ACKNOWLEDGMENTS

We thank Susan Kelly for the artwork and Marjorie Needham for preparation of the manuscript.

Registry No. DNS-DODA, 116234-53-8; DNS-Cl, 605-65-2; DODA, 2504-85-0; C_8-LAP, 101554-72-7; C_{12}-LAP, 101554-73-8; C_{16}-LAP, 101554-74-9.

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Homology-Dependent Underwinding of Duplex DNA in RecA Protein Generated Paranemic Complexes†

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Received March 14, 1988; Revised Manuscript Received June 15, 1988

ABSTRACT: RecA protein promoted formation of paranemic joints, in which a recA–ssDNA complex and a nicked circular dsDNA molecule are homologously aligned without net cross-strand interwinding, is accompanied by extensive underwinding of the dsDNA molecule. When the nick is sealed by DNA ligase, a highly negatively superhelical DNA molecule is formed. This underwinding has the following properties: (a) it occurs within 2 min; (b) it is completely homology dependent; (c) it does not require a homologous free DNA end. The resulting underwound DNA species is comprised of a heterogeneous population of topoisomers. The degree of unwinding exhibits a strong dependence on the fractional length of homology in the dsDNA molecule and indicates that paranemic joints can extend for at least 2900 base pairs. The rapid underwinding associated with paranemic joint formation is followed by a longer phase in which the dsDNA molecule is more extensively underwound.

The recA protein of *Escherichia coli* promotes homologous genetic recombination in vivo and in vitro [reviewed in Dressler and Potter (1982) and Cox and Lehman (1987)]. An early step in homologous genetic recombination is the homologous synapsis (pairing) of two DNA molecules (Holliday, 1964; Meselson & Radding, 1975). In vitro, a prerequisite step to pairing is the binding of recA protein to DNA (Shibata et al., 1979a; Cox & Lehman, 1981). RecA protein stoichiometrically binds (one recA monomer per four nucleotides) to single-stranded DNA (ssDNA)[1] to form a filamentous nu-

† This work was supported by National Institutes of Health Grant GM 32335. B.C.S. was supported by Training Grant GM 07215 from the National Institutes of Health. M.M.C. is supported by National Institutes of Health Research Career Development Award AI 00599.

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0006-2960/88/0427-7886$01.50/0 © 1988 American Chemical Society

cleoprotein complex (Dunn et al., 1982; Flory et al., 1984). In the presence of ATP, this complex binds dsDNA and pairs with homologous sequences (Shibata et al., 1979b; McEntee et al., 1979; Cox & Lehman, 1981; DasGupta & Radding, 1982).

As first described by Radding and co-workers (DasGupta et al., 1980; Cunningham et al., 1981; Bianchi et al., 1983), homologous pairing of two DNA molecules during the synapsis phase occurs with at least two distinct intermediates. The first, in which the homologous sequences of the two DNA molecules are aligned without net cross-strand interwinding, has been termed a paranemic joint. This intermediate is unstable when recA protein is removed by heat (DasGupta et al., 1980; Bianchi et al., 1983) or by protein denaturants (Riddles & Lehman, 1985). Formation of a paranemic joint can be demonstrated by the pairing of a recA–ssDNA complex with a dsDNA molecule that lacks a free end in the region of homology (DasGupta et al., 1980; Bianchi et al., 1983; Riddles & Lehman, 1985). Kinetic evidence (Riddles & Lehman, 1985) suggests that the paranemic joint is an intermediate in the formation of the second intermediate, a plectonemic joint. In a plectonemic joint, the complementary strands of the two paired DNA molecules are interwound. Unlike the paranemic joint, the plectonemic joint is resistant to protein denaturants (Riddles & Lehman, 1985) and has a strict requirement for a free end in the region of homology. If the DNA molecules lack this free end, net interwinding of the complementary strands is topologically prohibited, and plectonemic joint formation is blocked.

Little is known about the structure of the paranemic joint. We note three proposed models in the literature (Howard-Flanders et al., 1984; Christiansen & Griffith, 1986; Schutte & Cox, 1987). Until recently, published descriptions of these intermediates suggested that their size was limited to a few hundred base pairs (Bianchi et al., 1983; Christiansen & Griffith, 1986). Recently, we reported (Schutte & Cox, 1987) indirect biochemical evidence for the formation of long paranemic joints, exceeding several thousand base pairs. These results are supported in part by the direct observation of paranemic joints by electron microscopy that exceeded 1000 bp (Register et al., 1987). Stasiak et al. (1981) observed that in the presence of ATPγS the dsDNA molecule within the recA–DNA nucleoprotein filament is extended by as much as 50% relative to B-form DNA. Under the same conditions, similar extended complexes were observed when recA protein was bound to ssDNA (Koller et al., 1983). In the case of dsDNA, it has been demonstrated that this corresponds to an underwinding or reduction in helical twist of approximately 43% (Stasiak & Di Capua, 1982). Recently, we have shown that under conditions optimal for strand exchange (i.e., in the presence of ATP) recA protein underwinds dsDNA by 39.5% (B. F. Pugh and B. C. Schutte, unpublished results). With ATP or ATPγS, there is very little variation in the degree of underwinding observed in DNA from different recA nucleoprotein filaments, indicating a high degree of structural homogeneity (B. F. Pugh and B. C. Schutte, unpublished results). The ultrastructure of the recA nucleoprotein filament is not detectably altered by the pairing of the incoming dsDNA molecule, as shown by direct visualization of a paranemic joint by electron microscopy (Christiansen & Griffith, 1986; Register et al., 1987).

These observations suggest that the incoming homologous dsDNA molecule is underwound when it is paranemically paired to the recA–ssDNA complex. The rationale for this hypothesis is that since the DNA molecule in the recA–ssDNA complex is extended, the regions of the incoming dsDNA molecule paranemically paired with it must be equally extended. Extension of the incoming dsDNA molecule would require underwinding over the paired region. Evidence for underwinding of the dsDNA molecule paranemically paired to the recA–ssDNA complex is 2-fold. First, Wu et al. (1983) directly observed underwinding of the dsDNA molecule that was clearly related to the formation of a paranemic joint. However, the time course for this underwinding was slower than expected for an event associated with the synapsis phase of DNA strand exchange (Cox et al., 1983; Kahn & Radding, 1984; Riddles & Lehman, 1985). Second, we recently reported (Schutte & Cox, 1987) indirect evidence that suggests the dsDNA molecule is underwound upon formation of paranemic joints. This conclusion was based on the properties of a homology-dependent drop in ATP hydrolysis by the recA–ssDNA complex when paranemic joints are formed. Only a partial drop in the rate of ATP hydrolysis was observed when a F(I) DNA substrate (in which unwinding is topologically restricted) was paired with the recA–ssDNA complex as compared to the effect produced by the F(III) DNA substrate. This effect was kinetically competent to be an event associated with the synapsis phase. In this paper we extend these observations by providing direct evidence that the dsDNA molecule bound to the recA–ssDNA complex in a paranemic joint is underwound. In addition, we have measured the extent of this underwinding and used this information to estimate the length of these paranemic joints.

