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On RecA Protein-mediated Homologous Alignment of Two DNA Molecules

THREE STRANDS *VERSUS* FOUR STRANDS*

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The recA protein from *Escherichia coli* can homologously align two duplex DNA molecules; however, this interaction is much less efficient than the alignment of a single strand and a duplex. Three strand paranemic joints are readily detected. In contrast, duplex-duplex pairing is detected only when the incoming (second) duplex is negatively supercoiled, and even here the pairing is inefficient. The recA protein-promoted four strand exchange reaction is initiated in a three strand region, with efficiency increasing with the length of potential three strand pairing available for initiation. This indicates that a paranemic joint involving three DNA strands may be an important intermediate in *all* recA protein-mediated DNA strand exchange reactions and that the presence of three strands rather than four is a fundamental structural parameter of paranemic joints.

The recA protein is a central component in general genetic recombination and DNA repair pathways of *Escherichia coli*. Purified recA protein can homologously pair and exchange DNA strands *in vitro*. One of the most useful model systems, the three strand exchange reaction, utilizes circular single-stranded DNA (ssDNA)[1] and homologous linearized duplex DNA derived from bacteriophage (see Kowalczykowski, 1987; Cox and Lehman, 1987; Griffith and Harris, 1988; Radding, 1988 for reviews). This reaction can be divided into three steps. The first is the binding of recA protein to the ssDNA to form a highly structured nucleoprotein filament (Shibata *et al.*, 1979; Cox and Lehman, 1981a; Register and Griffith, 1985; Egelman and Stasiak, 1986). The single-stranded binding protein of *E. coli* (SSB) stimulates the formation of a stable and contiguous recA nucleoprotein filament that completely covers the ssDNA (Cox *et al.*, 1983; Kahn and Radding, 1984; Morrical *et al.*, 1986). RecA binding results in extension of the bound DNA so that the axial spacing between bases becomes approximately 5.1 Å (Koller *et al.*, 1983; Flory *et al.*, 1984; Williams and Spengler, 1986). In the next step of the reaction, the nucleoprotein complex binds a second DNA molecule (the dsDNA) and pairs it with the ssDNA in homologous alignment, forming an intermediate referred to as a paranemic joint (Shibata *et al.*, 1979; McEntee *et al.*, 1979; Cox and Lehman, 1981a; DasGupta and Radding, 1982). The incoming dsDNA becomes extended in length, and concomitantly underwound, to permit the homologous interaction (Wu *et al.*, 1983; Schutte and Cox, 1988). Both solution measurements (Schutte and Cox, 1987, 1988) and electron microscopy (Register *et al.*, 1987) provide evidence that this alignment can extend over thousands of base pairs. The final step in the reaction, the exchange of DNA strands, can occur if the linear duplex has a free 3′ end complementary to the ssDNA. The exchange occurs in the 5′ to 3′ direction relative to the circular ssDNA and requires ATP hydrolysis (West *et al.*, 1981a, 1981b, 1981c; Kahn *et al.*, 1981; Cox and Lehman, 1981a, 1981b; Cox *et al.*, 1983). The final products of the three strand exchange reaction are a nicked, circular heteroduplex DNA and a displaced linear ssDNA.

A very similar reaction can occur between two duplex DNA molecules, referred to as four strand exchange. Although this reaction may more closely mimic some of the *in vivo* reactions carried out by recA protein, it has not been as well characterized as the three strand exchange. Analogous to the three strand reactions, the first step in four strand exchange is the formation of a helical nucleoprotein filament on one of the two duplexes, hereafter referred to as DNA#1. There is a kinetic barrier (slow nucleation) to this binding of recA protein to duplex DNA at neutral pH (Pugh and Cox, 1987, 1988; Kowalczykowski *et al.*, 1987). A short single-stranded gap in DNA#1, however, serves as a nucleation site for recA protein binding, such that the rate-limiting step of binding to duplex DNA is overcome (Shaner *et al.*, 1987; Shaner and Radding, 1987; Lindsley and Cox, 1989). The filament coats the contiguous duplex DNA flanking the gap, and the duplex DNA within this nucleoprotein filament is underwound (*i.e.* has a reduced helical pitch) by 39.5% relative to B form DNA in the presence of ATP (Pugh *et al.*, 1989). As with ssDNA, the duplex is also extended approximately 50% relative to B form DNA, with a 5.1-Angstrom axial separation of base pairs. The ssDNA gap is required not only to initiate recA protein binding but also for subsequent steps in the reaction as outlined below.

In the next stage of four strand exchange, the recA protein-

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; FI DNA, supercoiled closed circular form of DNA as isolated from *E. coli* cells; FII DNA, the nicked circular form of the same molecule; FIII DNA, the linear form of the same molecule; FIV DNA, the relaxed, closed circular form of the same molecule; SSB, the single-stranded binding protein of *E. coli*; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; kb, 10^3 base pairs; bp, base pairs.

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gapped duplex DNA complex binds to a second, homologous duplex, DNA#2. A four strand exchange is initiated only if a homologous free end of DNA#2 overlaps the gap of DNA#1 (West *et al.*, 1981a, 1981b, 1981c, 1982); *i.e.* four strand exchanges begin as three strand exchanges. A three strand paranemic joint is a likely intermediate in this reaction (Fig. 1*a*). It is less clear, however, whether paranemic joints involving four strands are formed by recA protein. In studies in which the potential for pairing was restricted to four strand interactions and pairing was quantified by electron microscopy, Cassuto *et al.* (1982) reported that a low level of homologous pairing occurred between the two duplexes, while DasGupta *et al.* (1981) detected no such pairing. West and Howard-Flanders (1984) demonstrated that recA protein could promote four strand exchange past a double-stranded break in duplex DNA#2 (see Fig. 1*b*), so that exchange involving one of the two fragments of DNA#2 initiated in a four strand region. This result implies that recA protein can homologously align two DNA substrates in regions where both are duplex. More recently, Conley and West (1989) reported direct evidence of recA protein-promoted homologous pairing between regions of duplex DNA. This pairing appears to be nonproductive on its own because no strand exchange has previously been detected if the ends of DNA#2 are flush with the ends of the duplex region of DNA#1 (see Fig. 1*c*).

