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In vivo duplication of genetic elements by the formation of stem–loop DNA without an RNA intermediate

(inverted repeats/ColE1 plasmid/DNA polymerases/*Escherichia coli*/gene duplication)

ATSUSHI OHSHIMA, SUMIKO INOUYE, AND MASAYORI INOUYE

Department of Biochemistry, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, 675 Hoes Lane, Piscataway, NJ 08854

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ABSTRACT **Gene duplication through cDNA synthesis by reverse transcriptase is believed to have played an important role in the diversification of genomes during evolution. Here, we demonstrate that a genomic DNA sequence can be duplicated *in vivo* as a result of template switching. When an inverted repeat (IR) structure was inserted in a site downstream from a ColE1 plasmid origin of DNA replication, transformation of *Escherichia coli* cells with this plasmid resulted in the production of a new DNA fragment encompassing the region from the origin to the center of the IR structure. The structure of this DNA molecule is composed of a long stem–loop formed by a single-stranded DNA, in which the loop is formed by the IR structure. The DNA fragment is designated slDNA, for *s*tem–*l*oop DNA. The experiments in this study suggest that during DNA replication, template switching at the stem–loop structure formed by the IR structure gives rise to slDNA utilizing the nascent DNA strand or the parental strand as a template. The mechanistic implications of slDNA synthesis, and its possible roles in genome evolution, are discussed.**

Duplication of part of a genome is known to occur via an RNA intermediate that is reverse-transcribed into a complementary DNA (cDNA) by reverse transcriptase (see ref. 1 for a review). The consequential reverse flow of genetic information is considered to have played a major role in the evolutionary diversification of eukaryotic genomes. A similar mechanism may very well have been responsible for genomic evolution in prokaryotes, in the light of the recent discoveries of bacterial reverse transcriptases (see refs. 2 and 3 for reviews).

In the present study, we found that during the replication of plasmid ColE1 of *Escherichia coli*, a portion of a genome can be directly duplicated from the genome. This gene duplication thus requires neither an RNA intermediate nor reverse transcriptases and occurs during DNA replication. In the presence of an inverted repeat (IR) structure, the direction of DNA synthesis was found to be reversed by template switching at a certain frequency at the loop position formed by the IR structure. As a result, a DNA fragment containing specific genetic elements followed by the IR structure was produced. This fragment consisted of a single-stranded DNA that formed a long stem–loop structure, with the loop corresponding to that formed by the IR structure. The DNA fragment was thus designated slDNA, for *s*tem–*l*oop DNA. The seemingly simple mechanism of slDNA production, together with the frequent occurrence of IR sequences in both prokaryotic and eukaryotic genomes, implies that slDNAs may have played as significant a role in genome evolution as the production of cDNAs by reverse transcriptase.

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MATERIALS AND METHODS

Materials. Restriction enzymes and ribonucleases were purchased from either New England BioLabs or Boehringer Mannheim. [γ-^{32}P]ATP and [α-^{32}P]ddATP were from Amersham.

Bacterial Strain and Growth Condition. *E. coli* CL83 (4) was grown in L broth (5). *E. coli* cells harboring plasmids were grown in L broth containing kanamycin sulfate at 25 μg/ml.

Plasmid Construction. pUCK19 was constructed by inserting the 1.3-kilobase (kb) *Hin*cII fragment containing the kanamycin-resistance gene from Tn*5* (6) at the *Dra* I sites of pUC19 (7). At the single *Xba* I site, a 215-base-pair (bp) DNA fragment containing the 35-bp IR sequences (see Fig. 2) was inserted and the resulting plasmid was designated pUCK106. To construct pUCK106Δlac^{PO}, the 199-bp *Pvu* II–*Hin*cII fragment containing the *lac* promoter–operator region was deleted from pUCK106.

Preparation of slDNA. slDNA was isolated by the alkali/SDS method developed for the preparation of plasmid DNA (8). After treatment with ribonuclease A, the DNA preparation was applied to a 5% polyacrylamide gel for electrophoresis. slDNA was visualized by staining with ethidium bromide.

