just once. The molecules in the slower sedimenting minor peak (peak II) migrated at the position of OC monomers (Fig. 2*c*).

A LI molecule should yield an X-shaped molecule when it is digested with a restriction enzyme that cleaves the template DNA once, whereas an OC-OC catenane should yield two linear molecules (Fig. 3 *a* and *b*). While peak II DNA, which consisted of OC monomers, yielded the unit-length linear molecules by *Eco*RI cleavage, peak I DNA yielded, in addition, a species that migrated much slower than the linear molecules (Fig. 3*c*). Digestion of peak I DNA with *Xmn* I also yielded a species that migrated a little slower than the species formed by *Eco*RI digestion. These properties of the slower migrating products are those expected for X-shaped molecules formed by cleavage of LI molecules at different positions. The fraction of radioactivity found in the slower migrating molecules varied between 30% and 70% from experiment to experiment.

Locations of Termini of DNA Strands Where Synthesis Begins. When peak I and peak II DNAs were cleaved by *Eco*RI or *Xmn* I and the products were examined by alkaline agarose gel electrophoresis, similar sets of two fragments were found from both DNA (data not shown). Because the lengths of the two fragments produced by either digestion agree with the distances between the cleavage site and the replication origin, both ends of most DNA strands in these DNAs were located near the origin. To determine more precisely the locations of termini of newly synthesized strands, the X-shaped molecules and the unit-sized linear molecules formed by cleavage of peak I DNA with *Eco*RI were treated with *Rsa* I (Fig. 4 *a* and *b*). Peak II DNA, which consisted of OC monomers, was similarly cleaved. Then the products were denatured and hybridized with one of the strands of pSD71 DNA cloned into M13 DNA. Polyacrylamide gel electrophoresis of hybridized fragments after denaturation showed several fragments (a–d) of characteristic lengths, in addition to fragments expected from cleavage of pSD71 DNA (A_1, A_2, B, and C). Triplet fragments about 111 nucleotides long (a) were from the 5′ end of the leading strands (21). Because the amount of triplets formed by the cleavage was not affected by piperidine treatment (data not shown), the primer RNA must have been eliminated during incubation in the cell extract. The fragments about 130 nucleotides long (b) were from the lagging strands terminated at the *terH* site (13).

From the relative intensity of the bands, we judge that essentially all the leading strands had their 5′ ends at the origin. Strands that had their 3′ ends of the lagging strands at the *terH* site were about 60%, 40%, and 15% for LI molecules, OC-OC catenanes, and OC monomers, respectively. Besides these fragments, the lagging strands from the OC and

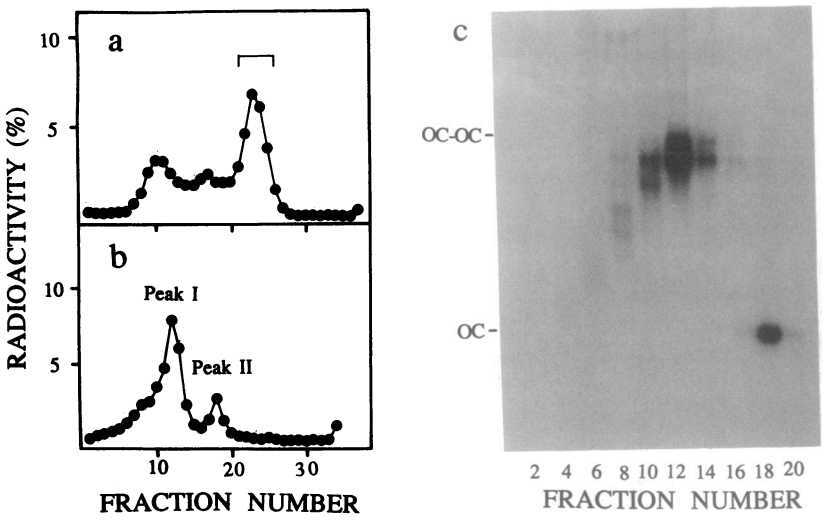

FIG. 2. Isolation of candidates for LI molecules. The products of step II (Fig. 1) were purified by gel filtration and fractionated by CsCl/ethidium bromide equilibrium centrifugation (*a*). The bracketed fractions were combined and dialyzed. A portion of the sample was centrifuged in a neutral sucrose gradient (*b*). Aliquots of fractions from the sucrose gradient were examined by neutral 1% agarose gel electrophoresis (*c*). Migration positions of the OC monomers and singly interlocked OC-OC catenanes are indicated.

