J. Mol. Biol. (1996) **264**, 696–712

JMB

Characterization of a Mutant RecA Protein that Facilitates Homologous Genetic Recombination but not Recombinational DNA Repair: RecA423

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A *recA* mutant (*recA423*; $Arg_{169} \rightarrow His$), with properties that should help clarify the relationship between the biochemical properties of RecA protein and its two major functions, homologous genetic recombination and recombinational DNA repair, has been isolated. The mutant has been characterized *in vivo* and the purified RecA423 protein has been studied *in vitro*. The *recA423* cells are nearly as proficient in conjugational recombination, transductional recombination, and recombination of λ *red*$^-$ *gam*$^-$ phage as wild-type cells. At the same time, the mutant cells are deficient for intra-chromosomal recombination and nearly as sensitive to UV irradiation as a *recA* deletion strain. The cells are proficient in SOS induction, and results indicate the defect involves the capacity of RecA protein to participate directly in recombinational DNA repair. *In vitro*, the RecA423 protein binds to single-stranded DNA slowly, with an associated decline in the ATP hydrolytic activity. The RecA423 protein promoted a limited DNA strand exchange reaction when the DNA substrates were homologous, but no bypass of a short heterologous insert in the duplex DNA substrate was observed. These results indicate that poor binding to DNA and low ATP hydrolysis activity can selectively compromise certain functions of RecA protein. The RecA423 protein can promote recombination between homologous DNAs during Hfr crosses, indicating that the biochemical requirements for such genetic exchanges are minimal. However, the deficiencies in recombinational DNA repair suggest that the biochemical requirements for this function are more exacting.

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Keywords: RecA protein; homologous recombination; DNA repair; replication restart; RecA–DNA complex

Introduction

The RecA protein of *Escherichia coli* is composed of 352 amino acid residues (M_r 37,842: Horii *et al*., 1980; Sancar *et al*., 1980) and plays a key role in both homologous genetic recombination and recombinational DNA repair. In both processes, two DNA molecules are aligned, DNA strands are exchanged, and the resulting crossover undergoes branch migration and resolution (Cox, 1993, 1994; Devoret, 1992; Kowalczykowski & Eggleston, 1994; West, 1992). RecA protein also plays a central role in the related processes of induction of the SOS response to DNA damage and SOS mutagenesis (Walker, 1984; Kowalczykowski & Eggleston, 1994; West, 1992; Roca & Cox, 1996). To induce the SOS response, RecA protein functions as a coprotease, facilitating an autocatalytic cleavage of the LexA repressor (Little, 1984, 1991).

Although recombination and recombinational repair are closely related processes, there are significant differences in molecular and thermodynamic requirements. During homologous genetic

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Abbreviations used: ATPγS, adenosine 5'-*O*-3-thiotriphosphate; poly(dεA), poly(1,N^6-etheno-deoxyadenosine); LD, linear dichroism; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; UV, ultraviolet; IPTG, isopropyl β-D-thiogalactoside; m.o.i., multiplicity of infection.

0022–2836/96/490696–17 $25.00/0

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recombination, DNA strand exchange is largely isoenergetic in that the number of base-pairs in the products and substrates is generally the same. Branch migration is relatively rapid in the absence of catalysis *in vitro* (Meselson, 1972; Thompson *et al*., 1976; Warner *et al*., 1979), although rates are strongly affected by the ions present in the reaction mixture (Panyutin & Hsieh, 1994). A high energy cofactor might be unnecessary in recombination except for DNA ligation. In contrast, the requirements for recombinational DNA repair are more exacting and efficiency is critical, since the failure to repair DNA damage could result in cell death. The system must be targeted to those locations in the DNA where repair is required. To make strand exchange efficient, the branch migration must be unidirectional, other DNA binding proteins present in the cell must be removed from the path of the migrating branch, and enough energy must be applied to ensure that the branch bypasses whatever type of DNA lesion might be present.

