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Enhanced recA Protein Binding to Z DNA Represents a Kinetic Perturbation of a General Duplex DNA Binding Pathway*

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recA protein binding to duplex DNA is enhanced when a B form DNA substrate is replaced with a left-handed Z form helix. This represents a kinetic rather than an equilibrium effect.

Binding to Z DNA is much faster than binding to B DNA. In other respects, binding to the two DNA forms is quite similar. recA protein binds to B or Z DNA with a stoichiometry of 1 monomer/4 base pairs. The final protein filament exhibits a right-handed helical structure when either B or Z form DNAs are bound. There are only two evident differences: the k_{cat} for ATP hydrolysis is reduced 3–4-fold when Z DNA is bound, and recA binding at equilibrium is less stable on Z DNA than on B DNA. At steady state, the binding favors B DNA in competition experiments. The results indicate that Z DNA binding by recA protein follows the same pathway as for recA binding to B DNA, but that the nucleation step is faster on the Z form helix.

The recA protein of *Escherichia coli* is essential for the central events of genetic recombination and for induction of the SOS repair pathway *in vivo* (1–3). These functions are reflected *in vitro* by the DNA strand exchange and lexA cleavage activities, respectively, exhibited by this protein (1–6). At the heart of these activities is the interaction of recA protein with DNA. Under conditions optimal for DNA strand exchange (pH 7–8), recA protein binds to single-stranded DNA much faster than to duplex DNA. The binding is stoichiometric, resulting in a filamentous nucleoprotein complex containing 1 recA monomer/3–4 nucleotides of DNA. In the electron microscope, this complex has a distinct right-handed helical structure (7–11). recA protein is also a DNA-dependent ATPase. When ATP (or the analogue ATPγS)[1] is present, the complex is extended about 50% relative to B form duplex DNA. This is the active species that interacts with a homologous duplex DNA to bring about strand exchange (1).

The direct binding of recA protein to duplex DNA is a relatively minor reaction at pH values above neutrality. This poor binding to duplex DNA, however, is a kinetic rather than an equilibrium effect. A general pathway for recA protein binding to duplex DNA has recently been outlined in some detail (12–14) (Fig. 1). The overall association pathway consists of at least three important steps: 1) a rapid pre-equilibrium that probably involves a weak interaction between recA protein and the DNA, 2) a nucleation step in which a recA monomer (or other small binding unit) binds tightly to the DNA, and 3) addition of additional recA protein to saturate the DNA (propagation), and form the nucleoprotein filament described above. The process requires ATP (or the analogue ATPγS). ATP is hydrolyzed in this complex with a k_{cat} of about 22 min^{-1} (12–14). The duplex DNA within the complex is extensively underwound so that 39.6% of the helical turns present in B form DNA are removed (15). Steps 2 and 3 are inextricably linked to the DNA underwinding (12).

The rate-limiting step in this process is nucleation (12) (step 2). The weak binding of recA to duplex DNA at higher pH values can be traced to the net uptake of 2 protons in the binding steps up to and including nucleation (12, 13). Propagation (step 3) is very rapid. Binding to form II DNA is stable even at pH values above 7, so that the apparent lack of binding observed in many studies reflects a long lag in binding rather than an inherent poor affinity. Virtually any sequence feature or structure that makes the DNA more "unwindable" will enhance the rate of nucleation, and thereby enhance the overall binding process (13). Binding is therefore enhanced by increases in DNA length (12), DNA ends (16), single-stranded gaps or tails (16–18), A-T-rich regions (14), underwinding of the DNA (12), and other structural perturbations (19, 20).

This is significant in light of observations that some types of DNA damage, as well as some altered DNA structures such as Z form DNA, are recombinogenic *in vivo* (2, 3, 21–23). In particular, a potential role for Z DNA in homologous genetic recombination has received much attention. A Z DNA forming sequence results in an increase in recombination frequency in *E. coli* when it is inserted into a plasmid (21). A repeating dT-dG sequence functions as an interaction site for aligning chromosomes in yeast (24). Recombination hot spots in eukaryotic genomes have often been traced to potential Z DNA forming sequences (25–29). These studies are complemented by observations *in vitro*. The recA-like protein rec1 from *Ustitago maydis* binds to Z DNA more readily than to B DNA (30–32). A strand exchange activity has been isolated from human cells by virtue of its tight binding to a Z DNA affinity matrix (33). Finally, the recA protein itself binds to Z DNA more readily than to B DNA as measured by filter retention of the DNA measured after 30 min of reaction (19).

There are at least two possible explanations for the enhanced binding of recA protein to Z DNA. The binding could

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[1] The abbreviations used are: ATPγS, adenosine 5′-(3-thio)triphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FI DNA, supercoiled closed circular form of a DNA molecule as isolated from *E. coli* cells; FII DNA, the nicked circular form of the same molecule; FIII DNA, the linear form of the same molecule; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

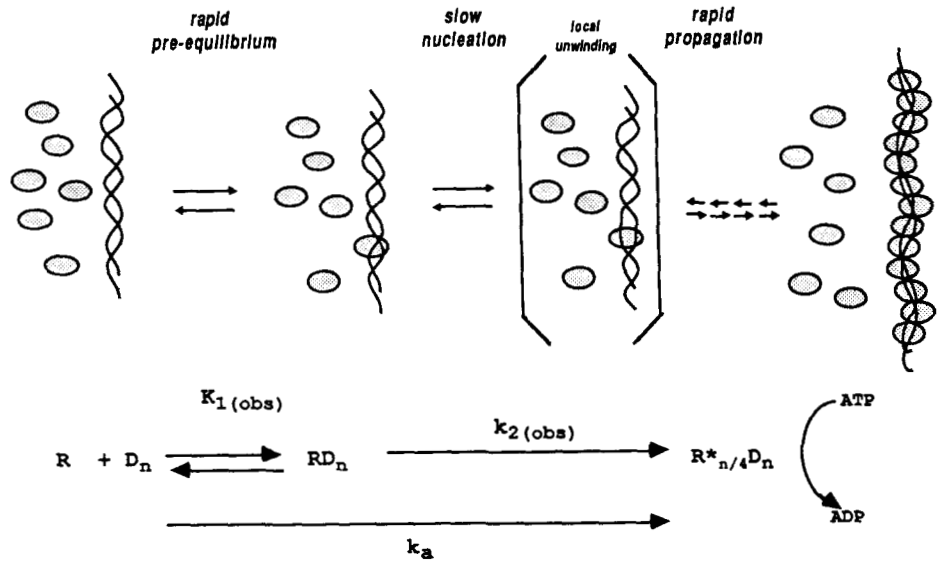


FIG. 1. **Binding pathway of recA protein to duplex DNA (from Ref. 13).** The general mechanism includes two kinetically important segments, a rapid pre-equilibrium and a slow nucleation step. *R* and *D* represent recA protein and DNA, respectively. The subscript *n* denotes the number of base pairs present in a given DNA molecule.

reflect an intrinsic high affinity for the Z form structure. Alternatively, binding to Z DNA could simply reflect an enhancement of the slow nucleation step in binding (*i.e.* a kinetic rather than an equilibrium effect) with little change in the overall pathway for binding or in the properties of the final nucleoprotein complex. We describe here the results of experiments that indicate that the second explanation generally accounts for the Z DNA-binding properties of recA protein.

