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Use of Structure-directed DNA Ligands to Probe the Binding of RecA Protein to Narrow and Wide Grooves of DNA and on Its Ability to Promote Homologous Pairing*

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We have used circular dichroism and structure-directed drugs to identify the role of structural features, wide and narrow grooves in particular, required for the cooperative polymerization, recognition of homologous sequences, and the formation of joint molecules promoted by recA protein. The path of cooperative polymerization of recA protein was deduced by its ability to cause quantitative displacement of distamycin from the narrow groove of duplex DNA. By contrast, methyl green bound to the wide groove was retained by the nucleoprotein filaments comprised of recA protein-DNA. Further, the mode of binding of these ligands and recA protein to DNA was confirmed by DNaseI digestion. More importantly, the formation of joint molecules was prevented by distamycin in the narrow groove while methyl green in the wide groove had no adverse effect. Intriguingly, distamycin interfered with the production of coaggregates between nucleoprotein filaments of recA protein-M13 ssDNA and naked linear M13 duplex DNA, but not with linear ϕX174 duplex DNA. Thus, these data, in conjunction with molecular modeling, suggest that the narrow grooves of duplex DNA provide the fundamental framework required for the cooperative polymerization of recA protein and alignment of homologous sequences. These findings and their significance are discussed in relation to models of homologous pairing between two intertwined DNA molecules.

Escherichia coli recA protein promotes recombination-like reactions between a variety of DNA substrates in at least three formal steps (Radding, 1991; Kowalczykowski, 1991; Griffith and Harris, 1988; Cox and Lehman, 1987). The recombination reaction is initiated *in vitro* when recA protein, guided by single-stranded binding protein (SSB)[1] under certain conditions, binds cooperatively in stoichiometric amounts to single-stranded DNA (ssDNA) to form a right-handed helical nucleoprotein filament (Flory *et al.*, 1984; Muniyappa *et al.*, 1984; Stasiak and Egelman, 1988). RecA protein exhibits low affinity under physiological conditions to linear duplex DNA, however, binding is enhanced by altering the structural and physical status of duplex DNA (Ohtani *et al.*, 1982; Kowalczykowski *et al.*, 1987; Pugh and Cox, 1987; Cox and Lehman, 1987). The binding of recA protein to duplex DNA is facilitated by the presence of single-stranded gap or tail in the duplex DNA (Radding, 1991).

Several lines of evidence suggest that the nucleoprotein filament of recA protein-ssDNA functions as an intermediate which directs the subsequent steps in homologous pairing and strand exchange reactions promoted by recA protein (Cotterill and Fersht, 1983; Flory *et al.*, 1984; Tsang *et al.*, 1985; Morrical and Cox, 1985; Muniyappa *et al.*, 1990). Studies on the structure of nucleoprotein filaments by electron microscopy and image processing have revealed a change in the conformation of DNA by its interaction with recA protein to give rise to a pitch of 95 Å and an axial separation of bases of 5.1 Å (Flory *et al.*, 1984; Stasiak and Egelman, 1988). These and a number of related observations have suggested that the continuous polymerization of recA protein on DNA occurs along the phosphate backbone (Leahy and Radding, 1986; Chabbert *et al.*, 1991). It is therefore expected that recA protein will interact preferentially with one side or other side of the helix thereby restricting the nature of the nucleoprotein filament. The filament thus formed is right-handed with a deep helical groove, with bases free for interaction with a second DNA molecule.

A prerequisite to recognition of homologous sequences, alignment, and strand exchange is believed to involve the merging of two right-handed helices: the nucleoprotein filament comprised of recA protein-ssDNA and the naked duplex DNA. Based on model building and electron microscopic observations Howard-Flanders *et al.* (1984) have proposed that the interwound three- and four-stranded helices function as key intermediates in the strand exchange reaction. Whereas this model is now widely accepted, the underlying molecular details of homologous recognition, alignment, and strand exchange are not completely understood. At this stage one can plausibly formulate a molecular model, as a working hypothesis, of homologous alignment that may involve wide or narrow grooves of two interacting right-handed helices.

The activity of recA protein in the *E. coli* cell depends on its binding to DNA which may be hindered by potential constraints such as the presence of adducts and protein components (Muniyappa *et al.*, 1991; Ramdas *et al.*, 1989, 1991; Huang and Friedman, 1991). Consequently, the features of the DNA helix that support the binding of recA protein, especially the role of wide and narrow grooves, is unclear. Correspondingly, we test whether the noncovalent binding of small ligands to the wide and narrow grooves affects polym-

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[1] The abbreviations used are: SSB, single-stranded binding protein; ATPγS, adenosine 5′-*O*-(thiotriphosphate); ssDNA, circular single-stranded DNA; Form I DNA, supercoiled closed circular DNA as isolated form *E. coli*; Form III DNA, linear duplex DNA.

erization of recA protein, its binding strength, and more importantly, the formation of joint molecules. We show that the nucleoprotein filaments formed on duplex DNA retain the ligand in the wide groove, but the ligand in the narrow groove was quantitatively displaced. Further, the efficient formation of joint molecules was prevented by the presence of distamycin in the narrow groove, but not methyl green in the wide groove, of duplex DNA. These observations strongly argue that the structural feature required for binding of recA protein to duplex DNA, recognition of homologous sequences between two right-handed helices, and the production of triple-stranded intermediates may involve narrow grooves of duplex DNA.

MATERIALS AND METHODS

Biochemicals, Enzymes, and DNA—RecA protein was prepared as described (Griffith and Shores, 1985), and its concentration was determined spectrophotometrically using an extinction coefficient ϵ_{280} = 0.63 (Tsang *et al.*, 1985). *E. coli* SSB was prepared as described, and its concentration was determined using an extinction coefficient ϵ_{280} 1.5 ml mg^{-1} cm^{-1} (Lohman *et al.*, 1985). Distamycin was a gift from Dr. N. Mongelli of Formatalia Carlo Erba, Milan, Italy. It was dissolved in water containing 20 mM NaCl, before use, and the concentration was determined spectrophotometrically using the extinction coefficient (in mM^{-1}) ϵ_{303} = 33. Methyl green (Sigma) was dissolved as described above, and its concentration was determined spectrophotometrically (ϵ_{650} = 77.8). ATPγS was obtained from Boehringer Mannheim. Form I [^{3}H]DNA (negatively supercoiled) from bacteriophages M13, φX174, and unlabeled M13 ssDNA were prepared as described (Cunningham *et al.*, 1980). Restriction endonucleases were purchased from New England Biolabs. The biochemicals were obtained from Sigma. Linear duplex DNA was prepared by cleaving Form I DNA by *Xho*I or *Hpa*I in reaction conditions as suggested by the manufacturer. The reactions were terminated with excess of EDTA, phenol extracted, and dialyzed. The concentration of DNA is expressed as moles of nucleotides.