Materials and Methods

Enzymes and Biochemicals. *E. coli* recA protein was purified by a previously described method (Cox et al., 1981). The purified protein was stored frozen at −70 °C. The concentration of the recA protein stock solution was obtained by the absorbance at 280 nm, with an extinction coefficient of $\epsilon_{280} = 0.59A_{280}$ mg^{-1} mL (Craig & Roberts, 1981). *E. coli* SSB protein was purified as described (Lohman et al., 1986) and stored frozen at −70 °C. The concentration of the SSB stock solution was determined by the absorbance at 280 nm, with an extinction coefficient of $\epsilon_{280} = 1.5A_{280}$ mg^{-1} mL (Lohman & Overman, 1985). T4 DNA ligase was purified by a published procedure (Davis et al., 1980). The concentration of the T4 DNA ligase stock solution was obtained by Bradford analysis (Bradford, 1976). DNA topoisomerase I was purchased from Bethesda Research Laboratories. Restriction enzymes were purchased from New England Biolabs or International Biotechnologies. The concentration of the ethidium bromide stock solution was determined by the absorbance at 480 nm, with an extinction coefficient of $\epsilon_{480} = 5600$ M^{-1} cm^{-1} (Waring, 1965). All other enzymes and biochemicals were purchased from Sigma.

[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; M13mp8(+) and M13mp8.1100(+), circular single-stranded genomes of bacteriophages M13mp8 and M13mp8.1100, respectively; Tris, tris(hydroxymethyl)aminomethane; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); EDTA, ethylenediaminetetraacetic acid; bp, base pair(s); SSB, *Escherichia coli* single-stranded DNA binding protein; F(I), negatively supercoiled closed circular form of a DNA molecule as isolated from *E. coli* cells; F(II), nicked circular form of the same DNA molecule; F(III), linear form of the same DNA molecule; F(IV), closed circular but relaxed form of the same DNA molecule; F(X), closed circular form of the same DNA molecule but underwound by 39.5%; F(P), closed circular form of a DNA molecule that has been underwound as a result of paranemic pairing of the nicked circular form of the same DNA molecule with a recA nucleoprotein filament and subsequent ligation of the nick [the degree of underwinding of F(P) DNA depends on the extent of homology with the DNA within the nucleoprotein filament as described in the text].

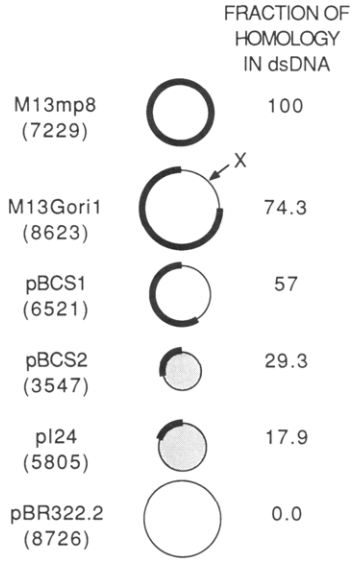


FIGURE 1: Duplex DNA substrates. Maps of duplex DNA molecules derived from bacteriophages M13mp8 and M13Goril and the plasmids pBCS1, pBCS2, pI24, and pBR322-2. X denotes the unique restriction endonuclease site *Xho*1. The total length of each molecule in base pairs is shown in parentheses. For duplex DNA molecules M13mp8, M13Goril, pBCS1, and pBR322-2 (open circles), thick lines represent regions of M13mp8 homology, while thin lines represent regions of heterology relative to M13mp8. M13mp85(+) is the ssDNA partner used to pair with these four dsDNA molecules in these experiments. For the molecules pBCS2 and pI24 (shaded circles), thick lines represent regions of M13mp8.1100 homology, and thin lines represent regions of heterology relative to M13mp8.1100. M13mp8.1100(+) is the ssDNA partner used to pair with these two dsDNA molecules. Fraction of homology in dsDNA refers to the fraction of the total length of the dsDNA molecule illustrated that is homologous with its ssDNA partner.

DNA. The DNA molecules from which the duplex DNA substrates were derived are shown in Figure 1. Single-stranded DNA derived from the bacteriophages M13mp8 and M13mp8.1100 were prepared by a published procedure (Messing, 1983). The bacteriophage M13mp8.1100 is the bacteriophage M13mp8 with a 1041-bp sequence (*Eco*RV fragment) from the *E. coli* galT gene inserted into the polylinker. F(I) DNA derived from the bacteriophages M13Goril (Kaguni & Ray, 1979) and M13mp8, as well as plasmid DNA, was prepared as described (Neuendorf & Cox, 1986; Messing, 1983; Davis et al., 1980). Stock concentrations of ssDNA and dsDNA were determined by absorbance at 260 nm, with 36 and 50 μg mL^{-1} A_{260}^{-1} conversion factors, respectively. DNA concentrations are reported as total nucleotides or as total molecules, as noted. Calculations of the degree of underwinding of different circular duplex topoisomers assume a helical periodicity of 10.5 bp per turn for B-form DNA.