Formation of slDNA dimers was performed as follows. slDNA eluted from the gel was solubilized in 10 mM Tris·HCl, pH 8.0/150 mM NaCl/10 mM $MgCl_2$. The slDNA solution was incubated in a boiling water bath for 3 min and then gradually chilled. All restriction digests were performed according to the conditions recommended by suppliers of the restriction endonucleases. In some experiments, DNA fragments generated by restriction enzyme digestions of slDNA were visualized by labeling at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase.

DNA Sequence Determination. The 3′ end and the loop regions of slDNA were labeled either with [γ-^{32}P]ATP at their 5′ ends by T4 polynucleotide kinase or with [α-^{32}P]ddATP at their 3′ ends by terminal deoxynucleotidyltransferase. DNA sequencing of these regions was carried out according to Maxam and Gilbert (9). DNA sequences of the other regions of slDNA were determined by the chain-termination method (10) using synthetic oligonucleotides as primers.

RESULTS

IR Structure Giving Rise to a New DNA Fragment. A DNA fragment of 215 bp containing long IR sequences was inserted into the single *Xba* I site at the polylinker site of pUCK19, a kanamycin-resistant derivative of pUC19 (Fig. 1). *E. coli* cells transformed with this plasmid (pUCK106) produced a distinct DNA fragment. The 215-bp fragment was basically derived from the *msr–msd* region of retron Ec67 spanning nucleotides 234–408 (see ref. 12). Note that part of the putative promoter of the retron and the entire gene for

Abbreviation: IR, inverted repeat.

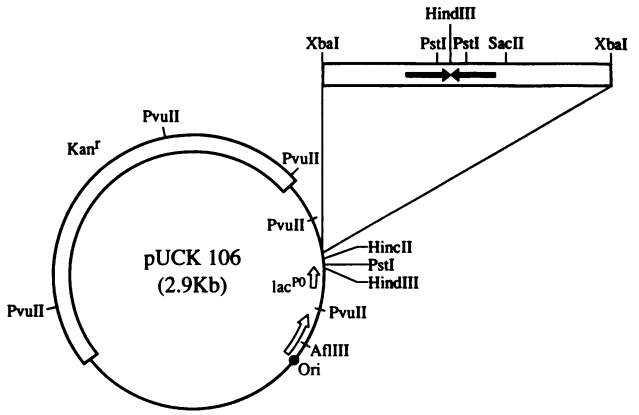

FIG. 1. Restriction map of pUCK106. The circular map corresponds to pUCK19, which is a kanamycin-resistant derivative of pUC19 (11). The kanamycin-resistance (Kan^r) gene is shown by an open bar in the circular map. At the single *Xba* I site, a 215-bp DNA fragment containing 35-bp IR sequences (see Fig. 2) was inserted; the fragment is shown by a straight open bar at upper right. Solid arrows indicate the IR structure. The origin of replication is indicated by a solid circle and a large open arrow indicates the direction of DNA replication from the origin. The position of the *lac* promoter–operator (lac^{PO}) is shown by a small open arrow.