Spectroscopic Measurements—Circular dichroism (CD) measurements were done using Jasco J20C spectropolarimeter. CD values were converted to nucleotide phosphate concentrations and are expressed as $\Delta\epsilon$ values. All experiments were done at 30 °C in a buffer containing 20 mM Tris-HCl (pH 7.5), 40 mM potassium glutamate, and 2.5 mM magnesium acetate (buffer A). Complexes of DNA-ligand were formed by adding the reactants in the order mentioned below: buffer A, DNA, ATPγS (or ATP regeneration system components where specified), and the respective ligand at the amount indicated. The reaction mixture was incubated for 10 min in 1-cm path length quartz cells in a final volume of 400 μl prior to recording the CD spectrum at the wavelengths indicated. Titration with recA protein was done by adding aliquots (1–8 μl) to the reaction mixture, incubated for varying time periods before recording CD spectra. The binding stoichiometry of ligands to DNA was measured as described (Dasgupta *et al.*, 1987). Similarly the association constant for recA protein-DNA was calculated as described (Menetski and Kowalczykowski, 1985).

Nitrocellulose Filter Assay for Joint Molecules—The assay was performed as described (Ramdas *et al.*, 1989). The standard recA protein assay buffer contained 33 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1.5 mM ATP, 6 mM phosphocreatine, 12 mM $MgCl_2$, 10 units of phosphocreatine kinase/ml, and 0.1 mg/mL bovine serum albumin. Eight μM M13 ssDNA was incubated with 0.5 μM SSB in the above reaction mixture for 5 min at 37 °C followed by 2.7 μM recA protein for 10 min to form nucleoprotein filaments of recA protein-ssDNA.

Five μM M13 linear duplex [^{3}H] DNA (prepared by digesting Form I DNA with *Hpa*I) was incubated with appropriate concentrations of distamycin or methyl green in 10 μl of buffer A or standard recA protein assay buffer (above) for 10 min at 37 °C. The formation of joint molecules was initiated by the addition of DNA complexed with appropriate ligands, or uncomplexed DNA, to the standard reaction mixture containing nucleoprotein filaments of recA protein-ssDNA. Under these conditions ATP was regenerated for over 60 min.

Coaggregation Assay—The production of coaggregates was assayed as described (Ramdas *et al.*, 1991). Briefly, the reaction mixture (30 μl) in a standard assay buffer contained 8 μM M13 ssDNA, 12 mM $MgCl_2$, and 2.7 μM recA protein, and an ATP regeneration system was preincubated for 20 min at 37 °C. M13 or φX174 linear duplex [^{3}H]DNA, complexed with distamycin or uncomplexed, was then added. The samples were removed from the water bath, at the indicated time intervals, and immediately centrifuged in an Eppendorf microcentrifuge for 25 s at 25 °C. Radioactivity was measured as described (Ramdas *et al.*, 1991).

Nuclease Protection Assay—Four μM M13 linear duplex [^{3}H]DNA was incubated in a standard recA protein assay buffer or buffer A with 1 μM recA protein or 1 μM distamycin or methyl green, where indicated, in the presence of 1 mM ATPγS instead of ATP regeneration system for 15 min at 37 °C. Aliquots of reaction mixtures (20 μl) were incubated with the indicated concentration of pancreatic DNaseI for 2 min at 37 °C. The reaction was stopped by adding EDTA to 25 mM, 20 μl of 1.75% perchloric acid, and 20 μl of 0.88 mg/ml heat- denatured calf thymus DNA. The tubes were kept on ice for 30 min and centrifuged in an Eppendorf microcentrifuge for 10 min at 4 °C. The amount of acid-soluble radioactivity was determined by assaying an aliquot of the supernatant by liquid scintillation.

Molecular Modeling and Energy Minimization—The initial coordinates of DNA were those of Drew *et al.* (1982). The atomic coordinates for distamycin and methyl green were generated employing standard geometry and refined using energy minimization procedure at all atom level by the method of Vinter *et al.* (1987). Using Desktop Molecular Modeler (distributed by Oxford University Press), models of DNA-distamycin and DNA-methyl green were built, initially by docking the respective ligands at a symmetric location into narrow and wide grooves, respectively, such that no particular site was favored. These models were subsequently optimized such that there were no bad contacts between the DNA and the ligand.

RESULTS

To test the effect of small ligands on the path of polymerization of recA protein, with respect to the wide and narrow grooves, we monitored under standard recA protein assay conditions, the binding of distamycin and methyl green to DNA by circular dichroism. A sensitive indicator of DNA conformation is the CD spectrum which has been intensively used as a probe to follow drug- and protein-induced structural changes in DNA. A number of studies involving drug-DNA interactions have helped to infer the role(s) of structural elements of DNA such as contacts in the wide and narrow grooves and sequence-dependent conformations in protein-nucleic acid interaction. We chose for our studies distamycin and methyl green, ligands that bind to narrow and wide grooves, respectively, since their interaction with DNA has been elucidated by crystallographic, model building, and other physicochemical methods (Zimmer and Wahnert, 1986; Dasgupta *et al.*, 1987).

Kinetics of Binding of Distamycin and Methyl Green to DNA—A large body of evidence suggests that distamycin, an oligopeptide chemotherapeutic agent, exerts its biological activity by binding to the narrow groove of B-DNA in a non-intercalative mode. The specificity of interaction involves AT-rich sequences, but the crystal structure of the complex of distamycin-DNA suggests a close structural complementarity with the narrow groove of B-DNA (Coll *et al.*, 1987). The formation of a complex between M13 linear duplex DNA and distamycin, under recA protein assay conditions, was confirmed by the appearance of an induced CD band in the spectral region between 300–360 nm (see below). The amplitude of the CD band, a characteristic feature of the polynucleotide, is consistent with observations made on DNA containing random nucleotide sequences. Distamycin itself has no ellipticity so that the positive CD band in the spectral region of 300–360 nm must be induced Cotton effects arising from the interaction of the chromophore with the asymmetric environment of the DNA. Likewise, methyl green which binds to the hydrophobic surfaces in the wide groove was analyzed by CD spectroscopy (Norden and Tjerneld, 1977). Methyl green by itself has no ellipticity, and its binding to DNA was

evident with the occurrence of a red-shift for the CD spectrum of DNA from 290 to 650 nm (see below). Similarly recA protein and ATP alone do not show ellipticity in these spectral regions.