The plasmid pBR322-2 is a dimer of the plasmid pBR322 (Bolivar et al., 1977) and was used as a heterologous substrate. The plasmid pI24 is the plasmid pBR322 with a 3456-bp region of the *E. coli* gal operon (including the 1041-bp galT sequence) replacing the 2014-bp *Eco*RI–*Pvu*II fragment of pBR322. The plasmid pBCS2 was constructed from the plasmid pBR322 by replacing the 1833-bp *Eco*RV–*Pvu*II fragment of pBR322 with the 1041-bp *Eco*RV fragment of pI24. Insertion of the 1041-bp *Eco*RV fragment was mediated by linker ligation (Lathe et al., 1984) with 12-bp *Sal*I linkers purchased from Bethesda Research Laboratories. Thus, the 1041-bp galT sequence contained in the plasmids pI24 and pBCS2 represents the only region of homology between these dsDNA molecules and the ssDNA derived from the bacteriophage M13mp8.1100. The plasmid pBCS1 was constructed from the plasmid pXF3 (Maniatis et al., 1982) by replacing the 307-bp *Bam*HI–*Cla*I fragment of pXF3 with the 3714-bp *Bam*HI–*Cla*I fragment from the bacteriophage M13mp8.

F(III) DNA was produced by cleaving bacteriophage F(I) DNA with an appropriate restriction endonuclease. F(II) was prepared from plasmid or bacteriophage DNA by two methods. For most experiments, F(II) DNA was produced by randomly nicking F(I) DNA with DNase I in the presence of ethidium bromide (Shibata et al., 1981). Greater than 99% of the DNA was present as nicked circles as determined from scanning densitometry of photographic negatives of DNA samples electrophesed in agarose gels and stained with ethidium bromide. This method introduces a single nick in the DNA molecule (Shibata et al., 1981). In the second method, F(I) DNA derived from the bacteriophage M13Goril was nicked specifically with the restriction endonuclease *Xho*I in the presence of ethidium bromide (DasGupta et al., 1981). This method produces 70–80% F(II) DNA and 20–30% F(III) DNA. F(IV) DNA was produced by ligation of F(II) DNA. Approximately 70% of the nicked molecules from any given preparation of F(II) DNA were ligated. F(II) DNA ligation was not detectably enhanced by longer incubation (data not shown).

F(X) DNA is a highly underwound form of covalently closed DNA originally described by Shibata et al. (1981). This DNA is underwound by 39.5% relative to B-form DNA (B. F. Pugh and B. C. Schutte, unpublished results) and migrates faster than supercoiled F(I) DNA in an agarose gel when electrophoresis is performed in the absence of ethidium bromide. F(X) DNA was prepared as described previously (Pugh & Cox, 1987) from M13Goril F(II) DNA that was nicked with the restriction enzyme *Xho*I in the presence of ethidium bromide. Forms of covalently closed DNA having intermediate levels of underwinding were prepared by incubating plasmid or bacteriophage F(I) DNA (100 μM) with 10 units of topoisomerase I and the indicated concentration of ethidium bromide in the buffer prescribed by the manufacturer. Reactions were carried out for 60 min at 37 °C in the dark. The reaction mixtures were then extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) and transferred to Eppendorf tubes containing gel loading buffer (described below).

Purified F(P) DNA derived from bacteriophage and plasmid DNA molecules was produced by a modification of the DNA unwinding assay (described below). The reaction volume was increased to 3 mL, and the concentrations of recA protein, SSB, ssDNA, and the F(II) DNA substrate were increased 1.5-fold for reactions containing either M13Goril, M13mp8, or pBCS1 F(II) DNA and 3-fold for reactions containing either pI24 or pBCS2 F(II) DNA. This increase in concentration had no effect on the extent of underwinding of the F(P) DNA (data not shown). T4 DNA ligase (0.1 mg/mL final) was added 3 min after initiation of the pairing reaction. After incubation for 1 min longer, cesium chloride and ethidium bromide were added. F(P) DNA was then purified by banding in a cesium chloride–ethidium bromide density gradient.

Reaction Conditions. All recA protein reactions were carried out at 37 °C in standard reaction buffer containing 25 mM Tris–acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 1 mM dithiothreitol, and 5% glycerol. Unless otherwise stated, reaction mixtures contained 2 μM recA protein, 0.8 μM SSB, 8 μM ssDNA (nucleotides), 1 mM ATP, an ATP regenerating system (13.5 units/mL pyruvate kinase, 2.31 mM phosphoenolpyruvate, 0.44 mM KCl), and a concentration of

F(II) dsDNA molecules that was equimolar relative to the concentration of ssDNA molecules. In reactions containing pBR322-2, pBCS1, M13Goril, and M13mp8 dsDNA, M13mp8(+) ssDNA was used. In reactions containing pBCS2 and pI24 dsDNA, M13mp8.1100(+) ssDNA was used. In all experiments recA protein, the ATP regenerating system, and DNA substrates were preincubated in reaction buffer for 10 min at 37 °C. Reactions were then initiated by the addition of a mixture of ATP and SSB.

DNA Unwinding Assay. This assay measures underwinding of the dsDNA substrate that results from the paranemic pairing of the dsDNA molecule with the recA–ssDNA complex. Aliquots (25 μL) were taken from a 0.25-mL reaction mixture at the indicated times and incubated with 1 μL of T4 DNA ligase (0.1 mg/mL final) for 1 min at 37 °C. The reaction was stopped by the addition of gel loading buffer containing 12 mM EDTA, 1% sodium dodecyl sulfate, 0.05% bromophenol blue, and 5% glycerol. Samples were then loaded onto 0.8% or 1.2% agarose gels. Electrophoresis was performed in one of the following buffers: (1) 40 mM Tris–acetate (pH 7.5), 1 mM EDTA, and the indicated concentration of ethidium bromide; (2) 50 mM Tris–phosphate (pH 7.2), 1 mM EDTA, and the indicated concentration of chloroquine. In all cases, ethidium bromide and chloroquine were added to both the gel and running buffer, and the buffer was circulated at a rate of 3 mL min^{-1}. Gels containing ethidium bromide were electrophoresed in the dark. Gels were stained in 0.4 μg/mL ethidium bromide for 30 min and then exposed to UV radiation to photolytically cleave covalently closed DNA. They were then restained in 0.1 μg/mL ethidium bromide for several hours. The restaining was extended to an overnight incubation for gels electrophoresed in the presence of chloroquine. Bands of DNA stained with ethidium bromide were illuminated with UV radiation and photographed. The photographic negatives were scanned by a laser densitometer. Band intensities were quantitated by integrating the area under the peaks from the densitometer tracings. The film response was tested by carrying out electrophoresis on different dilutions of a DNA sample. A film exposure was used such that the area under the peak in the densitometer trace was linearly related to the dilution.

