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DNA-induced Conformational Changes in RecA Protein

EVIDENCE FOR STRUCTURAL HETEROGENEITY AMONG NUCLEOPROTEIN FILAMENTS AND IMPLICATIONS FOR HOMOLOGOUS PAIRING*

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We have used circular dichroism as a probe to characterize the solution conformational changes in RecA protein upon binding to DNA. This approach revealed that RecA protein acquires significant amounts of α-helix upon interaction with DNA. These observations, consistent with the data from crystal structure (Story, R. M., Weber, I., and Steitz, T. (1992) *Nature* 355, 318–325), support the notion that some basic domains including the DNA binding motifs of RecA protein are unstructured and might contribute to the formation of α-helix. A comparison of nucleoprotein filaments comprised of RecA protein and a variety of DNA substrates revealed important structural heterogeneity. The most significant difference was observed with poly(dG)·poly(dC) and related polymers, rich in GC sequences, which induced minimal amounts of α-helix in RecA protein. The magnitude of induction of α-helix in RecA protein, which occurred concomitant with the production of ternary complexes, was 2-fold higher with homologous than heterologous duplex DNA. Most importantly, the stimulation of ATP hydrolysis by high salt coincided with that of the induction of α-helix in RecA protein. These conformational differences provide a basis for thinking about the biochemical and structural transitions that RecA protein experiences during the formal steps of presynapsis, recognition, and alignment of homologous sequences.

Homologous pairing and strand exchange promoted by *Escherichia coli* RecA protein has served as an instructive example to construct the formal steps of homologous genetic recombination (Radding, 1991; Kowalczykowski, 1991; Roca and Cox, 1990; Griffith and Harris, 1988; Cox and Lehman, 1987). The precise molecular mechanism(s) by which RecA protein executes the crucial steps of this process, *in vitro*, is still unclear. However, genetic, biochemical, and structural studies all converge to propel the notion that these processes occur within the physical framework of the nucleoprotein filament comprised of RecA protein and DNA (Radding, 1991; Kowalczykowski, 1991; Stasiak and Egelman, 1988).

Homologous pairing and strand exchange promoted by RecA protein is a dynamic process driven by ATP hydrolysis (Cox and Lehman, 1981; Riddles and Lehman, 1985). The ATPase activity of RecA protein is induced upon binding to a variety of ribo- and deoxyribopolymers, including homopolymers (reviewed in Kowalczykowski (1991)). By a series of elegant studies, Weinstock *et al.* (1981) have suggested that the binding of RecA protein to polynucleotides is necessary but not sufficient for ATP hydrolysis. In the absence of ATP hydrolysis, RecA protein can however bind to DNA and promote homologous pairing, but complete strand exchange is prevented (Menetski *et al.*, 1990; Muller *et al.*, 1990; Honigberg *et al.*, 1985). Several recent observations have suggested that hydrolysis of ATP facilitates the passage of strand exchange across regions of heterology in the recombining DNA molecules and also promotes the dissociation of RecA protein from the products of strand exchange (Menetski *et al.*, 1990; Roselli and Stasiak, 1991; Kim *et al.*, 1992a). More importantly, RecA protein-promoted reciprocal strand exchange shows an obligatory requirement for ATP hydrolysis (Kim *et al.*, 1992b). RecA protein is an allosteric enzyme; accordingly, the ATP-bound form shows higher affinity for DNA over the ADP-bound form (Radding, 1991; Kowalczykowski, 1991). It is conceivable that the molecular consequence of hydrolysis of ATP may set in an ordered series of conformational changes in RecA protein during the formal steps of presynapsis, synapsis, and strand exchange. An understanding of the mechanism of RecA protein-promoted DNA strand exchange, therefore, requires a definition of the sequential motion in the structure of DNA (Flory *et al.*, 1984; Stasiak and Egelman, 1988; Shutte and Cox, 1988; Rould *et al.*, 1992) in conjunction with detailed conformational changes that occur in RecA protein at various stages in the reaction.

The reaction(s) promoted by RecA protein is initiated by its binding to DNA, in the presence of ATP or ATPγS,[1] to produce an active helical nucleoprotein filament with a pitch of approximately 95 Å. Although RecA protein binds to ssDNA in the absence of nucleotide cofactors or in the presence of ADP, it produces "compressed" inactive filaments with a pitch of about 55–70 Å. Several lines of evidence have suggested that the cleavage of ATP promoted by RecA protein occurs only in the presence of DNA; however, two laboratories have shown the stimulation of intrinsic ATPase activity in the absence of DNA by high salt (Pugh and Cox, 1988; DiCapua *et al.*, 1990). In related studies it has been demonstrated that the morphology of nucleoprotein filaments formed in the presence of DNA and ATP or high salt is almost

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[1] The abbreviations used are: ATPγS, adenosine 5′-*O*-(thiotriphosphate); ssDNA, circular single-stranded DNA; Form I DNA, supercoiled closed circular DNA as isolated from *E. coli*; Form II DNA, nicked circular duplex DNA; Form III DNA, linear duplex DNA; bp, base pair; deg, degrees.

identical (DiCapua *et al.*, 1992). Taken collectively these data suggest that RecA protein exists in at least two conformational states that correlate with the "low" and "high" affinity forms of RecA protein as defined by Kowalczykowski (1991). Further evidence for the existence of two states of RecA protein was derived from proteolytic digestion (Kobayashi *et al.*, 1987), electron microscopy (Yu and Egelman, 1991, 1992), and biochemical and biophysical studies (Muench and Bryant, 1990, 1991; Kowalczykowski, 1991).

Although the three-dimensional description of RecA protein structure is immensely useful to our understanding of RecA protein-promoted homologous pairing and strand exchange (Story *et al.*, 1992), information on the dynamic activity of monomers of RecA protein in the polymer during these processes will be highly complementary. Here we have devised an assay system, using circular dichroism (CD), to monitor the conformational changes of RecA protein in solution, upon binding to DNA, and in ternary complexes comprised of homologous or heterologous duplex DNA. We have observed that the binding of RecA protein to single- and double-stranded DNA induced an altered conformation in RecA protein with increased α-helical content. These conformational differences indicate that the DNA binding motifs of RecA protein, which are disordered in the absence of DNA, take on α-helical configuration during the formation of nucleoprotein filaments. The binding of RecA protein to DNA is sequence nonspecific; therefore, the structure of nucleoprotein filaments is likely to be indistinguishable. Somewhat unexpectedly we found that the extent of induction of α-helix in RecA protein was dependent on the base composition of DNA. Although the structural basis for this difference is unclear, it might relate to the tilted base geometry in the respective nucleoprotein filaments (Norden *et al.*, 1992). In view of this, structural heterogeneity among nucleoprotein filaments, and the fact that some DNA sequences are excluded from homologous pairing and strand exchange perhaps suggest a possible basis for the pleiotropic nature of RecA protein. Further, we show that ternary complexes comprised of homologous duplex DNA induced significant amounts of α-helix in RecA protein when compared with heterologous duplex DNA. These conformational differences provide a structural basis for thinking about the biochemical changes that RecA protein experiences during the formal steps of homologous genetic recombination.