EXPERIMENTAL PROCEDURES

Reagents—recA protein of *E. coli* was purified as described (34). The concentration of recA protein in stock solution was determined by absorbance at 280 nm, using an extinction coefficient $E_{280} = 0.59$ A_{280} mg^{-1} ml (35). All DNA concentrations are reported in nucleotides. Poly(dG-m^5dC)·poly(dG-m^5dC), which served as an example of Z form DNA, was purchased from Pharmacia LKB Biochemicals Inc. Poly(dA-dT) was also purchased from Pharmacia LKB Biochemicals Inc. Solutions of poly(dG-m^5dC)·poly(dG-m^5dC) were prepared in 1 mM EDTA, 10 mM Tris-HCl buffer (pH 7.5), and then treated in S1 nuclease (10 units/μg of DNA) in a reaction buffer containing 40 mM sodium acetate (pH 4.8), 50 mM NaCl, and 1 mM $ZnSO_4$ to remove possible single-stranded regions in the DNA. Residual protein was removed by phenol extraction and ethanol precipitation. All DNA stock solutions were prepared in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. An extinction coefficient of 7,000 M^{-1} cm^{-1} at 255 nm was used for the concentration determination of this polynucleotide in the B conformation (36). The average length of the poly(dG-m^5dC) reported by the manufacturer was 1900 nucleotides measured by sedimentation analysis. After S1 nuclease treatment the preparation was examined by electrophoresis under nondenaturing conditions. More than 95% of the polymers were 700 to 2400 base pairs in length, and the average length was about 1700 base pairs. To make and stabilize the Z conformation, adjustment of salt concentration (to 10 mM Mg) was accomplished by introducing 1 M $MgCl_2$ into the DNA stock solution. Lower $MgCl_2$ concentrations were used for the samples prepared for electron microscopy, as described below. The DNA conformation was checked before and after the addition of $MgCl_2$ as described below. The plasmid pEAW3 (2426 base pairs) (15) was a gift of E. A. Wood in this laboratory and used for an example of B form DNA. Plasmid purification was performed as previously described (37). FII DNA (pEAW3) was prepared from FI DNA using DNase I (Sigma, type II) and ethidium bromide as described elsewhere (12, 13, 38). This procedure typically yields preparations containing 90–95% relaxed circular DNAs with single nicks (randomly placed). Some contaminating linear DNA is usually present. FIII DNA was generated by complete digestion with *Eco*RI restriction endonucleases, using conditions recommended by the enzyme supplier. To generate shorter fragments of pEAW3 (1.1 and 1.3 kilobase pairs), the DNA was also digested with the restriction enzyme *Ssp*I. The concentrations of duplex DNA (B form) were determined by absorbance at 260 nm, using 50 μg ml^{-1} A_{260}^{-1} as a conversion factor. Restriction endonucleases were purchased from New England Biolabs. Pyruvate kinase, lactic dehydrogenase, DNase I, NADH, phosphoenolpyruvate, and ATP were obtained from Sigma. MES buffer was from Research Organic Inc. ATPγS and Tris buffer were purchased from Boehringer Mannheim.

Conformations of DNA Polymers and Plasmids—The B to Z structural transitions for DNA samples were monitored at room temperature using a Jasco model J-41C spectropolarimeter. DNA samples were suspended in each of the reaction buffers used in experiments described in this paper, and the circular dichroism spectrum was recorded from 230 to 340 nm, as described elsewhere (39). Comparison with authentic left-handed Z DNA spectra confirmed that the (dG-m^5dC)$_n$·(dG-m^5dC)$_n$ duplex was essentially 100% in the Z conformation under all conditions used in this study. Prior to the addition of $MgCl_2$, this polymer was in the B conformation as determined by circular dichroism and comparison with spectra obtained with linearized plasmid DNA (pEAW3) and authentic B DNA spectra in the literature.

Reaction Conditions—Unless stated elsewhere (*e.g.* see Electron Microscopy, below), all reactions were carried out in solutions containing 25 mM buffer (MES-NaOH or Tris acetate), 10 mM $Mg(acetate)_2$, 5% glycerol, 1 mM dithiothreitol, 1 mM ATP, ATP-regenerating system (4.5 units/ml of pyruvate kinase, 4.5 units/ml of lactic dehydrogenase, 3 mM NADH, 3 mM phosphoenolpyruvate, 1 mM KCl), 10 μM DNA, and the indicated recA protein concentration. Reactions were performed in a volume of 0.5 ml in a 0.5-cm path length, self-masking quartz cuvettes at 37 °C. Reactions were started by addition of recA protein after all other components were incubated at 37 °C for 10 min to bring the mixture to thermal equilibrium. Each set of comparative experiments was done on the same day.

ATPase Assay—Absorbance measurements were obtained on a Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with two 6-position, thermojacketed cuvette holders attached to a constant temperature water circulator. Cell path length and band pass were 0.5 cm and 2 nm, respectively. The spectrophotometer and the coupled enzyme assay for ATP hydrolysis are described in detail elsewhere (12, 13, 40). In brief, regeneration of ATP from ADP and phosphoenolpyruvate is coupled to the conversion of NADH to NAD^+, which can be monitored spectrophotometrically by a decrease in absorbance at 380 nm. Due to the high concentration of NADH used in this study, absorbances were measured at 380 nm, instead of 340 nm (its absorbance maximum), so as to remain in the linear region of the spectrophotometer. High concentrations of NADH were necessary to ensure that a steady state (end point) was reached under all conditions. No component of the coupling system limited the observed rate of ATP hydrolysis. ATP is constantly regenerated in this system so that no product accumulation occurs. An extinction coefficient of $\epsilon_{380} = 1210$ M^{-1} cm^{-1} for NADH was used to obtain steady state velocities of ATP hydrolysis. All data were fit by linear regression and the standard deviations reported in the text.