Instrumentation. Absorbance measurements were carried out on a Perkin-Elmer Lambda 7 double-beam spectrophotometer equipped with two six-cell thermojacketed cuvette holders. Constant temperature during absorbance measurements was maintained by an external circulating water bath connected to the cuvette holders. Cell path length was equal to 1 cm when DNA and protein stock concentrations were being determined and 0.5 cm during ATPase assays. The band-pass was equal to 2 nm at all times. The densitometric scans were performed on an Zeineh soft laser scanning densitometer, SL-504-XL, from Biomed Instruments, Inc.

ATPase Assays. Hydrolysis of ATP was monitored by a coupled spectrophotometric assay (Morrical et al., 1986). Lactate dehydrogenase (13.5 units/mL) and 1.0 mM NADH were added to the ATP regenerating system to complete the coupling system. The conversion of NADH to NAD was then followed spectrophotometricaly at 340 nm. Rates of ATP hydrolysis, expressed as μM min^{-1}, were calculated from $-\Delta A_{340}$ per minute data with an extinction coefficient of ϵ_{340} = 6.23 mM^{-1} cm^{-1} for NADH. Error from all sources was generally less than 5% in experiments carried out on different days. Increasing the concentration of any of the coupling system components in no case affected the observed rate of ATP hydrolysis, so that data reflect the maximum velocity of ATP hydrolysis under all conditions used in this study. A short lag (less than 2 min) due to the coupling system is always observed in this assay before steady-state conversion of NADH to NAD is obtained. Reaction volumes of 0.4 mL were contained in 0.5-mL cuvettes having a 0.5-cm cell path length.

RESULTS

The dsDNA Substrate Is Underwound during the Formation of a Paranemic Joint. Our general approach, to look directly for underwinding of the dsDNA substrate during strand exchange, was similar to that employed by Wu et al. (1983). For these experiments, a circular ssDNA and a circular dsDNA molecule, nicked specifically within a heterologous region, were allowed to pair. The topological state of the dsDNA molecule paired to the recA–ssDNA complex was then trapped by sealing the nick with DNA ligase. Since these DNA substrates lack a free end in the region of homology, pairing occurs exclusively through a paranemic joint. Thus, when recA protein is removed after the nick has been sealed, the paired DNA molecules separate, and the topology of the dsDNA molecule is maintained in the form of supercoils. This underwound dsDNA molecule can be resolved from F(IV) DNA by agarose gel electrophoresis.

We note two important differences in the present study from the study by Wu et al. (1983). The first is the presence of SSB. In the presence of SSB the time required for the synapsis phase of DNA strand exchange is significantly reduced (Cox et al., 1983; Kahn & Radding, 1984; Muniyappa et al., 1984). The second difference is the use of only stoichiometric amounts of recA protein in all experiments. Shibata et al. (1982) have demonstrated that homologous ssDNA stimulates the direct binding of free recA protein to dsDNA. This "loading" of recA protein would interfere with this study, since direct binding of recA protein also extends (underwinds) dsDNA (Stasiak et al., 1981; Pugh & Cox, 1987). Therefore, all experiments were carried out in the presence of only stoichiometric amounts of recA protein relative to ssDNA. Under these conditions, direct binding of recA protein to dsDNA is minimized (Schutte & Cox, 1987).

The time course of underwinding in a typical experiment is shown in Figure 2. In panel A, the DNA substrates employed are M13mp8(+) ssDNA and M13Goril F(II) DNA, which contains a 6407-bp region of homology with M13mp8 and a 2216-bp region of heterology (Figure 1). For this experiment, M13Goril F(II) DNA was produced by nicking the DNA in the heterologous region, thereby preventing net interwinding of the paired DNA substrates. For comparison, DNA markers (M), with varying topology are included in the gels, as described in the legend of Figure 2. About 20–30% of the total dsDNA present in all lanes is linear F(III). This is a byproduct of the method of nicking used for these experiments. These molecules are not circularized at an appreciable rate under the ligation conditions used in this experiment. The time course for underwinding of the dsDNA substrate during binding to the recA nucleoprotein complex is shown in lanes a–m. As early as 1 min after initiation of DNA strand exchange, a fast-migrating DNA species appears that migrates near the F(I) DNA marker. In less than 2 min, all ligatable dsDNA molecules (approximately 70%) have been converted to this fast-migrating species. The time course for this underwinding is similar to the time necessary to complete the synapsis (pairing) phase of DNA strand exchange in the presence of SSB (Cox et al., 1983; Kahn & Radding, 1984; Riddles & Lehman, 1985). The underwinding is completely homology dependent (see below), and the pairing is topologically restricted to paranemic joints. This is consistent with