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). The activity of purified RecA protein was routinely assayed in the absence and presence of *E. coli* single-stranded binding protein as described (Muniyappa *et al.*, 1990). ATPγS was obtained from Boehringer Mannheim. Form I DNA (negatively supercoiled) and circular single-stranded DNA from bacteriophages M13, ϕX174, and M13 Goril 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 *Eco*RI in reaction conditions as suggested by the manufacturer. The reactions were terminated with an excess of EDTA, phenol-extracted, and dialyzed. The concentration of DNA is expressed as moles of nucleotides. The synthetic homopolymers were obtained from Pharmacia LKB Biotechnology Inc. and Sigma. They were dissolved in a buffer containing 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl. The concentrations of stock solutions were determined using molar extinction coefficients as shown in Table I.

Circular Dichroism Studies of RecA Protein Structure—CD spectra were recorded at 30 °C in a nitrogen atmosphere on a Jasco J20C or Jasco J500 spectropolarimeter using quartz cuvettes with a 0.1- or 0.05-cm path length. The reaction mixtures (600 μl) contained 20 mM Tris-HCl (pH 7.5), 20 mM NaCl, 10 mM $MgCl_2$ (where specified), 2 mM dithiothreitol, 0.5 mM ATPγS or 0.5 mM ATP (or 0.5 mM ADP where specified), and indicated amounts of DNA substrates. Reactions were carried out by starting with the above mixture containing DNA to which appropriate amounts of RecA protein were added in small volumes (1–10 μl) to minimize changes in the concentration of other components. The reaction mixture was incubated for 15 min prior to recording of CD spectra. In time course experiments under these conditions, we established that the reaction was completed in 10 min (Kumar and Muniyappa, 1992). The results of the spectra are the average of at least two scans and were corrected for an appropriate base-line scan obtained with buffer alone and buffer plus DNA. Because of strong absorbance of chloride ions in the buffer, CD spectra below 200 nm could not be determined. The percentages of α-helix were estimated from the measured ellipticities at 222 nm using the method of Zhong and Johnson (1992). The absorbance of samples at 222 nm containing all of the components including RecA protein, ATP, and DNA in 0.1-cm pathlength cuvettes was less than 0.51 in all of the measurements. The molecular ellipticity of DNA substrates used here, in the spectral region of 205–230 nm, was less than −500, and this value has been subtracted from the data in all of the experiments. The CD results are reported in terms of $[\theta]$ (molar ellipticities) in units as deg·cm^2·mol^{-1}. Molecular ellipticity was calculated with the formula $[\theta] = 10E(cl^{-1})$, where E is the observed ellipticity angle, l is the cell length, and c is the concentration of the protein in moles/liter.

TABLE I

Polynucleotide cofactors used for analyzing structural alterations in RecA protein

Polynucleotide	~Max	Extinction coefficient
	nm	$\times 10^{-4}$ M^{-1} cm^{-1}
Poly(dA)	260	1.54
Poly(dG)	260	1.15
Poly(dC)	260	0.74
Poly(dT)	260	0.87
Poly(rA)	260	1.54
Poly(rU)	260	0.99
Poly(rC)	260	0.74
Poly[d(A-T)]	260	1.14
Poly(dG)·poly(dC)	253	0.74
Poly(dI–dC)	251	0.69
Poly[d(A-C)]·poly[d(T-G)]	269	0.67
Poly(dG-m^{5}dC)·poly(dG-m^{5}dC)	254	0.84
Poly(dG·dC)·poly(dG·dC)	254	0.84
tRNA	260	0.83

Circular Dichroism Studies of RecA Protein Structure during Homologous Pairing and Strand Exchange—Nucleoprotein filaments comprised of RecA protein-M13 ssDNA were prepared as described (Muniyappa *et al.*, 1990; Honigberg *et al.*, 1985). Briefly, the reaction mixture contained 20 mM Tris-HCl (pH 7.5), 20 mM NaCl, 10 mM $MgCl_2$, 0.5 mM ATPγS (or 0.5 mM ATP); 0.26 μM RecA protein was incubated at 30 °C with 0.8 μM M13 ssDNA for 15 min. The reaction for the formation of joint molecules was initiated by the addition of 0.4 μM M13 or ϕX174 linear duplex DNA. CD spectra were recorded at the times indicated. Under these conditions, the formation of joint molecules was assessed by the nitrocellulose filter binding assay (Muniyappa *et al.*, 1990; Ramdas *et al.*, 1989). The extent of formation of joint molecules was greater than 80%. Because of strong absorbance by phosphocreatine and interference by phosphocreatine kinase, data could not be obtained in the presence of the ATP regeneration system.

ATPase Assay—The assay was done as described (Weinstock *et al.*, 1981). Briefly, reaction mixtures (28 μl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM [γ-^{32}P]ATP, 94 μg/ml bovine serum albumin, 8 μM RecA protein, and the indicated amounts of NaCl. A reaction with M13 ssDNA was included as a positive control. Reaction mixtures were incubated in Eppendorf tubes at 30 °C for 30 min. The reaction was terminated by the addition of a mixture containing 3 mM ATP, 3 mM ADP, and 25 mM EDTA and immediately kept on ice. Aliquots were spotted on polyethyleneimine-cellulose strips containing markers and developed in 1 M lithium chloride. The bands were identified with an ultraviolet lamp, cut out, and counted in scintillation fluid. In this solvent system, ATP bands near the origin, ADP migrates about halfway to the solvent front, and P_i runs ahead of ADP.

Nuclease Protection Assay—Ten μM M13 linear duplex [^{3}H]DNA

or single-stranded [^{3}H]DNA or ^{32}P-labeled poly(dG)·poly(dC) or ^{32}P-labeled poly(dG) was incubated in standard RecA protein assay buffer, in the presence of 0.5 mM ATPγS, with increasing amounts of RecA protein as described above. The nucleoprotein filaments were then treated with 1 unit of pancreatic DNase I 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.

Nitrocellulose Filter Binding Assay—NaOH-treated nitrocellulose filters (Sartorious; 0.22 μm) were used for filter binding assays. Uniformly labeled M13 duplex [^{3}H]DNA or ^{32}P-labeled poly(dG)·poly(dC) was incubated with varying amounts of RecA protein in a standard assay buffer in the presence of ATPγS, as described above. Reactions were done at 37 °C for 15 min, then filtered through NaOH-treated membranes, and rinsed with 5 ml of washing buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM KCl, and 20 mM $MgCl_2$. The bound radioactivity was monitored by a liquid scintillation counter.