The binding stoichiometry of distamycin and methyl green to topologically different DNA substrates, under recA protein assay conditions, was determined by CD spectral titration at several ligand concentrations that were 10-fold greater than stoichiometric amounts. The CD spectrum of distamycin-DNA showed a strong positive CD band at 325 nm and an intense negative band at 265 nm. Likewise, the CD spectrum of a complex of methyl green-linear duplex DNA showed a strong positive band at 650 nm and a less intense negative band in the spectral region of 460–580 nm (see below). The molar ellipticity at the induced positive CD band was plotted *versus* the input concentrations of respective ligands (Fig. 1). The binding appeared to be of first order at low ligand concentrations, and at high ligand concentrations the DNA was effectively saturated. Furthermore, the similarity in spectral changes indicated an identical mode of association of distamycin with these substrates. The calculated stoichiometric ratio was 4 base pairs per a molecule of distamycin and methyl green, respectively. These values are consistent with the values reported which were determined by using somewhat different experimental conditions (Zimmer and Wahnert, 1986). For the experiments described below, we used ligands in stoichiometric amounts.

RecA Protein Dislodges DNA-bound Distamycin—Fig. 2 shows the spectral changes in distamycin-induced positive CD band as a consequence of binding of recA protein to DNA. Before testing for the ability of recA protein to dislodge bound distamycin, we established the binding of recA protein to M13 linear duplex DNA. The binding of recA protein in stoichiometric amounts to DNA resulted in an intense positive CD band at 290 nm. Sequential addition of recA protein followed by 10 min of incubation, in the presence of a non-hydrolyzable analog of ATP (ATPγS), caused a striking displacement of bound distamycin from linear duplex DNA. Addition of increasing amounts of recA protein resulted in a large reduction in the amplitude of the positive CD band at 325 nm and a small decline in the amplitude of the negative CD band. These spectral changes were associated with the concomitant appearance of a positive band at 290 nm, a characteristic feature of nucleoprotein filaments comprised of recA protein-DNA. Interestingly, complete displacement of distamycin from linear duplex DNA required more than stoichiometric amounts of recA protein. By comparison of the time course of displacement of distamycin with varying concentrations of recA protein (two concentrations are shown here), we obtained evidence that binding of recA protein to a binary complex of linear duplex DNA-distamycin had reached an end point by 10 min (Fig. 3). These observations, therefore, suggest that the equilibrium strongly favors recA protein over distamycin. The results of all the experiments described below were obtained from end point reactions of 10- and 60-min incubations.

We investigated further whether nucleoprotein filaments of recA protein-DNA produced from binary complexes of

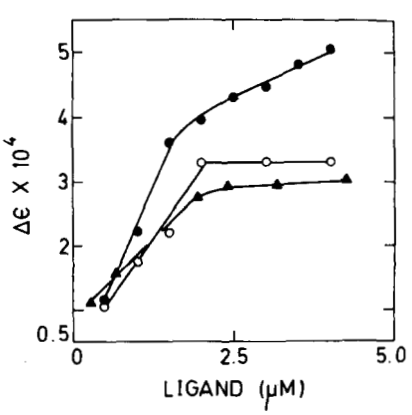

FIG. 1. **Determination of stoichiometry of binding of distamycin and methyl green to DNA.** The reactions were performed as described in the text. The figure represents the plot of maximum positive CD band *versus* the input ligand concentration. (○), Form I M13 DNA-distamycin; (●) Form III M13 DNA-distamycin; (▲) Form III M13 DNA-methyl green.

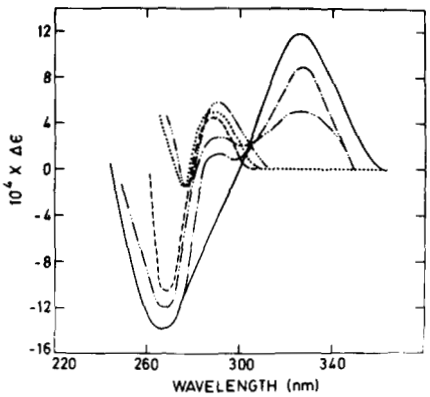

FIG. 2. **Displacement of distamycin from linear duplex DNA by recA protein.** The reaction was done, in the presence of ATPγ, as described under "Materials and Methods." In all the cases, the concentration of DNA and distamycin was 4 μM and 1 μM, respectively. CD spectra of Form III M13 DNA in the absence of distamycin and recA protein (- - - -); nucleoprotein filaments of recA protein-Form III M13 DNA (0.8 μM recA protein and 4 μM DNA) in the absence of distamycin(●●●); CD spectra of distamycin-Form III M13 DNA (——). All other reactions contained binary complexes of distamycin-Form III M13DNA which were incubated with recA protein for 10 min at 0.4 μM (●), 0.8 μM (●●), and 1.65 μM (●●●●) as described in the text.

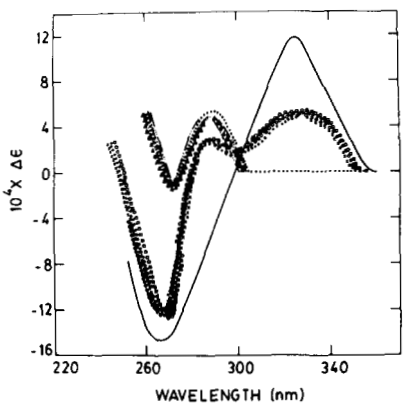

FIG. 3. **Time-dependent displacement of distamycin bound to DNA by recA protein.** The time course of the formation of a positive CD band at 290 nm, in the presence of ATPγS was performed as described under "Materials and Methods." CD spectra (●●●) of control reaction done in the absence of distamycin contained 4 μM M13 linear duplex DNA and 0.8 μM recA protein. The following reactions contained binary complexes of M13 linear duplex DNA and distamycin at 4 μM and 1 μM, respectively, which were incubated with varying amounts of recA protein for the times indicated prior to recording of CD spectra. Binary complexes incubated with 0.8 μM recA protein for 10 min (□□□□), 20 min (××××), 40 min (○○○○); 60 min (++++), and with 1.6 μM recA protein for 10 min (●); 20 min (●●); 40 min (ΔΔΔΔ); and 60 min (●●●●).

We investigated further whether nucleoprotein filaments of recA protein-DNA produced from binary complexes of

DNA-distamycin in the presence of ATPγS could be converted back into binary complexes of linear duplex-DNA distamycin. For this purpose we carried out displacement reactions as described above and then added 4-fold excess of distamycin. Under these conditions and with extended periods of incubation virtually, there was no indication of a shift of the positive CD band from 290 to 325 nm (data not shown). However, we are still puzzled by the fact that complete displacement of distamycin by recA protein requires more than the stoichiometric amount. Notwithstanding, it has been established that, in the presence of ATPγS, binding to DNA is complete in 5 min (Pugh and Cox, 1987) and nearly 70% of recA protein fails to transfer in 24 h (Kowalczykowski, 1991). This interpretation was further supported by the observation that recA protein failed to dislodge distamycin from linear duplex DNA, under identical conditions even after 60 min of incubation, in the presence of ATP regeneration system (data not shown).

