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Patrice Morel, Dmitry Cherny, Stanislav S. Ehrlich, Era Cassuto. Recombination-dependent repair of DNA double-strand breaks with purified proteins from Escherichia coli. Journal of Biological Chemistry, 1997, 272 (27), pp.17091-17096. 10.1074/jbc.272.27.17091 . hal-02684641

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 27, Issue of July 4, pp. 17091–17096, 1997
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Recombination-dependent Repair of DNA Double-strand Breaks with Purified Proteins from *Escherichia coli**

(Received for publication, March 12, 1997)

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We have developed an *in vitro* system in which repair of DNA double-strand breaks is performed by purified proteins of *Escherichia coli*. A segment was deleted from a circular duplex DNA molecule by restriction at two sites. 3′ single-stranded overhangs were introduced at both ends of the remaining linear fragment. In a first step, RecA protein catalyzed the formation of a D-loop between one single-stranded tail and a homologous undeleted supercoiled DNA molecule. In a second step, *E. coli* DNA polymerase II or III used the 3′ end in the D-loop as a primer to copy the missing sequences of the linear substrate on one strand of the supercoiled template. Under proper conditions, the integrity of the deleted substrate was restored, as shown by analysis of the products by electrophoresis, restriction, and transformation. In this reaction, DNA synthesis is strictly dependent on recombination, and repair is achieved without formation of a Holliday junction.

Double-strand breaks in DNA result essentially from exposure of the cells to ionizing radiations or defective repair of clustered single-strand lesions. In yeast, they also occur spontaneously during meiosis, particularly at homologous recombination hot spots (1). In the absence of an efficient repair system, the introduction of a double-strand break in a chromosome constitutes a lethal event.

In mammalian cells the repair of double-strand breaks seems to be predominantly achieved by nonconservative mechanisms such as direct strand rejoining (2, 3) or single-strand annealing (4), whereas in yeast or bacteria, homologous recombination appears to be the major way to overcome double-strand breaks. In *Saccharomyces cerevisiae*, most of the genes required for homologous recombination fall into the *RAD52* epistatic group (5 and references therein). Mutations in these genes render cells sensitive to ionizing radiations (6) and to chemicals known to induce DNA breakage but do not significantly affect UV sensitivity. In bacteria, the repair of double-strand breaks is an inducible SOS function and requires the presence of another DNA duplex with the same base sequence as that of the broken helix (7). Rapidly growing *Escherichia coli* cells have multiple copies of most of their genome and exhibit increased resistance to killing by x-rays, showing that breaks are repaired efficiently (8). The repair process is clearly conservative as the sequences of the participating DNA molecules are recovered.

Several models have been proposed for the repair of double-strand breaks by homologous recombination (9–11). The common feature is the exonucleolytic degradation on each side of the break, which exposes 3′ single-stranded (ss)[1] tails. One 3′ end invades the donor duplex to form a D-loop and primes DNA synthesis on one strand of the duplex while the other strand is displaced. According to Szostak *et al.* (10), as synthesis progresses, the displaced strand anneals to the noninvading 3′ ss end and is then used as template for repair synthesis, leading to the formation of two Holliday junctions. Resolution can occur with or without crossing over, and all products carry newly synthesized DNA. However, a number of genetic data obtained in a variety of organisms cannot be accounted for by a such a symmetrical mechanism. Belmaaza and Chartrand (12) proposed an alternative model in which the newly synthesized strand is released from the D-loop and anneals to the noninvading 3′ ss tail. In this case, only one strand of the donor duplex serves as template for DNA synthesis, and repair occurs without formation of a Holliday junction. This mechanism is strongly reminiscent of a reaction that occurs in T4-infected cells, which converts recombination intermediates into replication forks (13), and was substantiated by the biochemical studies of Formosa and Alberts (14). The *in vitro* reaction was strictly dependent on (i) the presence of the UvsX and gene 32 proteins (the T4 equivalents of *E. coli* RecA and SSB protein); (ii) T4 DNA polymerase and its accessory proteins; (iii) homologous ss and duplex DNA substrates, and was greatly stimulated by the T4 dda helicase.

The enzymes used in the T4 *in vitro* system have functional equivalents in *E. coli*, and there is little doubt that precise repair of double-strand breaks requires the cooperation of recombination and replication. In this report, we describe the reconstitution of a repair reaction with a mixture of purified *E. coli* recombination and replication proteins. The results support a mechanism in which RecA protein promotes one-sided invasion of a duplex donor by a linear homologous DNA bearing 3′ ss overhangs. The invading end serves as a primer for DNA synthesis by DNA polymerase II or III, which copy one strand of the duplex template. Repair is achieved without formation of a Holliday junction.

MATERIALS AND METHODS

Enzymes and Proteins—Restriction endonucleases, λ exonuclease, *E. coli* DNA gyrase, DNA polymerase I (Pol I) and T4 DNA ligase were purchased from New England Biolabs, Boehringer Mannheim, and Life

* This work was supported by Grant 1H6281-G341 from the Electricité de France and Grant ACC-SV8 from the Ministère de la Recherche (to E. C.) and by Grant 227 from the Program of International Scientific Cooperation (to D. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ss, single-stranded; SSB protein, single-strand binding protein; Pol, polymerase; RF, replicative form; wt, wild type; bp, base pair(s); ATPγS, adenosine 5′-*O*-(thiotriphosphate).

