*Proc. Natl. Acad. Sci. USA*
Vol. 78, No. 4, pp. 2100–2104, April 1981
Biochemistry

# recA protein promotes homologous-pairing and strand-exchange reactions between duplex DNA molecules

**(strand transferase/four-stranded pairing/genetic recombination)**

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*Communicated by Edward A. Adelberg, December 18, 1980*

**ABSTRACT** **Purified recA protein, product of the *recA*$^+$ gene, promotes homologous pairing between intact covalent circular duplex DNA and circular single-stranded DNA carrying a short hybridized fragment [West, S. C., Cassuto, E. & Howard-Flanders, P. (1981) *Nature (London)* 290, 29–33.]. In this paper we investigate the interaction of duplex fragments with circular single-stranded DNA carrying the hybridized fragment and find that recA protein promotes an efficient strand-exchange reaction between interacting DNA molecules. The exchange is dependent upon linear duplex DNA fragments that are homologous to, but extend beyond, the short fragment present on the hybridized DNA substrate. The reactions require stoichiometric amounts of recA protein and the presence of ATP.**

The early steps of genetic recombination involve pairing of homologous duplex molecules followed by cutting of the heteroduplex structure to produce a recombinant molecule (1–4). Most models for recombination favor the view that breakage occurs prior to homologous pairing and that initiation occurs by pairing of a single strand with its duplex partner (for review, see ref. 5). Other investigators have built molecular models which indicate that duplex DNA molecules may pair without the breakage of any phosphodiester linkages (6, 7), and evidence for four-stranded pairing has been obtained from the study of site-specific recombination (8). In *Escherichia coli*, genetic recombination is under the control of the *recA* gene. Purified recA protein is a single-strand DNA-dependent ATPase (9–11) that promotes homologous pairing between DNA molecules of various structures. Three experimental systems have been described. (*i*) Weinstock *et al.* (11) have shown that recA protein catalyzes the pairing of complementary single strands. (*ii*) Shibata *et al.* (10) and McEntee *et al.* (12) have shown that stoichiometric amounts of recA protein promote D loop formation between single-stranded DNA and homologous duplex DNA. (*iii*) More recently, Cassuto *et al.* (13) and Cunningham *et al.* (14) have demonstrated that recA protein also promotes the homologous pairing of duplex DNA molecules, if one of them contains a single-stranded region.

The ability of recA protein to join gapped and intact DNA molecules led us to develop an experimental system designed to investigate whether DNA molecules could pair without strand separation. We observed that recA protein joined an intact duplex with a homologous circular single-stranded molecule carrying a short hybridized fragment. We also found that recA protein did not promote strand separation of the short hybridized fragment from the single-stranded circle. Because the hybridized fragment was essential for pairing, we suggested that pairing occurs by a mechanism that results in a four-stranded structure (15).

These observations led us to the present study of homologous pairing of the hybridized substrate with duplex DNA fragments. We show that recA protein promotes a pairing reaction that results in efficient strand exchanges between DNA molecules. The exchange is initiated by recA protein acting at the free end of the duplex fragment by a mechanism presumably similar to that involved in the formation of D loops. While these experiments were in progress, DasGupta *et al.* (16) sent us their manuscript, prior to publication, in which they have reported electron microscopic observations that recA protein promotes the transfer of one strand from a linear duplex molecule to a single-stranded circular molecule.

## MATERIALS AND METHODS

**Enzymes and Proteins.** The purification of recA protein has been described (17). Protein concentrations described in the text refer to the concentration of recA protein monomers (1 $\mu$g = 25 pmol). The single-strand binding (SSB) protein of *E. coli* was purified as described (18). Restriction enzymes *Hpa* I and *Hae* III were purchased from Bethesda Research Laboratories, Rockville, MD.

**Preparation of DNA Substrates.** $\phi$X174 single-stranded DNA was prepared by phenol extraction of purified $\phi$X174 *am3* bacteriophage. $^3$H- or $^{32}$P-labeled $\phi$X174 covalent circular duplex DNA (form I DNA) was prepared as described (19).