RESULTS

Experimental Rationale—The three-dimensional structure of *E. coli* RecA protein has been determined to 2.3-Å resolution (Story *et al.*, 1992). The monomer of RecA protein consists of three distinct structural domains. A major central domain (residues 31–269) comprising parallel 8-stranded β-sheets flanked by α-helices harbors both nucleotide-binding and DNA-binding sites for single- and double-stranded DNA. Two smaller domains, one at the amino-terminal end (residues 1–30) and one at the carboxyl-terminal end (residues 270–352) of the monomer, protrude from the body of the molecule and are believed to play a dominant role in the polymer and interpolymer bundles (Story *et al.*, 1992; Story and Steitz, 1992). Genetic and structural studies have revealed that RecA protein contains two DNA-binding sites, and these are unstructured in the crystal lattice (Story *et al.*, 1992). The structure of the polymeric form (6 monomers/turn) of RecA protein determined in the absence of nucleotide cofactor and DNA has a helical pitch of 82.7 Å, which is intermediate between compressed inactive filaments and an extended active filament (Story *et al.*, 1992; Yu and Egelman, 1992). This suggests, therefore, that information about the conformation of RecA protein in the crystal structure is unlikely to be the structure of active polymer. For instance, soaking crystals with ATP resulted in their destruction (Story and Steitz, 1992 (cited in DiCapua *et al.*, 1992)). Since RecA protein is an allosteric enzyme, it is conceivable that it would exhibit DNA-dependent structural variation under a variety of experimental conditions, and a comparison of its conformation during these processes is valid and informative.

Structural Alterations in RecA Protein in the Absence of DNA—The CD spectra of proteins in the spectral region between 175 and 260 nm provide semiquantitative information about their secondary structure and are simpler to interpret than the data generated by x-ray crystallography or nuclear magnetic resonance (Blundell and Johnson, 1976; Wuthrick, 1986). The far-UV CD spectra of RecA protein were therefore monitored to determine its overall solution conformation under a variety of experimental conditions such as in the presence and absence of nucleotide cofactors, in the presence of $MgCl_2$, and in the presence of ethylene glycol. We have included ethylene glycol, which had no effect on the activities of RecA protein, to minimize interactions between nonpolar groups. In the first series of experiments, we monitored CD spectra of RecA protein in a standard buffer containing 0.5 mM ATP or 0.5 mM ATPγS, 50% ethylene glycol, and 10 mM $MgCl_2$. The CD spectrum of RecA protein, in the absence of DNA, displayed a maximum near 200 nm, considered to be the hallmark of proteins with partial helical content. The contributions from peptide bond chromophores are predominant in this region, and information about the secondary structure of the protein can be deduced from the CD spectra. Analysis of CD spectra for α-helix was done using the method of Zhong and Johnson (1992), which indicated that RecA protein in the presence of various nucleotide cofactors contained 21–31% of α-helix.

Nucleoprotein Filaments Comprised of RecA Protein and DNA Are Structurally Different—To examine the propensity of RecA protein to acquire α-helical structure in the presence of DNA, we used several DNA substrates with varying base compositions (natural as well as synthetic homopolymers) to determine if there is any sequence selectivity. The CD spectra of different nucleoprotein filaments comprised of RecA protein and natural DNA are illustrated in Fig. 1. Interaction of RecA protein, in stoichiometric amounts, with DNA containing natural sequences caused a distinct change in the overall shape of the CD spectrum with an increase in α-helical content. The binding of RecA protein to φX174 ssDNA or to M13 ssDNA increased the magnitude of ellipticity at 208 nm to a maximum of approximately −29,000 deg·cm^2·mol^{-1}. Other experiments done with varying concentrations of RecA protein/ssDNA attained a steady state at 1 RecA monomer/3–4 nucleotides (see below). The variation in the base composition of DNA substrates produced significant differences in the overall CD spectra of RecA protein, as evidenced by the intensity of the negative minima at 208 and 222 nm. RecA protein acquired a maximum of approximately 30% α-helix over the amount present in the native protein upon binding to natural DNA substrates at a steady state of 1 RecA monomer/3–4 nucleotide residues or base pairs. Under these conditions, with the natural DNA substrates tested, the order of efficiency of DNA cofactors in inducing α-helix in RecA protein follows the series: φX174 ssDNA > M13 duplex DNA ≥ M13 ssDNA. The net content of α-helix in RecA protein,

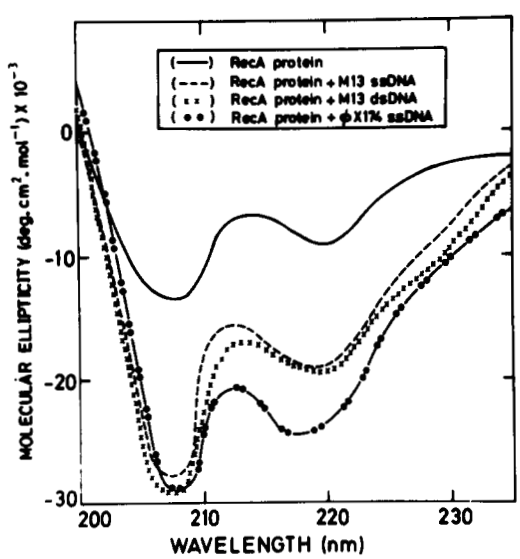

FIG. 1. **DNA-induced CD spectra of RecA protein.** Reactions were done using standard assay conditions as described under "Materials and Methods" and contained the following: 0.8 μM RecA protein, 10 mM $MgCl_2$, 3.2 μM DNA (or 6.4 μM duplex DNA), 50% ethylene glycol, and 0.5 mM ATPγS. Mixtures of RecA protein and the indicated natural DNA were incubated at 30 °C in the assay buffer for 15 min prior to the recording of the CD spectra. Each experimental spectrum represents the best fit of at least three determinations. The percentages of α-helix were calculated as described (Zhong and Johnson, 1992). The ellipticity of DNA substrate in this spectral region was near zero.

in solution, is approximately 21–31% depending upon conditions; upon binding to natural DNA substrates it acquired an additional 30%.