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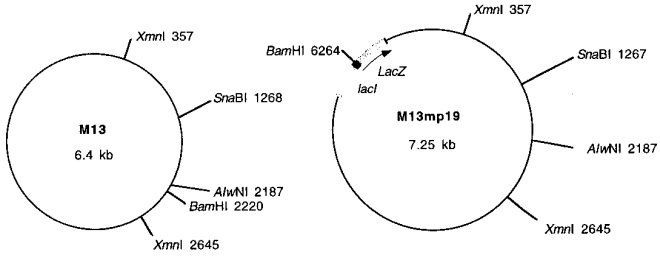

FIG. 1. **Partial restriction map of M13 wt and M13mp19.** Only the restriction sites relevant to this work are indicated. *kb*, kilobases.

Technologies, Inc. and were used according to specifications. *E. coli* SSB was from U. S. Biochemical Corp. RecA and helicase II (UvrD) were prepared as described (15, 16). Two preparations of Pol III* were used. One was a gift from Dr. A. Kornberg, who also provided the β subunit; the other was prepared in the laboratory as described previously (17). The Pol III holoenzyme was reconstituted by mixing Pol III* with β protein. DNA Pol II was a gift from Dr. M. Goodman. The relative activity of the polymerases used in these experiments was determined on viral M13mp19 DNA annealed to a 17-nucleotide primer (here, 1 unit is defined as the enzyme activity that incorporates 10 nmol of total nucleotides into acid-insoluble material in 30 min under the conditions of the assay).

DNA Substrates—All DNA concentrations are expressed as molarity of nucleotide residues. Double-stranded covalently closed DNA (RF I) from M13 wt and M13mp19 bacteriophages was prepared according to classical procedures (18). The M13mp19 DNA (7249 bp) uniformly labeled with either ^{3}H or ^{32}P was digested with *Xmn*I, *Alw*NI, and *Sna*BI restriction nucleases (*Alw*NI and *Sna*BI were used to reduce the size of the smaller *Xmn*I fragment and facilitate the purification of the larger one). The large fragment (4961 bp) was purified by sedimentation in a 35-ml 5–20% neutral sucrose gradient for 18 h at 4 °C and 26,000 rpm in an SW28 Beckman rotor. Aliquots of collected fractions were analyzed by agarose gel electrophoresis, and fractions containing the proper fragment were pooled and ethanol precipitated. The 2288-bp fragment was completely eliminated. The 4961-bp fragment was incubated with a saturating amount of λ exonuclease for 0.5, 0.75, and 1 min at 20 °C. In different preparations, this treatment yielded 3′ ss tails with an average length of 250–700 nucleotides, estimated from the amount of radioactivity rendered acid-soluble. The DNA was extracted with phenol and chloroform and precipitated with ethanol. Control DNA with 5′ ss tails was prepared by treatment of the 4961-bp fragment with exonuclease III for 1 min at 20 °C, which introduced ss tails of 500 nucleotides on average.

Joint Molecules—Typical incubation mixtures contained 40 μM ^{3}H or ^{32}P-labeled linear substrate with ss tails, 80 μM M13 wt RF I DNA, 20 μM RecA, 4 μM SSB, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM ATP, 2 mM dithiothreitol, and 100 μg/ml bovin serum albumin. Mixtures were incubated for 10 min at 37 °C, supplemented with EDTA (25 mM) for electron microscopic analysis, with 25 mM EDTA and 1% sodium dodecyl sulfate (SDS) for gel electrophoresis.

For invasion-primed synthesis, joint molecules made with ^{3}H-labeled linear substrate were purified by centrifugation in a 38-ml 5-to 20% sucrose gradient as described in the above paragraph. Fractions (0.4 ml) were collected and aliquots analyzed by agarose gel electrophoresis. Fractions containing joint molecules were pooled and ethanol precipitated. Purified joint molecules were free of the linear substrate but still contained significant amounts of M13 RF I DNA.

Electron Microscopy—Samples were passed through a Superose 6 column equilibrated with 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA to eliminate free proteins. The DNA-containing fractions were pooled and applied to a carbon film glow discharged in the presence of pentylamine vapors (19), stained with a 0.5–1% aqueous solution of uranyl acetate. Some samples were further shadowed with tantalum/tungsten with the electron gun of a Balzers MED 010 apparatus. The samples were observed with a Zeiss CEM-902 electron microscope in the annular dark-field mode (20). Image recording and length measurements of DNA molecules were performed with the built-in Kontron image analyzer and software.

Invasion-primed DNA Synthesis—Mixtures contained, in 10 or 20 μl, 25 mM Tris-HCl (pH 7.5); 10 mM $MgCl_2$; 2 mM ATP; 2 mM dithiothreitol; 100 μg/ml bovine serum albumin; 200 mM dATP, dGTP, and dTTP; 25 mM ^{32}P-labeled dCTP; 2 μM RecA; 0.4 μM SSB; 1 unit of T4 DNA ligase; 2 units of DNA polymerase; and, unless otherwise indicated, 5–10 μM purified ^{3}H-labeled joint molecules. *E. coli* DNA gyrase (1 or 2 units) was added when indicated (mixtures containing gyrase were also supplemented with 1.8 mM spermidine hydrochloride and 20 mM KCl). Incubation was for 5 or 30 min at 37 °C. In reactions with UvrD (100 nM), the protein was introduced 3 min after the start of primed synthesis to avoid the dissociation of joint molecules (16). The reactions were stopped with EDTA (25 mM), dialyzed for 45 min on Millipore VSWP membranes *versus* 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and analyzed by gel electrophoresis after the addition of SDS (1%).