Significant lags are observed before steady state ATP hydrolysis is achieved. The time lag (τ) in this system is determined by extrapolating the linear steady state rate back to the χ axis where zero ATP is hydrolyzed. The time value at the intersection with the χ axis is τ, and it represents the average time required to form a complete recA·double-stranded DNA·nucleoprotein complex. A system lag of approximately 1.0 min due to the coupling system was subtracted from all data. Small corrections (<1%) were also made for the low level of DNA-independent ATP hydrolysis.

Nitrocellulose Filter Binding—The binding of recA to Z form DNA at pH 6.47 was also measured using a nitrocellulose filter binding assay. Nitrocellulose filters from Schleicher & Schuell (BA85, 0.45 μm; 24 mm circles) were soaked in 0.4 M KOH for 10 min and rinsed with double distilled water. Prior to filtration of reaction samples, the filters were soaked in reaction buffer (25 mM MES-NaOH, pH 6.47, 10 mM $Mg(acetate)_2$, 5% glycerol, 1 mM dithiothreitol and 1 mM ATP).

recA protein (2 μM) was added to a reaction mixture containing 10 μM Z form DNA preincubated at 37 °C. The DNA was end-labeled with ^{32}P as described elsewhere (16). Samples (50 μl) were taken at various times and filtered through nitrocellulose filters, which were immediately washed with 1 ml of reaction buffer to remove DNA unbound by protein. After drying under a heat lamp, the bound ^{32}P-end-labeled DNA was quantitated by liquid scintillation counting. The background level of retention was determined in control samples that were identical except for the absence of recA protein. Background counts were less than 3 ± 1% of total counts in all experiments. The extent of retention of DNA molecules was calculated by dividing the background-corrected counts by the theoretical maximum number of counts retained if binding were complete. The maximum level of binding attainable was determined by spotting an appropriate aliquot of reaction mixture directly on a filter and drying it without rinsing. In these experiments, the maximum retention of DNA on filters due to recA binding was about 80% of the theoretical maximum, even in the presence of large excesses of recA protein. We attribute this to incomplete retention by the filters. No correction has been made for this effect, and we assume that the 80% retention of counts observed in many assays (see Fig. 2) generally represents complete binding.

Electron Microscopy—Reaction mixtures prepared for electron microscopy contained 30 mM Hepes buffer (pH 7.0), 5 mM $MgCl_2$, 70 mM KCl, 3 mM EGTA, 5.2 μM recA protein, 15 μM DNA (in nucleotides), and 2 mM ATP or ATPγS. Where band Z DNA were mixed, both were present at 15 μM (total 30 μM DNA). Reactions were carried out at room temperature, and components were added in the order: buffer and salts, recA, DNA, and finally ATPγS (or ATP). When ATPγS was used, reactions were mixed and left at room temperature for 30–60 min. Samples were then prepared for electron microscopy as described (41). When ATP was used, samples were mixed and left at room temperature for only 10 min before they were prepared for electron microscopy.

RESULTS

Experimental Design

The binding of recA protein to Z and B form DNAs was compared in side by side experiments involving the Z form polymer $(dG\text{-}m^5dC)_n\cdot(dG\text{-}m^5dC)_n$ and the B form linearized plasmid pEAW3. Both the kinetics of binding and the properties of the final bound state were investigated. Results were obtained under the conditions we normally use for strand exchange, and also in the presence of 100 mM K glutamate. The latter condition more closely approximates the environment *in vivo* (42), and has little effect on strand exchange[2] (43).

The kinetics of binding was monitored indirectly using the DNA-dependent ATPase assay as described (12, 40). For B DNA, this provides an accurate measure of DNA binding. Measurement of DNA binding by monitoring ATP hydrolysis is based on the assumption that recA monomers bound to duplex DNA hydrolyze ATP with a k_{cat} of 22 min^{-1}. Estimates of binding obtained in this way correlate very well with other measures of binding such as DNase protection, light scattering, and DNA unwinding assays (12, 13, 15). The ATP hydrolytic activity of recA protein is reduced when the Z form DNA is used as a cofactor. When adjustments are made for the lower apparent k_{cat} for ATP hydrolysis when recA is bound to Z DNA as described below, the observed rates of ATP hydrolysis again provide binding estimates that are consistent with filter-retention measurements of Z DNA binding.

[2] J. Kim, unpublished.

Kinetics of DNA Binding

The time course of ATP hydrolysis in the presence of pEAW3 or $(dG\text{-}m^5dC)_n\cdot(dG\text{-}m^5dC)_n$ is presented in Fig. 2 as a measure of the course of DNA binding. Experiments were done at both pH 6.47 and pH 7.5. At pH 6.47, both reactions reach a steady state of ATP hydrolysis after a lag. The lag for B DNA (14 min) was significantly longer than the lag for Z DNA (3 min). The lag for B DNA is consistent with results obtained previously (12, 13) and primarily reflects the slow nucleation step in binding. The lower lag observed with Z DNA indicates that binding to the Z polymer is faster than binding to the B DNA. Interestingly, the steady state rate of ATP hydrolysis is 3–4-fold lower when Z DNA replaces B DNA (12–13 *versus* 43 μM min^{-1}). As in the case of B DNA, the order of addition of reaction components had no effect on the time course of the reaction with Z DNA, indicating that ATP is required for binding. At pH 7.5, the effect is more dramatic (not shown). The lag for Z DNA is 12 min, whereas it is greater than 70 min for B DNA. The reaction with the linear B DNA at pH 7.5 never reaches the steady state rate observed at pH 6.47. Steady state binding to B DNA at pH 7.5 is reduced because of the presence of a slow but significant recA dissociation reaction at the higher pH that complicates the kinetics (16). With Z DNA, the steady state rates were comparable at the two pH values and remained constant for at least 2 h. These results indicate that recA protein binds to Z DNA faster than to B DNA, consistent with the observa-