The hybridized substrate for the strand-transfer assay was prepared by digesting $^3$H-labeled $\phi$X174 form I DNA with *Hpa* I, and the products were separated by 5% (wt/vol) polyacrylamide gel electrophoresis. Fragment 3 [392 base pairs (bp)] was eluted from the gel, and traces of ethidium bromide were removed by isobutanol treatment. The DNA was denatured by heating and rapid cooling, and the (−) strand was hybridized to circular (+) strands of unlabeled $\phi$X174 viral DNA. Incubation was for 45 min at 65°C in 0.6 M NaCl/0.6 M Na citrate, pH 7.0, in the presence of a 4-fold excess of fragments in order to fully saturate the viral DNA. Excess fragments were removed by sedimentation through a 35-ml sucrose gradient at 25,000 rpm for 18 hr in a Beckman SW 27 rotor (4°C).

Double-stranded DNA fragments were prepared by digesting $^{32}$P-labeled $\phi$X174 form I DNA with restriction enzymes. *Hae* III fragments 3 (872 bp) and 4 (603 bp) and *Hpa* I fragment 3 (392 bp) were purified by gel electrophoresis. All DNA samples were dialyzed extensively against 10 mM Tris·HCl, pH 7.5/1 mM EDTA.

**DNA Strand-Transfer Assay.** The basic reaction mixture (total volume, 100 $\mu$l) contained 0.73 nmol of hybridized substrate DNA, 20 mM Tris·HCl (pH 7.5), 25 mM $MgCl_2$, 2 mM dithiothreitol, 100 $\mu$g of bovine serum albumin per ml, and 2

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Abbreviations: ATP[S], adenosine 5′-*O*-(3-thiotriphosphate); form I DNA, covalent circular duplex DNA (replicative form I); bp, base pairs; SSB protein, single-strand binding protein.

mM ATP. In some reactions ATP was replaced or supplemented with 1 mM adenosine 5′-*O*-(3-thiotriphosphate) (ATP[S]). Duplex DNA restriction fragments, recA protein, and SSB protein were added as described. Incubation was for 60 min at 37°C. The reaction was stopped by the addition of 50 mM EDTA, and proteins were removed from the DNA by incubation for 15 min at 37°C with 200 μg of proteinase K per ml and 1% $NaDodSO_4$. All centrifugations were for 150–180 min at 45,000 rpm through 5–20% neutral sucrose in a Beckman SW 50.1 rotor at 4°C.

**Crosslinking of Duplex DNA.** DNA solutions were supplemented with 0.01 vol of a saturated solution of 4, 5′, 8-trimethylpsoralen in ethanol and irradiated (36 $kJ/m^2$) at 360 nm as described (20).

**ATPase Assay.** $^3H$-Labeled ATP (New England Nuclear; 40 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels) was added to the basic reaction mixture. After incubation, the reaction was stopped by the addition of excess EDTA, and the percentage of ATP hydrolyzed was determined by spotting 10-μl samples directly onto PEI/UV-254 thin-layer chromatography plates (21).

## RESULTS

**recA Protein Promotes Joint Molecule Formation.** recA protein promotes homologous pairing between duplex DNA molecules if one of them contains a single-stranded region (13, 14). When form I DNA and single-stranded circular DNA carrying a short hybridized fragment were incubated with recA protein, we found that approximately 15% of the form I DNA was retained by nitrocellulose filters which permit intact duplexes to pass unless paired to the hybridized substrate DNA. The presence of the hybridized fragment was essential for the formation of joint molecules (15). Under identical conditions, recA protein did not separate the hybridized fragment from the single-stranded circular DNA, indicating that it is not a helicase. However, when the form I DNA was replaced with duplex DNA fragments that were homologous to the hybridized fragment, we observed a new activity of recA protein—promotion of a reciprocal DNA strand-exchange reaction.

**recA-Promoted Strand Exchange.** The following experiments were designed to detect strand exchange, a step of genetic recombination that may follow homologous pairing during the formation of recombinant DNA molecules. The DNA substrate used in the strand-transfer reactions consisted of unlabeled single-stranded (+) viral DNA with hybridized $^3H$-labeled complementary (−) strand fragments (prepared from *Hpa* I restriction fragment 3). For simplicity, this DNA will be called the hybridized substrate.