Synthetic Holliday Junction—A synthetic Holliday junction carrying a consensus site for resolution by RuvC was constructed as described (21). The oligonucleotides were a gift from Dr. S. C. West. This structure has been shown to be cleaved by T4 endonuclease (21). Oligonucleotide

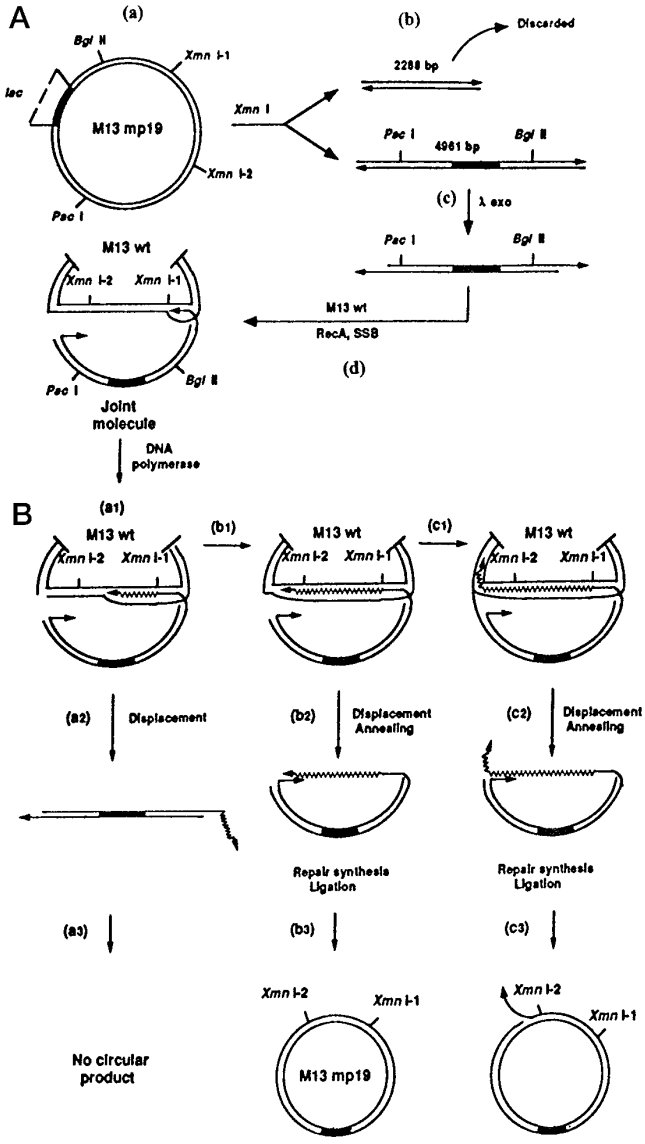

FIG. 2. **Experimental system.** Formation of joint molecules (*panel A*) and invasion-primed synthesis (*panel B*). *Panel A*: *a*, schematic representation of the structure of M13mp19 (7249 bp). The two possible sites of invasion, *Xmn*I-1 and *Xmn*I-2, are shown. *Bgl*II and *Pac*I are indicated for orientation. Here invasion is shown at *Xmn*I-1, but it can occur at *Xmn*I-2 with equal probability. The *lac* insert is absent in M13 wt. *b*, fragments produced by digestion with *Xmn*I. *Arrows* correspond to the 3′ end. *c*, treatment of the 4961-bp fragment with λ exonuclease followed by incubation with M13 wt RFI (6404 bp, only the relevant region is shown), RecA, and SSB (*d*) to produce joint molecules. *Panel B*: three stages of invasion-primed synthesis are illustrated in *a1*, *b1*, and *c1*. In each case, the expected result of strand displacement and, when applicable, annealing to the noninvading 3′ ss tail are shown in *a2*, *b2*, and *c2*. In contrast with *a2*, the intermediates in *b2* and *c2* can in principle sustain repair synthesis and ligation, leading to the formation of *b3* and *c3*. In all cases, the M13 wt template should be recovered intact. See "Results" for more details.

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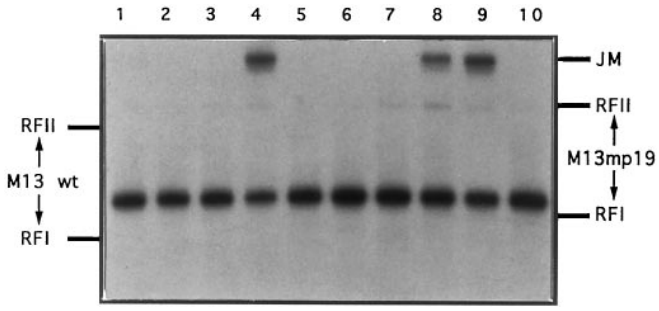

FIG. 3. **Requirements for the formation of joint molecules (*JM*).** The ^{32}P-labeled 4961-bp *Xmn*I fragment from M13mp19, digested with λ exonuclease (3′ ss tails) or exonuclease III (5′ ss tails), migrates slightly slower than the M13mp19 RF I marker. *Lane 1* contains the 4961-bp fragment with 5′ ss tails. All other lanes contain the 4961-bp fragment with 3′ ss tails. *Lane 2*, no RecA. *Lane 3*, no SSB. *Lane 4*, complete reaction. *Lane 5*, complete reaction with topoisomerase I. *Lane 6*, φX174 RFI DNA instead of M13 wt. *Lane 7*, M13wt/linear substrate (w/w) = 0.3. *Lane 8*, complete reaction with gyrase. *Lane 9*, complete reaction with ATPγS instead of ATP. *Lane 10*, no ATP.