These differences suggest that some features of RecA biochemistry may be more important for recombinational DNA repair than for homologous genetic recombination. The ATP hydrolytic activity of RecA, in particular, confers new properties to the DNA strand exchange reaction that might be critical in recombinational repair, but superfluous in some recombination reactions. *In vitro*, RecA protein promotes DNA strand exchange under some conditions in the presence of ATPγS, which is not hydrolyzed appreciably by RecA (Menetski *et al*., 1990; Rosselli & Stasiak, 1990). A similar reaction is observed in the presence of ADP·AlF_4^- (Kowalczykowski & Krupp, 1995) and with a RecA mutant protein (RecA K72R) which binds but does not hydrolyze ATP (Rehrauer & Kowalczykowski, 1993). The resulting reaction, however, loses the unidirectionality of the ATP-mediated reaction, does not bypass structural barriers in the DNA, and does not accommodate four DNA strands (Jain *et al*., 1994; Kim *et al*., 1992a,b; Shan *et al*., 1996). We have argued that impeding the ATPase function of RecA selectively inhibits those features of DNA strand exchange that are most important to recombinational repair (Cox, 1993). The association of the repair function of RecA with unidirectionality and the capacity to bypass structural barriers in strand exchange is hypothetical, inferred entirely from *in vitro* work. If we can really separate the recombination and repair functions of RecA *in vitro*, it might be possible to do the same thing genetically. The required mutant RecA proteins would confer sensitivity to UV light without inactivating the coprotease function, signaling a defect in recombinational DNA repair. However, the same mutant would have a negligible effect on the capacity of the cell to promote recombination during conjugation and transduction. In this report, we present the isolation and characterization of the first *recA* mutant exhibiting these properties.

Results

Experimental design

The mutant RecA423 protein was isolated and characterized *in vivo* and *in vitro*. Its phenotype provided a novel opportunity to explore enzymatic requirements for recombination *versus* recombinational DNA repair. The *in vivo* results are presented first, followed by an analysis of the DNA binding, ATP hydrolytic, and DNA strand exchange activities.

Phenotype and genotype of *recA423*

Isolation of recA423

PsiB protein, encoded by conjugative plasmids such as R6-5 and F, has an anti-RecA action: it prevents induction of SOS genes by inhibiting activation of RecA (Bagdasarian *et al*., 1986; Bailone *et al*., 1988; Golub *et al*., 1988). RecA protein mutants, such as RecA441 or RecA730, have been characterized as coprotease constitutive: they promote SOS induction in the absence of DNA damage (Wang & Tessman, 1985; Witkin & Kogoma 1984). RecA730 and RecA441 proteins are particularly sensitive to PsiB action, completely losing their coprotease constitutive activity in its presence. The anti-RecA activity of PsiB protein may result from direct interaction with RecA protein or a competition between PsiB protein and RecA protein for binding to single-stranded DNA.

The mutant *recA423* was isolated in a search for *recA* coprotease constitutive mutants insensitive to PsiB action. Into a host expressing PsiB polypeptide and carrying a *sfiA*::*lacZ* fusion as an indicator of SOS induction, *recA* coprotease constitutive mutants insensitive to PsiB are induced for SOS in the absence of DNA damage and express constitutively the *lacZ* reporter gene. They were detected as dark blue colonies on Xgal indicator plates. Ten dark blue colonies were found in a screen of 20,000. Since *recA* coprotease constitutive mutants may be defective in recombination (Tessman & Peterson, 1985), the mutant plasmids were introduced into appropriate hosts to determine which recombination phenotype was associated with PsiB resistance. We used four different recombination assays: (1) Hfr × F^- recombination following conjugation, (2) P1 transduction, (3) recombination between monomer circles of λ*bio* phage, and (4) intrachromosomal recombination between a *lac*$^-$ duplication. The isolated mutants fell into three classes: five were recombination proficient, four were as deficient in recombination as a Δ*recA* bacterium and one, *recA423*, had a recombination proficiency that varied with the recombination assay used.

The yield of recombinants formed in Hfr × F^- crosses or in P1 transduction assays was just slightly reduced in *recA423* recipients (Table 1). Similarly, the efficiency of plaque formation of λ*bio*,

Table 1. *recA423* discriminates between recombination substrates

[a]*recA* allele	[b]Efficiency of *leu::Tn9* recombination per 100 Hfr donor	[c]Average of Lac$^+$ papillae per colony	[d]Efficiency of *leu*$^+$ transduction per P1 phage	[e]Efficiency of plating per λbio phage
recA$^+$	3.5	24	3.0×10^{-5}	1
Δ*recA306*	$<10^{-4}$	$<10^{-4}$	$<10^{-8}$	$<10^{-4}$
recA423	2.0	0.06	1.5×10^{-5}	1
recA730	1.8	42	n.t.	1

[a] Cells were transformed with a miniF plasmid carrying the indicated *recA* alleles or with the vector plasmid (Δ*recA*).
[b] Hfr × F$^-$ recombination, [c]intrachromosomal recombination, [d]P1 transduction and [e]plating of phage λbio were measured as described in Materials and Methods.

a phage that requires RecA recombinase function to form multimeric DNAs that can be packaged, was not affected by the *recA423* mutation (Table 1).