To obtain further insight into the structural alterations in RecA protein upon interaction with polynucleotides of varying base composition, we used a series of synthetic single- and double-stranded ribo- and deoxyribohomopolymers. Under identical conditions in the presence of ATP or ATPγS, single-stranded polynucleotides also enhanced the overall magnitude of the shape of the CD spectrum; the order of efficiency of single-stranded homopolymers in inducing structural alteration in RecA protein was: poly(dC) > poly(dA) > poly(dT) > poly(dG) (Fig. 2*A*). Interestingly, the overall CD spectra of RecA protein in the presence of poly(dG) showed considerably diminished ellipticity at 208 nm. The addition of a 2-fold excess of poly(dG) failed to enhance the molar ellipticity, thereby eliminating the possibility that lower ellipticity at 208 nm was the result of partial binding of RecA protein. It is conceivable that the higher structures that poly(dG) might assume in solution perhaps limit the binding of RecA protein, and its greater affinity for poly(dA) and poly(dC) over poly(dT), all of which contain little or no secondary structure, is intriguing. Nevertheless, the studies of Norden *et al.* (1992) on helical nucleoprotein filaments of RecA protein-poly[d(ϵA)] yielded a pitch of 10.6–11 nm, while the pitch of RecA protein-poly(dT) complex showed a significantly smaller value of 9.2 nm. There exists, we believe, a distinct relationship among the base geometry, helical pitch of the nucleoprotein filament, and the alterations in secondary structure of RecA protein.

We have used a series of synthetic double-stranded homopolymers, including substrates with alternating DNA sequence, to exclude the potential of single-stranded homopolymer(s) forming unusual structures in solution and thereby impeding the binding of RecA protein. Typical CD spectra recorded for nucleoprotein filaments comprised of RecA protein and a series of DNA substrates under identical conditions is illustrated in Fig. 2*B*. The double-stranded poly(dI·dC) and poly[d(A-C)]·poly[d(T-G)] were as efficient as M13 ssDNA (at stoichiometric amounts) in inducing α-helix in RecA protein. In contrast, double-stranded homopolymers comprised of GC sequences such as poly(dG)·poly(dC), poly(dG-m^{5}dC)·poly(dG-m^{5}dC), and poly(dG-dC)·poly(dG-dC) were less efficient in inducing structural alterations in RecA protein.

Most of the experiments were done with the commercially available polynucleotides with an average length of 500–3000 bp. However, when sheared polymers of approximately 1 kilobase were employed, the magnitude of induction of α-helix in RecA protein was similar (data not shown). Taken together these results suggest that RecA protein binds to DNA cooperatively in a sequence-nonspecific manner, but the nature of nucleoprotein filaments is determined by base composition. It could be argued, however, that the synthetic homopolymers such as poly(dG) and double-stranded homopolymers, which are rich in GC sequence, are less efficient in the induction of α-helix because they are not completely bound by RecA protein. To exclude this possibility, we monitored the binding of RecA protein to increasing amounts of a given DNA substrate in the presence of ATPγS by a series of CD measurements. Under these conditions RecA protein binds strongly to DNA (Kowalczykowski, 1991). The binding of RecA protein to DNA substrates was followed by the appearance of a binding-induced negative Cotton effect at 208 nm. At this wavelength the DNA substrates exhibited no CD signal. For the sake of appropriate comparison with the stoichiometric determinations, we confirmed the appearance of the induced CD signal at 208 nm for RecA protein binding to M13 ssDNA. Fig. 3 illustrates the corresponding CD titration curves for a series of single-stranded homopolymers and tRNA when they are added incrementally to the standard assay buffer containing RecA protein. The most significant finding is that the induced CD signal at 208 nm for all the substrates tested, except tRNA (*closed squares*), seemingly saturated at an input stoichiometric ratio of approximately 1 RecA monomer/4 nucleotide residues. In other words, the

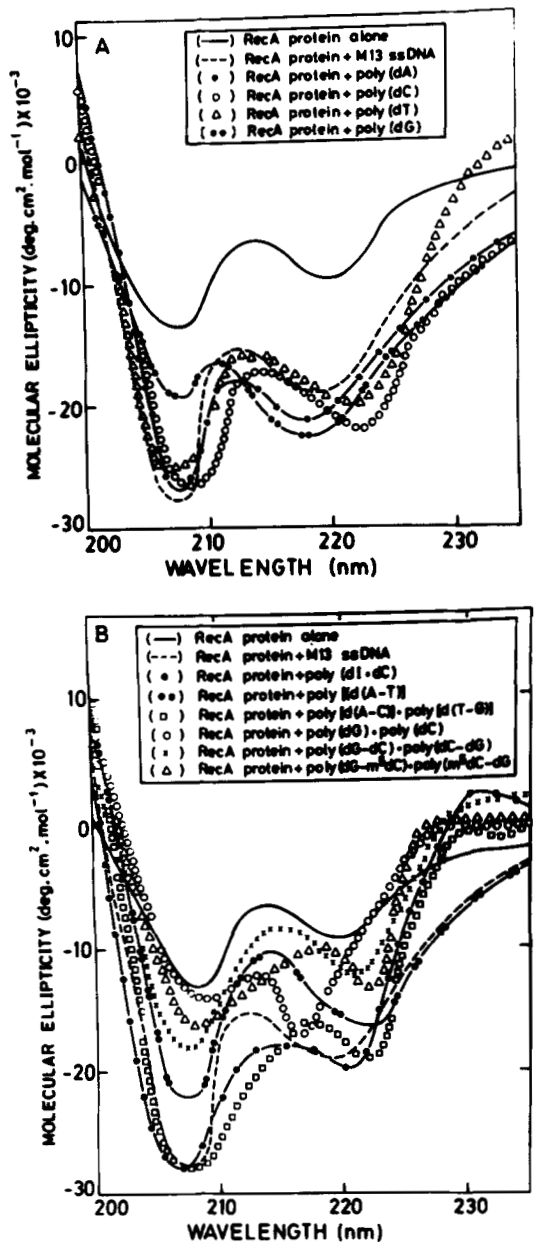

FIG. 2. **Differential induction of α-helicity in RecA protein by various synthetic homopolymers.** *A*, CD spectra of single-stranded homopolymers with RecA protein. The reactions were done in a standard assay buffer as described under "Materials and Methods" and contained 0.8 μM RecA protein, 3.2 μM indicated polynucleotide, 0.5 mM ATPγS, and 10 mM $MgCl_2$. *B*, CD spectra of double-stranded homopolymers with RecA protein. The reaction mixture contained 0.8 μM RecA protein, 3.2 μM indicated double-stranded DNA, 0.5 mM ATPγS, and 10 mM $MgCl_2$, and the assay was done as described above. Each experimental spectrum represents the best fit of three determinations. The ellipticity of all the homopolymers in this spectral region was near zero.

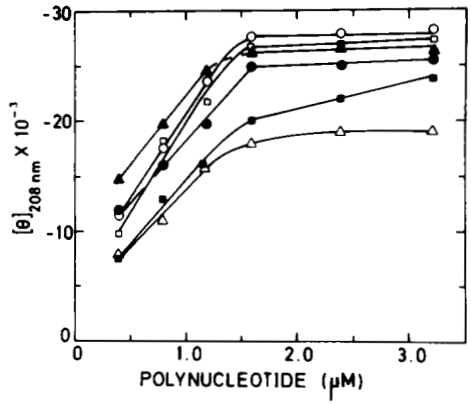

FIG. 3. **Stoichiometric binding of RecA protein to different polynucleotide substrates.** The reactions were done in a standard assay buffer containing 0.4 μM RecA protein and the indicated amounts of polynucleotide substrate. The maximal CD spectral band at 208 nm was plotted *versus* the input concentrations of the polynucleotide. ○, M13 ssDNA; ▲, poly(dA); ●, poly(dC); □, poly(dT); ■, tRNA; △, poly(dG).