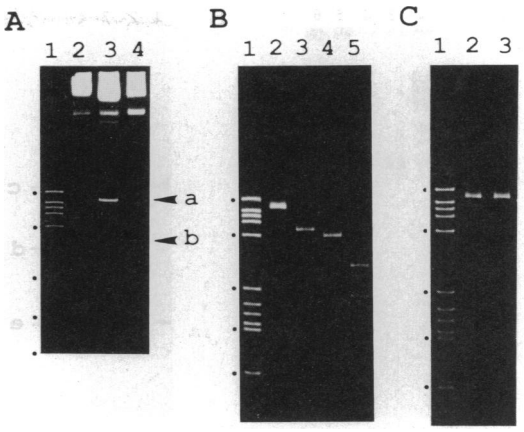

FIG. 3. Production of slDNA from pUCK106 and its characterization. (*A*) *E. coli* CL83 (4) was transformed with either pUCK19, pUCK106, or pUCK106Δlac^{PO}. After ribonuclease A treatment, DNA preparations were applied to a 5% polyacrylamide gel for electrophoresis. The gel was stained with ethidium bromide. Lane 1, *Hae* III digest of pBR322 (size markers; from the top, dots indicate 587, 434, 267, 184, and 124 bp); lanes 2–4, DNA preparation from cells harboring pUCK19, pUCK106, or pUCK106Δlac^{PO}, respectively. Plasmid pUCK106Δlac^{PO} was constructed from pUCK106 by deleting the 199-bp *Pvu* II–*Hin*cII fragment (see Fig. 1). A new band that appeared in lanes 3 and 4 is indicated by arrows a and b, respectively. (*B*) slDNA from pUCK106 was purified by polyacrylamide gel electrophoresis and digested with various restriction enzymes. The digests were analyzed by 5% polyacrylamide gel electrophoresis, and the gel was stained by ethidium bromide. Lane 1, *Hae* III digest of pBR322 (size markers); lane 2, slDNA without digestion; lane 3, *Xba* I digest; lane 4, *Hin*dIII digest; lane 5, *Pvu* II digest. (*C*) Heat denaturation of the slDNA from pUCK106. The purified slDNA as described above was solubilized in 10 mM Tris·HCl, pH 8.0/1 mM EDTA. The slDNA solution was incubated in a boiling water bath for 3 min and quickly chilled in an ice bath. Samples were analyzed as described in *A*. Lane 1, *Hae* III digest of pBR322 (size markers); lane 2, slDNA without heat treatment; lane 3, slDNA heat-denatured and then quickly cooled.

reverse transcriptase are excluded and that the IR sequences in the *msd* region are replaced with the 34-bp IR sequences shown in Fig. 2; in addition, there are a few sequence alterations in the *msr* region. As shown in Fig. 3*A*, lane 3, a new band appeared with an apparent molecular size of 560 bp (band a) when a plasmid DNA preparation from these cells was subjected to polyacrylamide gel electrophoresis. This band did not appear in the plasmid DNA preparation from cells transformed with the original pUCK19 vector (Fig. 3*A*, lane 2). This result indicates that the DNA insert of pUCK106 is responsible for the production of band a. Densitometric analysis of the ethidium bromide-stained bands revealed an approximate 1:10 molar ratio of band a to the plasmid DNA, an amount equivalent to 10–20 copies per cell (data not shown). In the gel system shown in Fig. 3*A*, plasmid DNA stays at the top of the gel.

Double-Stranded DNA Composed of a Single Molecule of a Single-Stranded DNA. The DNA fragment comprising band a was purified by polyacrylamide gel electrophoresis and analyzed by digestion with *Xba* I (Fig. 3*B*, lane 3), *Hin*dIII (lane 4), and *Pvu* II (lane 5), which yielded fragments of 120 and 440 bp, 150 and 430 bp, and 240 and 320 bp, respectively. This result clearly demonstrates that the DNA fragment consists of double-stranded DNA. However, to our surprise, when the DNA fragment was subjected to heat denaturation followed by quick cooling, it still migrated at the same position as the untreated fragment (Fig. 3*C*, compare lane 3 with lane 2). The quantitative recovery of the DNA fragment after the heat treatment was nearly complete, indicating that it has a "snap-back" structure composed of a single-stranded DNA molecule in a long stem–loop configuration.

To confirm the long stem–loop structure, the DNA fragment was slowly renatured after heat denaturation. This treatment is expected to promote the hybridization of two individual molecules of stem–loop DNA. The length of the resulting double-stranded DNA molecule would be approximately twice as long as the untreated stem–loop DNA. Indeed, as shown in Fig. 4*A*, lane 5, the treatment resulted in the appearance of a new band (a) in addition to the band corresponding to the untreated DNA fragment (band b; see lane 3 for the control). The estimated size of the DNA fragment in band a is 1150 bp, approximately twice the size of the fragment in band b (560 bp), as expected. *Xba* I

