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Extent of Duplex DNA Underwinding Induced by RecA Protein Binding in the Presence of ATP

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A method is described for the accurate determination of the superhelical density (σ) of highly underwound circular DNA molecules. Using this method, duplex DNA bound by RecA protein in the presence of ATP at pH 7·5 is found to be underwound by 39·6% ($\sigma = -0{\cdot}396$), corresponding to a helical periodicity of 17·4 base-pairs per turn. The underwinding is increased to 41% (17·9 base-pairs per turn) in the presence of low levels of ATPγS, in good agreement with the 18·6 base-pairs per turn reported previously. In spite of the extensive underwinding, the distribution of DNA topoisomers produced by RecA protein binding is small. This indicates a high degree of structural uniformity among RecA–double-stranded DNA complexes in the presence of ATP.

1. Introduction

The RecA protein of *Escherichia coli* promotes key steps in homologous genetic recombination. This is manifested *in vitro* by RecA protein-promoted DNA strand exchange reactions (for reviews, see Cox & Lehman, 1987; Radding, 1982; Dressler & Potter, 1982). Central to these activities is the binding of RecA protein to duplex DNA (dsDNA‖). In a process requiring ATP or the non-hydrolyzed analog ATPγS, RecA protein binds to duplex DNA co-operatively and contiguously. The DNA in the resulting nucleoprotein filament is extended and extensively underwound (Di Capua *et al.*, 1982; Stasiak & Di Capua, 1982; Pugh & Cox, 1987).

Most of what is known about the structure of this complex has come from electron microscopy. In the presence of ATPγS, RecA forms very stable complexes on duplex DNA that are approximately 50% longer than the DNA molecule itself prior to binding (Di Capua *et al.*, 1982; Stasiak & Di Capua, 1982). The RecA protein forms a right-handed helical complex with a pitch of 9 nm or one turn for every 18·6 base-pairs of DNA (Di Capua *et al.*, 1982; Egelman & Stasiak, 1986). Because binding of RecA protein requires underwinding of the DNA, RecA protein binding will be topologically limited in a covalently closed circular DNA molecule. By determining the fraction of DNA bound by RecA protein in the presence of ATPγS for a series of cccDNA molecules with known superhelical densities, Stasiak & Di Capua (1982) were able to obtain the superhelical density of completely bound DNA by extrapolation. This showed that the DNA helix in the complex follows the protein helix and has 18·6 bp per turn, corresponding to a superhelical density (σ) of $-0{\cdot}43$.

Since ATPγS inhibits DNA strand exchange, it is not clear whether this information applies to the physiologically active complex. RecA–dsDNA complexes formed in the presence of ATP are dynamic, making them appear relatively unstable. They can appear contiguous, fragmented, or absent altogether in the electron microscope depending on sample preparation procedures (Dunn *et al.*, 1982; Shaner *et al.*, 1987). Independent measurements *in vitro* by non-disruptive methods indicate extensive binding and underwinding of DNA in the presence of ATP (Pugh & Cox, 1987, 1988; Kowalczykowski *et al.*, 1987). In a nicked circular DNA (FII), this underwinding can be trapped topologically by ligating the nick while the RecA protein is bound (Shibata *et al.*, 1981; Pugh & Cox, 1987). The underwound DNA resulting after removal of all

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‖ Abbreviations used: dsDNA, duplex DNA; cccDNA, covalently closed circular DNA; bp, base-pair(s); kb, 10^3 bases or base-pairs.

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protein is called form X or FX DNA (Shibata *et al.*, 1981). An accurate determination of the extent of DNA underwinding in form X DNA would provide an important clue to the structure and dynamic state of this complex while it is engaged in ATP hydrolysis.