There should be no topological barrier against the strand exchange reaction depicted in Fig. 1*c*. If recA protein can homologously align the two duplexes, why then is a region of three-stranded pairing required to initiate a four strand exchange reaction? We have devised experiments aimed at answering this question. Using the underwinding of DNA#2 as a measure of homologous pairing as previously described (Wu *et al.*, 1983; Schutte and Cox, 1988; Conley and West, 1989), we compared recA protein-promoted duplex-duplex pairing with ssDNA-duplex pairing. The length of potential three strand pairing necessary to initiate four strand exchange was then examined. The results of these experiments show that the recA protein-promoted pairing of three DNA strands is a much more favorable reaction than the pairing of four DNA strands. These results are discussed in light of current models for the structure of paranemic joints.

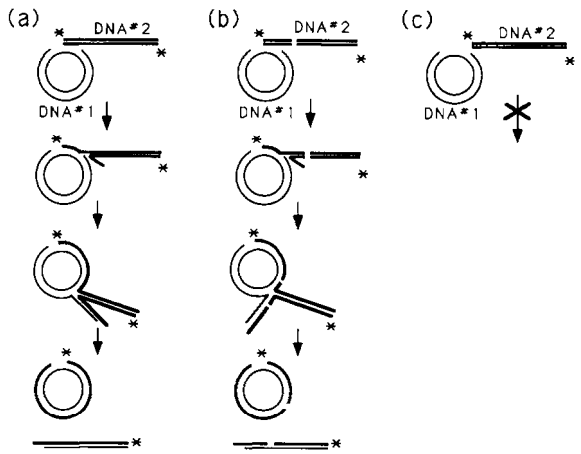


FIG. 1. **Four strand exchange.** *a*, recA protein can promote a four strand exchange reaction between two homologous duplex DNA substrates if DNA#2 has a free end complementary to the single-stranded DNA gap of DNA#1. *b*, four strand exchange can proceed through a double-stranded break in DNA#2, but not in DNA#1 (West and Howard-Flanders, 1984). *c* no recA protein promoted four strand exchange has previously been detected between two DNA substrates only homologous in duplex regions. As described below, however, we did detect a very modest reaction between the DNA substrates depicted in *c*. DNA#2 is end-labeled with ^{32}P (*) to aid in following the reaction.

MATERIALS AND METHODS

Enzymes and Biochemicals—E. coli recA protein was purified and stored as previously described (Cox *et al.*, 1981). The recA protein concentration was determined by absorbance at 280 nm, using a extinction coefficient of $\epsilon_{280} = 0.59\ A_{280}\ mg^{-1}$ ml (Craig and Roberts, 1981). *E. coli* SSB protein was purified as described (Lohman *et al.*, 1986). The concentration of the SSB stock solutions was determined by absorbance at 280 nm, with an extinction coefficient of $\epsilon_{280} = 1.5\ A_{280}\ mg^{-1}$ ml (Lohman and Overman, 1985). Restriction endonucleases and polynucleotide kinase were purchased from New England Biolabs. Eukaryotic topoisomerase I (10 units μl^{-1}, as defined by the manufacturer) was purchased from Biological Research Laboratories. Bacteriophage T4 DNA ligase was purified following published procedures (Davis *et al.*, 1980). The concentration of the T4 ligase stock solution was obtained by Bradford analysis (Bradford, 1976). Reverse transcriptase was purchased from Life Sciences. Pyruvate kinase, DNase I, and ATP were purchased from Sigma. Tris buffer and alkaline phosphatase were purchased from Boehringer Mannheim. The DNA sequence of M13mp8.437 was verified using the Sequenase version 2.0 sequencing kit from United States Biochemical Corporation. [γ-^{32}P]ATP, [α-^{32}P]dATP, [α-^{32}P]dGTP, and [α-^{32}P]dCTP were purchased from Amersham Corp.

DNA—The DNA molecules from which the duplex and ssDNA substrates were derived are shown in Fig. 2. Single-stranded DNA derived from bacteriophages M13mp8.437 (7666 bp) and M13mp8.1041 (8270 bp) was prepared by a published procedure (Messing, 1983). The bacteriophage M13mp8.437 is the bacteriophage M13mp8 with a 437-bp sequence (*Bam*HI fragment) from pGP704 (Miller and Mekalanus, 1988) inserted into the *Bam*HI site. The bacteriophage M13mp8.1041 is the bacteriophage M13mp8 with a 1041-bp sequence (*Eco*RV fragment) from the *E. coli galT* gene inserted into the *Sma*I site. The FI DNA derived from bacteriophages M13mp8, M13mp8.437, and M13mp8.1041, as well as plasmid DNA, was prepared as described (Davis *et al.*, 1980; Messing, 1983; Neuendorf and Cox, 1986). Stock concentrations of ssDNA and dsDNA were determined by absorbance at 260 nm, with 36 and 50 $\mu g\ ml^{-1}$ A_{260}^{-1} as conversion factors, respectively. DNA concentrations are expressed as total nucleotides.

The plasmid pJEL3 (8036 bp) was developed for a separate set of experiments and contains sequences from: pBR322 (5052 bp, Bolivar *et al.*, 1977), the 2-μm plasmid of *Saccharomyces cerevisiae* (1599 bp), the *galT* gene of *E. coli* (1041 bp), the kanamycin resistance gene from the transposon Tn 903 (268 bp, Oka *et al.*, 1981), and the ϕ10 promoter from bacteriophage T7 (76 bp, Tabor and Richardson, 1985). The DNA from pJEL3 was completely heterologous with the DNA from bacteriophage M13mp8 and was used in experiments requiring a heterologous DNA control.