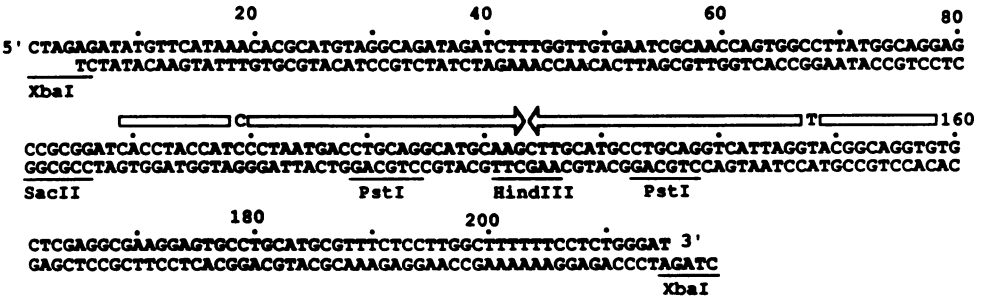

FIG. 2. DNA sequence of the 215-bp DNA fragment inserted at the *Xba* I site of pUCK19. The fragment was constructed from synthetic oligonucleotides. The IR sequences are indicated by open arrows. The mismatched positions are shown by open spaces in the arrows and the mismatched bases are inserted in the spaces.

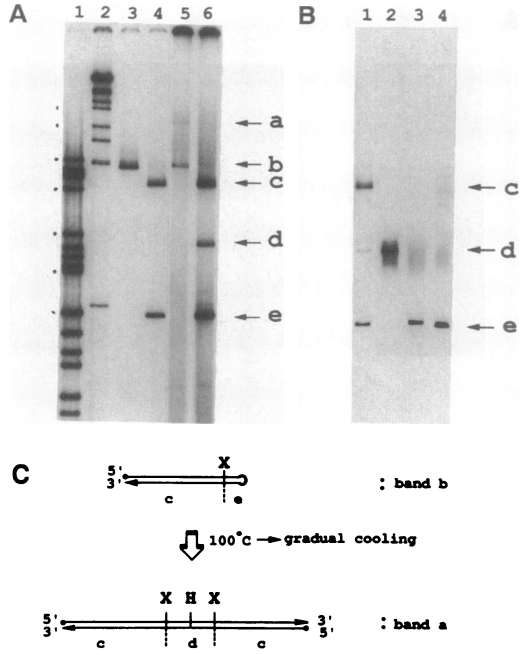

FIG. 4. Dimer formation of the slDNA from pUCK106. (*A*) slDNA was purified from pUCK106 as described in Fig. 3 and treated as described in *Materials and Methods*. The renatured slDNA was digested with *Xba* I and the DNA fragments thus generated were labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase. These products were applied to a 5% polyacrylamide gel. After electrophoresis, the gel was dried and subjected to autoradiography. Lane 1, *Hae* III digest of pBR322; lane 2, the *Eco*RI/*Hin*dIII digest of λ DNA markers (dots indicate 1370, 940, 587, 434, 267, 184, and 124 bp); lane 3, the slDNA from pUCK106 without treatment; lane 4, *Xba* I digest of the untreated slDNA; lane 5, the slDNA after heat denaturation followed by gradual cooling; lane 6, *Xba* I digest of the slDNA from lane 5. Bands are marked a–e at right. (*B*) Characterization of fragment *d* in *A*. Fragment d was gel-purified. Lane 1, same as lane 6 in *A*; lane 2, fragment d purified from the gel; lane 3, *Hin*dIII digest of the purified fragment d; and lane 4, purified fragment d that was heat denatured and quickly chilled as described in Fig. 3. (*C*) Schematic representation of bands a–e shown in *A* and *B*. X and H represent *Xba* I and *Hin*dIII sites, respectively. There are two other *Hin*dIII sites in fragment a, very close to the *Xba* I sites (within fragment c). These *Hin*dIII sites are not shown.

digestion of the DNA fragment in band b yielded two fragments migrating at positions c (440 bp) and e (120 bp) (Fig. 4*A*, lane 4). *Xba* I digestion of the products arising from gradual renaturation (bands a and b; lane 5) resulted in the appearance of an extra band at position d (240 bp) in addition to bands c and e (lane 6).