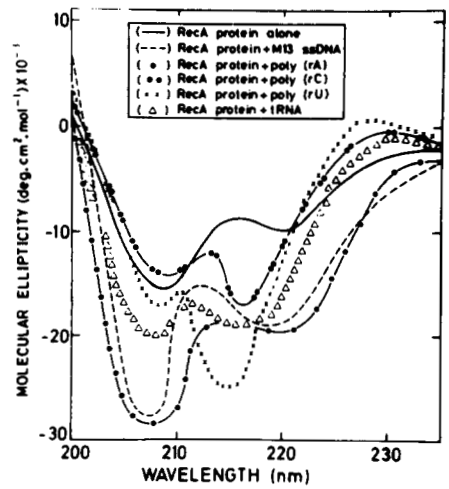

FIG. 4. **Induction of α-helicity in RecA protein by ribohomopolymers.** The reaction was done in a standard assay buffer as described in the legend to Fig. 2.

binding of RecA protein to poly(dG) yielded a site size of 4 nucleotide residues/monomer similar to that of M13 ssDNA. Thus the CD spectral data on the stoichiometric determination are consistent with the other methods (Tsang *et al.*, 1985; Morrical and Cox, 1985). In addition, these results are also in agreement with the site size for the binding of RecA protein to tRNA and homopolymers such as poly(dT) and poly(dA) as determined by neutron scattering measurements and activity assays (DiCapua *et al.*, 1992; Norden *et al.*, 1992).

To characterize further, we ascertained the efficiency of the binding of RecA protein as a function of input stoichiometric ratio to ^{32}P-labeled poly(dG) and poly(dG)·poly(dC) sequence by DNase I protection assay. As a control, we analyzed the nucleoprotein filaments comprised of RecA protein-M13 [^{3}H] DNA in a similar fashion. Consistent with the result described above, we obtained a site size of approximately 4 bp or nucleotide residues/monomer of RecA protein (data not shown). Similar stoichiometric ratios were also obtained by nitrocellulose filter binding assay (data not shown).

Induction of Structural Alterations in RecA Protein with Ribohomopolymers—RecA protein has been shown to bind, albeit with different affinities, to ribopolymers and consequently to serve as an effective cofactor in at least one of its activities, namely the cleavage of LexA repressor (McEntee *et al.*, 1981; Wang *et al.*, 1988; DiCapua *et al.*, 1992). These observations provided us a basis for examining the ability of ribohomopolymers and tRNA to induce structural alterations in RecA protein. Inspection of CD spectra of RecA protein with a series of synthetic ribohomopolymers revealed the order of efficiency as follows: poly(rA) > tRNA > poly(rU) > poly(rC) (Fig. 4). These observations, therefore, provide us with a situation from which to speculate that the type of activity that RecA protein promotes perhaps is modulated by the extent of induction of α-helix.

A caveat inherent in the CD analysis of DNA-induced structural alterations in RecA protein must be noted; the foremost is the contribution by DNA to the CD data. Although the molecular ellipticity of DNA alone was near zero and has been subtracted from the respective CD spectra, the contribution by DNA when bound by RecA protein cannot be excluded; however, this possibility exists for all of the DNA substrates tested. The comparison of CD spectra of Form I, Form II, and Form III M13 DNA, despite the differences in their topology, indicated no conformational differences.

TABLE II

Estimation of DNA-induced α-helicity of RecA protein in the presence of different nucleotide cofactors

The α-helical content was deduced from the measured ellipticities as described under "Materials and Methods." The CD spectra were recorded in a buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM of the indicated nucleotide cofactor, and 0.8 μM RecA protein in the presence of respective DNA substrates (3.2 μM).

	α-Helix		
	ATPγS	ATP	ADP
	%	%	%
RecA protein	30	22	20
RecA protein-M13 ssDNA	58	51	21
RecA protein-M13 duplex DNA	60	54	25
RecA protein-φX174 ssDNA	67	65	21
RecA protein-poly(dA)	63	55	24
RecA protein-poly(rC)	54	23	19
RecA protein-poly(rU)	44	21	24
RecA protein-tRNA	43	35	23
RecA protein-poly(dI-dC)	62	54	21
RecA protein-poly[d(A-T)]	50	49	26
RecA protein-poly(dG)·poly(dC)	32	25	23
RecA protein-poly(dG-dC)·poly(dG-dC)	41	35	21
RecA protein-poly(dG-m^{5}dC)·poly(dG-m^{5}dC)	40	24	23

Nonetheless, there are no appropriate standards available (*i.e.* DNA polymers with 18 bp/helical turn and an axial separation of bases of 5.1 Å) to verify this point.

Quantitative evaluation of CD spectra of RecA protein, under identical conditions, for the induction of α-helix is shown in Table II. Addition of a variety of DNA substrates, including several homopolymers, resulted in concomitant increase in the content of α-helix. The deduced amounts of α-helix, particularly in the presence of ADP, seem to differ from those of the crystal structure. A series of observations has revealed the occurrence of an inactive filament of RecA protein-ssDNA in the presence of ADP, which appeared to be similar to that of the filament formed in the absence of nucleotide cofactor (Stasiak and Egelman, 1988; Lee and Cox, 1990). Our data are consistent with the phenomenon that DNA-induced α-helix formation evidently occurs in the presence of ATP or ATPγS but not in the presence of ADP (Table II). Nonetheless, these data are not necessarily in conflict with those of the crystal structure. One of the differences may be simply due to the different experimental conditions, most

notably the omission of DNA and ADP during crystallization (the latter was, however, diffused into the crystal). The information about the crystal structure cannot be directly compared with the structure in solution, and it is important to obtain structural information in solution so as to correlate to the different activities of RecA protein. We believe the inability of poly(dG)·poly(dC) and related substrates, rich in GC sequences, to induce α-helix is important in view of a recent observation that high GC content in DNA substrates impedes RecA protein-promoted strand transfer activity *in vitro* (Gruss *et al.*, 1991). We speculate therefore that the basis for reduced strand transfer among DNA substrates with a high GC content perhaps is attributable to the inability of RecA protein to acquire critical amounts of α-helix.