Direct determination of the superhelical density of highly underwound DNA molecules has been hampered by technical limitations. The band counting method of Keller (1975) is precise but generally has been applied to DNA with modest superhelical densities. Electron microscopy has been shown to be accurate, but again for DNA molecules with a relatively low superhelical density (Sperrazza *et al.*, 1984). Electron microscopy has also been used to estimate very high degrees of underwinding by determining the extent of strand separation under denaturing conditions (Baker *et al.*, 1986), although the error in these observations is uncertain. In the present study, two techniques are combined to overcome these problems and provide a precise determination of σ when high levels of superhelical density are present. We demonstrate that the number of helical turns in duplex DNA bound by RecA protein is reduced by 39·6% under conditions optimal for RecA-mediated DNA strand exchange (pH 7·5, ATP).

2. Materials and Methods

(a) *Reagents*

RecA protein was purified to homogeneity as described (Cox *et al.*, 1981). The concentration of RecA protein in stock solutions was determined by absorbance at 280 nm, using an extinction coefficient of $\varepsilon_{280} = 0{\cdot}59\ A_{280}$ mg^{-1} ml (Craig & Roberts, 1981). The concentration of the ethidium bromide stock solution was determined at 480 nm, using an extinction coefficient of $\varepsilon_{480} = 5{\cdot}600$ M^{-1} cm^{-1} (Waring, 1965; Baase & Johnson, 1979).

The plasmid pEAW3 (2426 bp) is derived from pBR322 (Maniatis *et al.*, 1982). It was constructed by removal of the 18 bp *Bam*HI–*Eco*RI fragment from plasmid pT7-5 (2404 bp) (S. Tabor, personal communication) and inserting a 40 bp fragment containing a recognition site for the yeast FLP recombinase (E. A. Wood, unpublished results). The 8·7 kb plasmid pBR322-2 (a dimer of pBR322) was described by Pugh & Cox (1987). Plasmid purification was carried out as described (Davis *et al.*, 1980). The concentrations of dsDNA stock solutions were determined by absorbance at 260 nm, using 50 μg ml^{-1} A_{260}^{-1} as a conversion factor.

FII DNA was prepared from FI DNA using DNase I (Sigma, type II) according to the method of Shibata *et al.* (1981). Greater than 99% of the DNA was present as nicked circles as determined from scanning densitometry of photographic negatives of DNA samples electrophoresed into agarose gels and stained with ethidium bromide. This method generates FII DNA containing a single nick.

FX DNA was prepared at pH 7·5 in the presence of 10 mM-Mg^{2+} and 1 mM-ATP (*in vitro* conditions optimal for DNA strand exchange) by the pH-shift method described (Pugh & Cox, 1987). As adapted here, 200 μM-RecA protein and 20 μM-pEAW3 FII DNA were incubated for 30 min in a solution containing 10 mM-magnesium acetate, the ATP, 20 mM-sodium maleate (74% dianion, pH 6·35), 1 mM-dithiothreitol, 5% (v/v) glycerol, and an ATP regenerating system (2·24 units/ml pyruvate kinase, 2·34 mM-phosphoenolpyruvate, and 0·44 mM-KCl). The solution was shifted to pH 7·5 by dilution 1 : 1 into a solution containing 20 mM-Tris·HCl (50% cation, pH 8·1 before mixing) and all remaining components held constant except RecA protein and DNA. After 30 min, phage T4 DNA ligase (1 mg/ml) final concentration) was added and the incubation continued for 60 min. The solution was extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) and covalently closed DNA was isolated by banding twice in CsCl/ethidium bromide gradients (Maniatis *et al.*, 1982). Under these conditions the ligation efficiency is ~60% and the only detectable form of covalently closed DNA is form X. When 7 μM-ATPγS was added in addition to ATP, the efficiency of ligation was reduced to ~10%. Higher concentrations of ATPγS completely inhibited ligase activity. Again, form X DNA is the only covalently closed DNA detected.