1 was 5′ ^{32}P labeled prior to annealing. Reaction conditions were identical to those used for invasion-primed synthesis (see Fig. 5, *bottom lanes d–g*) except that joint molecules were replaced by 1 ng of synthetic Holliday junction. As a positive control, T4 endonuclease VII (10 units), a gift from Dr B. Kemper, was added to the mixture instead of the polymerases. Incubation was at 37 °C for 30 min. Reactions were analyzed by 6% neutral polyacrylamide gel electrophoresis (21).

Electrophoresis and Autoradiography—All samples contained 25 mM EDTA and 0.5–1% SDS. For agarose gels, electrophoresis was carried out in 0.8% gels (SeaKem GTG) in Tris acetate buffer at 50 V/30 mA for 18 h. The DNA was visualized by ethidium bromide staining. Dried gels were then exposed to x-ray films (Fuji RX). For neutral polyacrylamide gel electrophoresis, samples were electrophoresed through 6% polyacrylamide gels in TBE buffer and autoradiographed.

Transformation—E. coli strain JM109 (*supE* Δ(*lac-proAB*) *hsdR*17 *recA*1 F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) was rendered competent by a standard $CaCl_2$ procedure (18). The cells were incubated with DNA for 10 min at 0 °C followed by 10 min at 37 °C, plated in top agar containing indicator cells, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and isopropyl-1-thio-β-D-galactopyranoside onto LB plates placed at 37 °C for 18 h. In contrast with M13 wt, M13mp19 is able to perform α-complementation of the defective β-galactosidase gene of the host and forms blue plaques under these plating conditions (22).

RESULTS

The strategy developed to test for recombination-dependent repair is illustrated in Figs. 1 and 2. The major difference between phage M13 wt and its derivative M13mp19 is the *lac* insert carried by the latter. In addition, the *Bam*HI site at nucleotide 2220 in M13 wt has been moved to nucleotide 6264 in M13mp19 (Fig. 1). Treatment of M13mp19 RF I with *Xmn*I produces two fragments of 4961 and 2288 bp (Fig. 2*A*, *a* and *b*). The smaller fragment was discarded and the larger fragment subjected to controlled treatment with λ exonuclease, an enzyme specific for the 5′ ends of duplex DNA. Thus, 3′ ss tails were introduced on both strands of this fragment (Fig. 2*A*, *c*). We expected that in the presence of RecA, 3′ ss tails would invade supercoiled M13 wt RF I DNA to form a D-loop (23) and that the invading end would serve as a primer for DNA synthesis on the intact M13 wt template (Fig. 2*A*, *d*). If this were the case, elongation of the newly synthesized strand might provide a complete copy of the 2288-bp fragment, which, except for a single nucleotide change, is identical in M13 and M13mp19. In principle, if no Holliday junction is formed, displacement or unwinding of the elongated strand from its template and annealing to the noninvading 3′ ss tail may lead to conservative repair of the deleted M13mp19 DNA (Fig. 2*B*, *b1*, *b2*, and *b3*). Alternatively, if synthesis takes place on both strands of the donor duplex, resolution will be needed to release the repaired products.

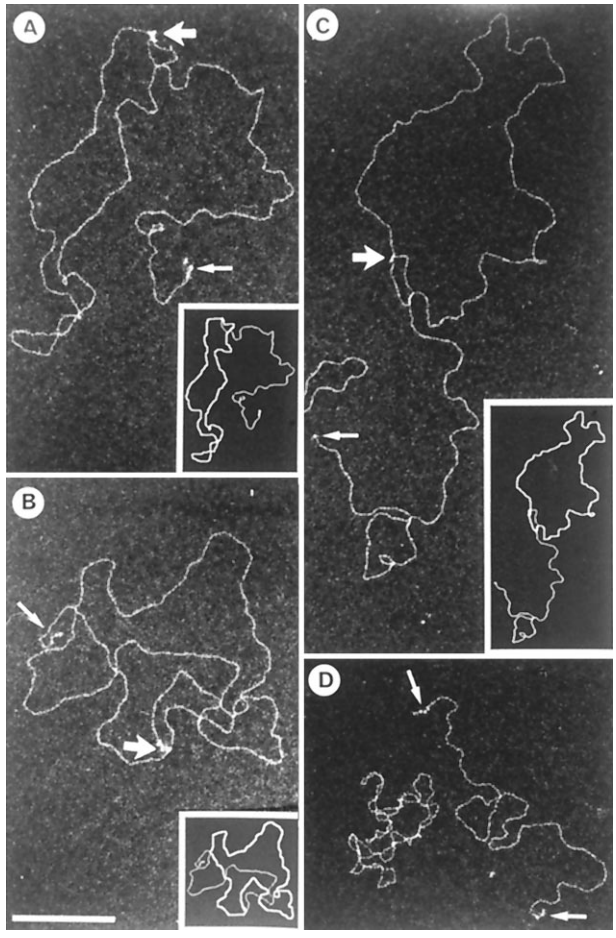

FIG. 4. **Electron microscopic visualization of joint molecules.** Grids were prepared and examined as described under "Materials and Methods." Examples of joint molecules are shown in *panels A*, *B*, and *C*. The input linear and supercoiled substrates are shown in *panel D*. *Thick arrows* indicate the position of the joint, *thin arrows* the 3′ ss overhangs. The *scale bar* corresponds to 200 nM. For clarity, the linear and circular DNAs in the *insets* are drawn, respectively, with a *thin* and *thick line*.