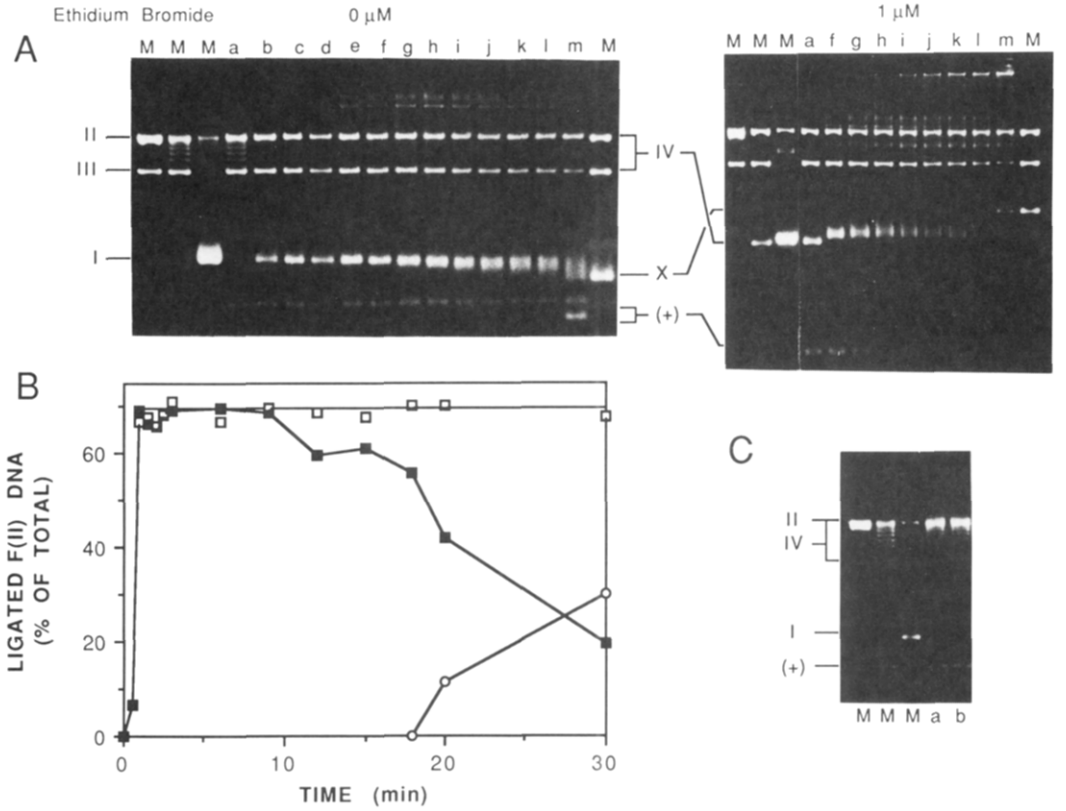


FIGURE 2: Underwinding of dsDNA resulting from paranemic pairing with the recA nucleoprotein complex. Reactions and DNA unwinding assays were performed as described under Materials and Methods. (A) Reaction mixtures contained 8 μM (nucleotide) M13mp8(+) ssDNA and an equimolar concentration of M13Goril F(II) DNA molecules (19 μM nucleotide) that had been nicked at a specific site. The two gels in (A) contain the indicated concentrations of ethidium bromide. DNA markers (M) from left to right are F(II) DNA, F(IV) DNA, F(I) DNA, and F(X) DNA. Migration of M13mp8(+) ssDNA is indicated by (+). As previously observed (Schutte & Cox, 1987), the ssDNA in some lanes migrates as a doublet, even though greater than 90% of these molecules are circular (data not shown). Lanes in each gel correspond to the following time points at which DNA ligase was added: (a) 0.5, (b) 1, (c) 1.5, (d) 2, (e) 2.5, (f) 3, (g) 6, (h) 9, (i) 12, (j) 15, (k) 18, (l) 20, and (m) 30 min. Since incubation was continued for 1 min after ligase was added, these time points actually represent a 1-min range greater than the indicated time. All lanes contain a small amount of F(III) DNA resulting from the method by which the DNA was nicked. (B) Band intensities in the agarose gels in (A) were quantitated as described under Materials and Methods. The fraction of the total starting F(II) DNA substrate that was ligated (□) was calculated for each time point from the gel run in the absence of ethidium bromide. The F(III) DNA band intensity was excluded from these calculations, since under these conditions covalent closure of these molecules was not detected (data not shown). The fraction of F(II) DNA converted to F(P) DNA (■) and F(X) DNA (○) was calculated as above except the intensities of their respective bands were obtained from the gel run in the presence of 1 μM ethidium bromide. (C) Gel unwinding assay for the heterologous control reaction. The reaction mixture contained 8 μM M13mp8(+) ssDNA and an equimolar concentration of molecules of pBR322-2 F(II) DNA (19.3 μM). Marker lanes (M), defined above, are from left to right F(II) DNA, F(IV) DNA, and F(I) DNA. Migration of these markers in the agarose gel run in the absence of ethidium bromide are indicated by II, IV, and I, respectively. Migration of M13mp8(+) ssDNA is indicated by (+). Lanes a and b are the time points 3 and 20 min, respectively.

the hypothesis that the dsDNA substrate bound to the recA–ssDNA complex is underwound during the formation of a paranemic joint. For purposes of this paper, we designate this underwound form of covalently closed DNA resulting from ligation of the dsDNA molecule immediately after pairing as F(P) DNA (for *P*aranemic).

At later times, the ligated dsDNA products migrate progressively faster, indicating a gradual increase in underwinding until the underwound DNA comigrates with the F(X) marker. This behavior is better demonstrated in the agarose gel run in the presence of 1 μM ethidium bromide. At this concentration, enough ethidium bromide is present to titrate out all negative supercoils and add positive supercoils to the F(I) DNA marker. However, some but not all of the negative supercoils have been titrated out of the more highly underwound F(X) DNA marker, and thus its migration is only retarded slightly [to titrate out all negative supercoils in F(X) DNA requires in excess of 10 μM ethidium bromide in an agarose gel (Pugh & Cox, 1987)]. In this gel system, the ligated dsDNA products are distinctly split into two groups. The F(P) DNA band [migrating near the F(I) DNA marker] fades with time after a peak at 2–5 min, and a new DNA band corresponding to a more highly underwound dsDNA species appears at 20 min that comigrates with the F(X) DNA marker. The F(P) DNA band fades somewhat before F(X) DNA appears. As the F(P) DNA band disappears, a faint smear of DNA appears representing intermediate topoisomerase between F(P) and F(X) DNA. This intermediate DNA suggests a slow, progressive increase in the superhelical density of F(P) DNA to the F(X) state. In panel B the band intensities for the ligated DNA species observed in panel A were quantitated. In all experiments, approximately 70% of the total starting F(II) DNA substrate was ligated. At 1.5 min all of the ligated DNA substrate migrates as F(P) DNA. The F(X) DNA product does not appear until 20 min.

In Figure 2, the F(III) DNA band in the gel containing 1 μM ethidium bromide appears to fade with a time course similar to the appearance of a DNA band at the well of the gel. We do not know the nature of this DNA species or of the reaction that produced it. It is not pertinent to the present study, since it occurs on a much longer time scale than is required for the formation of F(P) DNA. Several faint, slowly migrating bands are also observed in both panels and represent various topoisomers of the dimeric plasmid that is a minor

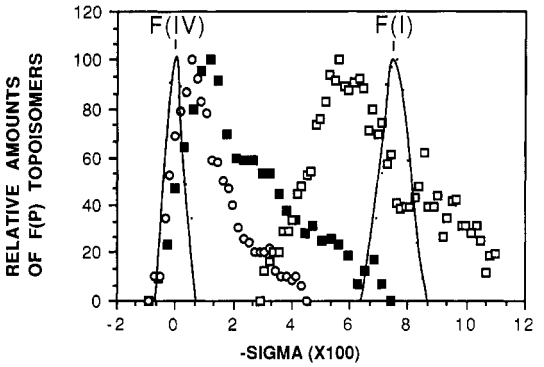


FIGURE 3: Comparison of the distribution of topoisomers comprising F(IV), F(I), and F(P) DNA. Data for this figure were obtained as described in the text. Representative distributions of F(IV) and F(I) DNA were taken from agarose gels containing pI24 dsDNA (data not shown). The distributions of topoisomers comprising F(P) DNA are shown for the following molecules: pI24 (○); pBCS2 (■); pBCS1 (□).

