650–654 Nucleic Acids Research, 1998, Vol. 26, No. 2

Binding of double-stranded DNA by *Escherichia coli* RecA protein monitored by a fluorescent dye displacement assay

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Received July 24, 1997; Revised and Accepted November 4, 1997

ABSTRACT

We have developed a new assay to characterize the double-stranded DNA (dsDNA) binding properties of RecA protein. This assay is based on measurement of changes in the fluorescence of a 4′,6-diamidino-2-phenylindole (DAPI)–dsDNA complex upon RecA protein binding. The binding of RecA protein to a complex of DAPI and dsDNA results in displacement of the bound DAPI, producing a decrease in the observed fluorescence. DAPI displacement is dependent on both RecA protein and ATP; dATP and, to a lesser extent, UTP and dCTP also support the DAPI displacement reaction, but dGTP, GTP, dITP and TTP do not. Binding stoichiometry for the RecA protein–dsDNA complex measured by DAPI displacement is 3 bp per RecA protein monomer in the presence of ATP. These results, taken together with data for mutant RecA proteins, suggest that this DAPI displacement assay monitors formation of the high affinity DNA binding state of RecA protein. Since this state of RecA protein defines the form of the nucleoprotein filament that is active in DNA strand exchange, these findings raise the possibility that the RecA protein–dsDNA filament may possess a homologous pairing capacity.

INTRODUCTION

RecA protein is a central element in both the recombination and repair pathways of *Escherichia coli* (1). Purified RecA protein can homologously pair and exchange DNA strands in vitro. One of the most useful model systems, the three-strand DNA exchange reaction, utilizes circular single-stranded DNA (ssDNA) and homologous linear duplex DNA substrates. In the presence of a nucleotide cofactor RecA protein polymerizes on the ssDNA to form a helical nucleoprotein filament (2,3). This nucleoprotein filament both aligns and pairs with a homologous region in the duplex DNA to form joint molecules. Consequently, the RecA protein filament must accommodate binding of two DNA molecules and must bring them sufficiently close together to promote exchange of DNA strands (4).

Despite the crucial importance of dsDNA binding in the DNA pairing process, the doubled-stranded DNA (dsDNA) binding properties of RecA protein are relatively poorly characterized. Analysis of the non-specific dsDNA binding properties of RecA protein has been hampered by the absence of a convenient method to monitor dsDNA binding. In contrast, analysis of the ssDNA binding properties (i.e. stoichiometry, affinity, effect of nucleotide cofactors, behavior of mutant RecA proteins, etc.) was greatly facilitated by the fluorescent etheno M13 ssDNA binding assay (5–9).

In this paper we describe a new method to detect binding of RecA protein to dsDNA. This assay is based on changes in the fluorescence of dsDNA complexed with a minor groove binding dye, 4′,6-diamidino-2-phenylindole (DAPI), upon RecA protein binding.

MATERIALS AND METHODS

Reagents

Chemicals were reagent grade and solutions were prepared using Barnsted NANO pure water. ADP, dADP, UTP, TTP, GTP and dATP were purchased from Sigma; ATP, dITP, dCTP, dGTP and TTP were purchased from Pharmacia Biotech Inc. or Boehringer Mannheim. DAPI was obtained from Molecular Probes and its concentration was determined using an extinction coefficient of 33×10^3/M/cm at 345 nm (10).

RecA proteins

RecA protein was purified from *E.coli* strain JC12772 (11) using a preparative protocol based on spermidine acetate precipitation (12; S.C.Kowalczykowski, in preparation). RecA K72R, RecA142 and RecA56 proteins were purified as described previously (13–15).

DNA substrates

Replicative form dsDNA from bacteriophage M13mp7 was prepared using the procedure described by Messing (16). The duplex DNA was linearized by digestion with EcoRI restriction endonuclease. Poly(dT) was purchased from Pharmacia Biotech Inc.