To investigate the strand-exchange activity of recA protein, we incubated it with the hybridized substrate and duplex DNA fragments (*Hae* III fragment 3) that were homologous to and overlapped the hybridized fragment (Fig. 1, substrate I). Fig. 2*A* shows the sedimentation at neutral pH of the starting substrates. Although the $^3H$-labeled *Hpa* I fragment was only 392 bases in length, it sedimented rapidly because it was hybridized to the intact φX174 DNA. The $^{32}P$-labeled duplex *Hae* III fragment 3 of 872 bp sedimented more slowly. Fig. 2*B* shows the results of incubation of hybridized substrate DNA with $^{32}P$-labeled homologous duplex fragments and 0.25 nmol of recA protein. The ratio of duplex fragments to hybridized substrate molecules was 1.4:1. When the reaction products were centrifuged in neutral sucrose, we found that approximately 50% of the $^3H$-labeled hybridized fragments were transferred from the φX174 viral strand and sedimented more slowly in the position of the duplex fragments. In addition, approximately 15% of the $^{32}P$-label was seen to sediment in the position of the hybridized substrate, indicating that 30% of the (−) strands of the duplex fragments had been transferred in the reverse direction. We

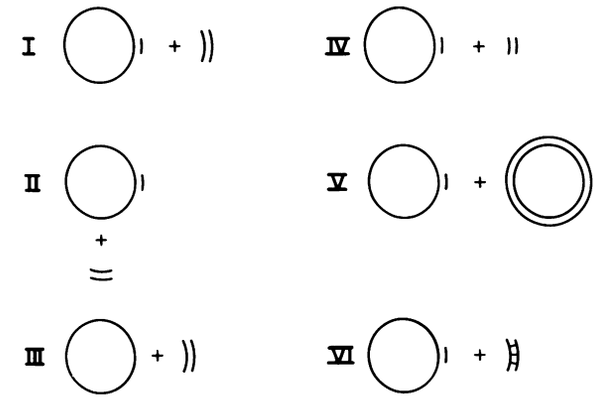


FIG. 1. DNA substrates used in the experiments. Substrates: I, Hybridized substrate reacting with homologous $^{32}P$-labeled duplex *Hae* III fragment 3 (872 bp); II, hybridized substrate with nonhomologous $^{32}P$-labeled duplex *Hae* III fragment 4 (603 bp); III, single-stranded viral φX174 (+) DNA with homologous $^{32}P$-labeled duplex fragment 3 (872 bp); IV, hybridized substrate with homologous $^{32}P$-labeled *Hpa* I duplex fragment 3 (392 bp); V, hybridized substrate with $^{32}P$-labeled φX174 form I DNA; VI, same as I, but the duplex fragment has been crosslinked. The hybridized substrate was single-stranded viral φX174 (+) DNA with hybridized $^3H$-labeled (−) strand of *Hpa* I restriction fragment 3 (392 bp). The angular position of each duplex fragment represents its relative position on the genetic map of φX174 DNA.

demonstrated that the hybridized fragment was hydrogen bonded to the (+) strand of the duplex fragment by sedimenting the reaction products through neutral sucrose for 9 instead of