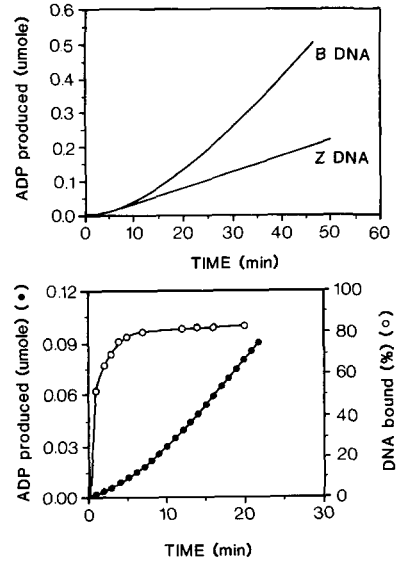


FIG. 2. **Binding of recA protein to duplex DNA.** The time course of ATP hydrolysis was monitored as a measure of recA protein binding to DNA and compared directly with binding measured by a filter retention assay. Reactions were carried out at pH 6.47 as described under "Experimental Procedures," and contained 2 μM recA protein, 10 μM DNA, 1 mM ATP. *Upper panel*, binding as measured by ATP hydrolysis with B DNA (FIII, pEAW3) or Z DNA (poly(dG-m^5dC)·poly(dG-m^5dC)); *lower panel*, recA protein binding to Z DNA, measured in side by side experiments with ATP hydrolysis (●) and DNA retention on nitrocellulose filters (○). Conditions were the same as those used in the *upper panel*.

tions made by Blaho and Wells (19).

The lower rate of ATP hydrolysis observed with Z DNA represents a decrease in the turnover number or k_{cat} for recA ATPase rather than a reduction in total binding of recA protein. In side by side experiments, the onset of steady state ATP hydrolysis using Z DNA as cofactor correlates very well with the completion of DNA binding as measured by a filter retention assay (Fig. 2). Rates of steady state ATP hydrolysis at pH 6.5 were measured at varying ATP concentrations in the presence of both DNAs. The resulting kinetic parameters are presented in Table I. The apparent K_M for ATP is similar for both the Z and B DNAs, but k_{cat} is reduced substantially on Z DNA, from 22 to 6–7 min^{-1}. The Hill coefficient for ATP hydrolysis was also reduced somewhat for Z DNA, suggesting an effect on cooperative interactions between recA monomers that might be related to the overall reduction in the ATPase k_{cat}. The binding stoichiometry for recA protein is very similar for B and Z DNA as described below.

Characterization of the Nucleoprotein Filaments

The steady state rate of ATP hydrolysis at pH 6.47 on B or Z DNA increased linearly with low recA protein concentrations, approaching an asymptote above which the rate became independent of protein concentration (Fig. 3). The titration end points are defined as the intersection of asymptotic lines drawn through the linear vertical and horizontal portions of each curve (not shown). This provides an apparent binding stoichiometry of 1 recA monomer/3.8 base pairs for B DNA and 1/4.1 base pairs for the Z polymer. The stoichiometries were not significantly altered when 100 mM K glutamate was present (Fig. 3, *C* and *D*). This property of the nucleoprotein filaments is essentially identical for the two DNA forms, and it again strongly indicates that the lower rates of ATP hydrolysis observed when recA protein binds Z DNA reflect an intrinsically lower k_{cat} for ATP hydrolysis.

The resulting nucleoprotein filaments were observed directly by electron microscopy (Figs. 4 and 5) in the presence of ATP or ATPγS at pH 7.0 in reactions carried out at room temperature. The filaments formed on the Z DNA (Fig. 4) were indistinguishable from those formed on B DNA (44). In both cases the filament had a distinct right-handed helical twist, with the same pitch (9.0 nm) and width (11–12 nm). The filaments formed with ATP were structurally identical to those formed with ATPγS, but they tended to be much shorter (~100 nm) and the total amount of bound recA protein was greatly reduced. Unbound recA protein in the form of small rings and short rods (45) was prominent in the background (not shown). To eliminate any possibility that the handedness of the filaments had been reversed during photographic processing, an equimolar mixture of the two DNAs was bound with a slight excess of recA protein in the presence of ATPγS. In the electron microscope, all of the complexes produced in this experiment were structurally identical right-handed helical filaments (Fig. 5).

Another structure observed in experiments carried out with ATPγS were bundles in which several right-handed filaments were wrapped about one another in superhelical twists with a left-handed sense (Fig. 4, *B* and *C*). These are probably equivalent to the elongated bundles observed by Brenner *et al.* (45) and Egelman and Stasiak (46), and generally represented less than 10% of the total recA protein on the grid. (Under the conditions of these experiments, these structures were much more common in the Z DNA reactions than in the B DNA samples.)

TABLE I

Kinetic parameters for DNA-dependent ATP hydrolysis by recA protein at pH 6.47

	B DNA	Z DNA	100 mM K glutamate	
			B DNA	Z DNA
k_{cat} (min^{-1})	22.4	6.4	20.6	8.5
K_m (μM)	170	190	220	260
Hill coefficient for ATP	2.8	2.3	2.1	1.2

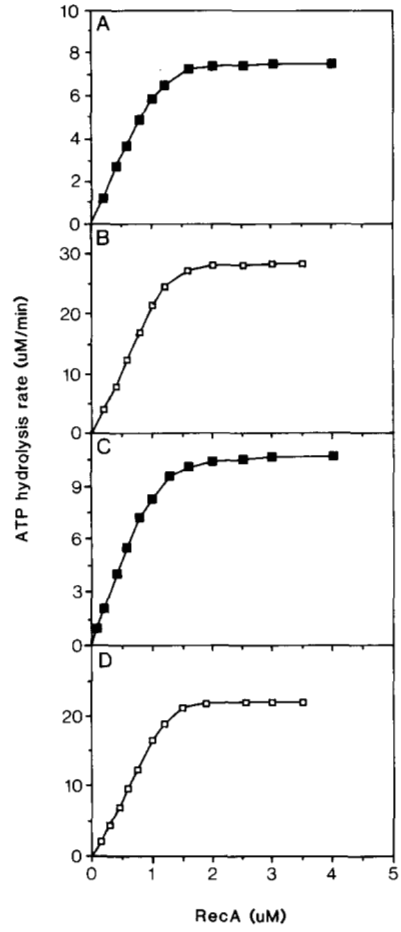


FIG. 3. **Titrations of DNA substrate with recA protein.** ATP hydrolysis reactions were performed at pH 6.47 with 10 μM DNA and recA protein at the indicated concentrations. The ATP hydrolysis rate is that observed after the reaction had reached steady state. The DNA substrates compared were: ■, Z DNA; □, B DNA. *A* and *B* are reactions in the absence of K glutamate; *C* and *D* are reactions in the presence of 100 mM K glutamate.