Structure of the DNA Fragment. The results described above can be interpreted as illustrated in Fig. 4*C*. The untreated DNA fragment yields fragments c and e following *Xba* I digestion, while *Xba* I digestion of the newly formed double-stranded DNA after heat denaturation and gradual renaturation yields a new fragment, d, in place of fragment e. Consistent with this hypothesis is the observation that the length of fragment d is approximately twice that of fragment e (Fig. 4*A*). Furthermore, as shown in Fig. 4*B*, lane 4, *Hin*dIII digestion of fragment d (lane 2) yielded a fragment almost identical to fragment e. In addition, heat denaturation of fragment d followed by quick cooling also yielded a fragment at position e (lane 4). These results unambiguously demonstrate the stem–loop structure of the DNA (see Fig. 4*C*). This DNA is thus designated slDNA. Note that the IR structure is designed to have a *Hin*dIII site at the center when two slDNA molecules hybridize with each other to form a double-stranded DNA (Fig. 4*C*).

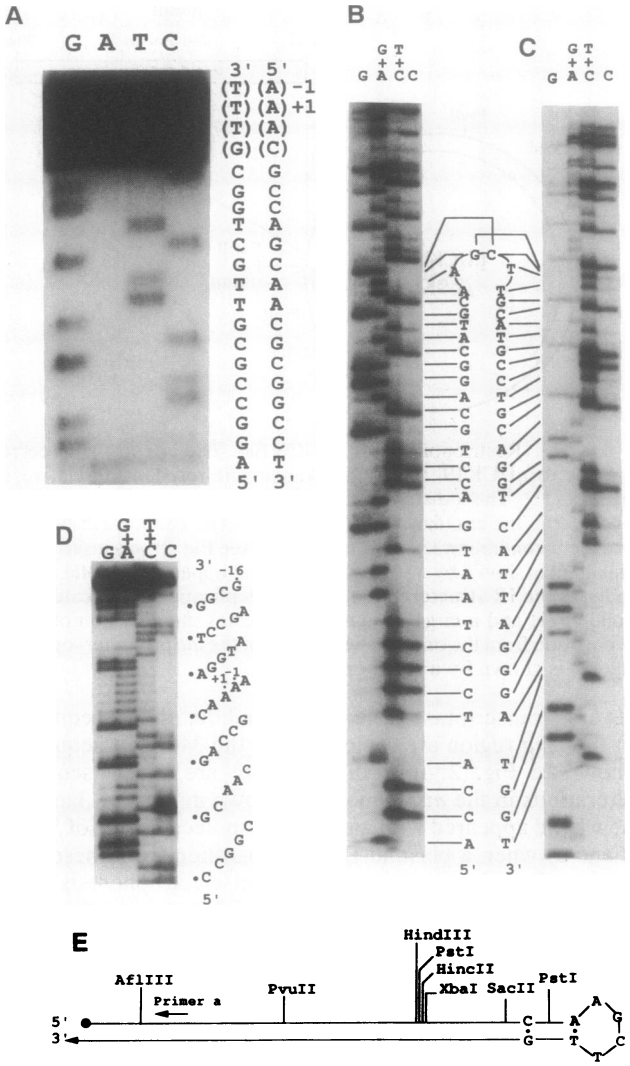

FIG. 5. Determination of the DNA sequence of the slDNA from pUCK106. (*A*) Sequencing of the 5′ end of the slDNA. Purified slDNA (0.2 μg) was used for sequencing by the chain-termination method (10). Primer a (5′-GGTTATCCACAGAATCAG-3′), which corresponds to the sequence 96 bp downstream from the origin (see *B*) was used as primer. (*B*) Sequencing of the 5′ end of the loop region of the slDNA. slDNA (0.5 μg) was digested with *Sac* II and the DNA fragments thus generated were labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase. The DNA fragment migrating at ≈40 bp was isolated and sequenced by the Maxam–Gilbert method (9). (*C*) Sequencing of the 3′ end of the loop region of the slDNA. The *Sac* II digest of the slDNA was labeled at the 3′ end with [α-^{32}P]ddATP by terminal deoxynucleotidyltransferase. The DNA fragment containing the loop region was isolated and sequenced by the Maxam–Gilbert method. (*D*) Sequencing of the 3′ end of the slDNA. The slDNA was digested with *Afl* III (see *E*). The 5′ ends were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled products were separated in a sequencing gel. The single-stranded DNA that migrated at 76 bases was isolated and sequenced by the Maxam–Gilbert method. The numbers represent the residue numbers from the origin of pUC19 (11). (*E*) Structure of the slDNA from pUCK106. The slDNA consists of a single-stranded DNA of 1138–1141 bases. The 5′ end of the slDNA appears to be heterogeneous; some start from +1 while others start from −1, +2, or +3. The +1 position corresponds to the origin of ColE1 DNA replication (13). At the 3′ end a sequence of 16 bases extends beyond the +1 position of the 5′ end. The loop is considered to be formed with the 4-base sequence AGCT, corresponding to the sequence at the center of the IR structure, where a *Hin*dIII site (AAGCTT) is designed to be placed. The base pair corresponding to the mismatch in the IR structure in pUCK106 was converted from C–T (in pUCK106) to C·G (in the slDNA) and is shown between the *Sac* II and *Pst* I sites. The position of primer a used for DNA sequencing in *A* is shown by an arrow.