In contrast, the *recA423* mutation severely affected intrachromosomal recombination. Intrachromosomal recombination was assayed in a host carrying two copies of the *lac* operon, each with a non-overlapping deletion. Recombination between the two defective *lac* genes produces Lac$^+$ recombinants, which can be scored as red papillae on white Lac$^-$ colonies on MacConkey-lactose indicator plates (Konrad & Lehman, 1975). A mutant deficient in recombination, such as Δ*recA306*, failed to produce any Lac$^+$ papillae out of 10^4 scored colonies, whereas *recA*$^+$ bacteria had an average of 24 papillae per colony (Table 1). Cells with *recA423* produced 0.06 papilla per colony on average.

Bacteria with recA423 *are UV sensitive*

The *recA423* bacteria were nearly as sensitive after UV-irradiation as bacteria devoid of RecA protein (Figure 1). *recA423* UV-sensitivity is not the result of a defect in SOS induction, since RecA423 protein has a constitutive coprotease activity and the *sfiA* gene is as efficiently induced in *recA423* as in *recA*$^+$ bacteria (Table 2).

One other major role of the RecA protein is to catalyze early steps in recombinational repair, such as homologous pairing and strand exchange. We used as a substrate for recombinational repair a UV-damaged F'*lac* plasmid transferred by conjugation from a UV-irradiated donor into an intact recipient. During conjugation, one strand of the F' plasmid is transferred into the recipient cell with a 5' → 3' polarity. Synthesis of the complementary strand, that takes place in the recipient, is discontinuous. Therefore, if the transferred strand is damaged, this process generates a gapped DNA molecule that can be repaired by recombination with an homologous region of the intact host chromosome. When the UV-irradiated F'*lac*$^+$ plasmid was transferred into a *recA*$^+$ *lacZ::kan* recipient, as many as 30% of the surviving F' were recombinants having cannibalized the chromosomal *lacZ::kan* allele. In contrast, when a *recA423* recipient was used, F' survival was lower and the recombinant fraction was less than 1%.

The transferred UV-damaged F'*lac* can also be repaired by an alternate mechanism, mutagenic bypass of a lesion. We found no difference between *recA*$^+$ and *recA423* in this process. The frequency of *lacI*$^-$ mutants among the surviving F'*lac* was the same, 10^{-3}, in the two hosts.

Moreover, *recA423* bacteria have a mutator phenotype. In *recA423* cells, the frequency of spontaneous Rif^R mutants is equal to that induced by 10 J/m^2 UV-irradiation in a *recA*$^+$ bacterium (Table 2).

We conclude that the *recA423* bacteria are not defective in repair other than recombinational