Marker topoisomers with $\sigma = 0$ to $-0{\cdot}31$ were prepared using 100 μM-FI pEAW3 DNA, 100 units of *E. coli* topoisomerase I ml^{-1} (Bethesda Research Labs), 50 mM-Tris·HCl (pH 7·5, 80% cation), 10 mM-$MgCl_2$, 50 mM-KCl, 0·1 mM-EDTA, 0·5 mM-dithiothreitol, and the indicated ethidium bromide concentration. Incubations were at 37 °C for 60 min. Reactions at high ethidium bromide concentrations (> 80 μM) required more topoisomersase I (typically 1000 units ml^{-1}) and longer incubations (3 h). Marker topoisomers were also generated under conditions described above but using FII DNA and $1{\cdot}6 \times 10^4$ units phage T4 DNA ligase ml^{-1} (New England Biolabs), instead of FI DNA and topoisomerase I. Both methods produced the same result. The most underwound topoisomer ($\sigma = 0{\cdot}31$) was obtained at 200 μM-ethidium bromide. Above this concentration of ethidium bromide, ligase and topoisomerase I were completely inhibited. To obtain higher superhelical densities ($\sigma = -0{\cdot}3$ to $-0{\cdot}4$), RecA protein was used to underwind the DNA under conditions permitting only a partial reaction of topoisomerase I. Such reactions contained 30 μM-FI pEAW3 DNA, 25 μM-RecA protein, 50 mM-Mes-KOH (pH 6·0), 20 mM-$Mg(CH_3COO)$, 2 mM-ATP, an ATP regenerating system (70 mM-phosphoenolpyruvate, 120 units pyruvate kinase ml^{-1} (type IV, rabbit muscle; Sigma), and approximately 10^3 units topoisomerase I ml^{-1}. Reactions were incubated at 37 °C. Portions were removed every 30 min for up to 3·5 h and treated with 0·5% (w/v) SDS, 25 mM-EDTA, and 50 μg proteinase K ml^{-1} (Sigma) for 10 min. The samples were then extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.).

(b) *One-dimensional gel electrophoresis*

Agarose gel electrophoresis was performed in TAE buffer (40 mM-Tris-acetate (pH 7·5), 1 mM-EDTA) and the indicated concentration of ethidium bromide. In all cases, ethidium bromide was added to both the gel and running buffer, the buffer was circulated at a rate of 3 ml min^{-1}, and run overnight in the dark. The gels were composed of 1·5% (w/v) agarose and were 0·3 cm thick. Gels stained with ethidium bromide were illuminated with ultraviolet radiation and photographed. The photographic negatives of the gels were scanned with a Zeineh Soft Laser scanning densitometer, SL-504-XL, obtained from Biomed Instruments, Inc.

