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Copy–choice recombination mediated by DNA polymerase III holoenzyme from *Escherichia coli*

(replication slippage/illegitimate recombination/direct repeats/inverted repeats)

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ABSTRACT **Formation of deletions by recombination between short direct repeats is thought to involve either a break–join or a copy–choice process. The key step of the latter is slippage of the replication machinery between the repeats. We report that the main replicase of *Escherichia coli*, DNA polymerase III holoenzyme, slips between two direct repeats of 27 bp that flank an inverted repeat of ≈300 bp. Slippage was detected *in vitro*, on a single-stranded DNA template, in a primer extension assay. It requires the presence of a short (8 bp) G+C-rich sequence at the base of a hairpin that can form by annealing of the inverted repeats. It is stimulated by (*i*) high salt concentration, which might stabilize the hairpin, and (*ii*) two proteins that ensure the processivity of the DNA polymerase III holoenzyme: the single-stranded DNA binding protein and the β subunit of the polymerase. Slippage is rather efficient under optimal reaction conditions because it can take place on >50% of template molecules. This observation supports the copy–choice model for recombination between short direct repeats.**

Recombination between short homologous sequences has been detected in all the organisms in which it has been sought, ranging from prokaryotes to higher eukaryotes (1–4). It often generates deletions but can also lead to duplications and cointegrate formation (5, 6). It has been reported that the frequency of deletion formation is affected by the length and G+C content of direct repeats, as well as the distance between the repeats and the proximity of inverted repeats (IRs) (7–12). The diversity of the genomes observed to undergo deletions and of the parameters that affect deletion frequency suggests that recombination between short repeats may take place in different ways (reviewed in ref. 13).

Two classes of models have been proposed to account for recombination between short direct repeats (14). The first is based on breakage and joining process of the DNA strands (15). The second model involves errors of the replication machinery, where a tip of the growing DNA chain is thought to dissociate from one repeat, anneal to another, and serve as a primer for further DNA synthesis (1, 16). It is akin to the model proposed to explain frameshift mutations (17) and is conceptually related to copy–choice recombination (ref. 18; reviewed in ref. 19). A strong support for the copy–choice model stems from a study of imprecise excision of the transposon Tn*10* in *Escherichia coli*, showing that the density-labeled parental DNA is not transferred to the recombinant progeny during this process (20). However, the experiments carried out *in vivo* offer only indirect evidence for the copy–choice model and do not establish which of the DNA polymerases present in the cell may be involved in this type of recombination.

Three DNA polymerases (Pol) are present in *E. coli* cells and presumably in most other prokaryotes (21). Pol III is responsible for the replication of the bacterial genome, whereas Pol I is mainly involved in DNA repair, and no clear role has yet been assigned to Pol II (22). It is well established that Pol I can slip between direct repeats *in vitro* (23, 24) and is therefore a candidate to generate genome rearrangements by a copy–choice process. However, Pol I intervenes in replication only periodically, during the lagging strand synthesis, and is not able to synthesize long DNA tracts (21), suggesting that any part of the genome is only occasionally exposed to this enzyme. This could limit the role of Pol I in the putative copy–choice recombination. These limitations do not apply to Pol III, which replicates the bacterial chromosome. There was however, no evidence, to the best of our knowledge, that Pol III holoenzyme (HE) can slip between the repeats.

Pol III HE is a complex enzyme, comprising at least 10 subunits (reviewed in refs. 25 and 26). The subunits are assembled in a three part structure: the core polymerase (subunits α, ε, and θ), the sliding clamp (or processivity factor, constituted of a β subunit dimer) and the clamp loader (or γ complex, constituted of γ, δ, δ′, χ, and ψ). The tenth subunit, the τ dimer, holds together two cores (each associated to one sliding clamp) and one γ complex. A subassembly of the subunits, known as Pol III*, contains all these elements except β. In this work we report that Pol III HE slips *in vitro* on a single-stranded DNA (ssDNA) template between short direct repeats that flank longer IRs. Slippage can occur with a high frequency, provided that the template structure and the reaction conditions are favorable. These results indicate that the main DNA replicase from *E. coli* may be able to carry out copy–choice recombination.

MATERIALS AND METHODS

Proteins. Pol III* was purified as described (27) from the *E. coli* strain SK 7772 (F⁻ *lacΔ, malA1, mtl-1, xyl-7, argH1, his-64, ilvD188, str-281, supE44, λS, uvrDΔ291*, provided by S. Kushner, University of Georgia). Two other samples of Pol III were tested and compared on SDS/PAGE and by enzymatic activity to our preparation: one (Pol III HE) was a gift of C. S. McHenry (University of Colorado); the other (Pol III*) was a gift of A. Kornberg (Stanford University), as was the β subunit of DNA polymerase. Protein concentrations and purity were estimated from the intensity of the bands after silver staining of SDS/PAGE.