FII DNA was prepared from FI DNA using DNase I and ethidium bromide (Shibata *et al.*, 1981). This method produces primarily FII DNA with a single random nick (approximately 90–95% of the total DNA) and a small amount (about 5–10%) of contaminating FIII DNA. FIII DNA was derived from FI DNA stocks by complete digestion with the appropriate restriction endonuclease. After digestion, residual protein was removed by extraction 1:1 sequentially with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), followed by ethanol precipitation.

Circular duplex DNA with a precisely defined gap was prepared as described (Lindsley and Cox, 1989). For simplicity, the gapped duplex molecules will be referred to as GD_x, where X is the length of the single-stranded region in nucleotides. Briefly, FIII M13mp8 linearized with *Bam*HI (for GD_{437}) or *Sma*I (for GD_{1041}) was denatured. The minus strand was annealed to a 15–20-fold excess of circular single-stranded M13mp8.437 or M13mp8.1041 DNA. The gapped duplex DNA that was formed was purified away from the remaining ssDNA on a small hydroxylapatite column. Approximately 100 μg of gapped duplex DNA was eluted from the column. The resulting gapped duplex DNA was at least 90% pure, with the main contaminant being FIII M13mp8. The gapped duplex DNA was then repurified from a 0.8% agarose gel (Maniatis *et al.*, 1982) using a unidirectional electroeluter (International Biotechnologies, Inc.) as previously described (Meyer-Leon *et al.*, 1988). Approximately 20–40 μg of gapped duplex DNA was eluted from the gel. The gapped duplexes were circular, with 7229 bp of duplex DNA and 437 or 1041 nucleotides of ssDNA. The duplex portion of these gapped duplexes was completely homologous with

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M13mp8 DNA, while the single-stranded region shared no homology with M13mp8 DNA.

The linear duplex DNA used in four strand exchange reactions was prepared from FI M13mp8.437 (for reactions with GD_{437}) or FI M13mp8.1041 (for reactions with GD_{1041}). The DNA was cut with restriction endonucleases such that at most one end of the linear duplex overlapped with the homologous sequence with the ssDNA gap of the appropriate gapped duplex (see Figs. 2 and 3). All of the linear duplex substrates, with the exception of pJEL3, shared 7.2 kb of homology with the duplex regions of the gapped duplexes. The FI M13mp8.437 DNA was cut with *Eco*RI and *Bam*HI, *Xba*I, or *Eco*RV, or only *Eco*RI, to produce linear duplex DNA substrates that overlapped with the gap of GD_{437} by 0, 15, 32, or 52 nucleotides (see Figs. 2 and 3). The plasmid pJEL3 cut with *Nco*I was used as a heterologous control. For reactions with GD_{1041}, the linear duplex DNA was derived from FI M13mp8.1041 cut with *Eco*RI and *Nco*I. This linear duplex overlapped the gap of GD_{1041} by 609 nucleotides. All of the linear duplex DNA substrates were end-labeled with ^{32}P on both strands. The *Eco*RI/*Eco*RV cut DNA was treated with phosphatase, to remove 5′ phosphates, and polynucleotide kinase, to add phosphate from [γ-^{32}P]ATP (Maniatis *et al.*, 1982). All of the other linear duplex DNA substrates were end-labeled using reverse transcriptase and the appropriate [α-^{32}P]dNTPs to add one labeled nucleotide to each end. The labeled linear duplex substrates were purified away from contaminating DNA fragments and unincorporated NTPs or dNTPs by electrophoresis through 0.8% agarose gels. The DNA was separated from the agarose using an electroeluter, as described above, yielding 10–20 μg of each labeled duplex DNA substrate in a typical preparation.

Reaction Conditions—All reactions were carried out at 37 °C in a standard reaction buffer containing 25 mM Tris-acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 1 mM dithiothreitol, 5% glycerol, 3 mM ATP and an ATP regenerating system (4.5 units/ml pyruvate kinase, 2.31 mM phosphoenolpyruvate, and 10 mM potassium glutamate). Protein and DNA concentrations are indicated for each experiment.

DNA Underwinding Assays—These assays measure underwinding of the dsDNA substrate (DNA#2) that results from the homologous pairing of DNA#2 molecules with the recA-ssDNA or recA-gapped duplex DNA complexes. Two different protocols were used to measure the underwinding of DNA#2, all of which trap negative supercoils formed in DNA#2 upon homologous pairing. The differences in the assays reflect differences in the topological state of the DNA#2 used in a particular experiment. When DNA#2 was a nicked circle (FII) the underwinding was trapped by ligating the nick. When DNA#2 was covalently closed (FI or FIV) underwinding was detected by treatment with topoisomerase I. All of the underwinding reactions contained 4 μM recA protein, 12 μM ssDNA (derived from M13mp8.437) or 24 μM GD_{437}, and 24 μM FI, FII, or FIV DNA derived from M13mp8 or pJEL3. SSB, when included, was at 1.2 μM. Reactions including recA protein, ssDNA or gapped duplex DNA, and ATP were incubated for 10 min at 37 °C prior to addition of SSB and the dsDNA to start the pairing reaction. Unless otherwise indicated, final reaction volumes were 20 μl. The complete reactions were incubated for a total of 3 min. For ligation reactions, 2 μl of T4 DNA ligase (0.2 mg/ml final) was introduced 2 min after the FII DNA was added, and the incubation continued for 1 min before the reactions were stopped. For reactions with FI DNA, 15 units of topoisomerase I were introduced 2 min after the FI DNA was added. The incubation continued for 1 min before the reactions were stopped. For reactions involving FIV DNA, FI DNA was incubated with 150 units of topoisomerase I in standard reaction buffer (40 μl of total volume) for 15 min prior to its addition to pairing reactions. This procedure converts all of the FI DNA into FIV DNA. To start the pairing reactions, 4 μl of this FIV DNA-topoisomerase I mixture was added to each pairing reaction mixture to give a final volume of 20 μl. After 3 min, the reactions were stopped. All of the reactions (20 μl) were stopped by the addition of 10 μl of gel loading buffer (25% glycerol, 5 mM EDTA, 0.005% bromphenol blue, 5% SDS). Samples were loaded in 1.4% agarose gels, electrophoresed at 2–2.5 V cm^{-1} for 16 h. Gels were then stained with ethidium bromide, and DNA bands were illuminated with UV irradiation and photographed.