DAPI displacement assay

The standard reaction buffer (buffer A) consisted of 25 mM sodium MES, pH 6.2, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP or dATP, 3 mM phosphoenolpyruvate,15 U/ml pyruvate kinase (Sigma) and 400 nM DAPI (Molecular Probes). The DAPI

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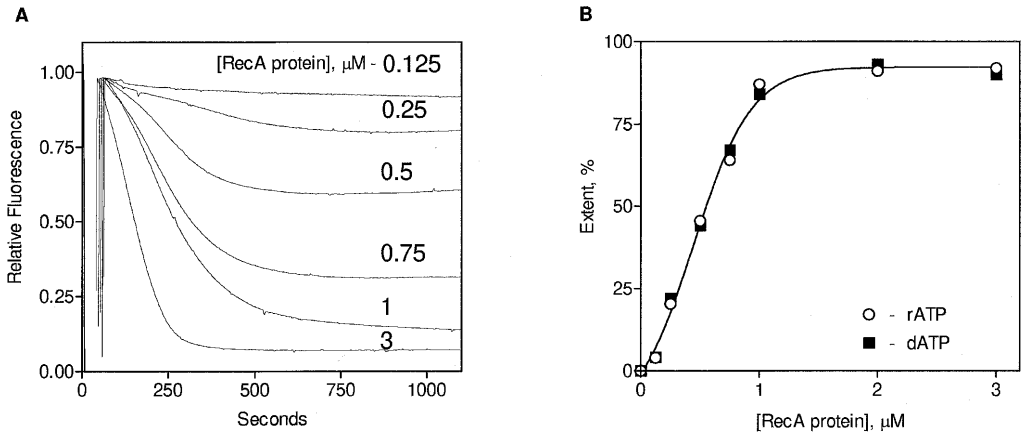

Figure 1. Dependence of DAPI displacement on RecA protein concentration. (**A**) Change in DAPI–DNA fluorescence after RecA protein addition. The concentrations of added RecA protein are indicated; data for 2 μM RecA protein are not shown. The DNA concentration in all experiments was 6 μM (nucleotides). (**B**) Maximum extent of DAPI displacement plotted as a function of RecA protein concentration using either ATP (○) or dATP (■) as cofactors.

concentration was chosen on the basis of our previous experience with binding of DAPI to dsDNA (10) to ensure that high affinity DAPI binding sites were saturated (which requires concentrations >200 nM) and to minimize any perturbation of RecA protein binding to the DAPI–dsDNA complex. The concentration of linear dsDNA was 6 μM (nucleotides). The reaction (400 μl total volume) contained standard buffer, linear M13 dsDNA and the indicated concentration of RecA protein.

Fluorescence measurements were carried out on a Shimadzu RF5000U spectrofluorophotometer. The excitation/emission wavelengths and the slit widths were 345/467 nm and 5/10 nm respectively. Reaction components were added as follows. After equilibration of the standard buffer to the indicated temperature any fluorescence background signal due to buffer components was set to zero. The dsDNA was added and the resulting fluorescence increased by ~6-fold due to formation of the DAPI–dsDNA complex. RecA protein was then added at the desired concentration and the decrease in fluorescence of the DAPI–dsDNA complex was monitored. The observed fluorescence change was divided by the initial fluorescence of the DAPI–dsDNA complex before RecA protein addition to obtain the fractional change in fluorescence, which is presumed to reflect the extent of DAPI displacemant. Unless otherwise noted, assays were conducted at 37°C.

To determine the effect of alternative NTP cofactors in the DAPI displacement assay the experimental conditions were modified as follows: the ATP regeneration system was omitted; reactions were started by adding NTP to a final concentration of 1 mM; the assay was conducted at 22°C to minimize NTP hydrolysis.

Salt titration: DAPI assay

Reaction mixtures (344 μl) containing 1 μM RecA protein and 6 μM dsDNA were incubated at 37°C until the fluorescence change reached a plateau. Then appropriate volumes of 5 M NaCl and H2O were added to make the total reaction volume 400 μl. Because the observed fluorescence of the DAPI–dsDNA complex is sensitive to changes in salt concentration (due in part to a decrease in apparent binding affinity of 100-fold per 10-fold increase in NaCl concentration; 17), whereas the fluorescence of free DAPI is not, the standard procedure was modified so that after partial salt-induced dissociation of the RecA–dsDNA complex the fluorescence data were normalized to the fluorescence value of the completely dissociated RecA protein–dsDNA complex at the same salt concentration in the same experiment. Complete dissociation of RecA protein from the complex with dsDNA was achieved by addition of an excess of poly(dT) (final concentration 15 μM) (see Fig. 2a for an illustration).

dsDNA-dependent ATP hydrolysis assay

The rate of ATP or dATP hydrolysis was measured by following oxidation of NADH spectrophotometrically at 340 nm as described previously (18). Buffer A, containing 15 U/ml lactate dehydrogenase and 0.2 mg/ml NADH, was used.