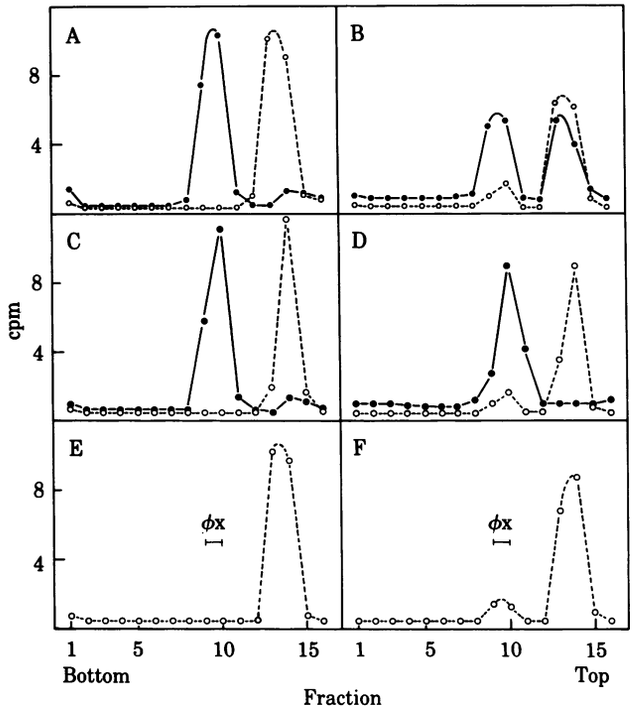


FIG. 2. Neutral sucrose sedimentation profiles showing strand transfer. ●, $^3H$ cpm × $10^{-2}$; ○, $^{32}P$ cpm × $10^{-3}$. (*A*) Hybridized substrate (0.73 nmol) with $^{32}P$-labeled homologous duplex fragments (0.32 nmol). (*B*) Same as *A* but with recA protein. (*C*) Hybridized substrate (0.73 nmol) with $^{32}P$-labeled nonhomologous duplex fragments (0.32 nmol). (*D*) Same as *C* but with recA protein. (*E*) Unlabeled φX174 viral DNA (0.73 nmol) with $^{32}P$-labeled homologous duplex fragments (0.32 nmol). (*F*) Same as *E* but with recA protein. Sedimentation is to the left. The sedimentation position of φX174 viral DNA (φX) was determined in control experiments.

3 hr. The $^{3}H$-labeled 392-bp and $^{32}P$-labeled 872-bp nucleotide fragments sedimented in the same position (data not shown).

To determine whether strand exchange was dependent on homology between the hybridized fragment and the duplex fragment, we performed a similar experiment with a duplex fragment (*Hae* III fragment 4) homologous to a different region of the $\phi$X174 viral strand (Fig. 1, substrate II). Because this duplex fragment was not homologous to the hybridized fragment, we refer to it as the nonhomologous fragment. Fig. 2*C* shows sedimentation of the DNA substrates. Fig. 2*D* shows the results obtained when the substrates were incubated with 0.25 nmol of recA protein. As in the preceding experiment, 15% of the $^{32}P$-label of the duplex fragments was now associated with the fast-sedimenting hybridized substrate. However, this interaction was at a site away from the hybridized fragment and did not initiate strand transfer of the $^{3}H$-labeled hybridized fragment.

In control experiments we incubated unlabeled $\phi$X174 viral single-stranded DNA with homologous duplex *Hae* III fragment 3 (Fig. 1, substrate III) in the absence or presence of recA protein (Fig. 2 *E* and *F*). After incubation with recA protein, we found approximately 15% of the $^{32}P$-labeled DNA was now hybridized to the $\phi$X174 single-stranded DNA. This corresponds to a transfer of approximately 30% of the complementary (−) strands from the duplex fragments to the single-stranded circles. This latter reaction is presumably similar to the strand-transfer reaction observed by DasGupta *et al.* (16).

**Requirements for the recA-Promoted Strand-Exchange Reaction.** recA protein is a single-strand DNA-dependent ATPase (9–11). The ATPase can be blocked by addition of the nonhydrolyzable ATP analogue ATP[S] (22). We next investigated whether ATP hydrolysis was required for recA-promoted strand exchange. Fig. 3*A* shows the results of a control experiment in which recA protein was incubated with the hybridized substrate and homologous duplex *Hae* III fragment 3. The results are similar to those presented in Fig. 2*B* and show transfer of 55% of the $^{3}H$-labeled hybridized fragments and 30% of the (−) strands of the $^{32}P$-labeled duplex DNA. However, the results presented in Fig. 3*B* show that strand exchange was completely blocked by the addition of 1 mM ATP[S] to an identical reaction mixture. Because this concentration of ATP[S] was sufficient to inhibit the recA protein ATPase (22), this result indicates that ATP hydrolysis is required for strand exchange.