Homologous Pairing Results in Increased α-Helix Formation—One of the intriguing aspects of strand exchange reaction is the mechanism by which RecA protein recognizes homologous DNA sequences. Howard-Flanders *et al.* (1984) proposed the notion that RecA protein carries at least two DNA-binding sites. Correspondingly, biochemical and structural analysis has shown that RecA protein can bind duplex and ssDNA or two duplex molecules simultaneously, irrespective of their complementarity (Radding, 1991; Kowalczykowski, 1991; Cox and Lehman, 1987). Nevertheless the ternary complexes do show differences depending on heterologous or homologous duplex DNA (Kumar and Muniyappa, 1992). To understand the molecular details of strand exchange reaction, it is important to analyze the structural transitions in RecA protein in ternary complexes. It is possible that RecA protein might undergo additional alterations in its structure upon formation of ternary complexes, and it is likely that there might be differences between heterologous and homologous complexes.

To determine the ability of a second DNA binding motif to influence the α-helical character of RecA protein, we compared the spectra obtained with the nucleoprotein filaments of RecA protein-M13 ssDNA in the presence of homologous or heterologous duplex DNA. To facilitate this set of experiments, we formed nucleoprotein filaments of RecA protein-M13 ssDNA as described under "Materials and Methods." The nucleoprotein filaments were then reacted with homologous (M13) or heterologous (φX174) linear duplex DNA; the former would support the formation of plectonemic, and the latter, paranemic joint molecules. The progress of the reaction was followed by recording CD spectra at the indicated time intervals. In Fig. 5, we have represented the negative Cotton effects at 208 and 222 nm, respectively. During the first few minutes of the pairing reaction, homologous DNA induced a large increase in the magnitude of $[\theta]_{208\,nm}$ to an extent of $-20{,}000$ deg·cm^2·mol^{-1}. However, with the progress of the reaction there was a biphasic decline in the net content of α-helix at 208 nm. A large increase in the content of α-helix is consistent with search and alignment of homologous sequences followed by strand exchange that occurs within these time limits (Shutte and Cox, 1988; Rould *et al.*, 1992; Adzuma, 1992). On the other hand, the reaction done with heterologous substrates showed minimal effect on the induction of α-helix in an identical reaction. The addition of more DNA in either case failed to increase the ellipticity as would be expected if the lower induction were the result of partial interaction of naked duplex DNA with nucleoprotein filaments. Since these reactions were done in the presence of ATPγS, it was unknown as to the extent of strand exchange. However, a recent study has shown that in the putative triple-stranded DNA intermediate, under conditions similar to the ones used in this study, the base-pairing status is essentially that of the

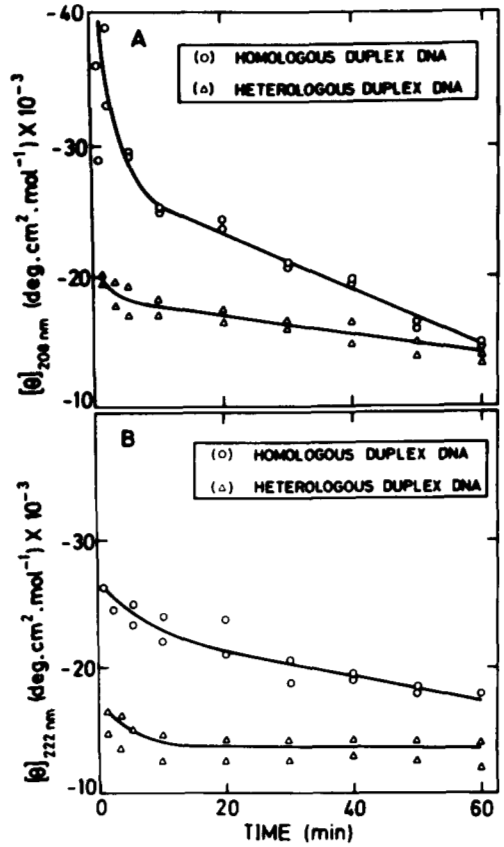

FIG. 5. **Formation of joint molecules results in increased α-helix formation in RecA protein.** Nucleoprotein filaments comprised of RecA protein and DNA were prepared in the presence of 0.5 mM ATPγS as described (Honigberg *et al.*, 1985). The reaction contained 0.26 μM RecA protein and 0.8 μM M13 ssDNA and was incubated at 30 °C for 15 min. The pairing reaction was initiated by the addition of 0.4 μM linear duplex DNA. The CD spectra were recorded at the indicated time intervals at 30 °C. The data represent changes in molar ellipticity at 208 and 222 nm as a function of time.

product of complete strand exchange (Adzuma, 1992). We therefore propose that the conformational changes that manifest at 208 and 222 nm are required for RecA protein to promote homologous pairing and strand exchange reactions.

Molecular Basis of Activation and ATPase Activity of RecA Protein by High Salt—The electrostatic forces between the terminal cation groups of protein and phosphates of the DNA backbone are believed to play a dominant role in the cooperative binding of RecA protein to DNA (Radding, 1991; Kowalczykowski, 1991; Roca and Cox, 1990). Correspondingly, the requirement for an anionic environment, in the form of DNA, could be mimicked by high salt to elicit the intrinsic ATPase activity of RecA protein (Pugh and Cox, 1988; DiCapua *et al.*, 1990). Although the exact molecular mechanism of this activation process is unknown, models have been proposed that invoke the binding of anions at specific DNA-binding sites on RecA protein (Pugh and Cox, 1988). We have investigated this issue and now provide a plausible molecular basis for the anion-induced ATPase activity of RecA protein. As illustrated in Fig. 6*A*, in the presence of increasing amounts of NaCl and 0.5 mM ATP we obtained a corresponding increase in helical content of RecA protein. Maximal induction of α-helix occurred at 2 M NaCl. To correlate the salt-induced induction of α-helix with the catalytic properties of RecA protein, we have analyzed it, under these conditions, for its ability to hydrolyze ATP to ADP and P_i. The intrinsic ATPase activity of RecA protein showed a measurable in-

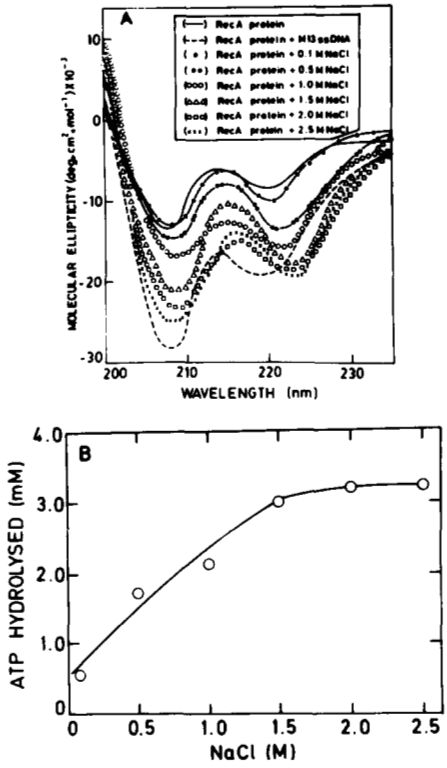

FIG. 6. **Salt-induced changes in the helical content and ATP hydrolysis of RecA protein.** *A*, NaCl-induced secondary structure in RecA protein. The reactions were done in a standard buffer, containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM ATP, 0.8 μM RecA protein, and NaCl at the indicated concentrations. The control reaction contained 3.2 μM M13 ssDNA. Reaction mixtures were incubated for 10 min at 30 °C prior to the recording of the CD spectrum, and each spectrum represents the best fit of at least two determinations. *B*, correlation between alteration in secondary structure and activation of ATP hydrolysis by RecA protein. Reactions were performed as described under "Materials and Methods."