M13 gene protein II (gp II) was purified to homogeneity as described (28); purity was estimated from the intensity of the band after Coomassie staining of SDS/PAGE and protein concentration was measured with the Bio-Rad protein assay.

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Abbreviations: Pol, *E. coli* DNA polymerase; Pol III HE, *E. coli* DNA polymerase III holoenzyme; Pol III*, *E. coli* DNA polymerase III* (holoenzyme minus the β subunit); SSB, *E. coli* single-stranded DNA binding protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; IR, inverted repeat.

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The overproducing strain was a gift of P. Model and K. Horiuchi (Rockfeller University). Sequenase was from United States Biochemical, and sequencing on ssDNA or double-stranded DNA (dsDNA) templates was carried out according to the protocol of the Sequenase Version 2 Sequencing Kit (United States Biochemical). ssDNA binding protein (SSB) was purchased from United States Biochemical. Restriction enzymes, exonuclease III, and T4 polynucleotide kinase were purchased from Boehringer Mannheim or New Englands Biolabs and used as recommended by the suppliers. Proteinase K was from Appligene (Strasbourg, France) or Boehringer Mannheim.

Chemicals. [α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq), [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (6000 Ci/mmol) were purchased from DuPont/NEN. Unlabeled nucleotides were from Pharmacia. Oligonucleotides were synthesized on an Applied Biosystems synthetizer.

Plasmids. pHP7f and pHP727FX plasmids were a gift from S. Bron (University of Groningen). They correspond to plasmids pHP3Ff and pHP327FX, respectively, but lack the pTA1060 replication origin (8). Recombination units FXa, FXb, and FXc (see Fig. 1 *C–E*) were constructed as follows. In a first step, synthetic oligonucleotides (whose sequences are available upon request) were annealed to form a duplex carrying a *Sna*BI site and single-stranded overhangs complementary to a *Nco*I site. They were then inserted into the *Nco*I site of the Cm gene of pHP7f. In a second step, the blunt-ended *Bgl*I fragment from pHP727FX, containing the palindrome and the K_m insert, was inserted at the unique *Sna*BI site of the three plasmids constructed in step 1.

ssDNA Templates. To prepare ssDNA templates, plasmid DNA was extracted by the alkaline lysis method, followed by phenol extraction, two successive centrifugations on CsCl density gradient, and dialysis against TE buffer, as described (29). A specific nick was introduced in the (+) strand of the f1 replication origin of the supercoiled plasmid (200 μg), using M13 gpII (20 units of DNA per μg), as described (30). Reaction was arrested with EDTA (20 mM), the products treated with proteinase K (200 μg/ml) for 10 min at 55°C, phenol extracted, and dialyzed against TE buffer. The nicked strand was then totally removed by exonuclease III digestion (10–40 units of DNA per μg for 1 h at 37°C). Exonuclease III, resulting nucleotides, and oligonucleotides were eliminated by gel filtration on FPLC Superose 6 (Pharmacia) after heat denaturation of the sample at 100°C for 1 min.

Synthetic heteroduplex used as a size marker was prepared *in vitro* by annealing circular parental ssDNA (minus strand, prepared as described above) from pHP727FX plasmid, with thermally denatured pHP7f dsDNA cleaved with *Eco*RI. After annealing, the heteroduplex was purified by gel electrophoresis from other products.

Primer Extension Reaction. The two primers used were designated 66, a 17-mer annealing 165 bases from the palindrome, and 1233, a 24-mer annealing 1235 bases from the palindrome. Unless otherwise specified, primer extension reactions contained (in 10 μl) 25–75 ng primed ssDNA, 33 units (75 ng) Pol III*, 20 ng β subunit in 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 100 μg of BSA per ml, 5–30 mM NaCl, 10% glycerol, 250 μM dNTP (each) if ^{32}P-labeled primer was used, or 250 μM dGTP and dTTP (each) and 50 μM (2.5 μCi) [α-^{32}P]dATP and [α-^{32}P]dCTP, if unlabeled primer was used. SSB was added to the mixture in a 4:1 ratio (wt/wt) relative to ssDNA (50 ng are issued from our Pol III* preparation). After 15 min at 37°C, the reaction was arrested by the addition of 25 mM EDTA and 500 μg of proteinase K per ml and further incubated 15 min at 50°C or 1 h at 37°C. When the reaction products were cleaved with restriction enzymes, proteinase K was inactivated by addition of 2 mM AEBSF [4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride] (ICN Biomedicals) and incubation for 10 min at room temperature. The reaction mixture was then dialyzed on micromembrane (Millipore VS 0.025 μM for 20 min) before cleavage.