Fig. 5*A* shows DNA sequencing of the 5′ end of the slDNA by the chain-termination method using primer a (Fig. 5*E*). Since alkaline treatment of slDNA labeled at its 5′ end with [γ-^{32}P]ATP removed little radioactivity from the DNA (data not shown), most of the RNA primer appears to have been removed in the slDNA. Heterogeneous initiation at the ColE1 origin has been reported at A^{+1}, A^{+2}, and C^{+3} (and probably at A^{-1} as well) (ref. 13; see ref. 14 for a review), which is in good agreement with the sequencing result shown in Fig. 5*A*. The DNA sequences of both strands of the slDNA were subsequently determined by the chain-termination method with appropriate primers and were found to be identical to those expected from the origin to the center of the IR structure inserted in pUCK106 (data not shown). The existence of the loop structure is shown in the sequencing gels of the DNA fragment generated by *Sac* II digestion of the slDNA (Fig. 5 *B* and *C*). It is evident that the slDNA is composed of a stem–loop structure forming a loop at the center of the IR structure. The loop is most likely to consist of the 4-base sequence AGCT (see Fig. 5*E*).

The 3′ end structure of the slDNA was determined by the Maxam and Gilbert method (9) using a fragment generated by *Afl* III digestion (Fig. 5*E*). Surprisingly, the 3′ end of the slDNA extended beyond the 5′ end to the G residue at position −16 (Fig. 5*D*), which coincides well with the *terH* site (position −17) known as the termination site of lagging-strand synthesis (15).

The structure of the slDNA is thus determined as shown in Fig. 5*E*. The slDNA consists of a single-stranded DNA of 1138–1141 bases, which forms a stem structure of 558–561 bp with a 4-base loop. The 3′ end of the slDNA is extended by 18–15 bases over the 5′ end of the slDNA of 1138–1141 bases. The restriction enzyme analysis in Fig. 3*B* agrees completely with the restriction map derived from the DNA sequence (Fig. 5*E*). The DNA sequence inserted in pUCK106 (Fig. 2) contains a 35-bp IR sequence with one mismatch at the 11th position from the end of the stem (indicated by an open space in the arrows in Fig. 2). In the slDNA, the mismatch is absent. At this position the T residue has been replaced with a G residue in the bottom strand in Fig. 5*E*.

Further Evidence for slDNA Synthesis. When the 199-bp *Pvu* II–*Hin*cII fragment containing the *lac* promoter–operator was deleted from pUCK106 (see Fig. 1), the resulting plasmid, pUCK106Δlac^{PO}, produced a slDNA that migrated faster than the slDNA from pUCK106 as shown at position b in lane 4, Fig. 3*A*. This slDNA was 360 bp long, which is shorter than the pUCK106 slDNA by a length nearly identical to the size of the deletion in pUCK106Δlac^{PO}. This supports the model for slDNA synthesis proposed above and also indicates that the *lac* promoter–operator is not essential for slDNA synthesis. This notion was further supported by the fact that the addition of isopropyl β-D-thiogalactopyranoside, an inducer of the *lac* operon, did not affect the production of the slDNA from pUCK106. However, the reason for the reduction of slDNA synthesis from pUCK106Δlac^{PO} is not known.