crease at 0.1 M NaCl, which steadily increased with increasing salt concentration and reached saturation at 1.5 M NaCl (Fig. 6*B*). To obtain quantitative data concerning the changes in α-helix at increasing amounts of NaCl, in the presence of ATP or ATPγS, we have estimated its content as described by Zhong and Johnson (1992). Consistent with the DNA-induced conformational alterations in RecA protein, there was a gradual increase, with increasing salt concentration, in the amount of α-helix (Table III). In conjunction with the studies of Pugh and Cox (1988) and DiCapua *et al.* (1990), these results provide compelling evidence that conformational changes in RecA protein, especially the induction of α-helix, are associated with the stimulation of ATPase activity. In an analysis similar to that described above, we also observed induction of α-helix in RecA protein in the presence of ATPγS instead of ATP (Table III). A simple interpretation of these results is that the high affinity state is a consequence of the ability of RecA protein to acquire crucial amounts of α-helix.

TABLE III

Quantitation of solution conformational states of RecA protein in the presence of increasing amounts of NaCl

The CD spectra were recorded in a buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM NaCl (if not mentioned), 0.8 μM RecA protein, 3.2 μM M13 ssDNA (were mentioned), and 0.5 mM ATP or ATPγS.

	α-Helix	
	ATPγS	ATP
	%	%
RecA protein	28	23
RecA protein-M13 ssDNA	60	53
RecA protein in the presence of		
0.1 M NaCl	36	27
0.5 M NaCl	40	41
1.0 M NaCl	48	48
1.5 M NaCl	55	53
2.0 M NaCl	60	56
2.5 M NaCl	60	57

DISCUSSION

A number of biochemical and structural studies have propelled the idea, originally proposed by Howard-Flanders *et al.* (1984), that RecA protein contains two DNA-binding sites, one for the binding to ssDNA and the second for binding to duplex DNA. Kubista *et al.* (1990) have identified a third weak ssDNA binding site in RecA protein. Previous studies have shown that binding of RecA protein is largely DNA sequence-nonspecific; however, it binds to DNA with different affinities, which are determined by the sequence composition of DNA (Kowalczykowski, 1991). This sequence-dependent binding of RecA protein to ssDNA is reflected by its ability to promote hydrolysis of ATP (Weinstock *et al.*, 1981; Brenner *et al.*, 1987; Amaratunga and Benight, 1988). Nonetheless, no information is available to correlate, at the molecular level, the observed differences between sequence-dependent DNA binding, hydrolysis of ATP, and possibly other activities of RecA protein.

A fundamental requirement of homologous pairing and strand exchange reactions promoted by RecA protein depends on its ability to bind to single-stranded DNA. Further, these processes are dependent on the structural state of the nucleoprotein filament comprised of RecA protein-DNA. A number of studies have shown that the binding of RecA protein to ssDNA is nonspecific, rapid, and cooperative resulting in the formation of a nucleoprotein filament. However, its affinity to different polynucleotide substrates varies considerably (reviewed in Kowalczykowski (1991)). The binding of RecA protein to ssDNA is impeded by secondary structure in ssDNA; this barrier to binding is overcome by *E. coli* single-stranded binding protein (Muniyappa *et al.*, 1984). Under physiological conditions the binding of RecA protein to duplex DNA is slow but could be enhanced by altering the physical status of DNA (Kowalczykowski, 1991). The nucleoprotein filament comprised of double-stranded or ssDNA-RecA protein has a right-handed helical form with a deep helical groove. The polymerization of RecA protein onto DNA and the initial recognition of homologous sequences occur through the minor grooves (Kumar and Muniyappa, 1992; Jain *et al.*, 1992).

The polymerization of RecA protein onto DNA and the morphology of nucleoprotein filaments comprised of RecA protein-DNA is governed by the nature of nucleotide cofactors. In the absence of ATP or in the presence of ADP, RecA protein forms a compressed nucleoprotein filament that is characterized by a helical pitch ranging from 55 to 70 Å, a diameter of approximately 120 Å, and an internucleotide distance of 1.8–2.5 Å (Radding, 1991; Stasiak and Egelman, 1988). By contrast, the nucleoprotein filament formed in the presence of ATP or ATPγS has a helical pitch of 90–95 Å, is 100 Å in diameter, is extended 1.5 times relative to duplex DNA, and contains about six monomers of RecA protein per turn. This unprecedented RecA protein-induced stretching of DNA results in the separation of bases from 3.4 Å in the B-form structure to 5.1 Å in the nucleoprotein filament (Ko-

walczykowski, 1991). A number of structural and biochemical studies have established the occurrence of at least two different polymeric states of RecA protein: a high affinity state, which exists in the presence of ATP or ATPγS and is characterized by the extended nucleoprotein morphology, and a low affinity state, which predominates in the absence of nucleotide cofactors or in the presence of ADP. In addition, RecA protein under certain conditions assembles spontaneously, in the absence of DNA, to produce helical polymers, but the general feature of the filament corresponds to the low affinity state. Further, the binding affinity of RecA protein for adenine dinucleotides corresponds with their ability to induce the high affinity state. The extended nucleoprotein filament promotes homologous pairing as well as supports the cleavage of LexA repressor whereas no function has been established for the compressed filament. RecA protein effectively hydrolyzes all of the NTPs in the presence of DNA, but processes such as DNA strand exchange and repressor cleavage occur efficiently only in the presence of adenine nucleotides such as ATP, dATP, and ATPγS. Correspondingly, it has been suggested that these nucleotide cofactors perhaps induce the high affinity state while other NTPs induce the low affinity state (Kowalczykowski, 1991; Roca and Cox, 1990).

Yu and Egelman (1992), while describing the nature of compressed nucleoprotein filaments, have also observed structural differences among nucleoprotein filaments formed on ssDNA in the absence of ADP. Our interpretation of these results would be that the differences in morphology relate to the conformational changes described here at the molecular level. Consistent with these changes, it has been shown that in the presence of a nucleotide cofactor, the bases are oriented perpendicular to the axis, while in its absence they are randomly oriented. The above studies suggest that there are likely to be conformational alterations associated with the formation of nucleoprotein filament.