Formation and Structure of Joint Molecules—To determine the requirements for optimal formation of joint molecules, we used a ^{32}P-labeled M13mp19 large *Xmn*I fragment with 3′ ss tails. Analysis by electrophoresis showed that the formation and yield of joint molecules depended on several factors (Fig. 3). As expected, the reaction required the presence of RecA, SSB, and ATP or ATPγS (adenosine 5′-*O*-thiotriphosphate), as well as homology between the linear substrate and the circular template (no joint molecules were formed if M13 wt RF I was replaced by φX174 RF I). Superhelicity of the template was also required, since either relaxation with topoisomerase I or nicking of the template (not shown) with gpII protein, the product of M13 gene II (24), considerably lowered the yield of joint molecules. In addition, the reaction was strictly dependent on the presence of 3′ ss tails on the linear substrate, as the undigested fragment (not shown) or the fragment carrying 5′ ss tails originating from digestion of the 4961-bp *Xmn*I fragment with exonuclease III did not produce joint molecules. This is consistent with the 5′ to 3′ polarity of the binding of RecA to ssDNA (25, 26) and of RecA-catalyzed branch migration (27).

Aside from these absolute requirements, the yield of joint molecules was strongly influenced by the total concentration of DNA and proteins and by the relative concentration of the substrates. The length of the ss tails seemed to have no effect within the available range (250–700 nucleotides), nor did the

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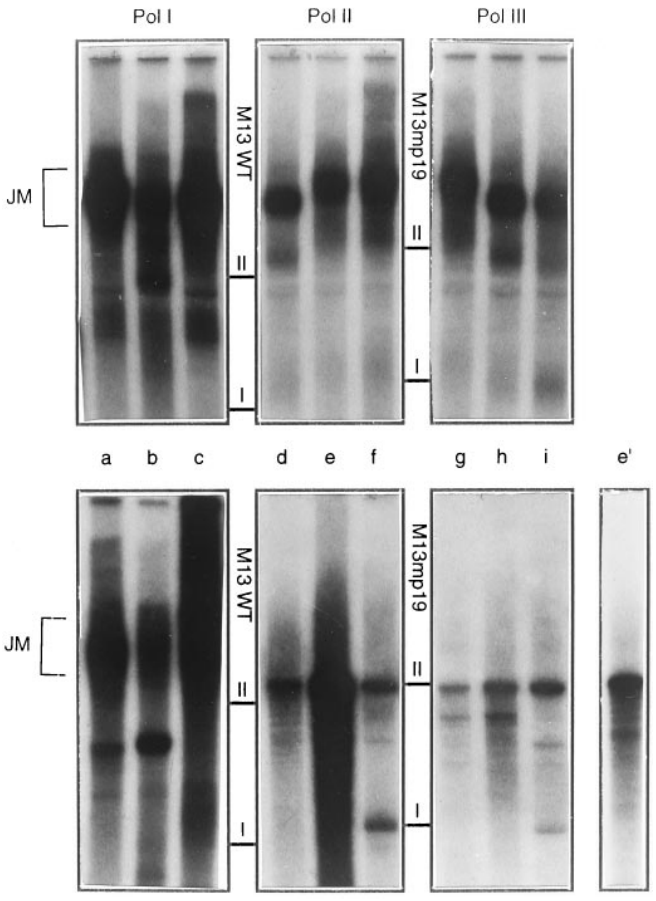

FIG. 5. **Invasion-primed synthesis with *E. coli* DNA POL I, II, and III.** Reactions were carried out as described under "Materials and Methods." *Top panels*, incubation was for 5 min at 37 °C. *Bottom panels*, incubation was for 30 min at 37 °C. Gyrase was present in *lanes c*, *f*, and *i*. UvrD was added in *lanes b*, *e*, and *h* 3 min after the start of incubation to avoid the dissociation of joint molecules (16). *Lane e′* shows a shorter exposure of *lane e*. Mixtures were supplemented with 25 mM EDTA, dialyzed, and electrophoresed after the addition of SDS (1%). *JM*, joint molecules. *I* and *II*, RF I and RF II, respectively.

addition of DNA gyrase. Under the optimal conditions described under "Materials and Methods," a substantial fraction of the input radioactivity was found in joint molecules (Fig. 3, *lane 4*).

Joint molecules were analyzed by electron microscopy after removal of free proteins. Out of several hundred DNA molecules examined, close to 30% of the linear molecules were joined to circular molecules, whereas no joint molecules were found in controls lacking RecA. As expected, all joint molecules resulted from one-sided invasion of the M13 wt template (see Fig. 4 for representative examples), since RecA-promoted invasion of a supercoiled DNA molecule by ssDNA induces unwinding of the duplex (28), thus preventing invasion at another site. Most ss tails were covered by RecA, but the coverage appeared to be somewhat irregular, possibly because of the presence of ATP (instead of ATPγS) in the reaction (29).