These results indicate that the final complex is essentially identical in structure when formed on B or Z DNA. Binding to left-handed Z form DNA ultimately results in a right-handed nucleoprotein filament. This suggests that the more rapid binding of recA protein to Z DNA may reflect a kinetic perturbation of a common association pathway rather than a distinct binding mechanism.

Kinetic Characterization of the Association Reaction

The τ Analysis—An equation derived for the binding mechanism of Fig. 1 has been described (13). It is based on an analysis developed for RNA polymerase by W. McClure (43, 44). The lag time for binding or τ is:

$$\tau = \frac{1}{k_2} + \frac{1}{k_a[R]}$$

where K_1 is the pre-equilibrium in step 1, k_2 is the nucleation

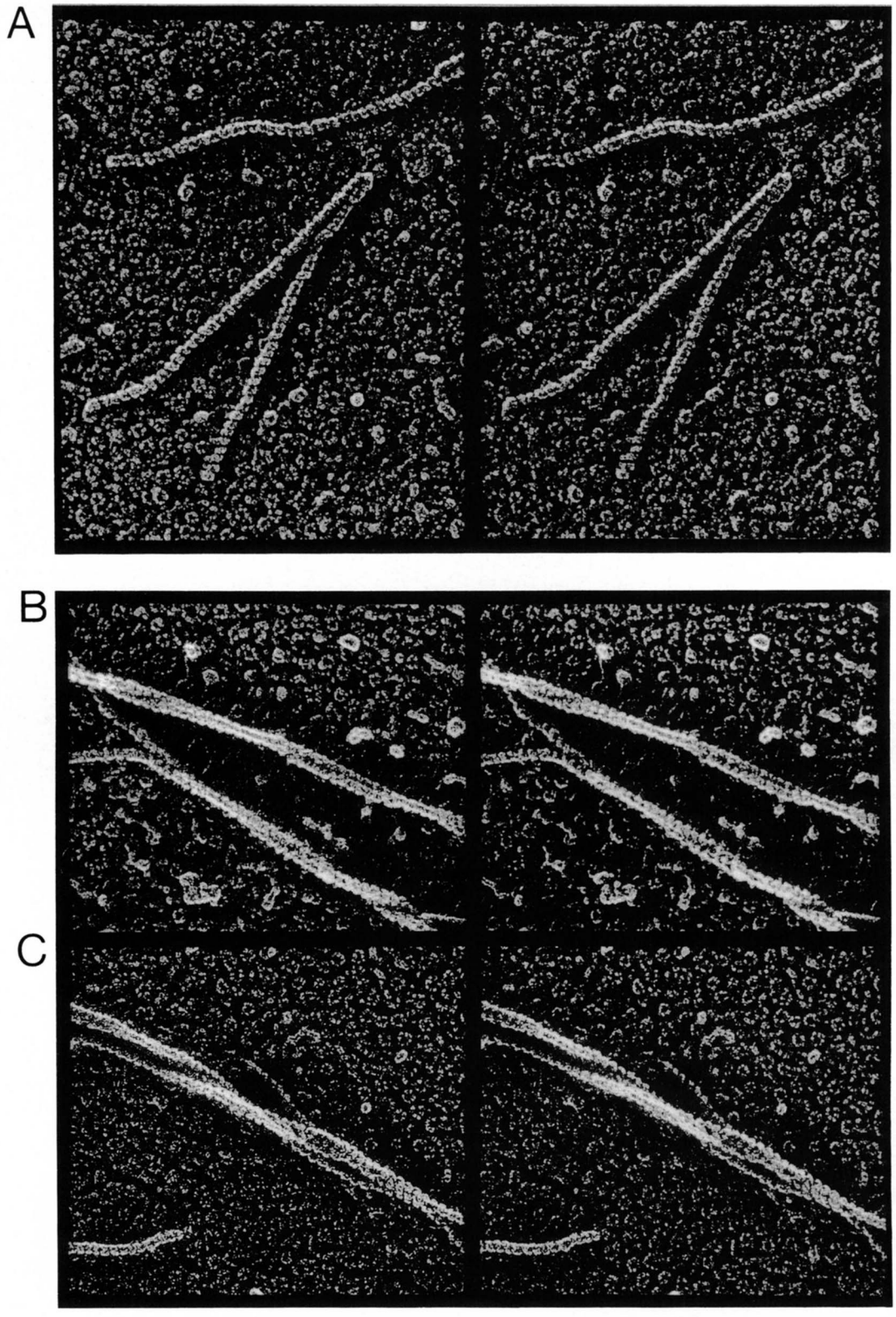


FIG. 4. **recA nucleoprotein filaments formed on Z DNA ((dG-m^{5}dC)$_n$·(dG-m^{5}dC)$_n$).** Images are presented in stereo. *Panel A* illustrates representative filaments. *Panels B* and *C* illustrate the bundles mentioned in the text. Magnification is ×177,000.

step, $k_a = K_1k_2$, and $[R]$ is the total concentration of recA protein. The individual constants can be evaluated by plotting τ *versus* $1/[R]$ (13, 46, 47). The reciprocal of the τ intercept is $k_{2(obs)}$ (min^{-1}) and the reciprocal of the slope is k_a. K_1 may be derived from the relationship $K_1 = k_a/k_{2(obs)}$. As is the case with B DNA, a line through the data points obtained in such a plot with Z DNA at pH 6.47 is linear and gives a positive τ intercept (Fig. 6). This pattern is consistent with the idea that association follows a similar pathway for the two DNAs. The experiment of Fig. 6 was also carried out in the presence of 100 mM K glutamate, with very similar results (not shown). The kinetic parameters derived from this analysis are given