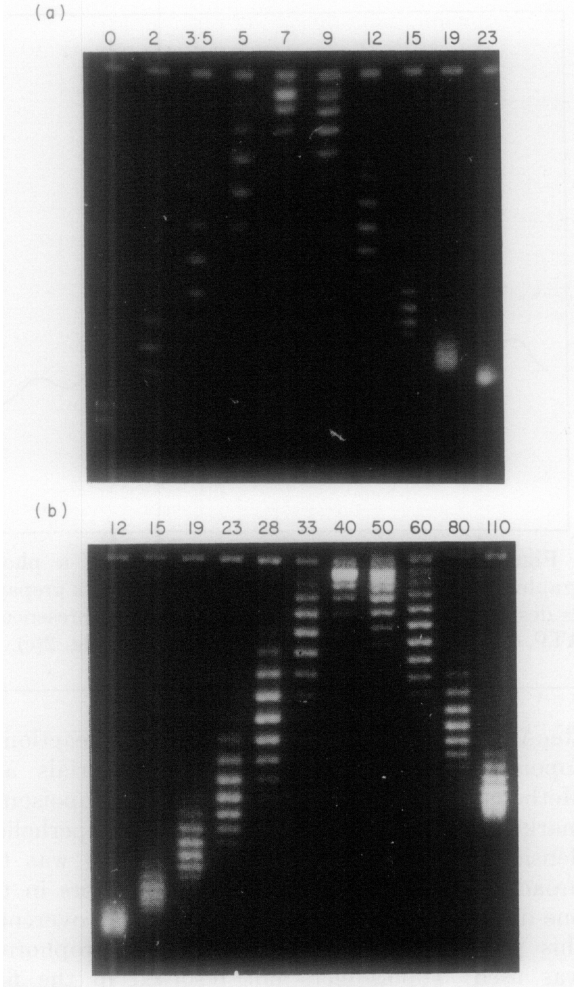


Figure 1. Topoisomers of pEAW3 formed in the presence of varying concentrations of ethidium bromide. One-dimensional electrophoresis was carried out as described in Materials and Methods. DNA samples were electrophoresed in the presence of either (a) 0·1 μM or (b) 1·1 μM ethidium bromide. The numbers above each lane correspond to the concentration of ethidium bromide (μM) used to generate each set of topoisomers.

(c) *Two-dimensional electrophoresis*

Agarose gel electrophoresis in the indicated concentration of ethidium bromide was performed essentially as described above. DNA topoisomers were mixed as indicated, precipitated with ethanol, and resuspended in a small volume of TE buffer (10 mM-Tris·HCl, 1 mM-EDTA (pH 7·6)). After adding loading buffer (5% glycerol, 1 mM-EDTA, 0·25% (w/v) bromophenol blue), 5 μl of the DNA mixture (at a concentration of 2 to 5 μg ml^{-1} final) was loaded into a 1/16 in diameter well. In an adjacent well, a topoisomer marker, delineating the minimum underwinding of the sample (right side in each panel; Fig. 2) was loaded. After 30 min of electrophoresis, a topoisomer marker, delineating the maximum underwinding of the sample (lower left side in each panel; Fig. 2), was loaded into the same well as the mixed topoisomers. After 17 h of electrophoresis, a section of the gel containing the 2 lanes of DNA was removed, rotated clockwise by 90°, and placed on the gel bed at the negative electrode end. The remainder of the gel bed was filled to a height of 0·3 cm with agarose containing the indicated concentration of ethidium bromide and electrophoresed in the 2nd dimension.

3. Results

The method used to examine the superhelical density of form X DNA involved an extension of the band counting method of Keller (1975). Accurate results were obtained by combining the band counting method with two-dimensional gel electrophoresis. Results obtained with one-dimensional electrophoresis are also presented to provide technical comparisons and background. As described in Materials and Methods, the form X DNA was generated in the presence of ATP at pH 7·5 and 37 °C.

(a) *The limitations of band counting with one-dimensional electrophoresis*

The band counting method relies on the agarose gel resolution of DNA topoisomers differing by a single superhelical turn. Overlapping sets of topoisomer markers are generated to span a range extending from $\sigma = 0$ (relaxed) to the superhelical density of the sample. Each marker set, produced as described in Materials and Methods, represents a narrow Gaussian distribution of topoisomers centered about a superhelical density characteristic of the conditions used to generate it. Quantification of this distribution by laser densitometry allows specific topoisomers to be identified from gel to gel. All the members of each set must be resolved in at least one gel. Often several gels, run in the presence of varying concentrations of intercalating agents, are used to resolve all sets. By counting bands from one set to another (lane to lane) in a given gel, and from one gel to the next as the resolution limit of a gel is reached, the number of superhelical turns in a sample can be determined accurately. This technique has been used to determine σ values for DNAs with natural superhelical densities such as *E. coli* plasmids (FI DNA) (Shure *et al.*, 1977; Schutte & Cox, 1988) and simian virus 40 (SV40) DNA isolated from monkey cells (Keller, 1975). The superhelical density of form X DNA is estimated to be six to ten times greater than FI DNA (Pugh & Cox, 1987). We attempted to extend the technique to higher superhelical densities by using a relatively small plasmid (pEAW3, 2426 bp) to generate form X DNA. With ten separate gels, 72 topoisomers ($\sigma = -0{\cdot}31$) were counted. Two of these gels are presented in Figure 1. Inhibition of DNA ligase and *Eco* topoisomerase I by ethidium bromide prevented the generation of marker topoisomer sets with greater superhelical densities. There was also an error of $\pm 0{\cdot}5$ to 1·0 superhelical turn associated with each gel comparison, so that the final number has an intrinsic error of about 5%. This experiment, therefore, could only establish a limit, demonstrating that form X DNA had a superhelical density greater than $-0{\cdot}31$.