Invasion-primed DNA Synthesis—In preliminary control experiments (not shown), the linear and circular starting substrates were incubated separately with Pol I, Pol II, or Pol III under the conditions described under "Materials and Methods" for invasion-primed DNA synthesis, or together without RecA. With Pol II and Pol III, no incorporation was observed with either substrate in the presence of RecA, although traces of radioactivity, supposedly due to self-priming, could be detected in the absence of RecA at the position of the linear substrate. However, in controls with Pol I, incorporation probably due to

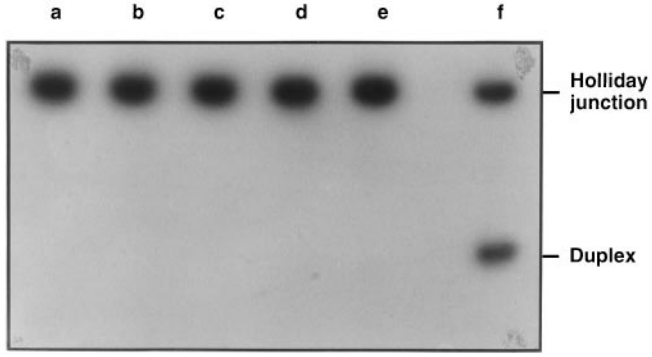

FIG. 6. **M13mp19-like species (forms I and II) are not due to contamination by a Holliday junction resolvase.** Synthetic Holliday junctions (1 ng) were incubated under the conditions described in Fig. 5, *lanes d–g*. Incubation was for 30 min at 37 °C. *Lane a*, Holliday junctions, no addition; *lane b*, with Pol II; *lane c*, with Pol II and UvrD; *lane d*, with Pol II and gyrase; *lane e*, with Pol III; *lane f*, with T4 endonuclease VII (10 units).

nick translation and/or strand displacement produced species migrating from the original positions of the substrates to the position of joint molecules and above. To minimize such events in further experiments with Pol I, joint molecules made with a ^{3}H-labeled large *Xmn*I fragment with 3′ ss tails were purified as described under "Materials and Methods." This step completely removed the free linear substrate, but a substantial amount of M13 RFI was still present in the preparation.

The ability of the invading 3′ ss tails to serve as primers for DNA synthesis was tested with the three DNA polymerases in the presence or absence of gyrase and helicase II (UvrD). T4 DNA ligase was present in all reactions, as were RecA and SSB, the former to favor annealing of potential complementary single strands, the latter because it stimulates Pol II and Pol III activity. Analysis of the products on agarose gels showed that with all three polymerases, radioactivity was found at the position of joint molecules after 5 min of incubation (Fig. 5, *top lanes*). With Pol I, however, incorporation also took place at and above the position of the starting substrates (*lanes a–c*), as observed in the controls. After a 30-min incubation (Fig. 5, *bottom lanes*), radioactivity at the position of joint molecules decreased in reactions with Pol II and Pol III (but not with Pol I), and a fraction of the incorporated label was found at the position of M13mp19 RF II DNA. In the presence of gyrase, radioactivity was also found at the position of M13mp19 RF I DNA. Gyrase or UvrD stimulated the formation of M13mp19-like species, but stimulation was not increased when both proteins were present in the same reaction.

Since M13mp19-like species could also result from contamination of any of the enzymes used in these experiments by an activity capable of resolving Holliday junctions, we incubated a synthetic junction with Pol II with or without UvrD or gyrase and with Pol III, under the exact conditions used for invasion-primed synthesis. As a positive control, the synthetic junction was also incubated with T4 endonuclease VII (21, 30). As shown in Fig. 6, the synthetic junction was resistant to all treatments with the exception of T4 endonuclease VII.

Restriction Analysis of M13mp19-like DNA—To verify the identity of the RF I and RF II M13mp19-like species, invasion-primed synthesis reactions containing gyrase were set up as in Fig. 5, *lanes f* and *i*, and electrophoresed on agarose gel. Bands at the position indicated by the RF I and RF II markers were cut out and the DNA electroeluted and cleaved with *Xmn*I or *Bam*HI. The former enzyme should produce two bands from M13mp19 DNA, the invading 4961-bp fragment and the 2288-bp fragment originally deleted (Figs. 1 and 2). These fragments were observed in all cases (Fig. 7, *lanes e–h*).

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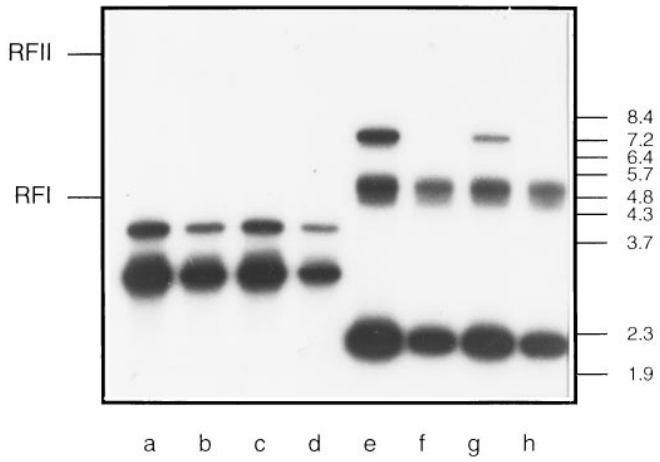