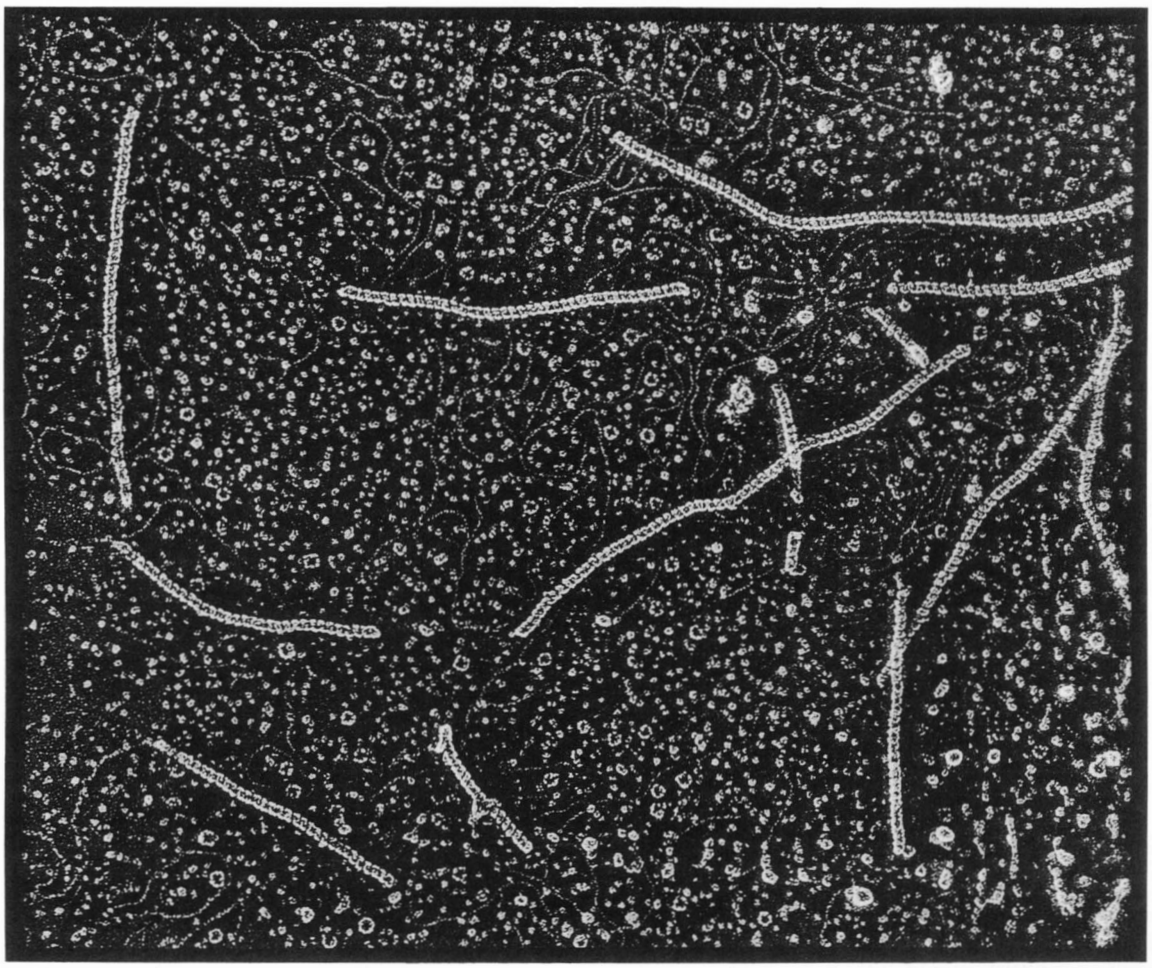


FIG. 5. **RecA nucleoprotein filaments formed on a mixture of Z and B DNAs.** A field view. RecA monomers and ring structures (see Ref. 45), as well as unbound DNA, are present in the background. Magnification is ×130,000.

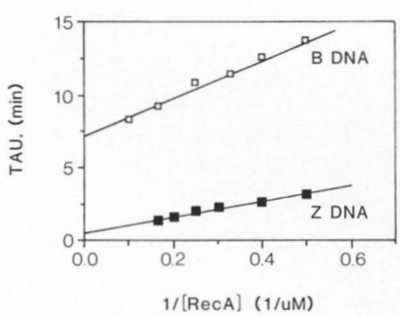


FIG. 6. **Tau plot.** The time lag (τ) in the approach to steady state ATP hydrolysis is plotted as a function of the reciprocal of the total recA protein concentration. Reactions were carried out at pH 6.47 under standard reaction conditions with 10 μM DNA (B DNA or Z DNA as indicated), 1 mM ATP, and the indicated recA protein concentration. These plots permit a calculation of K_1 and k_2 (Fig. 1) as described in the text.

TABLE II

Kinetic parameters for recA protein binding to linear duplex DNA

The kinetic parameters are obtained as described in the text. All reactions were carried out at pH 6.47. See "Experimental Procedures" for reaction conditions.

	No glutamate		+Glutamate[a]	
	B DNA	Z DNA	B DNA	Z DNA
$K_{1(obs)}$ ($M^{-1} \times 10^{-5}$)	2.3 ± 0.3	1.3 ± 0.1	2.9 ± 0.1	1.5 ± 0.2
$k_{2(obs)}$ (min^{-1})	0.16 ± 0.04	1.4 ± 0.2	0.09 ± 0.02	1.9 ± 0.1

[a] Reactions also contained 100 mM K glutamate.

in Table II. The most striking difference is a 10–20-fold increase in k_2 (when Z replaces B DNA). This is consistent with the straightforward conclusion that binding to Z DNA is faster because the rate-limiting nucleation step in binding is accelerated.

Salt Effects—As with most DNA-binding proteins, DNA binding by recA protein is affected by the ionic environment (Fig. 7). The B and Z DNA-dependent ATPase activities showed a different sensitivity to anion concentrations. The Z DNA-dependent reaction was relatively resistant to salt, being 50% inhibited at 200 mM KCl. Even less inhibition was observed in the presence of 200 mM K acetate. Glutamate exhibited a stimulatory effect on Z DNA-dependent ATP hydrolysis up to a concentration of 150 mM. B DNA-dependent ATPase activity, in contrast, exhibits a large inhibition with 200 mM KCl or K glutamate. A previous study (13) demonstrated that Na glutamate causes an increase in binding equilibrium constant (K_1) for the association of recA with duplex DNA but a decrease in nucleation ($k_{2(obs)}$) in binding to B form DNA. In the presence of glutamate the reduced activity in ATP hydrolysis was interpreted as a direct effect on the ATPase k_{cat} (13). No apparent changes in the binding stoichiometry of recA protein to B DNA or Z DNA were observed in the presence of 100 mM K glutamate (Fig. 3, *C* and *D*). From the analysis of the τ plot obtained in the