Salt titration: ATPase assay

Reaction mixtures (255 μl) containing 1 μM RecA protein, 6 μM dsDNA (nucleotides) and all components required for the ATP hydrolysis assay were incubated at 37°C until a steady-state rate was achieved. Then appropriate volumes of 5 M NaCl and H_2O were added to make the total volume 300 μl. The assay was performed using the multi-cell transport system of a Hewlett-Packard 8452A spectrophotometer.

RESULTS

Binding of RecA protein to dsDNA is accompanied by displacement of DAPI

The binding of RecA protein to dsDNA and ATP yields a ternary complex that is capable of displacing DAPI from dsDNA (Fig. 1A). Formation of this complex is detected as a decrease in DAPI fluorescence. As measured fluorimetrically, the time courses for DAPI displacement in the presence of increasing RecA protein concentrations are shown in Figure 1A. Both the observed rate and extent of this reaction increase with increasing RecA protein concentration until a plateau is reached (Fig. 1B). Saturation of the dsDNA occurs at an apparent DNA binding stoichiometry of ~6 nt, or 3 bp, per RecA protein monomer. The same DNA binding stoichiometry of 3 bp per RecA protein monomer was found when dATP was substituted for ATP (Fig. 1B).

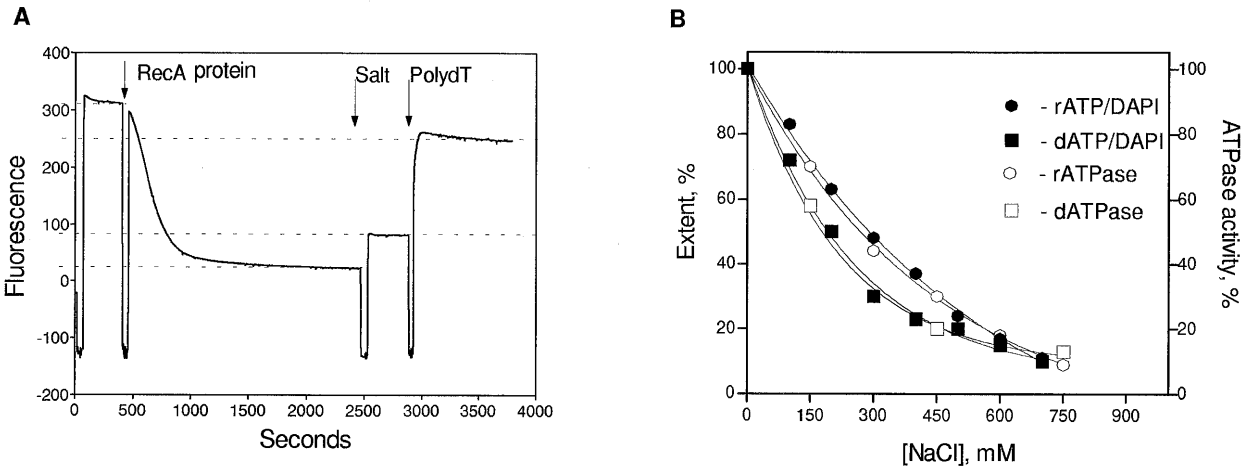

Figure 2. Experimental procedure used to measure dissociation of RecA protein–dsDNA complexes by increasing concentrations of NaCl. (**A**) A typical experiment conducted at 300 mM NaCl in the presence of ATP. The arrows indicate the time of addition of RecA protein, NaCl and poly(dT) respectively. The extent of DAPI displacement was calculated as the change in fluorescence of the DAPI–DNA complex after partial or complete dissociation of RecA protein due to addition of NaCl and poly(dT) respectively, normalized to the fluorescence value of the DAPI–DNA complex after poly(dT) addition. (**B**) Salt titration curves for DAPI displacement and dsDNA-dependent ATPase assays are shown. The extent of DAPI displacement was determined at each salt concentration from independent experiments. In the absence of NaCl, the final fluorescence of the DAPI–DNA complex after complete dissociation of the RecA protein due to addition of poly(dT) was reproducibly ~93–95% of initial value; reactions were in the presence of ATP (●) or dATP (■) and the concentration of DAPI was 200 nM. Salt titration data for the dsDNA-dependent ATPase (○) and dATPase (□) assays are shown.