FIG. 7. **Restriction analysis of the products of invasion-primed DNA synthesis catalyzed by Pol II and Pol III.** For details, see "Results." Primed synthesis reactions were carried out with Pol II and Pol III in the presence of gyrase, as in Fig. 5, *lanes f* and *i*, and electrophoresed. Bands at the position indicated by the M13mp19 RF I and RF II markers were excised. The DNA was electroeluted and digested with *Bam*HI or *Xmn*I and the digests electrophoresed. A *Bst*EII digest of λ DNA was run as a size marker. *Lanes a–d*, *Bam*HI. *Lanes e–h*, *Xmn*I. *Lanes a*, *b*, *e*, and *f*, Pol II reaction. *Lanes c*, *d*, *g*, and *h*, Pol III reaction. *Lanes a*, *c*, *e*, and *g*, digests of RF II-like DNA. *Lanes b*, *d*, *f*, and *h*, digests of RF I-like DNA. In *lanes e* and *g*, the extra fragment at the position of linear M13mp19 DNA (7249 bp) is presumably due to cleavage at only one of the two *Xmn*I sites. Markers on the *left* indicate the position of the DNA eluted from the excised bands prior to digestion.

*Bam*HI cuts both M13mp19 and M13 wt at one location, the former within the *lac* insert, the latter within the region corresponding to the 2288-bp *Xmn*I fragment (Fig. 1). Since the newly acquired 2288-bp fragment of M13mp19 must be a copy of the corresponding fragment in M13, repaired M13mp19 molecules should carry two *Bam*HI sites, and cleavage at both sites should yield two fragments of 4044 and 3205 bp. Again, the expected fragments were seen in all cases (Fig. 7, *lanes a–d*), confirming the structure of the "repaired" RF I and RF II species. In all digests (*lanes a–h*), the higher radioactivity in the smaller fragment indicates that synthesis occurred predominantly in the originally deleted region of the M13mp19 chromosome, as expected from the model in Fig. 2*B*.

Transformation—Reconstitution *in vitro* of viable M13mp19 phage from the large *Xmn*I fragment and M13 wt DNA was also examined by a transformation test. M13mp19 can be identified by its ability to perform α-complementation in a *lacI*^{q} *lacZ*ΔM15 host and to form blue plaques in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and isopropyl-1-thio-β-D-galactopyranoside, whereas M13 wt will form colorless plaques (22).

Primed synthesis reactions (scaled up to a total volume of 50 μl) were carried out with Pol I, II, and III, with and without gyrase or UvrD and electrophoresed on agarose gel alongside M13mp19 RF I and RF II markers. Bands at the positions indicated by the markers were excised and the DNA eluted by a modification of the freeze-squeeze method (31) and introduced into competent JM109 (*recA*) cells as described under "Materials and Methods." 93 to 97% of the total number of plaques (several hundred) issued from Pol II and Pol III reactions were blue. No blue plaques were seen for reactions with Pol I or when the cells were transformed with a mixture of the starting substrates incubated without RecA (Table I).

TABLE I
Yield of repaired DNA

The yield was estimated on the basis of the following considerations. One joint molecule can produce at best one molecule of M13mp19 (size 7.25 kilobase pairs), or 64% of input (w/w). As the primed synthesis reactions contained 80 ng of joint molecules, 100% repair would yield 51 ng of M13mp19 DNA. In preliminary experiments, we found that recovery of elution of M13mp19 RF I or RF II DNA from excised gel bands was close to 40% and that after phenol extraction, ethanol precipitation, and dialysis, the transformation frequency of the eluted DNA was 8×10^4 plaque-forming units/mg in strain JM 109 (*recA*). Therefore 100% repair would correspond approximately to 20 ng of M13mp19 DNA or 1.6×10^3 plaque-forming units. Results are the number of observed blue plaques/number expected for complete repair of joint molecules.

	RF I band	RF II band
	%	
Pol I		0
Pol I +Gyrase	0	0
Pol I +UvrD		0
Pol II		12
Pol II +Gyrase	4	13
Pol II +UvrD		23
Pol III		6
Pol III +Gyrase	2	9
Pol III +UvrD		13

DISCUSSION

In this paper, we describe a recombination-dependent *in vitro* system for repair of double-strand breaks. In a first step, RecA promoted the formation of joint molecules between a covalently closed duplex DNA donor and a deleted linear homologous substrate bearing 3′ ss tails, one of which invaded the donor DNA. In a second step, Pol II and Pol III used the invading end as a primer to copy the deleted sequences on one strand of the template.

As seen in Fig. 2*B*, the precision of the repair process should depend on the extent of DNA polymerization. Complete repair can only occur if the end of the newly synthesized strand is complementary (and therefore can anneal) to sequences contained in the noninvading ss tail of the linear substrate (*b2*). In such a case, repair synthesis and ligase must carry out the final steps (*b3*). If invasion-primed synthesis does not proceed far enough (*a1*), the product will correspond to the original linear substrate with one extended ss tail (*a2*). If, on the contrary, primed synthesis proceeds past the noninvading ss tail, the product will be an open duplex circle with an extra whisker (*c3*).