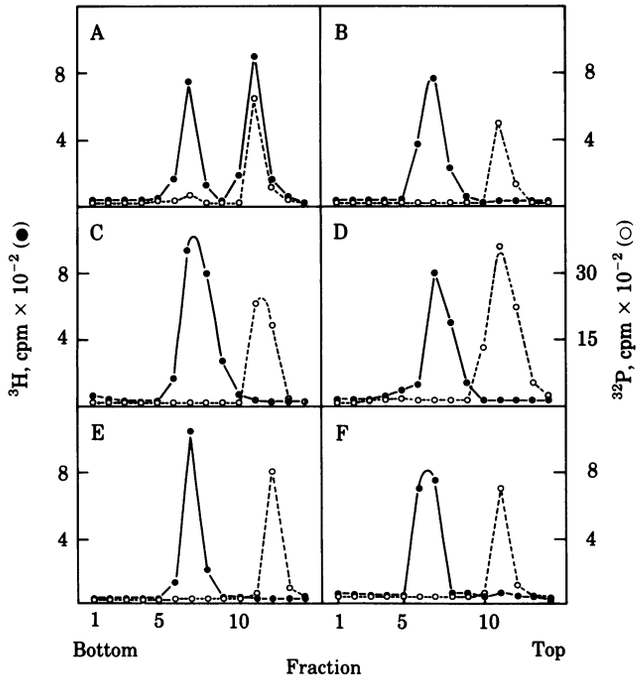


FIG. 3. Neutral sucrose sedimentation profiles of recA-promoted strand transfer reactions. (*A*) Hybridized substrate with 0.32 nmol of $^{32}P$-labeled homologous duplex fragments and 0.25 nmol of recA protein. (*B*) Same as *A* except that 1 mM ATP[S] was added at the start of the reaction. (*C*) Hybridized substrate with 2 nmol of $^{32}P$-labeled form I DNA. (*D*) Same as *C* but with 0.25 nmol of recA protein. (*E*) Hybridized substrate with 0.32 nmol of $^{32}P$-labeled *Hpa* I duplex fragments and 0.25 nmol of recA protein. (*F*) Hybridized substrate with 0.32 nmol of homologous crosslinked $^{32}P$-labeled duplex fragments and 0.25 nmol of recA protein. After incubation, samples *C* and *D* were treated with *Hae* III for 15 min at 37°C. Sedimentation is to the left.

Because homologous linear duplex fragments initiate strand transfer, we next determined whether strand transfer was observed after the interaction of homologous form I DNA with the hybridized substrate (Fig. 1, substrate V). Fig. 3 *C* and *D* shows the results of incubation of the hybridized substrate with $^{32}P$-labeled form I DNA in the absence or presence of recA protein. Because form I $\phi$X174 DNA sediments in neutral sucrose in almost the same position as the hybridized substrate does, we stopped the reaction by adding 1 mM ATP[S] and cut the form I DNA into small fragments with a restriction enzyme; the products were sedimented through neutral sucrose. In this way we would be able to detect any separation of the hybridized fragment from the substrate DNA. Strand separation was not observed.

We also reacted the hybridized substrate with recA protein and a $^{32}P$-labeled duplex fragment (*Hpa* I fragment 3) identical to the fragment hybridized to the substrate DNA (Fig. 1, substrate IV). Although this duplex was homologous to the hybridized fragment, it did not initiate strand exchanges (Fig. 3*E*).

If the transfer of one strand of the duplex fragment is required for transfer of the fragment from the hybridized substrate, one might expect the reaction to be inhibited by interstrand crosslinks that prevent separation of the duplex fragment. Therefore, we incubated recA protein with the hybridized substrate and crosslinked homologous duplex *Hae* III fragment 3 (Fig. 1, substrate VI). It was estimated from the yield of crosslinks in λ phage DNA (20) that the crosslinked fragments contained an average of 1 crosslink per 100 bp. Fig. 3*F* shows that strand transfer did not take place.

The results presented in Figs. 2 and 3 show that reciprocal strand exchange occurred between the hybridized substrate and linear duplex fragments. The reaction was dependent on recA protein and ATP and required that the duplex fragment was homologous to and overlapped the fragment hybridized to the substrate DNA.