(b) *The superhelical density of form X DNA*

To generate topoisomers of superhelical densities greater than $-0{\cdot}31$, DNA was underwound with

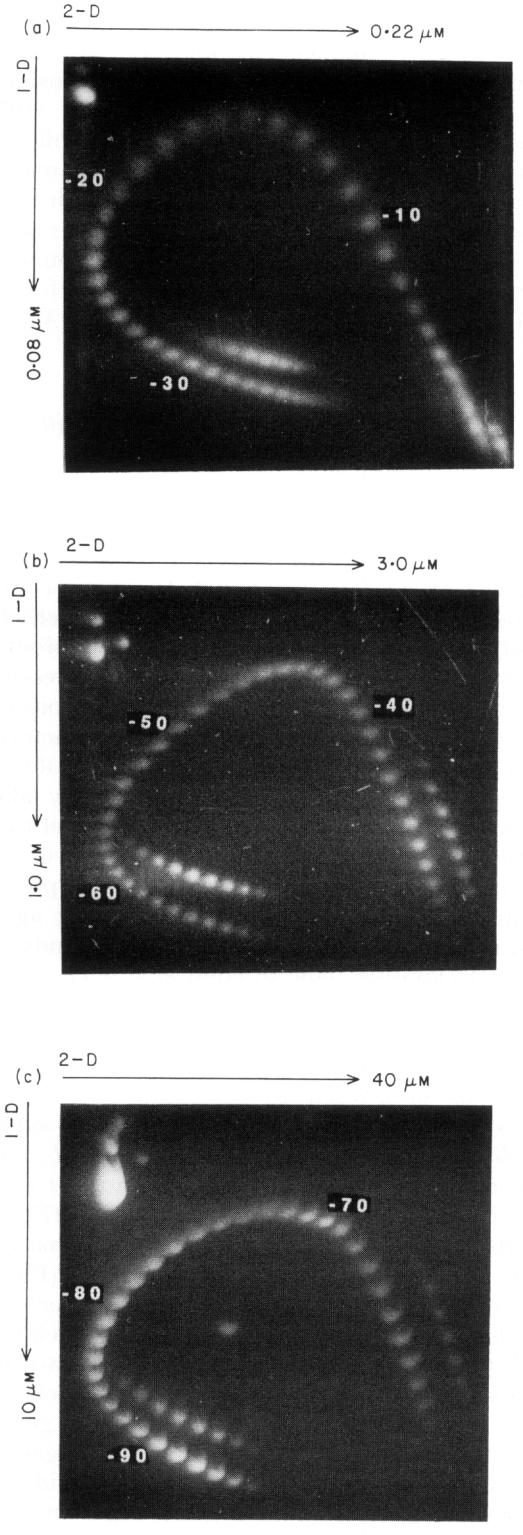


Figure 2. Two-dimensional agarose gel electrophoresis of topoisomers of pEAW3. Topoisomers were electrophoresed in the presence of the indicated concentration of ethidium bromide in the indicated dimension. Topoisomers, prepared over a range of concentrations (Fig. 1) were combined ((a) 0 to 28; (b) 23 to 110; (c) 110 to FX) and electrophoresed as described in Materials and Methods. Marker topoisomers (offset from the continuum on the right and left side of the gel, respectively) were prepared in the presence of the following concentrations (μM) of ethidium bromide: (a) 0, 23; (b) 23, 110; (c) 110. The lower left marker in (c) is FX DNA.