Four Strand Exchange Reactions—The substrates for the four strand exchange reactions are described above and shown in Fig. 3*b*. The four strand exchange reactions contained 3 μM recA protein, 12 μM gapped duplex DNA, and 6 μM ^{32}P-labeled linear duplex DNA. SSB, when included, was at 0.6 μM. The recA protein, gapped duplex DNA, and ATP were incubated for 10 min at 37 °C prior to addition of SSB and the ^{32}P end-labeled linear duplex DNA to start the reactions. Aliquots of 5 μl were removed from reactions at each time point, added to 5 μl of gel loading buffer (described above) to stop the reactions, and placed on ice. The zero time point of each reaction was simply an aliquot of the appropriate labeled linear duplex DNA substrate added to gel loading buffer. The volume of samples loaded/gel lane was constant for a particular time course but was adjusted from one time course to the next so that the total number of counts loaded/lane was approximately equal. The 0.8% agarose gels were electrophoresed at 2–2.5 V cm^{-1} for 12–16 h, transferred to Whatman 3M paper, dried, and autoradiographed at −70 °C. Densitometric scans of the gels were performed on a Zeineh Soft Laser scanning densitometer, SL-504-XL, from Biomed Instruments, Inc.

RESULTS

A Comparison of Three and Four Strand Pairing by RecA Protein—Homologous interaction of DNA molecules was examined in this study with the aid of a series of single strand, gapped duplex, and duplex DNA substrates. The DNAs from which the substrates were derived are illustrated in Fig. 2 and described under "Materials and Methods." Gapped duplexes with defined gap boundaries were constructed from these DNAs as shown in Fig. 3. These substrates permitted an unambiguous comparison of pairing interactions that involve either three or four DNA strands.

A recA protein filament formed on a ssDNA or gapped duplex (DNA#1) can bind a second duplex DNA molecule (DNA#2) and align the two (see Fig. 3*a* for diagram). Since DNA#1 is extended and underwound, homologous alignment requires the extension (and underwinding) of DNA#2. Underwinding of DNA#2 therefore becomes a relatively unambigu-

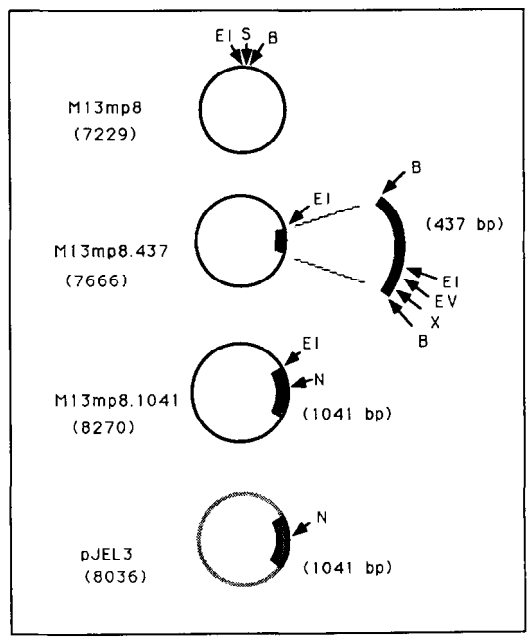


FIG. 2. **DNA substrates.** Maps of duplex DNA molecules derived from bacteriophages M13mp8, M13mp8.437, and M13mp8.1041, and plasmid pJEL3. Homologous sequences shared by different DNAs are indicated by similar *line thicknesses* or *shading*. The viral ssDNA from bacteriophages M13mp8.437 and M13mp8.1041 was also used in experiments described. *B, EI, EV, N, S,* and *X* denote the restriction endonuclease sites of *Bam*HI, *Eco*RI, *Eco*RV, *Nco*I, *Sma*I, and *Xba*I. Except for the two *Eco*RI sites shown on bacteriophage M13mp8.437, all restriction sites are unique. The total length of each molecule in base pairs is shown in *parentheses*. M13mp8.437 and M13mp8.1041 DNA molecules each share 7.2 kb of homology with M13mp8. The DNA sequences of bacteriophage M13mp8 and plasmid pJEL3 share no homology. M13mp8.1041 and pJEL3 are homologous through the 1041-bp *gal*T DNA fragment present in both DNA molecules as indicated.

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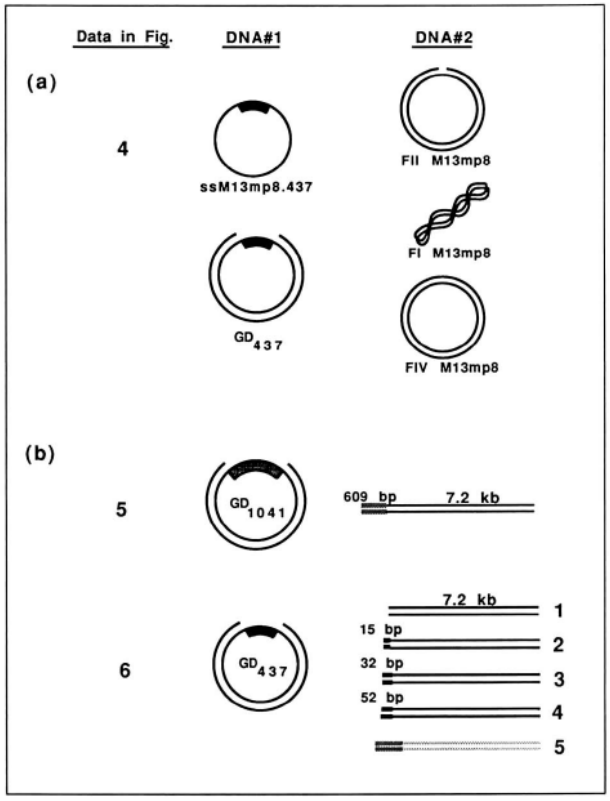


FIG. 3. **Combinations of DNA substrates used for experiments described in figs. 4–6.** *a* Three strand *versus* four strand pairing were compared by underwinding assays described under "Materials and Methods." RecA protein complexes formed on single-stranded M13mp8.437 DNA or GD_{437} were allowed to pair with FII, FI, or FIV dsDNA from M13mp8. DNA#1 is homologous to DNA#2 in the regions depicted by *thin black lines*. *b* four strand exchange reactions were performed between gapped duplex and linear duplex DNA substrates. The *numerals 1–5* beside the depictions of linear duplex substrates correspond to the reaction numbers in Fig. 6. Regions of homology between DNA substrates are shown by similar *shading*. DNA molecules are not drawn to scale.