Reaction products were analyzed by electrophoresis through 0.8% agarose gels (Seakem GTG or Ultrapure BRL) under native conditions [run in TAE buffer (40 mM Tris acetate/1 mM EDTA, pH 8.3) at 50 V for 16 h] or denaturing conditions (run in 30 mM NaOH/2 mM EDTA at 25 V for 20 h), or through 6% acrylamide sequencing gels [run in TBE buffer (90 mM Trisborate/2 mM EDTA, pH 8.3), at 1.8 KV/65 mA for 2–3 h]. DNA was visualized by direct exposure of the dried gels to x-ray films (Fuji RX or DuPont Cronex 4).

RESULTS

Experimental Approach. To examine the ability of Pol III HE to slip between direct repeats a simple *in vitro* assay was set up, consisting of a primer extension reaction on a circular ssDNA template (Fig. 1*A*). The template carries two direct repeats that flank a pair of IRs. Replication without slippage should generate a double-stranded molecule, termed parental, whereas slippage between the direct repeats should generate a heteroduplex, composed of one recombinant and one parental DNA strand. All the material between the direct repeats and one of the direct repeats, designated the recombination unit (Fig. 1*B*), is lost from the recombinant strand, and size analysis of the replication products can be used to follow this loss. We conjectured that pausing of Pol III immediately after replication of the repeat proximal to the primer may favor the slippage. It has been shown previously that Pol III can be arrested at palindromes, presumably by formation of hairpin structures (31) and the IRs were therefore introduced onto the template next to the direct repeats. To increase the hairpin stability and thus Pol III pausing a stretch of 8 G+C base pairs ("G+C clamp") was present at, or close to, the base of the palindrome in three of the four recombination units (Fig. 1 *C–F*).

Pol III Slips Between the Direct Repeats. DNA synthesis was carried out on ssDNA template to which a primer was annealed 165 bp from the IR. The products were separated by agarose gel electrophoresis, and visualized by autoradiography. Two bands were detected with all templates (Fig. 2*A*). The slower band corresponded to the double-stranded parental DNA (P), as judged by comparison with the appropriate standard, and the faster to a partially replicated DNA, due to the arrest of the synthesis at the hairpin (S, see below). An additional strong band, comigrating with the synthetic heteroduplex, was detected with FXb. Denaturing gel electrophoresis (Fig. 2*B*) indicated that the size of the newly synthesized strand of the putative FXb heteroduplex corresponds to that of the linear recombinant strand.

The synthesis of the entire parental duplex is somewhat surprising since Pol III is thought not to have a strand displacement activity, presumably required to traverse a long hairpin structure. A hairpin was present on most or all single-stranded template molecules, as judged by two lines of evidence: (*i*) a direct observation by electron microscopy and (*ii*) the ability of a restriction enzyme specific for dsDNA to cleave the single-stranded template at a site within the IRs (not shown). No helicase activity was observed when the enzyme was incubated with a 24-base-long oligonucleotide annealed to a ssDNA template (not shown). These results suggest that Pol III may be endowed with a strand displacement activity that can be detected only at hairpin structures.

To map precisely the Pol III arrest sites, the reaction products obtained with the FX template were analyzed on a sequencing gel (Fig. 3*A*). The major stops occurred at nucleotides 8, 9, and 11 within the hairpin (numbering starts at the beginning of IR) rather than at its base. Interestingly, the stop sites are located just ahead of and within the G+C clamp (Fig. 1*F*). This suggests that the clamp may prevent the hairpin from opening and thus arrest Pol III.