**Strand Transfer Requires Stoichiometric Amounts of Duplex Fragments.** In the absence of homologous duplex fragments, recA protein is unable to separate the hybridized fragment from the substrate DNA. To investigate the stoichiometry of the strand-transfer reaction, we incubated 0.73 nmol of hybridized substrate with 0.25 nmol of recA protein and varying amounts of homologous duplex fragments (*Hae* III fragment 3). Fig. 4 shows that the percentage of total strand transfer was directly proportional to the duplex fragment concentration. The maximum observed transfer of the hybridized fragment (60%) was attained at a ratio of 1.4 duplex fragments per hybridized substrate molecule (3.2 μM duplex fragments). Addition of excess duplex fragments did not increase the percentage of transfer.

**Kinetics of Strand Transfer.** A time course of the strand-transfer reaction is shown in Fig. 5. After a lag of about 5 min following addition of enzyme, the reaction proceeded at a linear rate for the next 30 min. The reaction was complete within 60

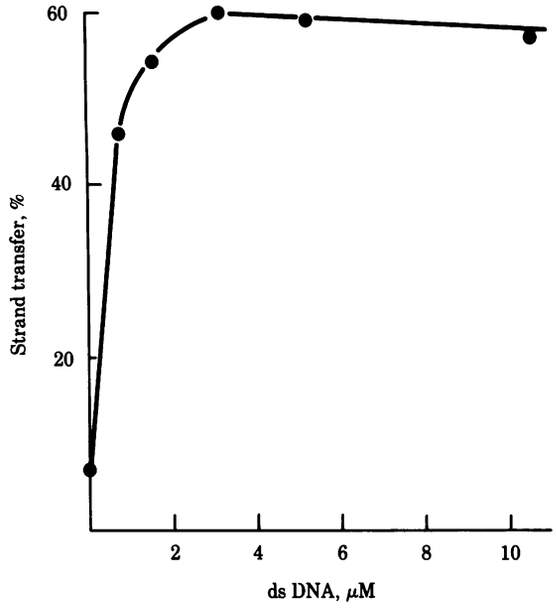


FIG. 4. Effect of duplex fragment concentration on the strand transfer reaction. Reaction mixtures were prepared with 0.25 nmol of recA protein and various amounts of homologous duplex fragments. Transfer of $^3$H-labeled fragments from the hybridized substrate was determined by centrifugational analysis. At a duplex fragment concentration of 2.3 μM, there was one duplex per hybridized substrate molecule. ds, Double stranded.

min. We determined the rate of ATP hydrolysis during this reaction and found it to be constant at a rate of 5 mol of ATP per min/mol of recA protein. Of the available ATP, 60% remained in the reaction mixture at the end of the incubation.

**Dependence of Strand Transfer on recA Protein Concentration.** Because strand transfer occurs after homologous pairing of the hybridized substrate with the duplex fragment, we

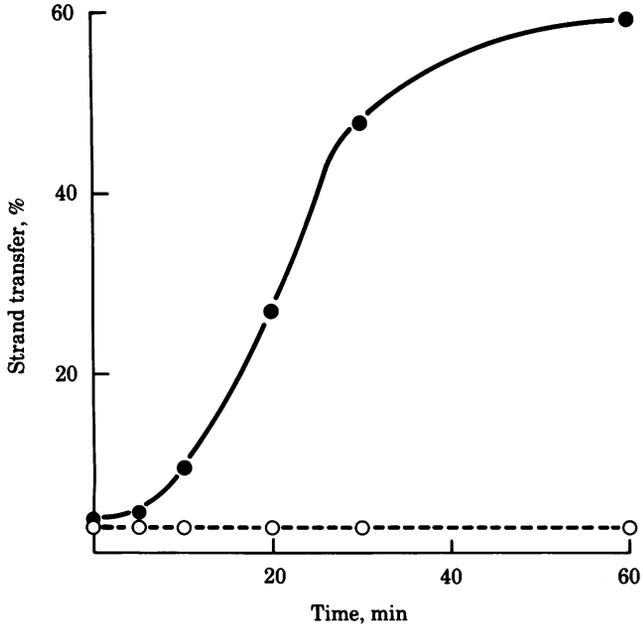


FIG. 5. Time course of the strand-transfer reaction. Reaction mixtures containing 0.25 nmol of recA protein, 0.73 nmol of hybridized substrate, and the presence (●) or absence (○) of 0.32 nmol of homologous duplex fragments were prepared. At various times of incubation, samples were removed, the reaction was stopped and the percentage of strand transfer was determined as in Fig. 4.