OC-OC molecules formed bands of around 210 and 300 nucleotides (c and d). When the OC and OC-OC molecules were digested with *Taq* I, which cuts the DNA 17 base pairs further downstream from the origin than *Rsa* I, the fragments formed were longer by the expected amount (data not shown). Therefore, these fragments must be the cleavage products of the lagging strands extended by about 80 and 170 nucleotides, respectively, beyond the *terH* site. These fragment families were not present in the cleavage products of the LI molecules.

Locations of Termini of DNA Strands Where Synthesis Ends. To determine the locations of the 3′ ends of the leading strands and the 5′ ends of the lagging strands of OC-OC and LI molecules, we isolated linear and X-shaped molecules formed by digestion of peak I DNA with *Eco*RI. These molecules and OC monomers (peak II DNA) were further digested with *Hin*fI, and the resulting fragments were analyzed after denaturation (Fig. 4*c*). Of particular interest are the fragments produced by cleavage on either side of the origin (at positions −312 and +71). In addition to the triplet

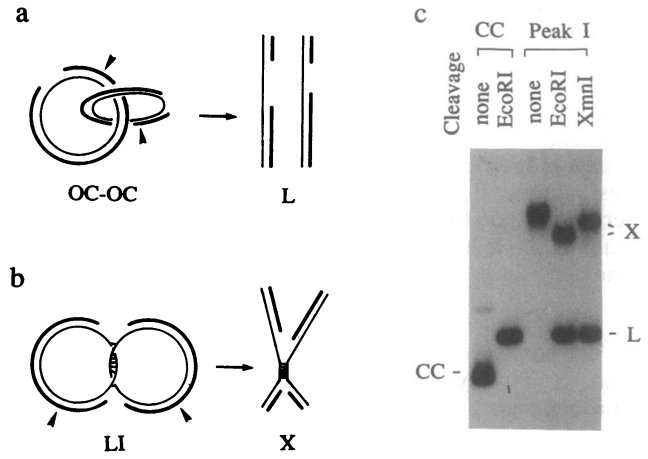

FIG. 3. Detection of LI molecules in peak I DNA. (*a* and *b*) Products of digestion of an OC-OC catenane and a LI molecule with a restriction enzyme that cleaves the template DNA once are schematically shown. (*c*) Products of digestion of peak I DNA with *Eco*RI or *Xmn* I were electrophoresed in neutral 1% agarose. Samples were supercoiled CC monomer, untreated or treated with *Eco*RI, and peak I DNA, untreated or treated with *Eco*RI or *Xmn* I.

fragments from the 5′ ends of the leading strands (a) and the fragments from the 3′ ends of lagging strands terminated at the *terH* site (b), we found a group of characteristic fragments 280–310 nucleotides long in the digest of all three species and named them T-group fragments. Analysis of hybridization with either one of the strands of pSD71 DNA showed that T-group fragments were exclusively from the leading strand (data not shown). The *Hin*fI digests were further treated with *Hae* II, which cleaves the *Hin*fI C segment 126 nucleotides from the upstream end. We found that each T-group fragment was shortened by about 130 nucleotides (data not shown), indicating that T-group fragments contained the 3′ ends of the leading strands. A large fraction of the T-group fragments from the OC monomers had lengths of about 310 nucleotides, indicating that the 3′ ends of the leading strand on the OC monomers were located very close to the origin. Cleavage of the OC-OC catenanes yielded T-group fragments of about 280 nucleotides in addition to the fragments of about 310 nucleotides. The 3′ ends of these fragments must be located about 30 nucleotides upstream of the origin. On the other hand, T-group fragments from LI molecules were 280 nucleotides or less. The OC-OC catenanes that gave the 280-nucleotide fragments were probably derived from LI molecules that had been converted to OC-OC catenanes during the purification process.