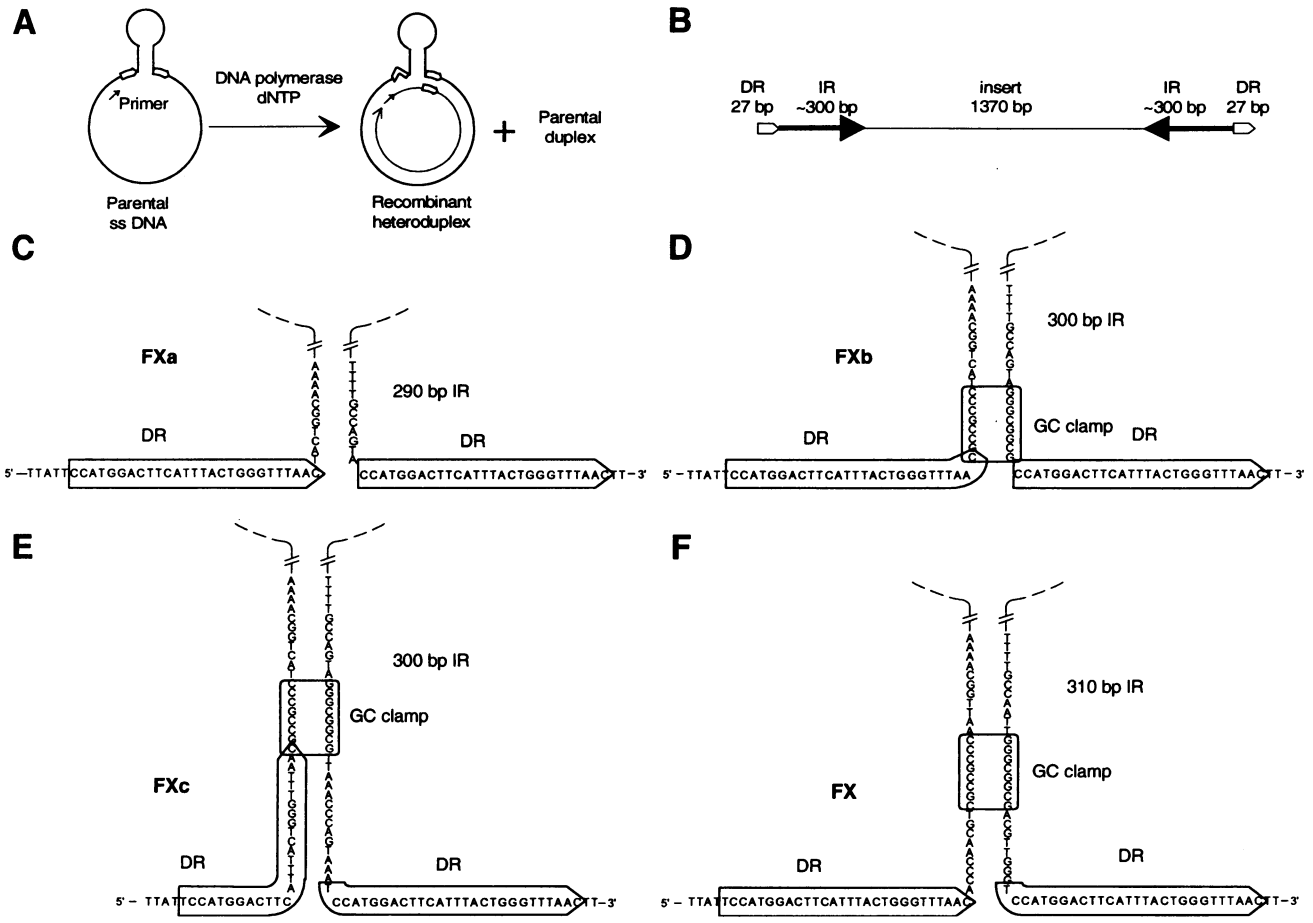

FIG. 1. Experimental system. (*A*) Schematic representation of the primer extension reaction used in this work. (*B*) Structure of the recombination units. The units are constituted of two 27-bp direct repeats (DR, open boxed arrows), flanking a pair of IRs varying from 290 to 310 bp (thick arrows) and a central 1370-bp region (insert). The units are inserted at the *Nco*I site of the plasmid pHP7f (4115 bp) and differ by the sequence at the base of the palindrome. (*C–F*) Sequences of the recombination units. Sequences of the DR (boxed arrows) and the beginning of the IR are represented. The remainder of each unit is indicated by the interrupted lines. The length of the IR is indicated. The G+C clamp, when present, is boxed.

The structure of the putative heteroduplex obtained with FXb was analyzed in more detail by restriction with three different enzymes (*Ase*I, *Rsa*I, and *Stu*I), which cleave the plasmid vector beyond the palindrome, relative to the primer used (Fig. 3*B Right*). Electrophoresis on a sequencing gel revealed in each case a band of the size corresponding exactly to that expected from slippage of the polymerase between the direct repeats, which eliminates one of the repeats and all of the intervening sequences (Fig. 3*B Left*). Double digestions with one of the three enzymes and an enzyme cleaving the vector between the primer and the palindrome (*Xmn*I) also gave segments of the expected size (212, 242, and 312 nucleotides for digestions with *Ase*I/*Xmn*I, *Rsa*I/*Xmn*I, and *Stu*I/*Xmn*I, respectively; data not shown).