A number of previous observations have indicated that the binding of recA protein to linear duplex DNA, under physiological conditions, is determined by several variables such as DNA sequence, the topological state, buffer, and others (Kowalczykowski, 1991). Thus, agents that perturb the native structure of DNA readily promote the loading of recA protein to DNA (Ohtani *et al.*, 1982; Lindsley and Cox, 1987; Thresher and Griffith, 1990). A strong prediction, therefore, is that recA protein should quantitatively dislodge distamycin from Form I DNA in the presence of ATP regeneration system. Before describing the ability of recA protein to dislodge the bound distamycin from M13 Form I DNA, it is pertinent here to consider the role of ATP hydrolysis on the kinetics of binding of recA protein to ssDNA. ATP is hydrolyzed by monomers of recA protein uniformly throughout the nucleoprotein filament but not simultaneously in all the monomers and dissociation of recA protein which occurs at both the ends of the filament is mediated by the ADP/ATP ratio (Brenner *et al.*, 1987; Lee and Cox, 1990). Correspondingly, in the presence of ATP regeneration system, the amplitude of positive CD band of Form I DNA-distamycin, at 325 nm, decreases with concomitant formation of a positive CD band at 290 nm (data not shown). Moreover, the pattern of recA protein- promoted displacement of distamycin from φX174 Form I or linear duplex DNA, with blunt or 3–4 unpaired bases, either at the 3′ or 5′ end, was similar to that of M13 linear duplex DNA in the presence of ATPγS (data not shown).

To assess the strength of binding of recA protein to DNA as a consequence of complex formation with distamycin, we plotted the CD values, obtained from end point titrations (60 min), for the increasing binding of recA protein *versus* the input amounts of recA protein (Fig. 4). Evidently, recA protein binds to duplex DNA that was previously complexed with distamycin as strongly as that of naked duplex DNA with an approximate association constant of 2.4×10^6.

Binding of Distamycin to Single-stranded DNA—While the results presented above clearly illustrate that recA protein efficiently dislodges the noncovalently bound distamycin from duplex DNA, it is unknown whether distamycin binds to M13 ssDNA. Further it is important that this issue should be addressed here since the formation of joint molecules involved a combination of substrates:nucleoprotein filaments comprised of recA protein-ssDNA and linear duplex DNA. To demonstrate the binding of distamycin to M13 ssDNA, we took advantage of its ability to induce a positive CD band at 325 nm. The results of such an experiment are shown in Fig. 5. Evidently, the pattern of displacement of distamycin from

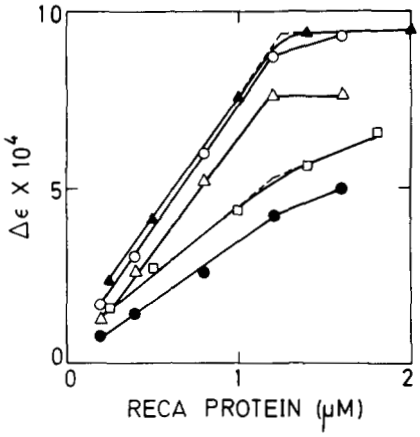

FIG. 4. **Binding of recA protein to binary complexes of DNA and distamycin.** The reactions were done as described in the text. The maximum CD spectral band at 290 nm obtained from incubating DNA alone (4 μM) or DNA complexed with distamycin (1 μM) for 60 min with recA protein was plotted *versus* the input concentrations of recA protein. Reactions were done with binary complexes of distamycin-DNA in the presence of 1 mM ATPγS: Form I M13 DNA (○); Form III M13 DNA (γ); M13 ssDNA (Δ). Control reaction, containing M13 ssDNA and recA protein, was done in the absence of distamycin: in the presence of ATPγS (▲) and in the presence of ATP regeneration system (●).

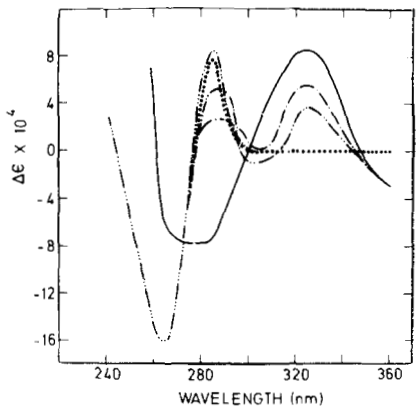

FIG. 5. **Release of distamycin from circular single-stranded M13 DNA by recA protein in the presence of ATPγS.** The reactions contained 6 μM M13 ssDNA, 1.5 μM distamycin, and 1 mM ATPγS and done as described in the text. CD spectra of (——) binary complexes of ssDNA-distamycin; ssDNA plus 1.2 μM recA protein (●●●). The remaining reactions contained binary complexes of distamycin-ssDNA which were incubated with recA protein at: 0.4 μM (●); 0.8 μM (●●); and 1.2 μM (●●●●●).

ssDNA with concomitant formation of a positive CD band, at 285 nm, was similar to that of linear duplex DNA-distamycin complex. Here again the amplitude of the distamycin-induced CD band decreases as a consequence of binding of recA protein. It is imperative that the binding of distamycin to ssDNA does not involve narrow grooves, perhaps to the regions of secondary structure, but is readily dislodged by recA protein. Interestingly, complete displacement of distamycin from ssDNA, unlike duplex DNA, occurred at stoichiometric amounts of recA protein.

Nucleoprotein Filaments of RecA Protein-DNA Retain Methyl Green—To get an insight into the effect of drugs that interact with the wide groove of duplex DNA on the binding of recA protein, we used CD spectroscopy to characterize the binding of methyl green to DNA. Binding of methyl green to linear duplex DNA was evident by the red-shift of the positive CD band from 290 to 650 nm and the negative CD band from

260 nm to a wide band in the region of 460–590 nm, respectively. The binary complex of linear duplex DNA-methyl green was then incubated with recA protein at stoichiometric amounts (0.8 μM) in the presence of ATPγS for 60 min, to permit the reaction to attain the end point, prior to recording of the CD spectrum. As illustrated in Fig. 6, a slight red-shift occurred upon binding of recA protein to the binary complex of methyl green-DNA but there was no blue-shift of CD spectrum to 290 nm. Time courses of this reaction varying from 10–60 min yielded similar results (data not shown). Because of the absence of a blue-shift, following the formation of nucleoprotein filaments of recA protein- DNA, but were resistant to DNaseI digestion (see below), we reasoned that the displacement process may require more than stoichiometric amounts of recA protein. We therefore incubated the binary complex with 1.2 and 2.0 μM recA protein under identical conditions as described above. Evidently under these conditions also, there was no indication of a blue-shift of CD spectrum suggesting that, unlike in the case of distamycin, recA protein failed to dislodge methyl green bound to linear duplex DNA. Similar kinetics were observed with Form I DNA-methyl green complex under identical conditions in the presence of ATP regeneration system (data not shown). Methyl green under these conditions does not bind to ssDNA (data not shown).