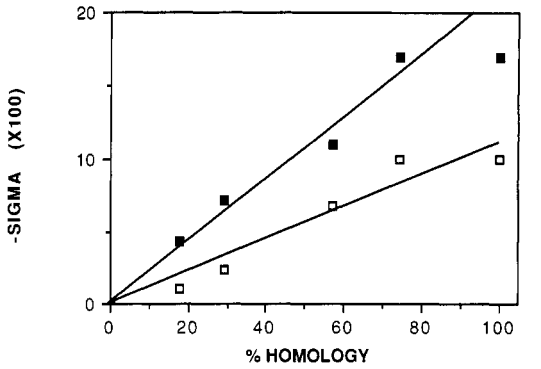


FIGURE 4: Relationship between extent of underwinding of the dsDNA molecule and the fraction of homology. Values for the maximum (■) and average (□) superhelical density, σ, were obtained as described in the text. F(P) DNA molecules and their present homology are as follows: pBR322-2 (0%); pI24 (17.9%); pBCS2 (29.3%); pBCS1 (57%); M13Goril (74.3%); M13mp8 (100%). Percent homology refers to the fraction of the total length of the dsDNA that was homologous with its ssDNA partner.

contaminant in all of these DNA preparations.

Panel C shows that under identical conditions the completely heterologous dsDNA substrate does not become underwound. This agarose gel was electrophoresed in the absence of ethidium bromide. In this reaction, heterologous pBR322–2 F(II) DNA was substituted for the chimeric M13Goril F(II) DNA. The efficiency of ligation of this DNA substrate was about 80% (data not shown). At both the 3- and 20-min time points the ligated dsDNA substrate comigates with the F(IV) DNA marker, demonstrating that no underwinding of this heterologous substrate has occurred. This shows that formation of F(P) DNA is homology dependent.

The Degree of Underwinding of F(P) DNA Is Strongly Dependent on the Fractional Length of Homology of the Duplex DNA Substrate. The degree of underwinding of F(P) DNA produced from six pairing reactions was measured by either of two methods as described in the supplementary material (see paragraph at end of paper regarding supplementary material). The fraction of homology in these six pairing reactions ranged from 0 to 100% (Figure 1). F(P) DNA was purified in order to eliminate interference on agarose gels from DNA bands corresponding to the ssDNA and unligated dsDNA molecules. The F(II) DNA substrates used to prepare F(P) DNA were produced by introducing random nicks in the DNA. Since some nicks were introduced in homologous regions, some potential existed for the formation of plectonemic (interwound) joints. However, in control unwinding assays carried out with these substrates, we detected no change in the intensity of the ssDNA band or aberrantly slow migration of any of the covalently closed dsDNA species under these conditions, as would be expected from the interwinding of the paired molecules (data not shown). Thus, we infer that strand transfer, which proceeds efficiently with homologous F(III) DNA under these conditions, is minimal here with the F(II) DNA substrates. The efficiency of the ligation reaction (about 70%, data not shown) used to produce F(P) DNA was constant for all substrates, indicating that nicks in homologous regions were ligated about as well as those in heterologous regions.

In Figure 3 the distribution of topoisomers of F(IV) and F(I) DNA are plotted along with the distributions of topoisomers for F(P) DNA produced from pairing reactions containing pI24, pBCS2, and pBCS1 dsDNA. The superhelical density of the topoisomers in the distributions was determined by the counting method (Keller, 1975). Each point corresponds to the amount of each topoisomer relative to the most abundant topoisomer in each distribution.

This plot provides two important results. The most striking result is the broad distribution of topoisomers comprising each F(P) DNA product. As previously observed (Depew & Wang, 1975), F(IV) DNA is composed of a small number of topoisomers resulting from thermal fluctuations within the DNA helix at the time of ring closure. As also shown previously (Shure et al., 1977), the distribution of F(I) DNA is somewhat wider. However, both these distributions are relatively narrow compared to the F(P) DNA distributions. The second result is that as the fraction of homologous sequences in each dsDNA substrate increases the distribution of topoisomers comprising F(P) DNA shifts to higher negative superhelical densities.

In Figure 4 we have plotted both the average and maximum superhelical densities versus the fraction of the total length of the dsDNA molecule that is homologous to the ssDNA molecule with which it was paired. Values for the superhelical densities of pI24, pBCS2, and pBCS1 F(P) DNA were obtained from Figure 3. Values for the superhelical densities of M13Goril and M13mp8 F(P) DNA were determined from the critical free dye concentration (Espejo & Lebowitz, 1976) of the least and most underwound species comprising these DNA samples. In all cases, the maximum superhelical density was obtained as the most underwound topoisomer observed. For pI24, pBCS2, and pBCS1 F(P) DNA, the most abundant topoisomer represents the average superhelical density in each distribution. Since individual topoisomers comprising M13Goril and M13mp8 F(P) DNA were not resolved, the average superhelical density for these distributions was calculated as the average of the least and most underwound topoisomers. The values of both the average and maximum superhelical densities for the completely heterologous substrate pBR322-2 were plotted as equal to zero, since ligation of this F(II) DNA substrate [under the same conditions used to generate F(P) DNA] resulted in a product that comigrated with relaxed F(IV) DNA (Figure 2).

An approximately linear relationship is observed between both the average and maximum superhelical densities and the fraction of homologous sequences in the dsDNA substrate (Figure 4). This strongly suggests that the underwinding is mediated only by the homologous regions of the dsDNA DNA. If underwinding also invovled an interaction with the heterologous regions, then all dsDNA substrates that contain sufficient homology to synapse with the recA–ssDNA complex would be expected to be underwound to the same extent. The data were fit by linear regression, and the slope of the lines

indicate that over the region of homology the dsDNA molecule that is bound to the recA–ssDNA complex in these paranemic joints is underwound by 12% (average) or up to 18% (maximum) relative to relaxed dsDNA.