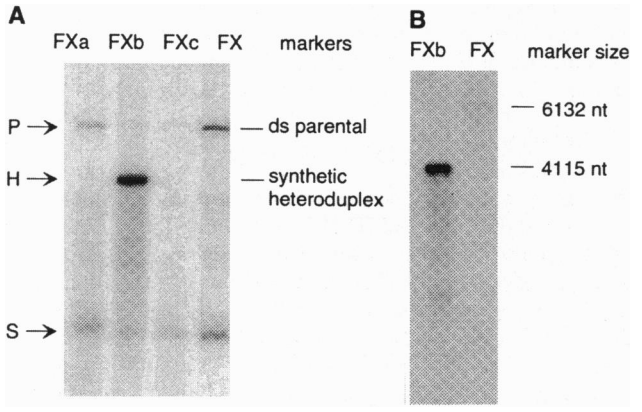

FIG. 2. DNA synthesis on ss templates carrying different recombination units. The reactions were carried out in the presence of labeled dNTPs, with primer 66 annealed to the templates, and analyzed by electrophoresis. (*A*) Neutral gel electrophoresis. The templates are indicated above the lanes. The size markers (revealed by ethidium bromide staining; not shown) were pHP727Fx nicked dsDNA (ds parental, lanes P) and a synthetic heteroduplex (lanes H) obtained by annealing of pHP727FX circular ssDNA to the complementary strand of pHP7f linear DNA. The fast-migrating band (S) is due to pausing of Pol III at the hairpin. (*B*) Alkaline gel electrophoresis. Size markers were 6132 bp pHP727Fx dsDNA and 4115 bp pHP7f dsDNA .

Formation of the FXb heteroduplex, in addition to parental double-stranded molecules, was also observed with two other enzyme preparations (Pol III* plus β, both from A. Kornberg, and Pol III HE, from C. S. McHenry). These results strongly indicate that the heteroduplex is due to Pol III and not to a contaminating activity. We conclude that Pol III can slip *in vitro*. The slippage is rather efficient, since the main reaction product can be a heteroduplex molecule (Fig. 2*A*, lane FXb).

Parameters Affecting Pol III Slippage. The above results suggest that pausing of Pol III at the bottom of the hairpin, and thus presumably within the direct repeat, may be a critical parameter for *in vitro* slippage. To test whether the enzyme-to-template ratio may affect the pausing and thus the efficiency of slippage, the concentration of the enzyme was varied while the template concentration was kept constant; the reaction products were then cleaved as indicated and analyzed on a sequencing gel (Fig. 4). At high polymerase concentrations the

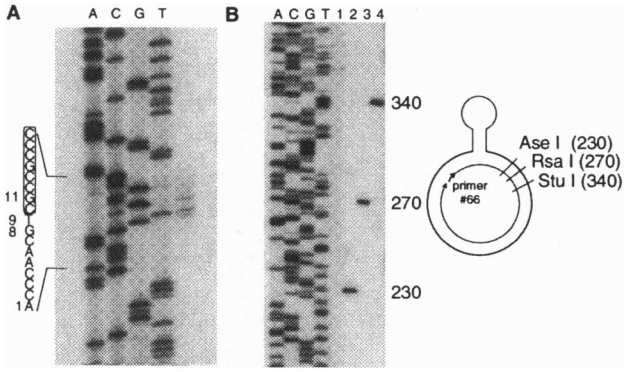

FIG. 3. Characterization of products formed by replication of recombination units FX and FXb. (*A*) The reaction was carried out with labeled primer 66 annealed to single-stranded pHP727FX DNA and the products were analyzed by electrophoresis on a sequencing gel. The sequence on the left of the gel corresponds to the beginning of the IR (number 1 is the first nucleotide of the IR), and the eight contiguous G+C pairs within the IR are boxed. The numbers refer to nucleotide position within the IR. (*B*) Restriction analysis of the FXb replication products. DNA synthesis was carried out in the presence of labeled dNTPs on single-stranded FXb template to which primer 66 was annealed, the products were purified, cleaved with the restriction enzymes, and analyzed by electrophoresis on a sequencing gel. Lanes: 1, uncleaved sample; 2–4, samples cleaved with *Ase*I, *Rsa*I, and *Stu*I, respectively. The numbers refer to the size, in bp, of the bands. To the right of the gel is represented schematically the heteroduplex formed by replication of FXb. Positions of cleavage sites used for heteroduplex characterization are indicated. Numbers in parenthesis refer to the distance (in bp) between the 5′ end of primer 66 and each restriction site.

parental molecules (P) were predominant while at low polymerase concentrations the heteroduplex (H) was the major product. The intermediate band (P+H+S) is formed by restriction of all products and served as an internal standard for the amount of material deposited on the gel. We conclude that heteroduplex formation is favored at low enzyme-to-DNA ratio. Similar results were obtained when the concentration of enzyme was kept constant and the DNA concentration was varied (not shown). It is possible that at higher ratios the polymerase may more easily traverse the entire hairpin, or pause within it (see Fig. 4, lanes 2 and 6).

The stability of duplex DNA could affect formation of parental and heteroduplex molecules by modulating the ease of penetration of the enzyme into the hairpin. Since the stability should depend on the salt content of the medium, DNA synthesis was carried out at different NaCl concentrations (Fig. 5*A*). Formation of parental molecules diminished with increasing salt concentrations and was completely inhibited at 50 mM NaCl, whereas heteroduplex formation increased slightly until 50 mM NaCl and was inhibited only at 100 mM. A similar effect was observed in the presence of potassium glutamate, where the parental and heteroduplex formation was inhibited at 200 mM and 400 mM, respectively (not shown).