The conformational rearrangement that RecA protein undergoes consequent to the formation of nucleoprotein filaments is evidenced by the dramatic changes in CD signals. While the major structural rearrangements may be necessary for RecA protein to form active extended nucleoprotein filaments, the deduced amounts of α-helix in RecA protein in the absence of DNA seem to differ from those of the crystal data. Nonetheless, these results are not necessarily at odds; the differences between these two studies may be simply due to differences in experimental conditions. The helical symmetry defined for the crystal structure (6 monomers per helical form of 82.7 Å) is similar to that of the helical symmetry of a self-polymer (5–6 monomers per helical turn of 70–75 Å), whereas the extended nucleoprotein filament has a helical pitch of 97 Å. More importantly the formation of extended nucleoprotein filaments requires the presence of natural DNA and ATP or ATPγS, both of which were absent from the crystal structure. Alternatively, the structural features of the monomer in the crystal correlate between the compressed inactive self-polymer and the active extended nucleoprotein filament.

Most importantly the results presented here enlighten the subtleties of the heterogeneity of the nucleoprotein filaments comprised of RecA protein and a variety of DNA substrates with varying base composition. The degree of differences is revealed by the extent of induction of α-helix in RecA protein by different DNA substrates. Among the substrates tested, except for poly(dG)·poly(dC) and related polymers that are rich in GC sequence, other substrates were able to induce varying amounts of α-helical structure. The question that arises from the above results is the following: what is the molecular basis of induction of different levels of α-helical structure by these DNA substrates if binding of RecA protein primarily involves the phosphate backbone? We propose that the DNA sequence induced conformational transition is likely due to base geometry differences in the respective DNA substrate(s).

It could be argued that the large increase in the content of α-helix in the presence of DNA may arise from self-polymerization of the RecA protein. This possibility may seem likely especially since 2 M NaCl, although nonphysiological, was as effective as polynucleotide(s) in inducing structural alterations in the RecA protein. However, it is important to note that these conditions abolish the binding of RecA protein to polynucleotide substrates. Our conclusion that binding of DNA to RecA protein results in the increase of α-helix but not as a consequence of self-polymerization or contributions from contacts between monomers in the helical filament is based on the following: RecA monomers can assemble spontaneously, under certain conditions, at a concentration of protein (>100 μg/ml) in the presence of polycations (Morrical and Cox, 1985; Heuser and Griffith, 1989). However, this process is inhibited by nucleotide cofactors (Griffith and Harris, 1988). The reaction conditions used in this study do not represent either of these situations; however, we cannot eliminate the possibility of increase in α-helicity due to interactions between monomers in the nucleoprotein filament. Nonetheless, in each experiment we have shown a CD spectra obtained for native RecA protein that has a molecular ellipticity of -12×10^{-3} deg·cm^2·mol^{-1} at 208 nm compared with -29×10^{-3} deg·cm^2·mol^{-1} when bound to natural DNA substrates. More importantly, we would not have observed DNA base composition-dependent changes in CD spectra, if self-polymerization or contacts between monomers is the cause for the increase in α-helicity of RecA protein.

It has been demonstrated that the basic motifs of several transcriptional activator proteins exist as aperiodic structures, which enable them to bind to DNA in a sequence-specific manner (O'Neil *et al.*, 1991; Patel *et al.*, 1990; Weiss *et al.*, 1990). A classic example is the DNA binding motif of λ repressor, which is disordered in the free protein. The arm interacts with the "back side" of the DNA helix and thereby determines the specificity of the repressor (Jordan and Pabo, 1988). However, the binding of RecA protein is quite different; its binding to DNA is sequence-nonspecific and has been shown to involve the phosphate backbone (Leahy and Radding, 1986). The data presented here suggest that both the base sequence and the presence of a nucleotide cofactor play important role(s) in determining the conformational state of RecA protein in different nucleoprotein filaments. Furthermore, the ability of high salt to stimulate ATPase activity of RecA protein is seemingly not a trivial phenomenon but is connected with the molecular changes in the three-dimensional structure of protein. One of the most intriguing questions, to our knowledge, which has not been addressed previously, is how multiple activities of RecA protein are manifested when it binds to DNA substrates. These results suggest that the ability of different nucleotide cofactors and DNA substrates to induce the extent of α-helix perhaps may coincide with a particular activity among many activities of RecA protein. More importantly, it appears that the binding of RecA protein to DNA is necessary but not sufficient for the manifestation of all of its activities. For instance, RecA protein binds to all of the DNA substrates with a stoichiometric ratio of 1 monomer/4 nucleotides to produce nucleoprotein filaments, but its ability to induce α-helical conformation (this study) and to promote strand transfer activity is im-

paired by substrates containing GC sequence (Gruss *et al.*, 1991).

Many of the conformational changes, the increase in the α-helical content in RecA protein during the formation of nucleoprotein filaments, and recognition of homologous DNA sequences can be rationalized with the data from the crystal structure (Story *et al.*, 1992). Consistent with the proposal of Story *et al.* (1992), it is possible that, in the presence of ATP and DNA, the allosteric state of RecA protein would be different from that of the crystal structure. Natural DNA sequences such as M13 and φX174 and synthetic homopolymers such as poly(dA), poly(dC), and poly(dT) were much more potent than poly(dC)·poly(dG) and related polymers rich in GC sequences in inducing α-helicity in RecA protein. It has been suggested that the proteins that bind to DNA take on α-helical conformation over other types of secondary structures. Such extensive conformational differences, which result in increased α-helicity, may be the basis for the occurrence of two different states of RecA protein: a high affinity state and a low affinity state. Thus, it is likely that, in the absence of DNA, the basic motifs of RecA protein exist in flexible nonhelical conformation so as to curtail intradomain repulsive electrostatic interactions. In an anionic environment, such as in the presence of DNA, interaction between the positively charged motifs of the proteins and the phosphodiester backbone stabilizes the conformation resulting in the overall increase in the α-helical content in RecA protein. Correspondingly, heterologous duplex DNA induces significantly lower amounts of α-helix in RecA protein (Fig. 5). This partial helical conformation may be sufficient to allow RecA protein to efficiently search for homologous sequences. Upon location of homologous sequences, the second DNA binding motif of RecA protein perhaps becomes completely α-helical to permit specific interactions.

In summary, the binding of both DNA and a nucleotide cofactor set in a "conformational domino effect" (Milburn *et al.*, 1990) in RecA protein suggesting that the whole molecule is conformationally linked and that a change induced in one domain of the molecule may be propagated into an adjacent domain. These observations, we believe, have immense implications in interpreting the atomic structure (Story *et al.*, 1992), *vis à vis* the dynamic state of RecA protein in the polymer. In addition, or more importantly, these observations provide a basis for thinking about the biochemical changes that RecA protein experiences during the formal steps of homologous genetic recombination.

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