Rsa I digestion of the OC and OC-OC molecules yielded fragments derived from lagging strands extended by 80 or 170 nucleotides from the *terH* sites (Fig. 4 *a* and *b*), *Hin*fI digestion of these molecules yielded C fragments (positions −312 to +71) from the lagging strands, covering the origin (Fig. 4*c*). No such fragments were found in either digest of the LI molecules. These fragments must be derived from lagging strands extended from the *terH* sites. In addition, because some OC and OC-OC molecules had lagging strands terminated at the *terH* sites, the extension was not necessary for segregation. We could not detect unique-size products with the 5′ ends of the lagging strands in the digests of OC, OC-OC, and LI molecules with *Hin*fI (Fig. 4*c*) or *Bst*EII (cleaves at position −359) (data not shown). Therefore, the ends may be located in many sites.

Segregation of LI Molecules. To examine whether LI molecules could be precursors for OC monomers and OC-OC catenanes, we incubated peak I DNA in a mixture for *in vitro* replication containing KCl at about 40 mM and rifampicin at 10 μg/ml (Fig. 5). As a control, the purified CC-CC catenanes

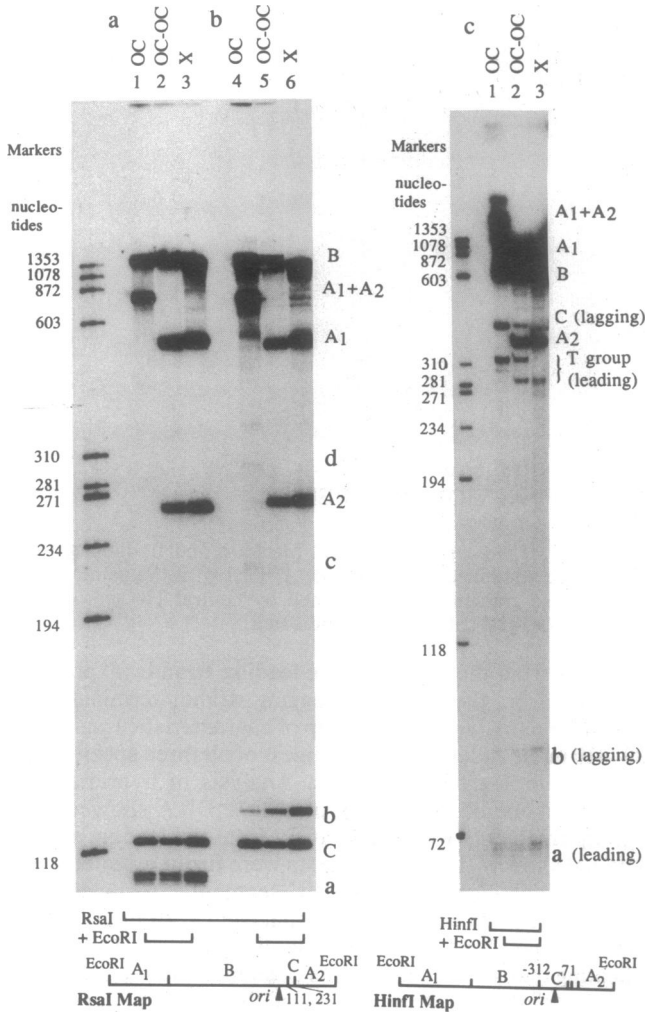

FIG. 4. Fragments of newly synthesized DNA strands cleaved by *Rsa* I or *Hin*fI. Peak I DNA was digested with *Eco*RI and unit-length linear molecules that derived from OC-OC catenanes and X-shaped molecules from LI molecules were separated. These molecules and OC monomers of peak II DNA were cleaved with *Rsa* I or *Hin*fI. The *Rsa* I fragments were hybridized with one or the other strand of pSD71 DNA. Those hybridized with the template DNA for leading-strand synthesis (*a*) or for lagging-strand synthesis (*b*) were separated. These fragments were denatured and analyzed by electrophoresis in a 6% polyacrylamide gel containing 8 M urea. The *Hin*fI fragments were denatured and analyzed by electrophoresis in a 5% polyacrylamide gel containing 8 M urea (*c*). The strand assignments of the isolated *Hin*fI fragments that were made by hybridization to separate strands of pSD71 DNA are shown in parentheses. Fragments A_1, A_2, and B hybridized almost equally to both strands. The markers were *Hae* III fragments of ϕX174 DNA. The cleavage maps with *Rsa* I or *Hin*fI of pSD71 DNA linearized at the *Eco*RI site are shown at the bottom.

formed in an extract supplemented with fraction II at 40 mM KCl were also incubated. These molecules were a mixture of catenanes of various interlocking numbers. The distribution of the catenanes with respect to interlocking number was not significantly changed by the incubation, as shown by the analysis of molecules after their conversion to OC-OC catenanes (refs. 1 and 2; Fig. 5, lanes 2, 4, and 6).