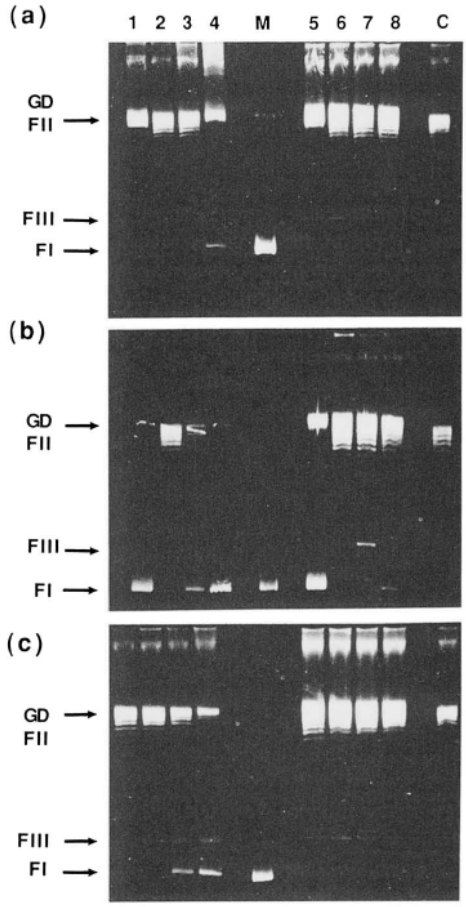


FIG. 4. **Three *versus* four strand pairing detected by underwinding assays.** Pairing reactions were performed as described under "Materials and Methods" using ligase and FII DNA (*a*), topoisomerase and FI DNA (*b*), and topoisomerase and FIV DNA (*c*). Reactions used for *lanes 1–4* included ssDNA from M13mp8.437; reactions used for *lanes 5–8* included GD_{437} DNA. A schematic of DNA substrates is shown in Fig. 3. Reactions used for *lanes 1* and *5* lacked ligase or topoisomerase (in *panel c*, these lanes represent zero time points taken immediately after all reaction components were added, since the topoisomerase had already been added to make the FIV DNA). Reactions for *lanes 2* and *6* lacked recA protein; reactions for *lanes 3* and *7* lacked SSB. The reactions used for *lanes 4* and *8* contained all reaction components. The marker (*M*) is FI DNA derived from M13mp8. The control lanes (*C*) lacked only the ssDNA or the gapped duplex DNA. For all reactions, the DNA was allowed to pair for a total of 3 min. *GD*, gapped duplex DNA; *FII*, circular duplex DNA derived from M13mp8 containing a single nick; *FIII*, contaminating linear duplex DNA from M13mp8; *FI*, supercoiled, double-stranded circular DNA from M13mp8. The ssDNA from M13mp8.437 is not visible in these photographs.

ous manifestation of homologous pairing. The underwound state of DNA#2 can be trapped with either ligase or topoisomerase, depending on the initial topology of DNA#2. We wanted to compare the differences in the underwinding of DNA#2 when DNA#1 was single-stranded *versus* duplex. Previous studies have shown that the homologous pairing between a recA protein-ssDNA complex and duplex DNA occurs within 2 min (Schutte and Cox, 1987, 1988). At longer time points, recA protein may be transferred from the ssDNA to the incoming homologous duplex, as seen by the formation of more highly underwound DNA (Schutte and Cox, 1988). To insure that the only DNA underwinding that we detected was due to homologous pairing, and not to the direct binding of recA protein, pairing reactions were stopped after a total of 3 min, and the recA protein concentration was such that essentially all of the protein present was bound to the single-stranded or gapped duplex DNA. In the experiments shown in Fig. 4, the ratio of DNA to recA protein was three nucleotides (or bp) per monomer, consistent with the known DNA-binding site size (three or four nucleotides or bp) for recA protein (Cox and Lehman, 1987; Radding, 1988). Control reactions confirmed that recA protein did not directly bind to DNA#2 (see below).

Three strand pairing reactions were performed to insure that the results were the same as those previously obtained for similar substrates (Schutte and Cox, 1988). These are presented in *lanes 1–4* in Fig. 4 and are consistent with previous results. Approximately the same fraction of DNA#2 molecules were underwound when either FII DNA and ligase, or FIV DNA and topoisomerase, were used (compare *panels a* and *c*, *lanes 1–4*). When DNA#2 was FI, a larger fraction of DNA#2 molecules remained underwound after topoisomerase I treatment, indicating that pairing was more efficient (*panel b*). RecA protein and SSB were both required for optimal pairing (compare *lane 4* with *lanes 2* and *3*). Schutte and Cox (1988) found that DNA#2 became underwound by an average of 12% (a maximum of 18%) relative to B form DNA in similar pairing reactions. In the gel system used for the present experiments (*i.e.* no intercalating dyes added), DNA underwound by 12% migrates approximately with FI DNA (approximately 7% underwound), derived from the same plasmid or bacteriophage. Therefore, FI DNA was a useful marker

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for the underwound DNA product (DNA#2).