Formation of the high-affinity DNA-binding state is required for DAPI displacement

Displacement of DAPI from dsDNA promoted by RecA protein requires ATP; there is no change in fluorescence in the absence of a nucleotide cofactor or in the presence of ADP (Table 1). Also, as shown in Table 1, both the extent and rate of DAPI displacement are dependent on the nucleoside triphosphate. The middle column ($\tau_{1/2}$) is the half-time required for DAPI displacement. For comparison, the right column in Table 1 represents the relative fluorescence increase (RFI) values obtained from εM13 ssDNA binding experiments for the same NTP as published earlier (9). It is evident that the ability of each NTP to support DAPI displacement from dsDNA by RecA protein correlates with its ability to induce formation of the high-affinity DNA-binding state of RecA protein (as indicated by an RFI value >2.0) (9), but not with its ability to be hydrolyzed. For example, TTP, GTP and dGTP, though hydrolyzed by RecA protein, do not induce the high-affinity DNA-binding state (9,19) and they are ineffective in DAPI displacement. ATPγS, a non-hydrolyzable ATP analog, also supports DAPI displacement but, because of an unexpected dependence of this reaction on Mg^{2+} concentration, we will address this issue in a subsequent paper (in preparation).

Binding of mutant RecA proteins to dsDNA

Since mutant RecA proteins that are defective in homologous recombination also fail to undergo induction of the characteristic high affinity DNA binding state, it was of interest to test the behavior of mutant RecA proteins in the DAPI displacement assay. The RecA142 protein is defective in genetic recombination *in vivo* and shows relatively little dsDNA-dependent ATPase and no DNA strand exchange activity *in vitro* (14). A second mutant RecA protein, RecA K72R, retains the ability to promote homologous pairing *in vitro* in the presence of dATP but not ATP (13). A third mutant RecA protein, RecA56, possesses none of the *in vitro* enzymatic activities of RecA protein other than the ability to bind ssDNA (15).

Table 1. Effect of nucleotide cofactors on RecA protein-promoted displacement of DAPI

Cofactor	Extent (%)	$\tau_{1/2}$ (s)	RFI[a]
dATP	67	60	2.55
ATP	67	165	2.25
UTP	38	192	2.13
dCTP	31	412	2.55
dITP	9.8	817	ND
dGTP	0	ND[b]	1.3
TTP	0	ND	1.91
GTP	0	ND	1.75
ADP	0	ND	1.8
None	0	ND	1.8

DAPI displacement in the presence of each nucleotide cofactor was conducted at 22°C as described in Materials and Methods. Note that to permit comparison among each of the NTPs and NDPs the ATP regenerating system was omitted. This omission results in lower extents of DAPI displacement than reported in Figure 1, where the regenerating system was used. Reactions contained 6 µM dsDNA (nucleotides) and 1 µM RecA protein.

[a]Data from Menetski *et al*. (9).

[b]ND, not determined.

Both the extent of DAPI displacement and the half–time required for DAPI displacement ($\tau_{1/2}$) for each mutant RecA protein are summarized in Table 2. The data demonstrate a range of reaction extents that generally correlate with activity of the particular RecA protein. The extent of displacement for RecAwt protein is 69%, whereas RecA56 protein shows no displacement at all. In particular, RecA K72R shows a marked preference for dATP, as reported previously (13). These data also clearly demonstrate a preference for dATP as the nucleotide cofactor for RecA protein–mediated displacement of DAPI (20,21). This preference is particularly evident when the kinetic parameter $\tau_{1/2}$ is compared; for each

protein the half–time for DAPI displacement is reduced. Unexpectedly, RecA142 protein showed both an extent of displacement and a $\tau_{1/2}$ that were very close to the values for RecAwt, in spite of being phenotypically *RecA*$^-$ *in vivo* and deficient in DNA strand exchange *in vitro*. However, the *in vitro* reactions were conducted at pH 7.5 and when DNA strand exchange activity was examined under the reaction conditions used for the DAPI displacement assay (pH 6.2), RecA142 protein was found to promote DNA strand exchange in a pH–dependent manner and its dsDNA–dependent ATPase activity was also restored (unpublished observations).