It appeared interesting to test whether the processivity of Pol III affects its capacity to slip between the repeats. Two factors are essential for the processivity of the enzyme: the SSB protein and the β subunit of Pol III HE. To test the effect of SSB, we added it in ratios varying between 2 and 4 (wt/wt) relative to template DNA (our Pol III preparation contained some contaminating SSB, which allowed DNA synthesis even when no SSB was added). Formation of heteroduplex (H) and partially replicated molecules (S) was enhanced at high SSB/template ratios, while the formation of parental molecules was inhibited (Fig. 5*B*).

The β subunit acts as a dimer that forms a ring around dsDNA, thus preventing the dissociation of Pol III (32–34). Addition of increasing amounts of β to the reaction stimulated synthesis of all molecular forms, but the formation of heteroduplex was stimulated to a greater extent than that of parental molecules (Fig. 5*C*). This result, together with that obtained with SSB, suggests that although Pol III should dissociate from its template at the base of the palindrome, factors known to enhance the Pol III processivity favor the slippage.

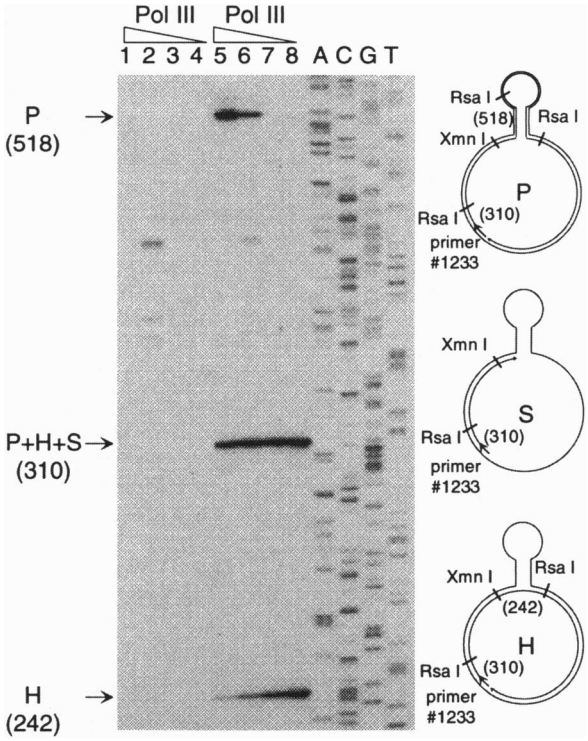

FIG. 4. Effect of variation of the polymerase/DNA ratio on heteroduplex formation. Primer extension reactions were carried out in the presence of labeled dNTPs on single-stranded FXb template (75 ng per reaction) to which primer 1233 was annealed. Pol III amounts were as follows: lane 1, 90 units; lane 2, 45 units; lane 3, 33 units; lane 4, 22 units. The products were cleaved and analyzed on a sequencing gel. Cleavage was either with *Xmn*I (lanes 1–4) or with *Xmn*I and *Rsa*I (lanes 5–8). To the right of the figure are indicated the structure of the expected products (P, H, and S refer to the parental, heteroduplex, and partially replicated molecules, respectively) and the position of the relevant restriction sites. The numbers indicate the size of the informative restriction segments. To the left of the figure are indicated the positions of these segments (P for parental, H for heteroduplex, and P+H+S for a segment derived from all products).

DISCUSSION

We report here that the main *E. coli* replicative polymerase, Pol III holoenzyme (Pol III HE), can slip with high frequency on a ssDNA template, between direct repeats of 27 bp. Slippage gave rise to a product missing one of the repeats and the region between the repeats. It was observed with three different enzyme preparations, from laboratories of A. Kornberg, C. S. McHenry, and our own, and required presence of subunit β. This argues against the possibility that a contaminating activity was generating the slippage product. An appropriate ssDNA template was required to detect the slippage.