The results of duplex:duplex pairing experiments are shown in *lanes 5–8* of Fig. 4. Duplex DNA#1 had a 437 nucleotide gap as a nucleation site for recA protein binding. The ssDNA in the gap was completely heterologous to DNA#2 (see Fig. 3*a* for diagram). Therefore, homologous interactions are only possible between the duplex regions of DNA#1 and DNA#2. When recA protein, or recA protein plus SSB, were bound to DNA#1, no pairing could be detected between the nucleoprotein complex and the FII DNA#2 by ligation (*panel a, lanes 5–8*). All of the ligated DNA ran as a ladder of topoisomers beneath the FII DNA (corresponding to relaxed FIV DNA), regardless of whether all reaction components were included (*panel a, lane 8*) or recA protein, SSB, or DNA#1 were left out of the reaction mixture (*panel a, lanes 6, 7* and *c*, respectively). No rapidly migrating bands, indicative of underwound DNA#2, were detected. This reaction has been repeated numerous times and pairing under these conditions has never been detected. However, when DNA#2 was negatively supercoiled (*FI*) and eukaryotic topoisomerase I was used, pairing was detected (*panel b, lanes 7* and *8*); *i.e.* the topoisomerase did not relax some of the FI DNA#2 in the presence of the recA·DNA#1 complex. Although the reaction conditions were different, this result is similar to that of Conley and West (1989). The pairing we detected required recA protein and was greatly enhanced by SSB (compare *lane 8* with *lanes 6* and *7*). The fraction of DNA#2 molecules underwound here, however, was much less than that observed in the three strand pairing reactions (compare *lanes 4* and *8, panel b*). If the FI DNA#2 was heterologous to DNA#1, the topoisomerase completely relaxed DNA#2 to a FIV DNA ladder (data not shown), indicating an absence of pairing and confirming the result in *panel b, lanes 7* and *8*. The results of reactions shown in the control lanes depicting reactions carried out in the absence of ssDNA or gapped duplex indicate that recA protein did not directly bind to DNA#2 within the time period of the reaction. Such binding would have resulted in underwinding of the DNA.

Duplex-duplex pairing was detected if DNA#2 was supercoiled and topoisomerase was used but was not detected if DNA#2 was nicked and ligase was used. Was the topology of DNA#2 the critical factor, or did the use of two different enzymes introduce an artifact? The results of an experiment shown in Fig. 4, *panel c* indicate that DNA#2 must be supercoiled to detect duplex-duplex pairing by an underwinding assay. In this experiment, the FI DNA was treated with topoisomerase I *before* it was paired with the recA-gapped duplex DNA complex. *Lanes 1* and *5* (*panel c*) show that the topoisomerase fully relaxed the FI DNA to a FIV DNA ladder prior to the pairing reaction. No underwinding of the FIV DNA was detected in reactions when DNA#1 was duplex (*lanes 7* and *8*) but was easily detected when DNA#1 was single-stranded (*lanes 3* and *4*). Therefore, duplex-duplex pairing is not observed unless DNA#2 is supercoiled prior to the pairing reaction. When DNA#2 is relaxed, only three strand pairing is observed. Even when DNA#2 is supercoiled, three strand pairing is more efficient than four strand pairing.

The Length of Homologous Three Strand DNA Overlap Required for Four Strand Exchange Reactions—Homologous pairing is a prerequisite for recA protein-promoted strand exchange. One way to study the effectiveness of duplex-duplex pairing, therefore, is to examine four strand exchange reactions initiated in regions of three strand pairing (see Figs. 1*a* and 3*b* for diagram) in which the length of the potential three strand interaction is limited. Since the underwinding assay only detected four strand pairing when DNA#2 was supercoiled, the four strand exchange reaction provides an alternative method to detect pairing of relaxed homologous duplexes.

In vitro four strand exchange reactions are nearly as rapid and as efficient as three strand exchange reactions if the appropriate substrates are chosen (West *et al.*, 1981a, 1981b, 1981c, 1982). The results of a time course of a four strand exchange reaction, where 609 nucleotides at the end of DNA#2 were complementary to the ssDNA gap of DNA#1, are shown in Fig. 5. On an ethidium bromide-stained agarose gel, the reactants and products of this reaction would be indistinguishable (see *schematic* of four strand exchange reaction in Fig. 1). In these reactions, the linear duplex DNA substrate was labeled at both ends with ^{32}P. One strand of the labeled linear substrate was transferred to the circular strand of the gapped duplex, while the other strand was transferred to the linear strand. The final products were a labeled, circular gapped duplex and a labeled linear duplex. The gapped duplex and the linear duplex can be separated on an agarose gel. However, the labeled linear duplex product was not separated from the labeled linear duplex substrate. Therefore, if a four-strand exchange reaction proceeded to 100% products, 50% of the DNA label would run as gapped duplex DNA and 50% would run as substrate. The results shown in Fig. 5*b* indicate that the reaction proceeded to about 60% of the total possible reaction. The reaction was greatly inhibited when SSB was not included (see Fig. 5*a*). West *et al.* (1981a, 1982) reported that SSB decreases the final extent of four strand reactions. Although their findings appear to contradict our results, the difference is most likely explained by the different order of addition of reaction components used in these experiments. We have found that SSB only stimulated the four strand

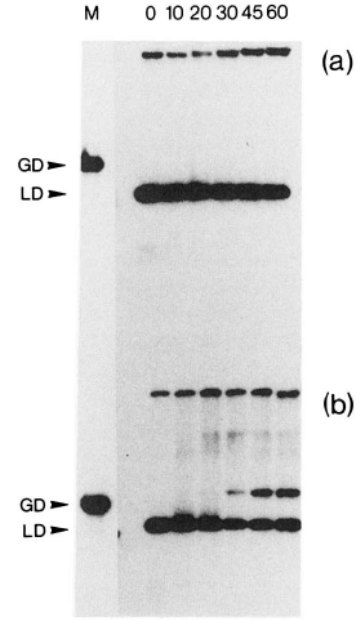


FIG. 5. **Four strand exchange reactions between gapped (*GD*) and linear duplex DNA substrates.** Time courses of four strand exchange reactions in which SSB was not included (*a*), and SSB was included at 0.6 μM (*b*). The gapped duplex, GD_{1041}, and a ^{32}P end-labeled linear duplex (which homologously overlapped on one end with the ssDNA gap by 609 nucleotides) were reacted with recA protein as described under "Materials and Methods." Aliquots of the reaction were removed as indicated, stopped by the addition of SDS to a final concentration of 2.5%, subjected to agarose gel electrophoresis, and autoradiographed. The marker (*M*) is ^{32}P-labeled GD_{1041}, which ran approximately with one of the products of four strand exchange. The second product ran with the starting material, *LD*, or linear duplex DNA substrate.