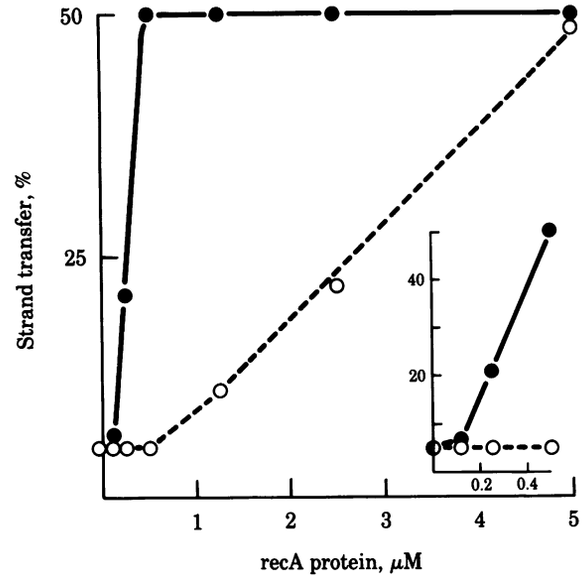


FIG. 6. Effect of recA and SSB protein concentrations on the strand-transfer reaction. Reaction mixtures were prepared with 0.5 nmol of homologous duplex fragments and varying concentrations of recA protein. The percentage of strand transfer was determined as described in Fig. 4. ●, Without SSB protein: ○, with 1 μM SSB protein.

presume that recA protein must interact with both DNA species. The results (Fig. 6) show that the overall reaction was influenced strongly by the ratio of recA protein to DNA. No strand transfer occurred until a certain concentration of recA protein was present, after which the extent of transfer increased sharply. In the presence of 0.73 nmol of hybridized substrate and 0.32 nmol of duplex fragments, strand exchange occurred with 0.05 nmol of recA protein (approximately equivalent to one monomer per 12 nucleotides of hybridized substrate). Addition of excess recA protein did not increase strand transfer.

In the same experiment, we added 0.1 nmol of SSB protein and varied the amount of recA protein. The presence of this concentration of SSB protein was sufficient to block strand transfer by 0.05 nmol of recA protein. Increasing concentrations of recA protein were able to promote strand transfer. However, in the presence of 0.1 nmol of SSB protein, 10-fold more recA protein was required to promote the maximum extent of strand transfer.

## DISCUSSION

We have shown that recA protein promotes homologous pairing of closed circular duplex DNA with single-stranded circular DNA carrying a short hybridized fragment (15). Pairing was dependent upon the presence of the hybridized fragment but did not involve initial separation of the fragment from the single-stranded DNA. The present experiments extend previous work by showing that reciprocal strand exchanges can occur following homologous pairing. The results presented in Fig. 2 *E* and *F* show that recA protein promotes the pairing of single-stranded circular DNA with duplex DNA fragments. We found that 30% of the complementary (−) strands from the duplex fragments became bound to the circular DNA. Interaction of duplex fragments with the hybridized substrate at the site of the hybridized fragment results in a transfer of approximately 50% of the hybridized fragments to the interacting duplex DNA. This strand-exchange reaction requires stoichiometric amounts of recA protein sufficient to cover the single-stranded DNA and is driven by ATP hydrolysis. Exchange occurs only when the duplex fragments are homologous to and overlap the hybridized fragment.