The observed degree of underwinding can be used to estimate the length of paranemic joints. This involves a comparison with the extent of underwinding observed for DNA in recA nucleoprotein complexes. Direct binding of recA protein to dsDNA or ssDNA in the presence of ATPγS results in an extension of the DNA, corresponding to underwinding of dsDNA by approximately 43% relative to B-form DNA (Stasiak et al., 1981; Stasiak & DiCapua, 1982; Koller et al., 1983). Under conditions employed here (with ATP instead of ATPγS), recA protein bound directly to dsDNA underwinds it by 39.5% (B. F. Pugh and B. C. Schutte, unpublished results). RecA protein complexes are extended to a similar degree when bound to ssDNA (Koller et al., 1983), and we assume that the ssDNA is held in a configuration equivalent to one of the two DNA strands in the recA–dsDNA complex. We therefore expect that the dsDNA molecule would have to be underwound by 39.5% if this DNA were in simultaneous homologous alignment throughout its length with the ssDNA in the recA nucleoprotein filament. The degree of underwinding of the incoming duplex observed here suggests that an average of 30% (0.12/0.395) and a maximum of 45% of the homologous sequences in the dsDNA molecule are in homologous register at any given moment in these paranemic complexes. For the longest homologous region tested, this corresponds to paranemic joints with average and maximum lengths of 1900 and 2900 bp, respectively.

The extent of underwinding observed for the M13mp8 dsDNA substrate (100% homology) appears to be somewhat less than expected given the linear relationship established by the other dsDNA substrates. We do not know whether this is attributable to a topological barrier to paranemic joint formation, to a fundamental limit in paranemic joint length, or to the somewhat decreased sensitivity of the method used to estimate the degree of underwinding of this DNA.

The Extent of the Homology-Dependent Decrease in ATP Hydrolysis Is Directly Related to the Length of Homology in the F(II) DNA Substrate. In addition to DNA underwinding, paranemic joint formation is also manifested by a homology-dependent drop in the rate of ATP hydrolysis by the recA–ssDNA complex (Schutte & Cox, 1987). When a constant concentration of recA–ssDNA complexes was titrated with an increasing concentration of a homologous F(III) DNA substrate, the full drop in ATP hydrolysis was achieved at a stoichiometry of one dsDNA molecule for each recA–ssDNA complex. The extent of the drop in ATP hydrolysis for each linear dsDNA substrate tested was linearly dependent on the length of homology in the dsDNA substrate. The only F(II) DNA substrate tested in the previous study was derived from the completely homologous molecule. Although the extent of the drop in ATP hydrolysis with this dsDNA substrate was equivalent to that of the linear form of the same DNA molecule, an excess of the F(II) DNA substrate was necessary to obtain the full drop in ATP hydrolysis. To better relate the underwinding data to previous results, a constant concentration of recA–ssDNA complexes was titrated with each of the F(II) DNA substrates used in the present study, and rates of ATP hydrolysis were monitored.

Two representative titration curves are shown in Figure 5A. In all ATPase assays the steady-state rate of ATP hydrolysis was achieved within 2 min after the initiation of strand exchange. This corresponds well with the time course for the

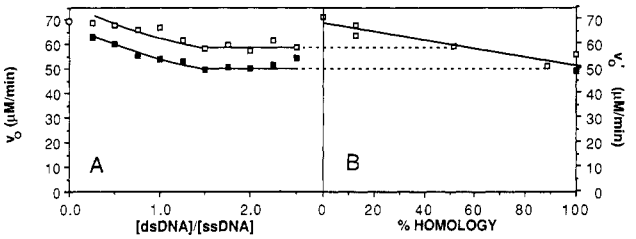


FIGURE 5: Relationship between the homology-dependent decrease in ATP hydrolysis and the fraction of homology in the ssDNA: effect of F(II) DNA. (A) ATPase reactions were performed as described under Materials and Methods. Each point represents a separate experiment, except the point at zero duplex DNA (○) which is an average of four assays. Duplex DNA concentrations are expressed as the ratio of total dsDNA molecules to total ssDNA molecules in the final reaction mixture. The F(II) DNA substrates shown are pBCS1 (□) and M13Goril (■). (B) The parameter v_0' represents the limiting value of v_0 at saturation with respect to a given duplex DNA substrate (dashed line). Each point represents the average v_0 value of five different titration points taken from the plateau region of each titration curve (data not shown). F(II) DNA substrates (□) and their percent homology are as follows: pBR322-2 (0%); pI24 and pBCS2 (12.6%); pBCS1 (51.4%); M13Goril (88.6%); M13mp8 (100%). The value of v_0' from the titration curve with M18mp8 F(III) DNA is also shown (■). Percent homology refers to the fraction of the total length of the ssDNA that was homologous with its dsDNA partner.

underwinding of these same dsDNA substrates and suggests that these two effects are associated with the same event. As observed previously (Schutte & Cox, 1987), each of the titration curves exhibits saturation with respect to the drop in ATP hydrolysis. However, as also previously observed (Schutte & Cox, 1987), an excess of these F(II) DNA substrates was necessary to obtain the full drop in ATP hydrolysis, suggesting that there is a topological barrier to the formation of paranemic joints between two circular DNA molecules.

We have defined the value of the rate of ATP hydrolysis at the "plateau" region of the titration curves as v_0, (Schutte & Cox, 1987). In Figure 5B, the values of v_0' for each titration curve are plotted versus the percent homology in the ssDNA molecule. (Since these results are an effect on ATP hydrolysis, a property of the recA–ssDNA complex, the x axis here is the fraction of the total length of the ssDNA molecule that is homologous to the dsDNA substrate with which it was paired.) As seen in the previous study (Schutte & Cox, 1987), a linear relationship is observed. The data were fit by linear regression, and the slope of the line was equal to a 31% drop in ATP hydrolysis over the region of homology. This value is comparable to the 33% drop in ATP hydrolysis observed previously with different dsDNA molecules (Schutte & Cox, 1987). Thus, the similarity of the properties of the underwinding of the dsDNA molecule bound to the recA–ssDNA complex and the drop in ATP hydrolysis with the same F(II) DNA substrates suggest that these two effects are associated with a common event: the formation of paranemic joints.