Stability of the RecA protein–dsDNA complex to dissociation by salt

By analogy with the salt titration experiments used to define the stability of RecA protein–ssDNA complexes, we wished to determine the relative affinity of RecA protein for dsDNA using the DAPI displacement assay (see Materials and Methods and legend to Fig. 2A). Salt titration curves using ATP and dATP as cofactors were generated at a DAPI concentration of 200 nM, as shown in Figure 2B. The effects of both DAPI concentration and the ratio of RecA protein to DNA on the stability of RecA protein–dsDNA complexes were also investigated (Table 3). The salt titration midpoints (ST_{mp}) for the ATP and dATP complexes measured at 200 nM DAPI were ~295 and 190 mM NaCl respectively; at 400 nM DAPI the STmp values were 350 and 230 mM NaCl respectively. This dependence of ST_{mp} values on the DAPI concentration indicates that DAPI can stabilize the RecA protein–DNA complex. For comparison, salt titration experiments using dsDNA–dependent ATPase activity as a measure of dsDNA binding (in the absence of DAPI) revealed a good agreement with ST_{mp} values derived from the DAPI assay performed at 200 nM DAPI.

These results were unexpected because for all previously examined activities of RecA protein dATP is a better cofactor than ATP (22). To determine whether this unusual inversion of effectiveness for ATP versus dATP could be attributed to the lower pH conditions used the stability of RecA protein–εM13 ssDNA complexes as defined by ATPase activity was measured at pH 6.2 (Table 3, right column). It is clear that the RecA protein–ssDNA complex formed with dATP is more stable than that formed with ATP; thus the increase in stability of the RecA protein–dsDNA complex that is afforded by dATP, relative to ATP, is specific to dsDNA and not to the lower pH conditions.

Table 2. Behavior of mutant RecA proteins in the DAPI displacement assay

Protein	Cofactor			
	ATP		dATP	
	Extent (%)	$\tau_{1/2}$ (s)	Extent (%)	$\tau_{1/2}$ (s)
RecA wt	69	165	69	60
RecA142	54	280	70	70
RecA K72R	19	1200	59	940
RecA56	0	ND	0	ND

The DAPI displacement reaction in the presence of either ATP or dATP was conducted at 22°C as described in Materials and Methods. Reactions contained 6 μM dsDNA (nucleotides) and 1 μM RecA protein.

Table 3. Stability of RecA protein–dsDNA complexes to dissociation by salt

Cofactor	Salt titration midpoint (mM NaCl)			
	DAPI assay		ATPase activity	
	200 nM DAPI	400 nM DAPI	Linear M13 dsDN-dependent	εM13 ssDNA -dependent
ATP	295	350 (440)[a]	290(400)[a]	330
dATP	190	230	180	410

Salt titration midpoints were determined using either the DAPI displacement or the DNA-dependent ATPase assays as described in Materials and Methods. The RecA protein and DNA concentrations for both assays were 1 and 6 μM (nucleotides) respectively.

[a]Using a RecA protein concentration of 2 μM.

DISCUSSION

In this paper we describe a new assay to measure the interaction of RecA protein with dsDNA. The assay is based on displacement of a fluorescent, nucleic acid binding ligand from dsDNA upon complex formation with protein. This concept seems quite general and such an assay may find applicability for the study of other dsDNA binding proteins. This assay is an extension and improvement of a conceptually similar one used previously (23). DAPI is a useful reagent for this assay in that it binds in the minor groove of dsDNA in regions containing two or more consecutive A:T base pairs and binding does not perturb the B-form conformation of the DNA duplex (24). The binding of DAPI is sufficiently tight (~20–50 nM) to permit saturation of the preferred AT binding sites, but is obviously not so strong as to preclude binding by RecA protein. Furthermore, though DAPI will bind to GC-containing sites, the observed binding affinity is ~1000-fold weaker (17,25). Consequently, the fluorescence increase upon DNA binding is linear with respect to DAPI concentration and all of the high affinity DAPI-binding sites are saturated at the concentrations used herein (10).

Binding of RecA protein to the dsDNA–DAPI complex in the presence of ATP results in displacement of DAPI, which is easily monitored by a decrease in DNA-bound DAPI fluorescence. It is not entirely clear whether DAPI displacement is a direct effect of RecA protein binding to the minor groove of dsDNA or whether this displacement is an indirect result of unwinding and extension of dsDNA by RecA protein. However, the first possibility is more likely because: (i) RecA protein is known to bind along the minor groove of the DNA duplex (26–29); (ii) unwinding of DNA by ethidium bromide intercalation does not result in displacement of DAPI from a DAPI–DNA complex (unpublished observations).