All templates tested carried a ≈300-bp IR, forming a hairpin structure, which promotes pausing of Pol III HE (Fig. 2 and ref. 31). The stop sites, analyzed in detail with a template that did not allow slippage (FX, Fig. 1*F*), were localized 8–11 bp within the hairpin, at a sequence containing eight consecutive G+C pairs, designated G+C clamp. This shows that the enzyme can penetrate into the hairpin. It is likely that the G+C clamp interferes with the opening of the hairpin and thus promotes pausing of the enzyme. Slippage was observed on a

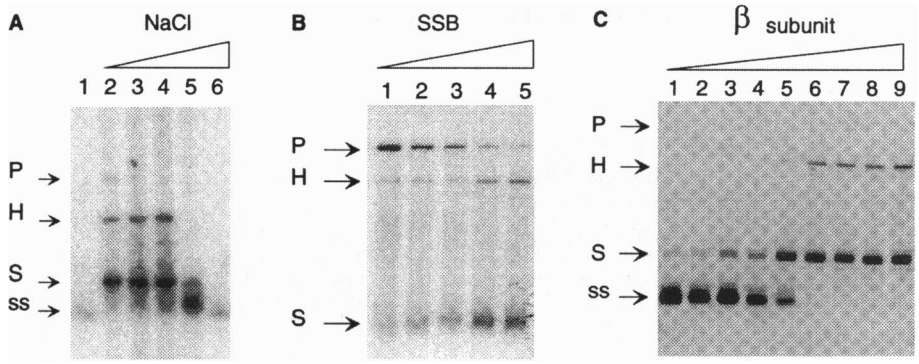

FIG. 5. Factors affecting Pol III slippage. Labeled primer 66 (*A* and *C*) or 1233 (*B*) was annealed to the ss FXb template, DNA synthesis was carried out under standard conditions except for the factor under study and the products were analyzed by agarose gel electrophoresis. P, parental dsDNA; H, recombinant heteroduplex DNA; S, stop at the palindrome; ss, parental ssDNA. (*A*) NaCl concentration. Lanes: 2, 30 mM; 3, 50 mM; 4, 100 mM; 5, 200 mM; 6, 400 mM. Lane 1, primed ssDNA. Primed ssDNA concentration, 15 ng/μl. (*B*) SSB concentration. SSB was added to the reaction mixture containing all the ingredients except Pol III and preincubated for 5 min at 37°C before the addition of Pol III. Amounts of total SSB are as follows: lane 1, 50 ng; lane 2, 62.5 ng; lane 3, 75 ng; lane 4, 100 ng; lane 5, 150 ng. Primed ssDNA concentration, 2.5 ng/μl. (*C*) β subunit concentration. Amounts of added β are as follows): lane 1, none; lane 2, 0.01 ng; lane 3, 0.03 ng; lane 4, 0.09 ng; lane 5, 0.3 ng; lane 6, 0.8 ng; lane 7, 2 ng; lane 8, 7 ng; lane 9, 20 ng. Primed ssDNA concentration, 2.5 ng/μl.

template that carries the G+C clamp at the very bottom of the hairpin, next to direct repeats (FXb; Figs. 1*D* and 2). No slippage was detected if the G+C clamp was removed (FXa; Figs. 1*C* and 2*A*). Taken together, these observations suggest that slippage takes place when Pol III HE pauses within, or at the end, of the first direct repeat.

A high enzyme-to-template ratio lowers the frequency of slippage (Fig. 4). The ability to replicate through a long duplex DNA (the 300-bp hairpin) suggests that the enzyme possesses an intrinsic strand displacement activity that, to the best of our knowledge, was not previously recognized, or that all of the preparations tested contain a helicase contaminant. The latter hypothesis is unlikely, since we have not been able to detect a helicase activity in our preparation by a direct test and have prepared the enzyme from an *E. coli* strain lacking Helicase II, known to copurify with Pol III (C. S. McHenry, personal communication). Furthermore, hairpin traversal was observed with three different Pol III preparations. It is conceivable that the strand displacement activity is due to the ability of Pol III HE to take advantage of transient opening of the duplex ends, and thus to progress step by step inside the duplex. It has been shown that, upon finishing a replication cycle, Pol III* dissociates from β and falls off the duplex, but is able to rapidly reassociate if β clamp is present (35). At high concentrations, the enzyme might undergo such a dissociation/reassociation cycle quickly enough to successively add new nucleotides at the 3′ DNA end while the base of the hairpin structure is temporarily open.

Our model for the slippage is depicted in Fig. 6. Pol III HE is arrested upon replication of the first direct repeat, by the hairpin structure stabilized by the G+C clamp (step 1). Pol III* then dissociates from the β clamp (step 2). High salt concentrations may favor the slippage reaction, not only by stabilizing the hairpin structure, but also by promoting the dissociation reaction (35). Upon dissociation of Pol III*, the tip of the new DNA strand can unpair from the first repeat and anneal with the second (step 3). Dissociation of Pol III* and annealing of the tip with the second repeat are both reversible. Loading of the β clamp on the duplex formed at the second repeat allows reassociation of Pol III* at this site and resumption of DNA synthesis (step 4), which renders the slippage process irreversible. Excess of β stimulates slippage (Fig. 5*C*), possibly by accelerating loading of Pol III* at the second repeat.