Discussion

Early in the strand exchange reaction carried out by recA protein, the homologous dsDNA molecule paired with the recA–ssDNA complex is underwound. This underwinding is completely homology dependent and occurs in two phases: a rapid initial phase that is complete within 2 min of the initiation of strand exchange and then a longer phase in which the underwinding of the dsDNA molecule increases. The time course for the initial phase of the underwinding and its homology dependence indicate that this underwinding of the dsDNA molecule is occurring in the synapsis phase. Moreover, since underwinding of the dsDNA molecule occurs in the absence of a free end in the region of homology, this event is

associated with the formation of paranemic joints. The resulting underwound DNA species, F(P), is composed of a heterogeneous mixture of underwound topoisomers. The extent of underwinding is strongly dependent on the fractional length of the dsDNA molecule that is homologous to the ssDNA molecule with which it is paired. Thus, only the homologous region of the dsDNA molecule is contributing to the overall underwinding of the molecule. This further suggests that the entire region of homology is in some way detected, though not necessarily at the same time (see below). The degree of underwinding observed (an average of 12% over the length of homology) is incompatible with simultaneous alignment of all available homologous sequences. The underwinding is nevertheless extensive and provides strong evidence for the formation of paranemic joints in which two DNA molecules are paired over 1000 or more base pairs.

The dsDNA molecule paired with the recA–ssDNA complex in a paranemic joint is underwound. Wu et al. (1983) carried out a similar set of experiments in the absence of SSB and observed underwinding of the dsDNA substrate with a time course that was significantly longer than observed here. It is possible that the underwinding of the dsDNA substrate observed in both studies reflects the formation of a paranemic joint. The longer time course for underwinding in the earlier study may simply reflect the greater time necessary to form paranemic joints in the absence of SSB (Wu et al., 1983; Bianchi et al., 1983). SSB is also required to observe the drop in ATP hydrolysis associated with paranemic joint formation (Schutte & Cox, 1987).

A longer underwinding phase in which the dsDNA molecule is even more extensively underwound is evident in this work as well as that of Wu et al. (1983). The final product is a DNA species that comigrates with F(X) DNA. F(X) DNA is a highly underwound form of dsDNA which can result from the direct binding of recA protein to dsDNA (Shibata et al., 1981; Pugh & Cox, 1987). As previously suggested (Bianchi et al., 1983), this further underwinding may reflect direct binding of recA protein to the dsDNA molecule, although we have no evidence that this occurs with the stoichiometric levels of recA protein employed here. Further work is necessary to evaluate the significance of this slower underwinding reaction.

Two aspects of the present work are especially important. These are (1) the correlation between the degree of underwinding and the length of homology and (2) the measured extent of the observed underwinding. The former suggests that underwinding is occurring only over the homologous regions of the dsDNA molecule. This relationship provides evidence against three alternative explanations for these observations. First, if underwinding also involved an interaction with the heterologous regions of the DNA, then all dsDNA substrates that contain sufficient homology to synapse with the recA–ssDNA complex would be expected to be underwound to the same extent. Second, if pairing were restricted to a few hundred base pairs, the underwinding induced by pairing would not increase for dsDNA substrates with homologous regions more extensive than those in the plasmids pBCS2 and pI24. Finally, if the underwinding were induced through direct binding of recA protein to the dsDNA molecule, the underwinding would not be expected to be limited to homologous sequences.

The average and maximum superhelical density of the F(P) topoisomers was approximately equal to −0.12 and −0.18, respectively, over the region of homology regardless of its length. This corresponds to a paranemic joint in which on average 30% and at most 45% of the homologous region of the dsDNA molecule is in homologous alignment (assuming the dsDNA molecule in the paranemic joint is underwound by 39.5%). For the paranemic joint formed between the dsDNA molecule and the ssDNA molecule derived from the bacteriophages M13Goril and M13mp8, these values correspond to average and maximum lengths of approximately 1900 and 2900 bp, respectively. This is significantly longer than the mean length of distinguishable paranemic joints observed in electron micrographs by Register et al. (1987), although longer joints may have been present. The values for the length of paranemic joints estimated in the present study should be considered lower limits, since a topological barrier to the formation of paranemic joints appears to exist when two circular DNA molecules are paired (Schutte & Cox, 1987). A final evaluation of underwinding in a paranemic joint will require resolution of the degree of underwinding when linear dsDNA molecules are used.

The extent of underwinding of F(P) DNA apparently reflects pairing of only a fraction of the available homologous sequences. However, the dependence on the length of homology indicates that all of the available homology is detected in a paranemic joint. This could be rationalized if homologous contacts in a paranemic joint are discontinuous. Alternatively, continuous paired regions might exist, but the position of the pairing could change rapidly throughout the homologous region. Periodic exposure of sequences located in homologous regions may explain how DNA ligase is able to seal DNA nicks within these regions during pairing. The migrating paranemic joint invoked by this hypothesis may also explain the heterogeneity of underwound species comprising F(P) DNA. Evidence suggesting that such migration occurs has been provided by Register et al. (1987). At this time both possibilities, and potentially others, can be rationalized in terms of these data.

Paranemic joints can extend over thousands of base pairs. In the paranemic complex both of the paired DNA molecules are extended and, in the case of dsDNA, are underwound. These facts must be accommodated in a structural model for paranemic joints. Three models have been represented to date. They differ primarily in the structural relationship of the two aligned DNA molecules. The incoming dsDNA molecule has been proposed to be either wrapped around the other DNA in a four-stranded (in this case, a three-stranded) structure (Howard-Flanders et al., 1984), bound in alternating right- and left-handed configurations (Christiansen & Griffith, 1986), or aligned approximately parallel with the other DNA molecule with periodic homologous contacts in the major groove (Schutte & Cox, 1987). At present, each of these structural models can potentially be accommodated with the results presented here, and further work is necessary to rigorously distinguish between them. The structure of a paranemic joint is important not only as a central clue to the mechanism of recA protein promoted strand exchange but as a potential model for DNA pairing in meiotic synaptonemal complexes in eukaryotic cells.

ACKNOWLEDGMENTS

We thank Theresa Cornwell of this department for supplying us with the bacteriophage M13mp8.1100 and the *E. coli* strain containing the plasmid pI24 and also for providing DNA sequence data. The bacteriophage M13Goril was kindly provided by Jon Kaguni.

SUPPLEMENTARY MATERIAL AVAILABLE

Detailed descriptions of the methods used to determine the superhelical densities of pBCS1, M13Goril, and M13mp8 F(P)

DNA and photographs of the agarose gels from which these data were derived (8 pages). Ordering information is given on any current masthead page.

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