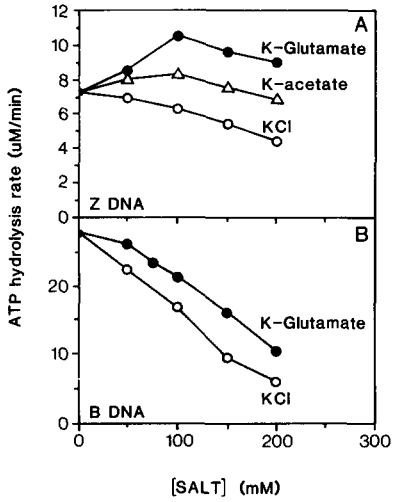


FIG. 7. **Effect of salt on DNA-dependent ATPase activity of recA protein.** ATP hydrolysis reactions were performed at pH 6.47 in the presence of 2 μM recA protein, 10 μM DNA, 1 mM ATP, and indicated concentration of various salts. *A*, reactions with Z DNA; *B*, with B DNA.

presence of 100 mM glutamate we observed an increase in K_1 for binding of recA protein to Z DNA but a decrease in the rate of the nucleation step, k_2, similar to trends seen with B DNA. $K_1 = 1.5 \pm 0.2 \times 10^5$ M^{-1} and $k_2 = 1.9 \pm 0.1$ min^{-1} for Z DNA in the presence of K^+ glutamate (100 mM). For B DNA under the same conditions, $k_2 = 0.09$ min^{-1}; $K_1 = 2.9 \pm 0.1 \times 10^5$ M^{-1}. The increased rate of recA-mediated ATP hydrolysis on Z DNA in the presence of K glutamate might result from a stabilization effect on the recA·Z DNA complex and/or a direct effect on ATPase activity. The results in Fig. 3 indicate that the effects of glutamate in these experiments reflect a change in the intrinsic k_{cat} for ATP hydrolysis rather than a decrease in the amount of recA protein bound, consistent with earlier observations (13). The same may be true for the effects of KCl, although we have not investigated this directly.

pH Effects—As noted previously (12, 45), the duplex DNA-dependent ATPase activity of recA protein is strongly dependent on pH. After the reaction had reached an apparent steady state, the rate of ATP hydrolysis was measured over a time span of 2 h. The values obtained at pH values above 7.0 on B DNA represent only an estimate of the lower limit of the steady state rate of ATP hydrolysis, because binding to B DNA is very slow under these conditions (12, 13). As shown in Fig. 8*A*, the pH profile of DNA-dependent ATP hydrolysis was similar for B and Z DNA except for the relatively low level of Z DNA-dependent activity at the lower pH values. To determine the effect of pH on individual reaction steps, time lags in approaching steady state ATP hydrolysis were measured at pH values ranging from 6.0 to 6.75 and τ plots were constructed. The individual constants K_1 and k_2 were determined from these plots at each pH. The lag time for binding at a given recA concentration increased with pH for both B and Z DNA, but the sensitivity to pH was much greater with B DNA than with Z DNA (not shown). To determine the net change in the number of protons involved in each step of binding reaction, the logarithms of kinetic rate constants or equilibrium constants were plotted as a function of pH as described in Ref. 13. A plot of log K_1 *versus* pH was linear for Z DNA, with a slope of 1.3 ± 0.1 (Fig. 8*B*). This indicates that ~1 proton is released in forming what we believe to be an initial weak binding complex. A nonlinear dependence of log K_1 on pH was observed for B DNA (Fig. 8*B*). The slope

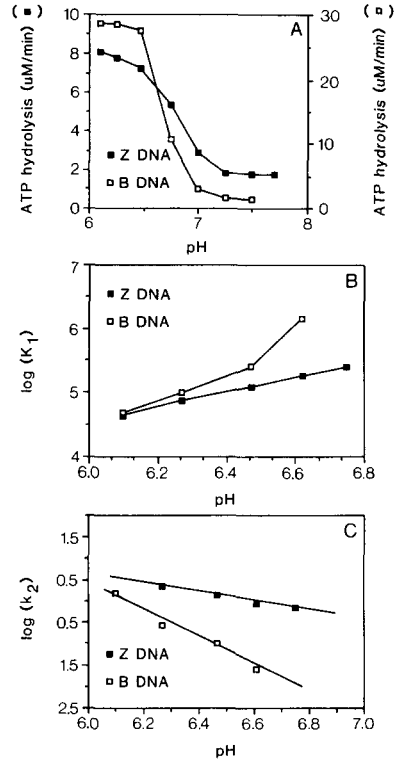


FIG. 8. **Effect of pH.** ATP hydrolysis reactions were carried out with 2 μM recA protein and 10 μM DNA using MES-NaOH (pH 6.1–6.8) or Tris acetate (pH 7.0–7.5) buffer. *A*, ATP hydrolysis rates were determined to be at steady state as described under "Experimental Procedures" for each reaction. Above pH 7.0, indicated rates of ATP hydrolysis in the presence of B DNA never reached a true steady state within the 2-h time period of the experiment due to the long binding lag under these conditions. The rates indicated therefore represent lower limits. *B* and *C*, dependence of $K_{1(obs)}$ and k_2 on pH, respectively. $K_{1(obs)}$ and k_2 were obtained at the different pH values by methods described in the text.

was near 1.0 at lower pH values as reported previously (13), but appeared to increase at higher pH values. In Fig. 8*C*, $k_{2(obs)}$ also exhibits a linear dependence on pH. The slope of this plot for Z DNA (1.2 ± 0.1) indicates that approximately 1 proton is taken up by the recA protein during the nucleation step. For B DNA the slope was 3.0 ± 0.2, which is consistent with previous observations (13). These data indicate that the nucleation step on Z DNA is much less sensitive to pH than it is with B DNA. As a result of these differences, the overall pH sensitivity of the reaction is less with Z DNA, leading to a large advantage in binding rates for Z DNA relative to B DNA at pH values above neutrality. The major difference again shows up in the rate-limiting nucleation step, k_2.

The Relative Affinity of recA Protein for B or Z Form DNA

The evident difference in the k_{cat} for ATP hydrolysis on B *versus* Z DNA provides a convenient characteristic to distinguish the two complexes in mixed solutions. This assay was used to characterize binding in competition experiments at pH 6.47.

In these experiments, linear pEAW3 DNA was replaced with nicked circular (form II) pEAW3 DNA. Binding to B DNA was first examined with linear pEAW3 DNA and with nicked (form II) pEAW3 DNA in parallel experiments. The substitution of the form II DNA for the linear DNA had no effect on patterns of ATP hydrolysis at this pH. The advantage of the nicked circle is that DNA binding can be confirmed independently by ligating the nick in the presence of recA

protein and determining if the DNA is underwound as described previously (12).