The synthesis of slDNA was not dependent upon the primary sequence of the IR structure used for pUCK106. Interestingly, the pUC7 vector by itself, which has an IR structure at the polylinker site (11), was also able to produce an slDNA corresponding to the DNA fragment from the origin to the center of the polylinker site (data not shown). These results indicate that any IR structure is able to promote slDNA production. The stability of the secondary structure formed by IR structures is most likely a major determinant in the efficiency of slDNA synthesis.

DISCUSSION

The present study demonstrates the production of a stem–loop DNA species, slDNA, during plasmid DNA replication. Based on the structure of slDNA, we propose the following model for slDNA synthesis. DNA synthesis initiates from the origin by the mechanism known for ColE1 plasmid DNA replication (13, 15). The DNA replication then proceeds by the same mechanism as chromosomal DNA replication (13). When contiguous synthesis of the leading strand initiating from the origin reaches the IR structure, most of this leading-strand synthesis proceeds through the IR structure, resulting in the replication of the entire plasmid genome. However,

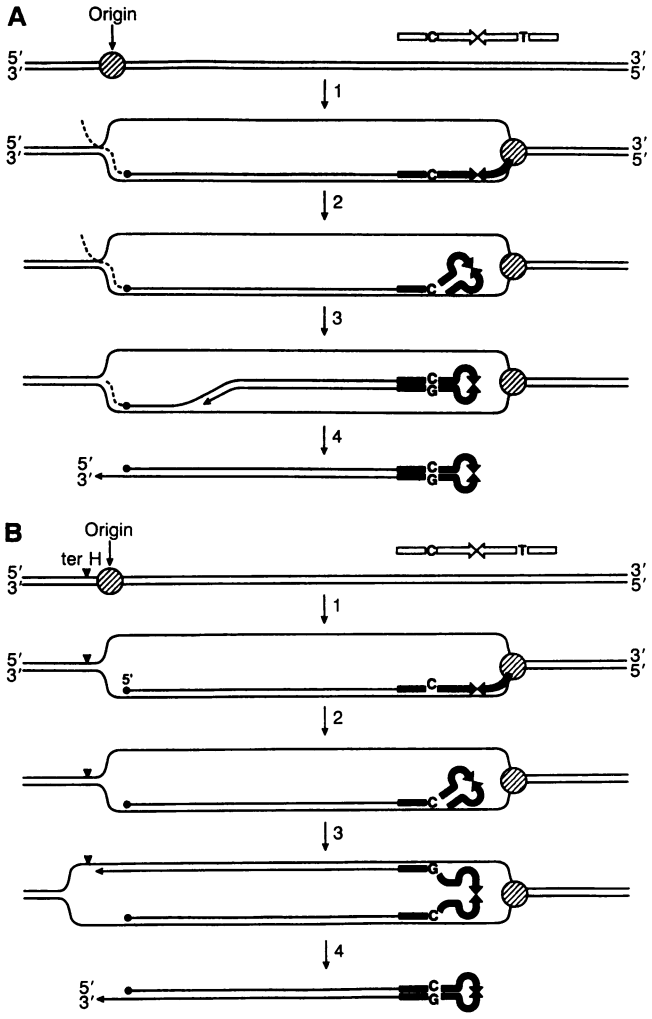

FIG. 6. Possible models of slDNA synthesis. The double-stranded DNA around the origin of the ColE1 DNA replication is shown at the top. Hatched circle represents the DNA replication complex that initiates replication from the origin. Open arrows on the DNA strand indicate the position of the 35-bp IR structure (see Fig. 2). The mismatched base pair (C–T) in the IR structure is indicated within the arrows. At step 1, the DNA replication fork proceeds from the origin (+1 position) to the position indicated by the hatched circle. The newly synthesized leading strand is shown extending from the origin (small filled circle) to the replication fork. The DNA replication complex reaches a point immediately before the mismatched T residue in the IR structure that is shown by solid arrows. At step 2, the 3′ end of the nascent strand detaches from the DNA replication complex and a secondary structure is formed by the IR structure. At step 3, DNA synthesis reinitiates from the 3′ end of the stem–loop structure, using either the nascent strand (model A) or the upper parental strand (model B). At step 4, DNA synthesis proceeds beyond the origin by 16 bases. In model A, the primer RNA remains attached at the 5′ end of the DNA and may be used as template. Subsequently, the RNA template may be removed, resulting in the formation of slDNA. In model B, DNA synthesis terminates at the *terH* site by a mechanism similar to that known for the termination of lagging-strand synthesis (15).

part of the leading strand forms a stem–loop structure at the IR sequences. As a result, leading-strand synthesis is disrupted or terminated within the stem structure (Fig. 6*A*, steps 2–3). The frequency of this disruption probably depends upon the stability of the stem–loop structure formed by the IR. However, DNA chain elongation resumes from the newly formed 3′ end, now utilizing the nascent leading strand as a template. Thus, the direction of DNA synthesis is reversed by template switching, to duplicate the DNA segment from the origin to the center of the IR structure (Fig. 6*A*, steps 4–5).