Displacement of DAPI by RecA protein is ATP dependent and saturates at an apparent binding stoichiometry of 3 bp per RecA protein monomer. This is in agreement with previously published data based on: electron microscopy, 3 bp/monomer (30); sedimentation analysis and ethidium bromide fluorescence, 3 bp/monomer (26); linear dichroism, 3 bp/monomer (33); light scattering and nuclease protection, 4 bp/monomer (32); nuclease protection, 2 bp/monomer (31); dsDNA-dependent ATPase assay, 3 bp/monomer (20; unpublished observations); HPLC, 3 bp/monomer (34). In contrast to our assay, a related displacement assay which used circular dichroism to monitor displacement of distamycin from the minor groove of dsDNA upon RecA protein binding has several disadvantages (29): complete displacement of distamycin from dsDNA required greater than stoichiometric

amounts of RecA protein (about twice as much); RecA protein failed to displace distamycin from dsDNA in the presence of ATP; and displacement occurred displacement occurring only in the presence of ATPγS.

As inferred from data reported in Table 1 and 2, there is a strong correlation between the extent of DAPI displacement and the ability of RecA protein to promote DNA strand exchange. Displacement occurs in the presence of dATP and, in fact, is more effective, both in terms of extent and rate, than in the presence of ATP. This correlation is further corroborated by the findings that UTP sustains a moderate amount of DAPI displacement (38%), but ITP is poor (<10%); UTP can support the DNA strand exchange activity of RecA protein (35) but dITP cannot (19). The ability to promote DAPI displacement also correlates with the ability to induce the high affinity DNA binding state of RecA protein, as manifested by an RFI value >2 (9,22). This latter correlation is not unexpected, since induction of this high affinity DNA binding state is required for DNA strand exchange (22).

The experiments with mutant RecA proteins provide additional support for the interpretations made above. RecA K72R protein shows both a low extent of DAPI displacement (19%) and a low RFI in the presence of ATP, but exhibits close to a wild-type level of displacement (59%) and a high RFI in the presence of dATP. This mutant protein can promote DNA strand exchange only in the presence of dATP (13). Thus both DAPI displacement and changes in εM13 ssDNA fluorescence reflect the structural changes that accompany formation of the protein–DNA complex that defines the high-affinity DNA-binding state of RecA protein (22,36).

The *relative* stabilities of both the ATP- and dATP-bound complexes of RecA protein and dsDNA were determined from salt titration experiments: the midpoint for dissociation is ≈295 and 190 mM NaCl for the ATP and dATP complexes respectively. This order of affinities is the opposite of that observed for complexes of RecA protein with ssDNA and is the first example where ATP serves to stabilize RecA protein–DNA complexes more effectively than dATP. However, dATP facilitates faster kinetics of RecA protein binding to dsDNA (see $\tau_{1/2}$ values in Table 2). This result is consistent with the observation that the lag phase which precedes steady-state hydrolysis of dsDNA-dependent NTPase activity is shorter for an equivalent concentration of dATP than for ATP (20,37).

Thus this DAPI displacement assay provides a convenient means for monitoring the binding of RecA protein to dsDNA and evidence that RecA protein within the dsDNA–RecA protein filament is in the high affinity DNA binding state. Previously, formation of this high affinity DNA binding state was defined with regard to properties that are typically manifest upon ssDNA binding (22). These properties include: an increase in ssDNA binding affinity upon binding ATP (or any suitable analog); extension of ssDNA by ~50% relative to the length of B-form DNA; and a consequent increase in fluorescence upon binding to εM13 ssDNA. Furthermore, studies of both wild-type and mutant RecA proteins in the presence of various NTPs and ATP analogs established a very simple correlation (36): when complete manifestation of these characteristics is evident, this high-affinity DNA-binding state of RecA protein is proficient in DNA strand exchange; in contrast, when any one of these characteristics is lacking then the resultant (low affinity DNA binding) state of RecA protein is incapable of DNA strand exchange. Thus the finding that the RecA protein–dsDNA complex displays analogous characteristics raises the possibility that this nucleoprotein filament might also be active in DNA pairing reactions. Though unknown at this time, this possibility bears further examination.

ACKNOWLEDGEMENTS

We thank Piero Bianco, Alex Mazin, Bill Rehrauer and Bob Tracy for their critical reading of the manuscript. This work was supported by NIH grant AI-18987.

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