Distamycin-bound DNA Is Protected from DNaseI Digestion—The foregoing studies have illustrated that the binding of recA protein to a binary complex of DNA-distamycin resulted in the displacement of the latter while binding to a complex of methyl green-DNA produced a ternary complex. To characterize further we used an enzymatic probe, DNaseI, to generate physical evidence about the nature of these complexes. DNaseI cleaves naked DNA to a mixture of oligo- and mononucleotides, but DNA gets protected once its bound by protein(s). Further, as revealed by the crystal structure, the specificity of cleavage arises from the interaction of DNaseI in the narrow groove of B-DNA (Suck *et al.*, 1986). This observation predicts that DNA containing distamycin should be resistant to cleavage by DNaseI. Correspondingly, the complex of distamycin-DNA was protected as judged from the decrease in susceptibility to DNaseI digestion (Fig. 7). As a control the complex of methyl green-DNA was analyzed in a similar fashion. By contrast to the complex of distamycin-DNA, the digestability of methyl green-DNA was similar to that of naked duplex DNA. To confirm that methyl green-DNA complex was capable of supporting the binding of recA protein, through the narrow groove, we formed ternary complexes of methyl green-DNA-recA protein and subjected them to DNaseI digestion. The binding of recA protein to such a complex resulted in protection. Taken together, these results indicate that the presence of distamycin in the narrow groove of duplex DNA suppressed while methyl green in the major groove afforded no protection.

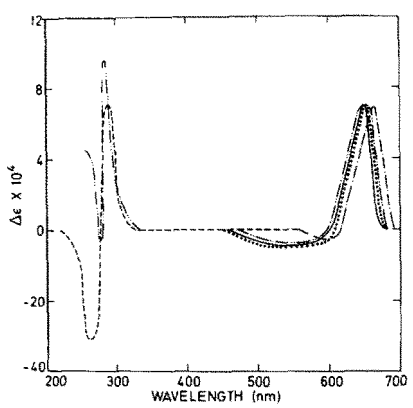

FIG. 6. **Binding of recA protein to binary complexes of DNA-methyl green.** The reactions were done in the presence of 1 mM ATPγS containing 4 μM linear duplex DNA and 1 μM methyl green as described in the text: CD spectra of Form III M13 DNA in the absence of methyl green and recA protein (- - - -); nucleoprotein filaments comprised of recA protein-Form III M13 DNA, in the absence of methyl green (●●●; CD spectra of a binary complex of Form III M13 DNA-methyl green in the absence of recA protein (— —). All the remaining reactions contained binary complexes of Form III M13 DNA-methyl green which were incubated with recA protein at: 0.8 μM (●); 1.2 μM (●●); and 2 μM (●●●●).

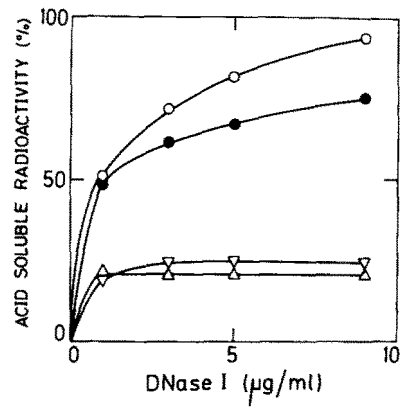

FIG. 7. **Protection of linear duplex [^{3}H]DNA from digestion by DNaseI in the presence and absence of ligands.** M13 linear [^{3}H]DNA was incubated in a standard assay buffer with the respective ligands, followed by recA protein where indicated, as described under "Materials and Methods." Naked linear [^{3}H]DNA (○); binary complex of linear [^{3}H]DNA and methyl green (●); ternary complex of recA protein-linear [^{3}H]DNA-methyl green (▽); binary complex of linear [^{3}H]DNA-distamycin (△).

Effect of Distamycin and Methyl Green on the Formation of Joint Molecules—The molecular details underlying the mechanism of recognition of homologous sequences by the nucleoprotein filaments of recA protein-ssDNA has remained elusive. From the biological perspective it is possible to use the principle of selective recognition of specific elements by structure-directed drugs as probes to interfere with recognition and homologous alignment promoted by recA protein. We therefore tested the possibility of inhibitory effect(s) of distamycin and methyl green, which bind to narrow and wide groves respectively, on the formation of joint molecules.

To evaluate the effect of distamycin on the formation of joint molecules by recA protein, we combined the binary complexes of M13 linear duplex DNA-distamycin, the latter at the indicated amounts, with nucleoprotein filaments of recA protein-ssDNA. Five min later we terminated the reaction by adding an equal volume of a solution containing 0.15 M NaCl, 0.015 M sodium citrate, and 25 mM EDTA. After taking an aliquot for the determination of total radioactivity, we added 5 ml of the above solution and assayed for joint molecules as described under "Materials and Methods." As shown in Fig. 8*A*, in the absence of distamycin the extent of formation of plectonemic joint molecules was greater than 90%. However, with the duplex DNA containing increasing amounts of distamycin there was a gradual decline in the yield of joint molecules, and at stoichiometric ratio (1 μM) their formation was less than 30%.

To ascertain that distamycin was exerting its inhibitory effect by not poisoning the system, we introduced naked duplex DNA into an ongoing reaction between distamycin-bound linear duplex DNA and nucleoprotein filaments of recA protein-ssDNA. The results shown in Fig. 8*B* reveal that

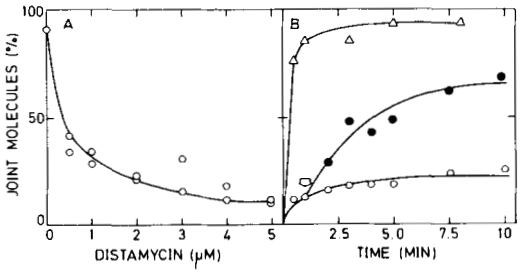

FIG. 8. **The effect of distamycin on the formation of joint molecules.** *Panel A*, effect of increasing concentrations of distamycin. The reactions were performed as described under "Materials and Methods." Nucleoprotein filaments of recA protein-ssDNA were formed in the presence of ATP regeneration system. Similarly, binary complexes of distamycin-Form III M13 DNA were formed by incubating 5 μM Form III M13 [^{3}H]DNA with the indicated amounts of distamycin at 37 °C for 10 min. The formation of joint molecules was initiated by combining binary complexes with the nucleoprotein filaments. Five min later, the reaction was terminated by the addition of an equal volume of a solution containing 1.5 M NaCl and 0.15 M sodium citrate. The assay for joint molecules was done as described (Ramdas *et al.* 1989). *Panel B*, time course of the formation of joint molecules. The reaction was performed as described above. Binary complexes of Form III M13 DNA-distamycin were formed by incubating 3 μM distamycin with 5 μM Form III M13 DNA at 37 °C for 10 min. The assay for joint molecules was done as described above. Control reaction contained 5 μM Form III M13 DNA (Δ); binary complex of Form III M13 DNA-distamycin (O); binary complex of Form III M13 DNA-distamycin (●), but we added at the time indicated by the *arrow* 4 μM of uncomplexed Form III M13 DNA.