Alternatively, template switching at step 2 may occur to the upper parental strand as shown in Fig. 6*B*. These two models are not mutually exclusive and template switching may happen during slDNA synthesis. The structure of the extended 3′ end of the slDNA can be explained by model B if slDNA synthesis is terminated by a mechanism similar to that known for ColE1 (15). In model A, the 3′ end is extended beyond the 5′ end of the slDNA by using the primer RNA still attached at the 5′ end as a template, which is eventually removed (see Fig. 6*A*).

Template switching is probably carried out by DNA polymerase I. In this respect, it is interesting that DNA polymerase I has been shown to be associated with highly repetitive chromosomal IR structures of *E. coli* (16). The fact that the C–T mismatch in the IR structure was replaced with a C·G pair (see Fig. 6) indicates either that the slDNA synthesis initiated before the site of the mismatch or that the mismatch was repaired during DNA synthesis.

A model for the formation of an slDNA-like structure associated with DNA polymerase I functioning at the 3′ end of a nicked DNA strand has been proposed (17). However, at least the 5′ half of the slDNA in the present study is likely to be synthesized by the mechanism known for ColE1 DNA replication [which uses DNA polymerase III (13, 15)], since slDNA synthesis started from the origin of ColE1. Furthermore, and more important, template switching (steps 2 and 3 in Fig. 6) in slDNA synthesis is directly associated with the formation of the loop structure at the IR sequences.

It remains to be shown whether slDNA is produced during chromosomal DNA replication. However, considering the relatively simple mechanism for slDNA synthesis proposed above, we speculate that slDNA production may be widely prevalent during both prokaryotic and eukaryotic chromosomal DNA replication. The chromosomal genetic elements followed by IR structures may always be subject to duplication into slDNA at a certain frequency, depending upon the stability of the IR structures and the properties of the DNA polymerases. There are many IR structures (≈1000 copies in *E. coli*), known as REPs (*r*epetitive *e*xtragenic *p*alindromic sequences) or PUs (*p*alindromic *u*nits) (18–20). These structures appear to be associated with specific cellular components, including DNA polymerase I, and may play a significant role in chromosomal organization (16, 19). Approximately 6% of the human genome consists of *Alu* elements, whose transcriptional products have been shown to contain substantial secondary structure (21).

Since slDNA synthesis, in contrast to cDNA synthesis, does not require RNA intermediates or reverse transcriptase activity, slDNA may be produced more frequently than cDNA. Thus slDNAs might have played a major role similar to that of cDNA in the genomic evolution of both prokaryotes and eukaryotes, by duplicating genetic elements which then were dispersed or rearranged within the genome.

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