Analysis of the results of primed synthesis (Fig. 5) indicates that the three kinds of products may be formed. Only the DNA species migrating at the position of M13mp19 RF I in the presence of gyrase reflect complete repair. Ligase and gyrase were present in sufficient concentration to close and supercoil an amount of nicked M13 DNA corresponding to several times the amount of DNA in the reaction mixtures, as determined in preliminary assays. We therefore assume that the M13mp19 RF II-like species that are refractory to ligation, hence to supercoiling, correspond to duplex circles with short ss gaps and/or ss tails (Fig. 2*B*, *b2*, *c2*, and *c3*). It should be noted that species with long whiskers, which would migrate well above the M13mp19 RF II marker, will not be taken into account in this assay. Smears (or even bands) between the RF I and RF II markers seem to be produced by displacement from the joint molecules of newly synthesized strands too short to anneal to the noninvading ss tail. Radioactivity at and above the position of joint molecules is likely to reflect synthesis in progress or displacement failure.

The results of restriction analysis and transformation experiments confirm the general structure of the repaired DNA but do not guarantee that the newly synthesized strand is a perfect copy of its template. The region deleted in the starting linear substrate is essential for phage viability. Gross rearrangements during invasion-primed synthesis would interfere with the life cycle of the phage and are therefore unlikely. But

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mismatches, for example, would not be detected by restriction analysis and would be eliminated in the cells upon transformation, as would other errors that can be repaired in the absence of RecA. Imperfect products, such as circles with short tails or gaps, could easily be trimmed or filled in the cells and produce viable phage.

In vivo, repair synthesis is thought to be carried out by Pol I or Pol III, depending on the size of the repair patch, and in some cases by Pol II (32). In the present experiments, the results obtained with Pol I are open to several interpretations: the label found around the position of joint molecules could result from primed synthesis without release of the new strand, but also reflects incorporation at nicks, as observed in the controls. Moreover, because of its low processivity and accessory degradative activities, Pol I could also produce new strands too short to anneal to the noninvading 3′ ss tail. Whatever the reason, M13mp19-like forms were never detected in our experiments with Pol I, which confirms that the RF I and RF II species obtained with Pol II and Pol III are not due to nick translation or strand displacement. In addition, the results in Fig. 6 eliminate a potential contamination by a Holliday junction resolvase. The activities of Pol II and Pol III in the repair of double-strand breaks are similar, although the former is apparently more efficient. Whether these differences among the three polymerases hold true *in vivo* remains to be determined.

Both UvrD and gyrase increased the yield of M13mp19-like structures but do not seem to act in a concerted or cooperative way since stimulation by either protein was not improved by the addition of the other. The effect of UvrD is reminiscent of that of the T4 dda helicase which stimulates recombination-dependent DNA synthesis *in vitro* (14). Interestingly, both dda and UvrD are known to stimulate, respectively, UvsX- and RecA-promoted strand exchange (16, 33, 34), and neither has been shown to play a direct role in replication. However, UvrD does stimulate DNA synthesis by Pol III at an artificial replication fork (35). In the T4 reaction (14), dda increased both the rate of polymerization and the rate of reannealing by UvsX at the back of the D-loop, thus expelling the newly synthesized strand and obviating the need for a topoisomerase. A similar mechanism could operate in the *E. coli* system, with UvrD acting in conjunction with RecA to release the new strand. In such a context, there would be no accumulation of positive supercoils ahead of the fork and no obvious role for gyrase. In the present experiments, gyrase may stimulate repair by maintaining the superhelicity of the M13 RF I template, thus facilitating secondary invasion by prematurely displaced newly synthesized strands. These delayed D-loops would be immediately destroyed by UvrD (16), which may explain why the effects of the two enzymes are not additive.

Whether the type of repair described here operates in the cells is an open question. Two important components of the normal replication machinery, primase and replicative helicase, are absent in the T4 or the *E. coli in vitro* reactions, and neither dda or UvrD has been shown to play a direct role in replication. However, the requirements for invasion-primed synthesis at the site of a double-strand break are probably quite different than those for chromosomal replication initiated at *OriC*. In addition, the introduction of double-strand breaks in the chromosome leads to SOS induction, which may modify the structure, the function, or the interactions of some of the proteins involved in replication/repair. For example, SOS induction has been shown to produce a smaller form of the sliding clamp of Pol III (36). It is also conceivable that UvrD may stimulate repair synthesis by removing proteins bound to the D-loop, such as RecA. In support of this hypothesis, UvrD stimulates the turnover of tightly bound *E. coli* proteins acting in the repair of UV lesions (37).

In conclusion, *in vitro* repair of double-strand breaks (or gaps) with *E. coli* proteins is dependent on homologous recombination, on Pol II or Pol III, is stimulated by gyrase or UvrD, and can be achieved in the absence of resolution. At the present time, it appears to be consistent with the features of the model for one-sided primed synthesis. This model was proposed to explain double-strand break repair without crossing over in a number of eukaryotic systems (12 and references therein). In recent papers, a similar model was invoked for asymmetric recombinational repair of double-strand breaks in *Ustilago maydis* (38) and in yeast (39). The present results suggest that such a mechanism may be relevant for all organisms.

Acknowledgments—We are grateful to M. Goodman, B. Kemper, A. Kornberg, and S. C. West for generous gifts of Pol II, T4 endonuclease VII, Pol III*, and β subunit and oligonucleotides, respectively. We thank C. S. McHenry for advice in the preparation of Pol III*; D. Canceill, J. Hejna, and A. Aucouturier for participation in this preparation; E. Delain for kindly allowing us to use his electron microscope facilities; B. Michel, P. Noirot, and W. Wackernagel for enlightening discussions; D. Canceill, M. Dutreix, and B. Michel for critical reading of the manuscript; and D. Laoudj for skillful help with the artwork.

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