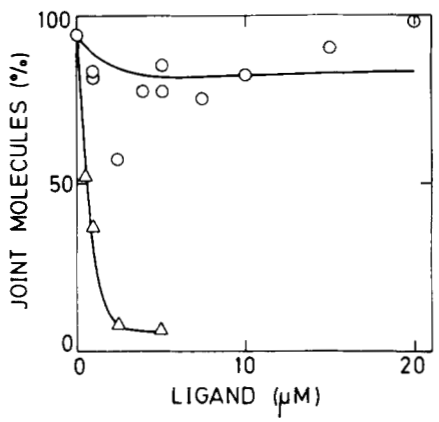

FIG. 9. **Effect of methyl green on the formation of joint molecules.** The preparation of binary complexes of Form III M13 DNA with the respective ligands and nucleoprotein filaments of recA protein-ssDNA was done as described under "Materials and Methods." The formation of joint molecules was initiated by combining the binary complexes nucleoprotein filaments of recA protein-ssDNA. Form III M13 DNA-methyl green (O); Form III M13 DNA-distamycin (Δ).

freshly added naked duplex DNA acts as a potential template for the formation of joint molecules.

Having discovered that distamycin bound to the narrow groove inhibited the formation of joint molecules we have addressed the issue of the possible inhibitory effect of methyl green, in the wide groove, on the formation of joint molecules. Consistent with the results described above distamycin in stoichiometric amounts completely abolished the formation of joint molecules whereas methyl green, even at 5-fold excess of its stoichiometric amount, had no inhibitory effect (Fig. 9). These results suggest that the nucleoprotein filament recognizes homologous sequences in the duplex DNA through the narrow groove and facilitate the merging of two right-handed helices to produce a triple-stranded structure.

Distamycin Inhibits the Production Homologous Coaggregates—Because of strong inhibition of the formation of joint molecules with linear duplex DNA complexed with distamycin, we wished to examine the possibility of distamycin interference with the production of coaggregates, believed to be the intermediates in the pathway to the formation of joint molecules, *in vitro*. The hallmark of the phenomenon of coaggregation is the ability of nucleoprotein filaments of recA protein-ssDNA to recruit both homologous as well as heterologous naked duplex into stable networks of DNA (Radding, 1991; Kowalczykowski, 1991). As shown in Fig. 10, in the control experiment the extent of the production of coaggregates between linear M13 duplex DNA and nucleoprotein filaments of recA protein-ssDNA is comparable to a number of previous observations. Interestingly, linear φX174 DNA complexed with distamycin appeared to be equally efficient in supporting the production of coaggregates. In contrast coaggregation between linear M13 DNA complexed with distamycin and nucleoprotein filaments of recA protein-ssDNA was measurable but remained constant throughout the incubation period. This correspondence provides a formal evidence that events inherent to the production of coaggregates, with heterologous DNA might be different with the events in the pathway to the formation of coaggregates with homologous DNA culminating in the formation of joint molecules. Finally, the formal possibility exists that these two events, namely coaggregate production and the formation of joint molecules with homologous combination of substrates, may be related directly by their requirement of narrow grooves of duplex DNA.

DISCUSSION

The determination of the path of polymerization of recA protein on single- and double-stranded DNA continues to be the focus for a more detailed understanding of the conformation of the nucleoprotein filament. The characterization of nucleoprotein filaments by electron microscopy and image processing have revealed an apparently striated structure with a wide helical groove. The protein filament follows the helical path of the DNA wherein the DNA resides within the perimeter of the filament (Stasiak and Egelman, 1988). The nucleoprotein filament contains approximately six monomers of

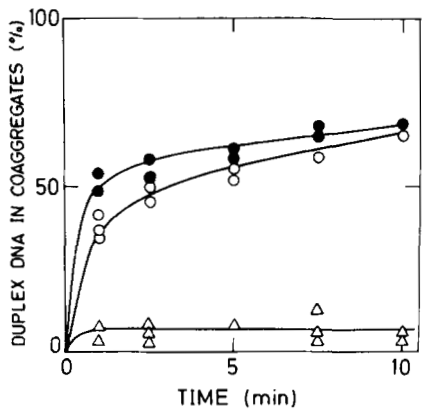

FIG. 10. **Distamycin interferes with the production of homologous coaggregates.** Reactions (30 μl) were prepared with 10 μM linear M13 duplex [^{3}H]DNA minus distamycin (O), 10 μM M13 [^{3}H]DNA plus 2.5 μM distamycin (Δ), and 10 μM φX174 linear [^{3}H] DNA (Form I DNA cleaved with *Xho*I) with 2.5 μM distamycin (●). The formation of nucleoprotein filaments of recA protein-M13 ssDNA was done as described under "Materials and Methods." The production of coaggregates was initiated by combining duplex DNA and nucleoprotein filaments and the assay was done as described under "Materials and Methods."

recA protein (Flory *et al.*, 1984; Tsang *et al.*, 1985; Bryant and Lehman, 1985; Lauder and Kowalczykowski, 1991) with a pitch of about 95 Å and 18 base pairs/turn (Flory *et al.*, 1984; Stasiak and Egelman, 1988). This unprecedented recA protein-induced stretching of DNA results in the separation of bases from 3.4 Å in the B-DNA structure to 5.1 Å in the nucleoprotein filament (Flory *et al.*, 1984; Stasiak and Egelman, 1988). Several lines of evidence have indicated that polymerization of recA protein occurs along the same face of the DNA helix, to the sugar phosphate backbone, with bases exposed (Leahy and Radding, 1986, Chabbert *et al.*, 1991). The N-7 guanine in the DNA complexed by recA protein, in the presence of ATPγS was found to be more reactive to dimethyl sulfate than in the free DNA (DiCapua and Muller, 1987). In the absence of other DNA-binding proteins, recA protein polymerizes on DNA in a 5′ to 3′ direction, and a polarity appears to determine the directionality of strand exchange at least with respect to the initiating ssDNA (Register and Griffith, 1985; Lindsley and Cox, 1988). The path of polymerization of recA protein with respect to the grooves of DNA is unclear, however. One could, therefore, predict two mutually exclusive possibilities: binding of recA protein to double-stranded DNA may either follow wide groove to wide groove or narrow groove to narrow groove mode of orientation. As mentioned above, since recA protein interacts with sugar phosphate backbone, both these possibilities in principle are plausible. The goal of the present study is to obtain an insight into the nature of other recognition elements involved in binding of recA protein to double-stranded DNA.