The peak I DNA used was composed of 60% LI molecules, the rest being OC-OC catenanes (lane 8). Analysis of the products of a 30-min incubation (lane 11) showed that about 40% of the total labeled material was converted to CC-CC catenanes, about 10% to CC-OC catenanes, about 15% to CC molecules, and about 5% to OC molecules. Most CC-CC catenanes were singly interlocked. During incubation about

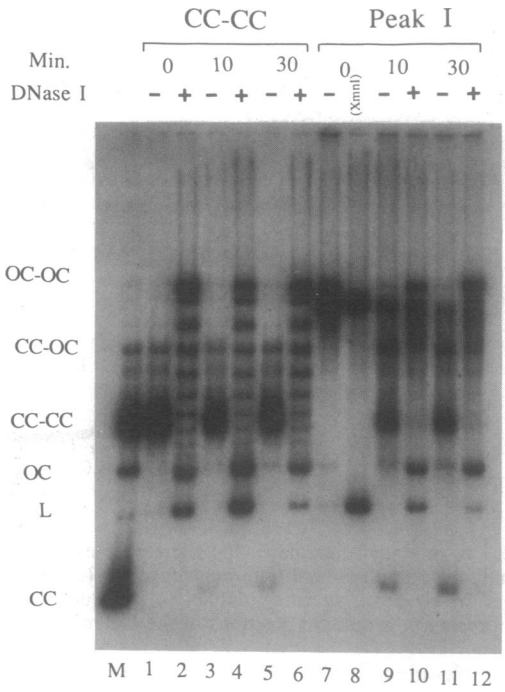

FIG. 5. Products of CC-CC catenanes and peak I DNA incubated in a cell extract. The following samples were analyzed by 1% agarose gel electrophoresis. The ^{32}P-labeled CC-CC catenanes were made by incubating pSD71 DNA for 30 min at 30°C in a cell extract supplemented with 1/20th volume of fraction II in the presence of about 40 mM KCl. They were isolated by centrifugation (7). The catenanes thus made (lane 1) or peak I DNA (lane 7) was incubated in cell extract in the presence of 40 mM KCl and rifampicin (10 μg/ml) at 30°C for 10 min (lanes 3 and 9) or 30 min (lanes 5 and 11). The aliquots of the products (lanes 1, 3, 5, 9, and 11) were incubated with DNase I (10 ng/ml) and supercoiled pSD71 DNA (20 μg/ml) for 10 min at 25°C (lanes 2, 4, 6, 10, and 12, respectively). Under the cleavage condition, supercoiled pSD71 DNA was converted to mostly OC and partly linear molecules. The amount of LI molecules in peak I DNA was estimated by digestion with *Xmn* I (lane 8). The lane for markers (M) contained a mixture of reference materials. Positions of three kinds of catenanes in which two daughter units are interlocked once [prepared as described (7)] are indicated. The CC units in these molecules are supercoiled.

30% of the input material was degraded to acid-soluble material. Because the amount of labeled material in catenanes and monomers in the products exceeded that of OC-OC catenanes in the peak I DNA, a part of the products must have been derived from LI molecules in peak I DNA. However, it is difficult to deduce the extent and process of the conversion of LI molecules to the products, because of the degradation of a significant fraction of the input molecules. Nonetheless, the absence of significant changes in the interlocking numbers of the control molecules shows that decatenation is unlikely to be involved in formation of monomers.