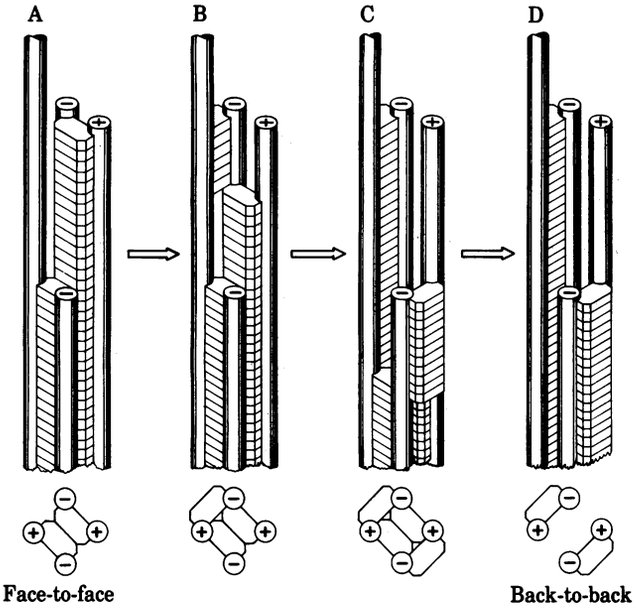


FIG. 7. Diagramatic representation of strand exchanges between the hybridized substrate and a homologous duplex fragment. Each diagram shows the hybridized substrate on the left and the duplex fragment on the right. Helical turns and unpaired bases are omitted for clarity. Diagrams: A, recA protein promotes four-stranded face-to-face pairing with the base pairs of both molecules in register over an unknown distance; B, strand exchange is initiated at the end of the duplex fragment, and the (−) strand of the duplex is transferred to the single strand of the hybridized substrate forming a heteroduplex; C, strand transfer is processive—as one strand of the duplex fragment is transferred to the single strand, the hybridized fragment is transferred to the remaining strand of the duplex; D, the end product of the reaction is a complete strand exchange forming two heteroduplexes. The molecules are now back-to-back, resulting in their separation. The switch in pairing is presumably the same as that proposed by Wilson (figures 5 and 7 of ref. 7), in which intercoiled parental homoduplexes with major groove pairing (face-to-face) undergo a strand-exchange reaction. Heteroduplexes are formed which untwist and separate because narrow groove pairing (back-to-back) is not stable.

Neither homologous fragments of the same size nor homologous form I duplex DNA initiate exchanges.

Although we do not know the exact mechanism of this strand-exchange reaction, it appears that recA protein may act on the hybridized substrate and duplex fragments through several successive and distinct reactions leading to the observed strand exchange. recA protein promotes the formation of joint molecules from two duplexes, if one contains a single-stranded region (13, 14). This reaction may depend on recA protein binding cooperatively to the single-stranded region and loading the adjacent duplex (17). recA protein also forms D loops between single-stranded and duplex molecules (10, 12). We visualize that reactions similar to these may bring the base pairs of the hybridized substrate and the duplex fragment into register, as shown diagramatically in Fig. 7, diagram A. Where homologous contacts are made, recA protein enables the two helices to assume a local four-stranded configuration (synapsis). Four-stranded pairing involving contacts in the wide groove (face-to-face pairing) has been proposed on the basis of studies with molecular models (6, 7) and has been invoked for site-specific recombination by the *int* protein of phage λ (8). The results of the present experiments indicate that the duplex fragment has to extend beyond the hybridized fragment for reciprocal exchanges to occur. This indicates that strand transfer is initiated by recA protein at the end of the duplex and is presumably the same reaction that occurs in D loop formation. A switch in pairing may occur so that the (−) strand of the duplex fragment base pairs with the (+) single strand (Fig. 7, diagram B). The transfer occurs in a processive manner, the bases rotating through 90° (or 270°) about the axis of the sugar phosphate backbone. As one strand of the duplex fragment is transferred to the single strand of the substrate DNA, the hybridized fragment is transferred to the remaining strand of the duplex fragment (Fig. 7, diagram C). The end product of the reaction is complete reciprocal strand exchange. The molecules, now back-to-back (Fig. 7, diagram D), are no longer held together and separate. This model provides an explanation for the transfer of strands in a crossed strand exchange, which involves a shift in base pairing by rotation of the two duplexes, and differs from several published models, which assume strand separation prior to homologous pairing (1–4).

This work was supported by grants from the National Institutes of Health of the U.S. Public Health Service, GM 11014, CA 06519, and AM 09397.

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