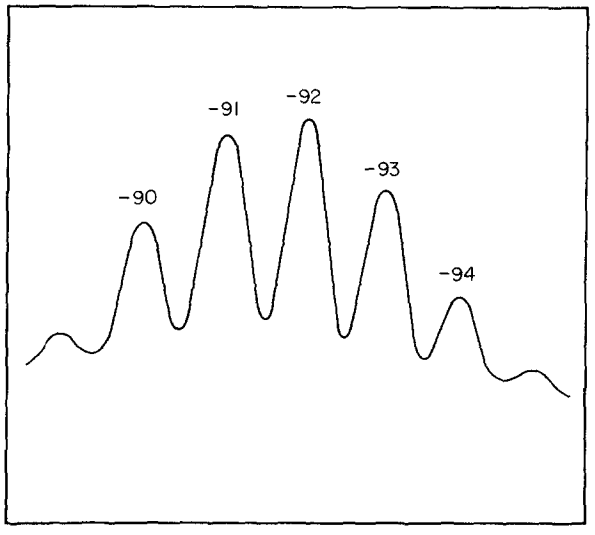


Figure 3. Scanning densitometer trace of a photographic negative of form X DNA. The DNA was prepared as described in Materials and Methods in the presence of ATP, and is the same as the DNA shown in Fig. 2(c).

RecA protein under conditions of partial reaction of topoisomerase I as described in Materials and Methods. This produced a range of topoisomer markers that extended up to the superhelical density of form X, but the distribution was too broad for resolution of all the topoisomers in the one-dimensional gels described above. To overcome this problem, two-dimensional gel electrophoresis was used. Topoisomers not resolved in the first dimension are separated in a second dimension run in the presence of a different concentration of ethidium bromide. This permitted the resolution of over 30 topoisomers on a single gel, and counting bands from the relaxed form of pEAW3 to form X required only three gels (Fig. 2). This also reduced the degree of error significantly. In this range, 92 topoisomers were counted to reach the average superhelical density of the pEAW3 form X species, and 95 topoisomers were counted to reach the maximally underwound topoisomer in the form X group. This corresponds to an average σ of $-0{\cdot}396$ ($\pm 0{\cdot}005$) and a maximum of $-0{\cdot}411(\pm 0{\cdot}005)$ for form X DNA. A superhelical density of $-0{\cdot}396$ translates into a helical periodicity (average) of 17·4 base-pairs per turn or an unwinding angle of 13·6° per bp. Thus, in the presence of ATP, RecA protein underwinds DNA by 39·6%. On the basis of DNA binding stoichiometry of one RecA monomer per 4 bp (Pugh & Cox, 1987; Shaner & Radding, 1987), this corresponds to 4·4 RecA monomers per turn of the filament. On the basis of stoichiometry of one RecA monomer per 3 bp (Di Capua *et al.*, 1982), this corresponds to 5·8 RecA monomers per filament turn.

Significantly, the range of topoisomers found in this form X DNA is narrow (Fig. 3). In spite of the size of the complexes (at least 600 RecA monomers per complex if all DNA is bound) and extensive degree of DNA underwinding, nearly 90% of the

DNA is found in only four topoisomers. The distribution is Gaussian. This indicates a high degree of structural homogeneity from one complex to the next, even in the presence of ATP.

The tight topoisomer distribution is not unique to the conditions used to generate this FX DNA (pH shift, RecA protein in excess). The fraction of DNA converted to form X was reduced somewhat when stoichiometric levels of RecA protein were used (1 RecA per 3 to 4 bp) but the FX topoisomer distribution was unaffected (data not shown). A similar tight distribution of form X topoisomers was also found for form X DNA formed at pH 6·35 (data not shown).

Stasiak & Di Capua (1982) determined the helicity of DNA complexed with RecA protein in the presence of ATPγS to be 18·6 bp per turn or an unwinding angle of 15° per bp. This corresponds to a σ value of −0·43. We find that in the presence of ATP and limiting amounts (7 μM) of ATPγS (higher amounts completely inhibit ligation or topoisomerase activity), four additional helical turns are removed from pEAW3 and σ increases to −0·41 (not shown). This corresponds to a helicity of 17·9 bp per turn, approaching the value obtained by Stasiak & Di Capua (1982). This demonstrates that there is a small but real increase in DNA underwinding in the presence of ATPγS relative to that found with ATP. The distribution of topoisomers found for form X DNA produced in the presence of ATPγS is essentially identical to that observed in the presence of ATP (data not shown). The results of Stasiak & Di Capua (1982) were obtained with higher levels of ATPγS than we were able to use, and it is possible that the slight difference in the two sets of results ($\sigma = -0{\cdot}41$ *versus* −0·43) is significant.