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exchange reaction if it was added last, after the recA nucleoprotein filament had completely formed on the gapped duplex DNA.

Is the three strand pairing region required for four strand exchange? If so, how long must it be for an efficient reaction? These questions were answered by four strand exchange reactions between substrates diagrammed in Fig. 3*b*. The results of time courses of these reactions are shown in Fig. 6. The length of homology between duplex regions of all substrates was 7.2 kb. One end of DNA#2 was complementary to the ssDNA gap of DNA#1 for 0, 15, 32, and 52 nucleotides for reactions 1–4, respectively. The results of reaction 5 demonstrate that sequence homology between DNA#1 and DNA#2 was critical for these reactions; no sign of pairing or strand exchange products was visible when they were heterologous. The results of reaction 1 show that a very inefficient four strand exchange reaction did occur, even though neither end of DNA#2 was complementary to the ssDNA gap of DNA#1. To our knowledge, this is the first report of recA protein-promoted four strand exchange between DNA substrates homologous only between duplex regions. The product band first appeared at 30 min. After 90 min the reaction had proceeded to approximately 5% of the total possible reaction. This result

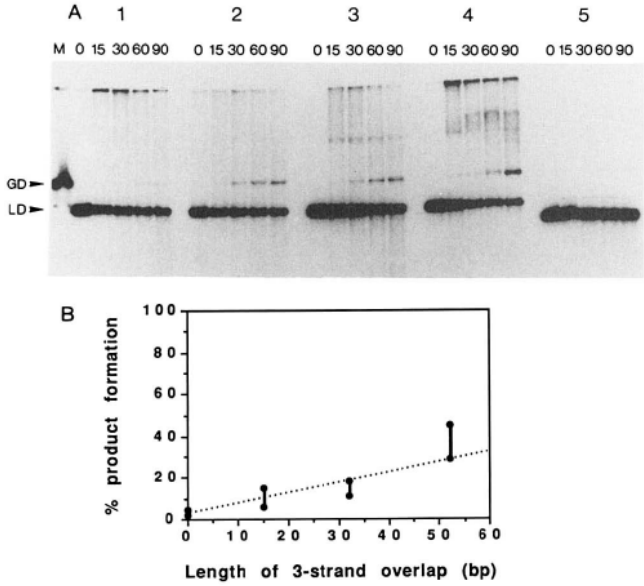


FIG. 6. **The length of three strand pairing required for four strand exchange.** *a*, time courses of four strand exchange reactions between substrates depicted in Fig. 3 are shown. Strand exchange reactions were performed as described under "Materials and Methods," with ^{32}P end-labeled linear duplex DNA and GD_{437}. SSB was included in all reactions at 0.6 μM. The length of potential three strand pairing, between an end of DNA#2 and the single-stranded gap of DNA#1, was 0 bp (*1*), 15 bp (*2*), 32 bp (*3*), and 52 bp (*4*). The linear duplex DNA in *reaction 5* was completely heterologous to GD_{437}. Aliquots of the reactions were removed at the time indicated, stopped by the addition of SDS to a final concentration of 2.5%, subjected to agarose gel electrophoresis, and autoradiographed. The marker (*M*) is ^{32}P-labeled GD_{437}, which runs with one of the products of four strand exchange. The second product runs with the starting material, *LD*, or linear duplex DNA substrate. *b*, the amount of the gapped duplex DNA product band at 90 min was quantitated for each reaction by densitometric scanning. As described in the text, the product band that runs with the gapped duplex DNA marker represents only half of the total product DNA. Therefore, to calculate the percent product formation, the amount of DNA running as gapped duplex was doubled before dividing by the total amount of DNA in the lane. The autoradiograms from two experiments run under identical conditions were scanned, as indicated by the two data points for each three strand overlap length.

has been repeated several times, with several preparations of gapped duplex DNA, all twice purified. Some exchange was detected in all of these experiments, but the result shown is the maximum level of exchange observed.

The reaction efficiency increased when the length of potential three strand homologous pairing was 15 bp (*reaction 2*), and further increased for 32 and 52 bp (*reactions 3* and *4*). The product band appeared more rapidly (by 15 min) and was a larger percentage of the total input DNA as the length of potential three strand pairing increased. Quantitation of the extent of the reactions at 90 min is shown in Fig. 6*b*. From the present data, it is impossible to discern whether the extent of strand exchange increases linearly with an increase in three strand overlap length, or by a higher order function. The limit of this increase was not determined, although reactions in which the overlap is several hundred base pairs typically proceed to 50–70% completion. Extrapolation of the line in Fig. 6*b* suggests that just over 100 bp of overlap is required for an optimal reaction. The results of reactions shown in Figs. 5 and 6 cannot be directly compared because the gapped duplex DNA substrates differed between the two experiments. The complex interaction between recA protein and SSB on the gapped duplex DNA makes the simple comparison of reactions, involving duplex molecules with different ssDNA gap lengths, impossible. We can say, however, that an increase in the length of DNA complementarity between one end of DNA#2 and the single-stranded gap of DNA#1, up to at least 52 bp, increases the efficiency of recA protein-promoted four strand exchange.