The proposed model can easily explain why the penetration of Pol III HE into the hairpin prevents slippage. If the replication is carried out past the first direct repeat, the tip of the new strand is not complementary to the second repeat and would not anneal to it. Exonucleolytic removal of the noncomplementary nucleotides could be carried out by the ε subunit of Pol III, but it might be kinetically unfavorable under the experimental conditions used. The reaction is therefore drawn toward the formation of a parental molecule. This hypothesis could be tested by addition of an exonuclease active on ssDNA to the Pol III preparation.

The role of SSB, which stimulates slippage at high concentration (Fig. 5*B*), is not obvious. SSB is known to enhance Pol III HE and Pol III* processivity, possibly by removing secondary structures on the ssDNA. However, processivity of two

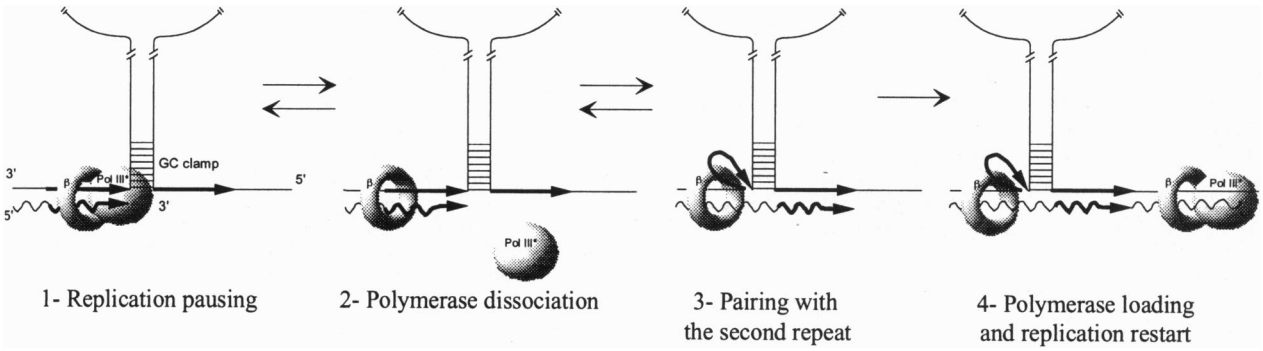

FIG. 6. Model for Pol III HE-mediated copy–choice recombination. A part of the recombination unit FXb is represented. The single-stranded template is drawn as a straight line, and the newly synthesized strand as a wavy line. Direct repeats are shown as thick arrows, IRs are annealed in a hairpin structure and G+C clamp is shown as a series of lines. Pol III* is represented as a sphere and β clamp as a ring, not respecting the relative dimensions. Stages 1, 2, and 3 are thought to be reversible.

polymerase subassemblies, Pol III core and Pol III′, is either diminished or unaffected by SSB, respectively (36, 37). This suggests that the increased processivity of the holoenzyme in presence of SSB may be due to factors other than the removal of secondary structures from the template. It is conceivable that SSB confers a higher affinity for DNA upon Pol III HE, possibly by interaction with one or several of the subunits; for instance, binding of the γ subunit to SSB has been recently observed (38). In our case, elimination of secondary structures on the second direct repeat could facilitate its pairing with the tip of the new strand. However, the sequence of the direct repeats suggests no obvious propensity to form secondary structures. Alternatively, SSB could promote arrest of Pol III HE at sites conducive to slippage or even stimulate reassociation of Pol III* with β. Further work would be required to clarify the role of SSB in the slippage process.

Slippage of Pol III HE leads to the formation of a heteroduplex molecule composed of one parental and one recombinant strand. Since the recombinant is due exclusively to replication, which has "chosen" to use one rather than the other region of the template, the process is a clear example of copy–choice recombination. How general might such recombination be? One of the key features of the process appears to be the arrest of the enzyme at appropriate locations. Studies of different parameters that may affect the arrest *in vitro*, such as the length and sequence of the IRs, as well as the presence of proteins with an affinity for DNA (M. A. Petit, personal communication), could provide insight into this question. Beyond the arrest of the enzyme, precise topology of the direct repeats, such as sequence, length, and their relative distance, as well as their position in respect to the arrest site, are likely to have an effect on the process. Furthermore, in our assay only one DNA strand is made, whereas *in vivo* Pol III HE synthesizes coordinately the two strands (26). Extension of the assay to such an *in vitro* system would be required to generalize the observations reported here. Nevertheless, the rather high frequency of Pol III HE slippage under optimal conditions, as well as the conjunction of *in vitro* results and several *in vivo* studies, in *E. coli* (20, 39) and yeast (40, 41), suggest that copy–choice recombination may well be carried out by the main replicative polymerase in living cells. It is interesting to note in this context that the length of direct repeats studied here, 27 bp, is at the borderline of that required for homologous or illegitimate recombination in *E. coli* (13), suggesting that a copy–choice process may be involved in both.

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