Using distamycin and methyl green, drugs that bind to the narrow and wide grooves, respectively, we show that the path of cooperative polymerization of recA protein occurs along the narrow grooves of duplex DNA. This conclusion is consistent with the studies of DiCapua and Muller (1987) who showed that the accessibility of reactive groups to dimethyl sulfate in DNA bound to recA protein was 2-fold higher in the wide groove whereas the N-3 of dA in the narrow groove, which may be in contact with recA protein, was protected. In addition, wide groove modification by methylation and glycosylation, as in the phage T4 DNA, neither interfered with the formation of helically ordered nucleoprotein filaments nor with ATPase and reannealing activities of recA protein (Dombroski *et al*, 1983; Yonesaki and Minagawa, 1988).

The ligands that bind to DNA are likely to distort the overall structure of DNA and thereby may influence its ability to interact with proteins. Studies done to assess the strength and stoichiometry of binding of recA protein to DNA complexed with the respective ligands yielded stoichiometric ratios and approximate association constants similar to that of uncomplexed DNA. In summary these studies, guided by the wealth of information available on the binding of recA protein, establish that recA protein binds to the sugar phosphate backbone along the narrow grooves of duplex DNA. Nonetheless, the deduced binding to the narrow groove should not be considered to imply that recA protein binds exclusively to the narrow grooves. At present there is no supporting evidence available, and at this level, therefore, the unambiguous resolution of this issue should await additional work.

The biochemical characterization of homologous pairing and strand exchange reaction promoted by recA protein and a variety of its homologues has identified several critical steps that are important for the overall process of homologous genetic recombination. One of the intriguing aspects, however, concerns the search and the alignment of homologous sequences which have become the focus of renewed interest as a result of the characterization of triple-stranded DNA, an early intermediate in strand exchange promoted by recA protein and its homologues (Radding, 1991). These findings are consistent with the model proposed by Howard-Flanders *et al.* (1984) that interwound three- and four-stranded helices play an important role in the process of exchange of strands. A variety of observations have provided evidence for the occurrence of a putative triple-stranded intermediate (Radding, 1991). Although these studies have abetted an important feature of the above model but do not address the mechanistic origin of recognition required for the merging of two right-handed helices.

What is the fundamental framework of duplex DNA, in addition to sequence homology, that determines the recognition between two helices? One approach to addressing this question is to use structure-directed drugs as probes. Our finding that recognition between two helices involving narrow grooves provides new insights for understanding the location of various strands and specific interactions in the interwound triple-stranded DNA (Hsieh *et al.*, 1990; Umlauf *et al.*, 1990; Rao *et al.*, 1990). The presence of a ligand in the wide groove had no adverse effect, a conclusion supported by two sets of experimental data: first, the non-interference of methyl green in the cleavage of DNA by DNaseI, while distamycin interfered with cleavage of DNA (Mendoza *et al.*, 1990; this work). Second, the binding of recA protein and the ability of nucleoprotein filaments of recA protein to form joint molecules with duplex DNA saturated with methyl green was not affected. These observations, therefore, persuasively argue that the narrow groove of duplex DNA plays a prominent role in homologous alignment and formation of joint molecules. It is conceivable, however, that narrow grooves may be required only for the initial recognition and for the proper assembly of triple-stranded DNA intermediate(s) and then may become dispensable. It is also likely that the triple-stranded DNA intermediates may use the wide grooves prior to strand switching. Although recA protein readily displaces bound distamycin from the narrow groove of duplex DNA, it failed as a nucleoprotein filament which lead us to question what determines this differential activity? We propose that recA protein has the ability to displace distamycin when it binds to DNA *in cis* but not in *in trans*, therefore, suggesting that displacement may require the primary but not the secondary DNA-binding site of recA protein.

We find it intriguing that the nucleoprotein filaments of recA protein-ssDNA show a remarkable specificity for the narrow grooves of B-DNA. We believe that the narrow groove recognition by the nucleoprotein filament offers new ionic interactions between basic residues of recA protein bound to ssDNA and phosphate groups further along the narrow grooves of duplex DNA in an arrangement similar to that proposed for oligoarginine, oligolysine, and nucleoproteins with B-DNA (Feughelman *et al.*, 1955; Wilkins, 1956).

The role of narrow groove in protein-nucleic acid interaction is not unprecedented. In the case of phage 434 repressor, the sequence-specific recognition occurs by a combination of water-mediated hydrogen bonding and sequence-dependent deformability of DNA in the narrow groove (Aggarwal *et al.*, 1988). The cleavage of DNA by DNaseI is through the narrow groove of duplex DNA (Suck *et al.*, 1986). Other examples of narrow groove recognition are derived from the structural analysis of *E. coli* glutamyl-tRNA synthetase complexed with the narrow groove of t-RNA (Rould *et al.*, 1989) and the class of DNA junction-resolving enzymes (Bhattacharya *et al.*, 1991).

Our current understanding of structural information of triple-stranded DNA emanates from several physicochemical