Both DNAs were present at concentrations of 10 μM (20 μM total when they are mixed). The recA protein concentration (1.25 μM) is sufficient to bind about 10 μM of DNA. When both DNAs are present, the recA protein is sufficient to bind no more than half of the DNA present.

The results of an experiment with both DNAs present is shown in Fig. 9*A*, and compared with results obtained with each DNA alone. Notably, the steady state rate of ATP hydrolysis in the mixture is exactly the same as that found for B DNA alone, suggesting that the recA protein is binding the B DNA in preference to the Z polymer. The same result is obtained when 100 mM K glutamate is added (Fig. 9*B*). DNA underwinding assays confirmed that the B DNA was bound in the B/Z mixtures. Underwinding was measured at times when ATP hydrolysis had reached steady state. Most of the ligated B DNA circles were extensively underwound and the result was exactly the same in the presence and absence of the Z polymer (not shown). This result indicates that binding to B DNA is more stable than binding to Z DNA. Interestingly, the presence of the Z polymer reduced the lag in binding to B DNA, suggesting that Z DNA somehow facilitates binding to B DNA in-*trans*. The preference for B DNA is clear even when the Z DNA is added 20 min prior to the B DNA (Fig. 9).

To make sure the evident preference for the B DNA did not simply reflect the greater length of this DNA, this experiment was repeated using either pEAW3 cleaved into 1.3- and 1.1-kilobase pair fragments or poly(dA-dT) (average length ~700 nucleotides). The rate of binding to these DNAs alone varied somewhat (pEAW3 fragments were bound somewhat slower, the poly(dA-dT) faster) as expected based on previous results (12, 13). In the challenge experiments the results were similar to those in Fig. 9, with a clear preferred binding to the B form DNA in each case (not shown).

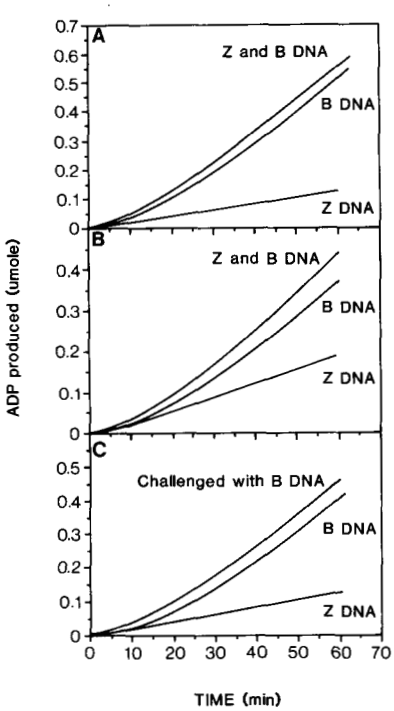


FIG. 9. **Binding competition.** *A*, a binding competition reaction was performed with 1.25 μM recA protein, 10 μM B DNA, 10 μM Z DNA, and 1 mM ATP at pH 6.47. ATP hydrolysis was initiated by addition of recA protein. Control reactions contained either 10 μM B or Z DNA alone. *B*, reaction conditions are identical to *A* but contain 100 mM K glutamate. *C*, challenge experiment. After incubation of 1.25 μM recA protein, 10 μM Z DNA, and 1 mM ATP at pH 6.47 for 30 min, 10 μM B DNA was added to the reaction mixture. The monitoring of ATP hydrolysis was started immediately after addition of B DNA and this addition defines $t = 0$. In the Z DNA control, the same procedure was used except that buffer solution (pH 6.47) was added in place of the B DNA. In both cases, ATP hydrolysis that occurs prior to $t = 0$ (~0.06 μmol) is subtracted from the data shown. In the B DNA control, the 30-min preincubation was carried out in the absence of DNA, and B DNA was added at $t = 0$.

DISCUSSION

Enhanced binding of recA protein to Z DNA does not reflect a special affinity of recA protein for left-handed DNA. The enhancement is a kinetic effect. The kinetic properties of the reaction suggest a common binding pathway for B and Z DNA. The final nucleoprotein filament found on Z DNA has a right-handed helical structure and is structurally indistinguishable from the filament formed on B DNA in the electron microscope. The rate-limiting nucleation step in binding is much faster for Z than for B DNA, leading to the overall faster binding rate. However, binding is more stable on B DNA. When the two DNA forms are mixed in the presence of limiting amounts of recA protein, the recA is ultimately found on the B DNA. The intrinsic affinity of recA protein for B DNA appears to be greater than the affinity for Z DNA, at least in the case of the Z structure formed by the $(dG\text{-}m^5dC)_n \cdot (dG\text{-}m^5dC)_n$ polymer under our experimental conditions.

This result is in no way inconsistent with the observation that Z DNA is recombinogenic *in vivo*. It does provide a more precise molecular explanation for this property of Z DNA. This left-handed helix provides a rapid loading point for recA protein. Z DNA, in fact, appears to slightly enhance binding of recA protein to B form duplex DNA even in-*trans*. *In vivo*, Z DNA may provide a loading point for recA both in-*cis* and in-*trans*, leading to significant local enhancements in recombination efficiency.

We have no direct evidence that bears on the structure of the DNA bound within the nucleoprotein filament when the substrate is initially in the Z form. *All* of the nucleoprotein filaments examined by electron microscopy in this study had a right-handed helical form. Recent results indicate that recA protein binds the DNA along the phosphate backbone (49), and in the presence of ATPγS the number of striations in the helical filament are identical to the number of helical turns in the bound DNA (11). It is reasonable to assume the bound Z DNA is held in a right-handed configuration underwound with respect to B DNA, *i.e.* that both B and Z DNA have the same configurations when bound in this complex. Recent results using circular and linear dichroism suggest that the Z form of the poly(dG-m^5dC) may be maintained in recA nucleoprotein filaments under some conditions, but that a right-handed form is favored at equilibrium in these filaments (50). This study did not include electron microscopy, and it is not clear whether the protein filaments were in a right- or left-handed configuration when bound to DNA in the Z form.

Several properties distinguish the complex formed on Z DNA. Both the k_{cat} and Hill coefficient for ATP hydrolysis are reduced when Z DNA replaces B DNA as cofactor. The overall stability of the filament is also reduced on Z DNA. These properties require further study.

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