DISCUSSION

When CC molecules of ColE1 DNA replicated in a cell extract at high KCl concentration (step II), LI molecules accumulated together with OC and OC-OC molecules. For the typical LI molecule, the 5′ end of the leading strand is at the origin and its 3′ end about 30 nucleotides away from the origin. The 3′ end of the lagging strand is at the *terH* site and its 5′ end is at various sites, some of which are located >300 nucleotides from the *terH* site. If ends of newly synthesized strands stay on the template strands, the parental strands can pair over a maximum of about 13 nucleotides, and thus they

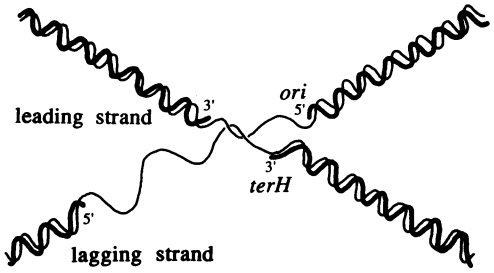

FIG. 6. Origin–terminus region of a typical LI molecule with a gap 30 nucleotides long in the leading strand and a larger gap in the lagging strand.

intertwine with each other only once (Fig. 6). Therefore, if the parental strands of the molecule separate without changing the topological relation, the molecule must be converted into a singly interlocked OC-OC catenane. If instead they separate while swiveling around each other once, they must give two OC molecules. Thus, the typical LI molecules are very likely to be the immediate precursors of the OC and OC-OC molecules. In fact, when the KCl concentration of the reaction mixture was lowered, practically all the LI molecules were converted into CC and CC-CC molecules (step III). In addition, when purified LI molecules were incubated in a cell extract at low KCl concentration, some of them were converted to CC monomers and to singly interlocked CC-CC catenanes. These molecules are the expected products of segregation from LI molecules, followed by elongation and circularization of the newly synthesized strands.

Some purified LI molecules were susceptible to degradation in a cell extract, while the molecules as they were made appeared to be stable. Similar observation has been made for the early replicative intermediates containing 6S DNA (Y. Sakakibara and J.T., unpublished results). The stability of LI molecules in the extract may be maintained by binding of replication proteins. If some of these proteins participate in the process of segregation, segregation may well be coupled with elongation of the DNA chains. The products of DNA synthesis in a cell extract under standard conditions are predominantly CC monomers together with a small amount of singly interlocked CC catenanes (7), whereas when DNA synthesis is stimulated by supplementing fraction II, CC monomers and multiply interlocked CC-CC catenanes are the main products (Fig. 5). Reduction of the extent of interlocking of CC-CC catenanes in both conditions was very inefficient. It seems that when the relative activity of a helicase is high, the parental strands will separate before reduction of the extent of intertwining, thus favoring formation of multiply interlocked catenanes. On the other hand, when the relative activity of a topoisomerase is high, the extent of intertwining will be reduced before separation of the parental strands, thus favoring formation of monomeric molecules. If the helicase activity associated with the DnaB protein in a replisome complex were involved in the process of segregation, the process would be difficult to separate from elongation of a DNA chain.

In principle, the intertwining number of a LI molecule can be changed by a type I topoisomerase acting on single-stranded regions in both parental strands, or by a type II topoisomerase acting on double-stranded replicated regions on both parental strands. We have observed that neither topoisomerase I (22) nor DNA gyrase (23) could produce monomers efficiently from LI molecules in purified peak I DNA under the conditions where OC-OC catenanes could be separated efficiently into monomers (data not shown). Even if one of these enzymes, or another topoisomerase, such as topoisomerase III (24–26) or IV (27), alone or together with a helicase allows segregation of LI molecules, the finding does not necessarily mean that the enzyme(s) is a participant in segregation under replication conditions.

In explaining the effect of the high concentration of KCl that allows accumulation of LI molecules, one has to propose a salt-sensitive process that operates at or immediately before segregation. A simple explanation is to suppose an enzyme uniquely required for segregation and suppressed at high salt conditions. Alternatively, the process would be carried out by normal components for DNA chain elongation but a unique structure of the DNA for segregation would make them sensitive to the high salt condition.

For a molecule whose replication proceeds bidirectionally, the ends of an elongating strand meet at a place other than the origin region. Nonetheless, the immediate precursor for segregation probably has a short unreplicated region where intertwined template strands pair by hydrogen bonds. Therefore, we could envisage essentially the same mechanism for segregation of daughter molecules as occurs after unidirectional replication.

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