4. Discussion

When RecA protein binds to duplex DNA in the presence of ATP under conditions optimal for DNA strand exchange, the number of helical turns in the DNA is reduced by 39·6%. This corresponds to a DNA unwinding angle of 13·6° per bp and a helical twist of 17·4 bp per turn. Upon the addition of small amounts of ATPγS, there is a relative increase of ~4% in these parameters, to an underwinding of 41% and a helical twist of 17·9 bp per turn. There is a narrow distribution of topoisomers in form X DNA, indicating a high degree of structural uniformity among RecA–dsDNA complexes in the presence of ATP.

The method used to obtain these data is very precise and the equipment required is widely available. The technique was applied to a plasmid 2426 bp in length with a σ value of −0·396. With ethidium bromide as the intercalating agent during electrophoresis, we estimate that these two-dimensional gels should be able to resolve topoisomers with σ value as high as −0·5. These topoisomers may not resolve easily if much larger DNA molecules are used, placing one potential constraint on the procedure.

The degree of underwinding in the RecA–dsDNA complex is an important parameter for understanding this structure. There are two additional observations with mechanistic significance. These are (1) the difference in the underwinding observed with ATP as opposed to ATPγS, and (2) the limited distribution of form X topoisomers. The increase in underwinding induced by ATPγS is small but real. The result with ATP must reflect the state of a complex actively engaged in ATP hydrolysis. ATP is hydrolyzed by monomers throughout this complex (Brenner *et al.*, 1987; Schutte & Cox, 1987; Pugh & Cox, 1987) with a k_{cat} value of ~20 to 25 min^{-1} (Pugh & Cox, 1987). The slight decrease in DNA underwinding relative to that found within the very stable ATPγS filament may reflect some degree of RecA dissociation at steady-state. Alternatively, it could reflect a slight collapse of the filament for a subset of the RecA monomers in it (e.g. a few RecA monomers may be in a different conformation at any given point in time by virtue of binding to ADP during the ATP hydrolytic cycle). The homogeneity in the degree of underwinding in form X DNA implies that the structural cause of the ATP–ATPγS underwinding difference is essentially constant from one complex to the next. If dissociation is occurring, it is limited and constant among complexes at steady-state. In electron micrographs, RecA–dsDNA complexes formed with ATP often appear fragmented and heterogeneous, and they are not extended relative to the length of the bound DNA (Shaner *et al.*, 1987). Our data indicate that these electron microscopic observations must reflect the harsh conditions used for sample preparation and do not accurately depict the state of these complexes in solution. On the basis of observations of RecA protein complexes on ssDNA, Egelman & Stasiak (1986) concluded that the state of the RecA filament changes little when ATP replaces ATPγS. Our results indicate that the difference is indeed small.

The structural uniformity in form X DNA also provides some information about the dynamic state of the complexes. The observation of two types of complexes in electron micrographs, the extended complexes described in the Introduction (with ATPγS), and "collapsed" filaments observed without nucleotide cofactors or sometimes with ATP, might suggest the possibility that the filaments expand and contract in concert with an ATP hydrolytic cycle that is synchronized for RecA monomers within a filament (see e.g. data of Dunn *et al.*, 1982; Williams & Spengler, 1986). The structural uniformity we observe indicates that either this does not occur, or the "collapsed" state is kinetically insignificant at steady-state.

The mechanism of RecA protein-promoted DNA strand exchange has not yet yielded to extensive efforts to elucidate it. The DNA underwinding observed in the present study provides a structural

constraint for these ongoing mechanistic considerations.

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