DISCUSSION

The recA protein-promoted pairing of three DNA strands is much more efficient than the pairing of four strands. Homologous pairing of a recA protein-duplex DNA complex with a second duplex DNA *in vitro* was detected by underwinding assays only if the second duplex was already negatively supercoiled. Even when DNA#2 was supercoiled, the three strand complexes were formed more efficiently than four strand complexes. The efficiency of four strand exchange reactions increased as the length of potential three strand pairing between the DNA substrates was increased. These results suggest that a three-stranded paranemic joint may be an important intermediate for *all* recA protein-mediated strand exchange reactions. They further suggest that the presence of three strands rather than four may be a fundamental structural parameter of paranemic joints formed by recA protein.

When recA protein homologously aligns two DNA sequences, both DNA substrates are extended and underwound (Williams and Spengler, 1986; Wu *et al.*, 1983; Schutte and Cox, 1988). Since supercoiling or underwinding of duplex DNA is energetically unfavorable, the recA protein-promoted pairing reaction is expected to be facilitated by a supercoiled DNA substrate. Indeed, the initial binding of recA protein to circular duplex DNA occurs more rapidly if the DNA is negatively supercoiled (Pugh and Cox, 1987). The homologous pairing of two duplex molecules was detected by an underwinding assay only if DNA#2 was supercoiled. RecA protein-promoted pairing between single-stranded and homologous duplex DNA was detected regardless of the topological state of the duplex.

The efficiency of four strand exchange increased if DNA#2 had an end that was complementary to the single-stranded gap of DNA#1. A 15-nucleotide three strand pairing region visibly increased the rate and final extent of the exchange reaction. The extent of the reaction continued to increase as

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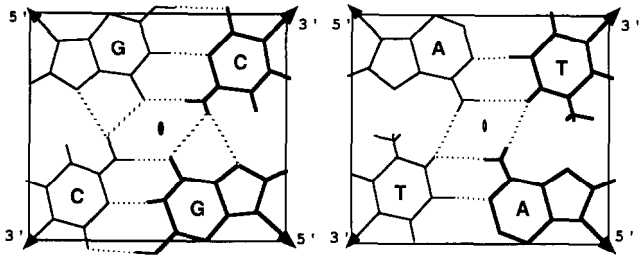


FIG. 7. **Model for four strand homologous alignment proposed by McGavin (1971) and Wilson (1979).**

the three strand pairing region was lengthened to 52 nucleotides. These results suggest that a region of three-stranded pairing greatly enhances the productive pairing of duplex substrates. As judged by recombination *in vivo*, recA protein is able to detect homology between sequences as short as 20 bp in length (Watt *et al.*, 1985). Therefore, it is not surprising that a 15–52 nucleotide region of homologous three-stranded interaction might contribute to the pairing phase of the four strand exchange reaction.

While this manuscript was in preparation, Conley and West (1990; Conley *et al.*, 1989) communicated to us the results of an experiment very similar to that in Fig. 6. Their conclusions are also similar, although the length of overlap required for optimal reaction in their experiment is 37 base pairs, significantly less than we have estimated. This may simply reflect a difference in the assays employed. The assay of Conley and West (1990) measures joint molecule formation and may detect some unproductive complexes that would not appear as products in our assay.

McGavin (1971) and Wilson (1979) proposed that homologous recognition of two duplex DNA substrates could occur by the formation of a tetra-helix (see Fig. 7). The nucleotides within each original duplex would be held together by Watson-Crick base pairs, while the two duplexes would align in their major grooves. Two (for A–T) or four (for G–C) hydrogen bonds would hold the base tetrads together. The specificity of tetrads in this model is derived from the regular rectangular structure formed only when two A–T or two G–C base pairs align (McGavin, 1971). According to this model, the number of hydrogen bonds involved in sequence recognition would be halved for three strand alignment. Therefore, this model suggests that the pairing of four strands should be more stable than the pairing of three strands. The result we obtained for recA protein-promoted homologous pairing *in vitro* is inconsistent with this expectation. The predominance of the three strand interaction may indicate that a three-stranded paranemic joint exists in this system that has no four-stranded counterpart. This does not mean that the four strand interaction is insignificant. Even a weak interaction between two homologous duplexes may facilitate homologous alignment in general and help maintain that alignment once strand exchange has been initiated.

Recent evidence suggests that paranemic joints with some true triple helical character are formed when recA protein-ssDNA complexes pair with homologous duplexes (Cox *et al.*, 1989).[2] DNA triple helices are not unprecedented, but it is our expectation that a triple-helix formed by recA protein will have a novel structure. The most common stable triple helical conformation observed to date in DNA is one involving two homopyrimidine strands and one homopurine strand (Felsenfeld *et al.*, 1957; Wells *et al.*, 1988; Htun and Dahlberg, 1988; Maher *et al.*, 1989). The two pyrimidine (like) strands are aligned antiparallel. Whereas these structures have been cited as possible models for recombination intermediates, they lack the key structural properties that must be present in a paranemic joint formed by recA protein. In the recA system, 1) base pairs (or triplets) are separated by 5.1 Å, 2) like strands must be parallel, and 3) pairing must be independent of sequence. The paranemic joint formed by recA protein is stabilized by bound recA protein and need not conform to any known, stable DNA conformation.

UV-induced single-stranded DNA gaps increase homologous recombination *in vivo* (Rupp *et al.*, 1971). Single-stranded DNA is also present in *E. coli* during conjugation and DNA repair at replication forks. The recBCD enzyme and the protein products of other *rec* genes may act to produce ssDNA. Regions of ssDNA *in vivo* may have two roles in recA protein-promoted DNA recombination and repair: 1) providing sites of increased affinity for recA protein as reported previously (West *et al.*, 1980; Shaner *et al.*, 1987; Lindsley and Cox, 1989), and 2) increasing the strength of homologous pairing interactions by providing regions of potential three-stranded DNA alignment.

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Note Added in Proof—Experiments related to those described here are published in the accompanying article (Conley and West, 1990).

[2] S. W. Umlauf, M. M. Cox, and R. B. Inman, unpublished results.

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