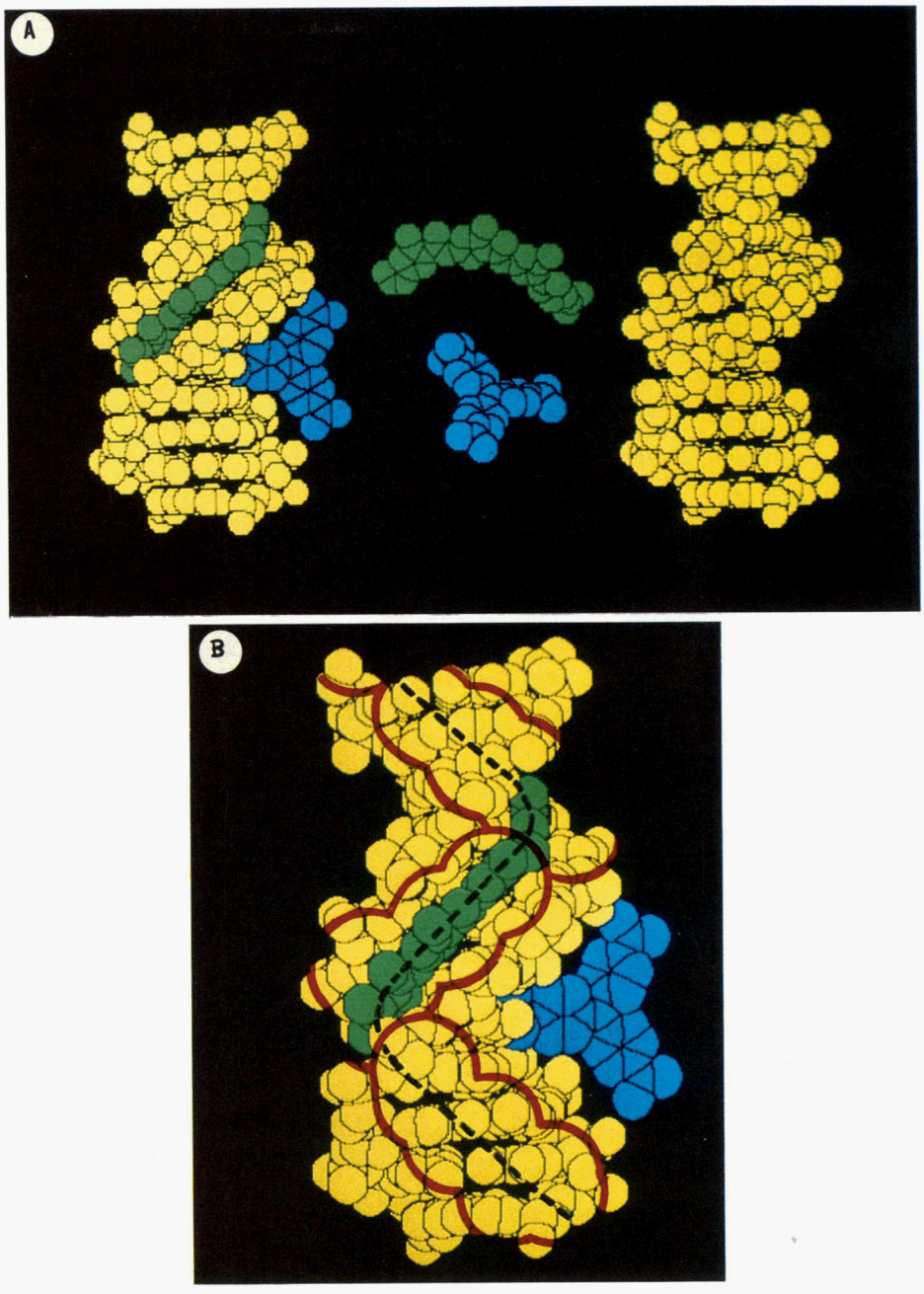

FIG. 11. **A possible model for the noncovalent intermediates formed between B-DNA- ligands and B-DNA-nucleoprotein filaments of recA protein-ssDNA.** *A*, computer drawn space filling of a B-DNA segment consisting of 12 base pairs (*right*); the ligands, distamycin (*above*), methyl green (*below*), and a B-DNA segment (*left*) with methyl green in the wide groove and distamycin in the narrow groove. *B*, a schematic representation of the general nature of the interaction presumed to occur between B-DNA segment complexed with distamycin and methyl green and a nucleoprotein filament of recA protein-ssDNA. The monomers of recA protein are drawn as a multidomain structure based on stoichiometry and the three-dimensional reconstruction of recA protein filament formed on DNA in the presence of ATPγS (Stasiak and Egelman, 1988). The *broken line* represents the helical path of single-stranded DNA.

studies including fiber diffraction (Moser and Dervan, 1987 and references therein). In the model proposed by Arnott and Selsink (1974) for the triple-stranded DNA, the third strand (*i.e.* the second pyrimidine strand) is located in the wide groove of a normal Watson-Crick duplex stabilized by Hoogsteen base pairing. Interestingly, the sugar-phosphate backbone of the additional homopyrimidine or homopurine strand aligns parallel to the purine or pyrimidine strand of the duplex DNA. The biological importance of these three-stranded structures remains to be determined, however. By contrast, in recA protein-promoted formation of triplex structure the third strand positions in the narrow groove of duplex DNA while the wide groove is relatively unaffected. We suggest, therefore, that the type of groove contacts and the polarities of the paired strands in triple-stranded DNA made by recA protein from random sequences, may conceptually have a different structure. Interesting parallels can be drawn from our findings on the path of polymerization of recA protein and the formation of joint molecules with duplex DNA complexed with distamycin. Both of these processes seemingly involve the narrow groove of B-DNA. These ideas are attractive *a priori* in view of the findings of Jain *et al.* (1992) who by substituting 7-deazaguanine for guanine in both the strands of the duplex DNA have demonstrated the dispensability of interstrand hydrogen bonding involving guanine N-7 in the major groove.

To represent our view on the interaction of nucleoprotein filament of recA protein-ssDNA with B-DNA complexed with distamycin and methyl green, we have employed computer modeling. As illustrated in Fig. 11, the linear shape of distamycin in the narrow groove presents a steric barrier for the cooperative polymerization of recA protein and the formation of joint molecules. Alternatively, distamycin might simply inhibit the ability of recA protein to unwind the second DNA molecule that is required for the alignment of two helices. On the other hand, the orientation of methyl green in the wide

groove, also consistent with the earlier space filling model (Norden and Tjerneld, 1977), reveals no such hindrance. On the basis of the above information and since there exists reasonable correlation between the structure of nucleoprotein filaments of recA protein on single- and double-stranded DNA (Stasiak and Egelman, 1988), we find it justified to superimpose schematically two helical structures, the nucleoprotein filament of recA protein-ssDNA and naked B-DNA, so as to generate a triple-stranded structure. This model is speculative but provides an integrated view of the findings presented here with all the available evidence on the interaction of nucleoprotein filaments with duplex DNA. Details of the exact arrangement of recA protein and DNA in the triplex structure should await crystallization of the complex.

The proposed arrangement of nucleoprotein filament of recA protein-ssDNA with B-DNA along the narrow grooves raises several obvious questions. The foremost question is the proposal of Howard-Flanders *et al.* (1984), based on lack of evidence on strand separation prior to pairing, that homologous alignment may involve wide grooves which is compatible with the model developed by McGavin (1977). However, application of sensitive methods of detection have revealed that recA protein unwinds heterologous duplex DNA during the search for homology (Rould *et al.*, 1992). Correspondingly, Wilson (1979) has proposed that two duplex molecules could specifically associate through their narrow grooves with no topological constraints, establish homologous alignment, and execute reciprocal exchange of strands to form heteroduplex DNA.

In summary these results support the concept that the narrow grooves of duplex DNA play an important role both in the cooperative polymerization of recA protein on DNA and in the recognition and alignment of homologous sequences. Correspondingly, there are a variety of observations, including model building studies, which support this conclusion. Elucidation of detailed mechanistic requirements between two right-handed helices, utilizing structure-directed ligands, may provide a complete scenario of the complexity of recognition of homologous sequences and strand exchange, much the same way as the mutant protein(s) provide insights into the workings of a wild type protein.

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