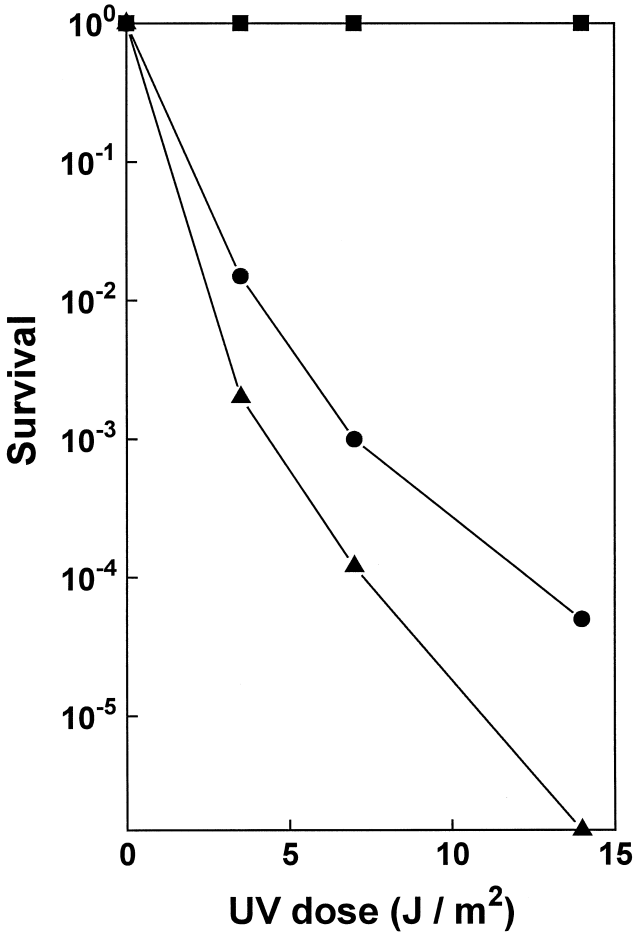

Figure 1. *recA423* bacteria are UV sensitive. Survival of GY7313: Δ*recA306* (▲) and its *recA*$^+$ (■) or *recA423* (●) derivatives was measured after exposure to the UV doses indicated on the abscissa.

Table 2. *recA423* is proficient for SOS repair

recA allele	*sfiA* induction (β-gal units/mg protein) UV-dose (J/m²) 0	5	SOS mutagenesis (Rif^R mutants per 10^7 survivors) UV-dose (J/m²) 0	1	2	10
recA423	754	1740	25	24	31	n.a
recA730	4937	n.t.	40	n.t.	n.t.	78
recA$^+$	45	1520	0.6	3	12	26

Bacteria GY7313: *sfiA*::*lacZ*$^+$ carrying the *recA* alleles indicated in column 1 were grown in YM9C supplemented with adenine (0.01%) up to an $A_{650} = 0.3$, centrifuged, resuspended in buffer and exposed to UV.

[a] *sfiA* induction. Bacteria were then incubated for 60 minutes before β-galactosidase was assayed.

[b] SOS mutagenesis. Cell survival and induction of Rif^R mutants were measured as described (Sedgwick & Goodwin, 1985).

n.t., Not tested.

n.a., Not applicable because of the low survival of *recA423*.

repair. The *recA423* bacteria appear to be selectively impaired in recombinational repair of UV-damage in spite of their capacity to carry out conjugational and transductional recombination.

The recA423 *bacteria are impaired in recovery of DNA replication after UV irradiation*

The capacity to recover a stalled replication fork and fill the discontinuities left by reinitiation of DNA synthesis beyond a lesion is essential to the survival of a UV-damaged cell. We measured the rate of DNA synthesis in *recA423* and *recA*$^+$ bacteria following UV irradiation. Exponentially growing cultures were therefore exposed to 10 J/m^2 and the rate of DNA synthesis determined by pulse labelling with [^{3}H]thymidine. DNA synthesis was inhibited after UV-irradiation in *recA423* and *recA*$^+$ bacteria (Figure 2). In *recA*$^+$ bacteria, the rate of DNA synthesis began to increase ten minutes after the initial inhibition whereas, in *recA423*, only partial recovery occurred (Figure 2). We determined that the reduced rate of DNA synthesis in *recA423* was not due to an uncontrolled postirradiation DNA degradation (data not shown). The poor recovery of DNA replication in UV-damaged *recA423* bacteria may be a consequence of a defect in recombinational repair and of the inability for *recA423* bacteria to recover an intact chromosome after UV-irradiation.

Localization of recA423 *mutation*

The *recA423* gene has a single base-pair substitution, G to A, at nucleotide 558 changing Arg$_{169}$ to His. Another mutation, *recA1203* isolated by Tessman & Peterson (1985), also changes residue 169 but to cysteine. The two mutations confer a protease constitutive phenotype but *recA1203*, in contrast to *recA423*, is deficient in Hfr × F$^-$ recombination (Tessman & Peterson 1985). Residue 169 is located at the beginning of the α-helix F near the L1 loop (Story & Steitz, 1992). Loop 1 was proposed to be involved in the binding to DNA (DiCapua *et al*., 1989; Egelman & Yu, 1989; Story *et al*., 1992; Malkov & Camerini-Otero, 1995; Wang & Adzuma, 1996). A mutation at position 169 might

Table 3. Wild-type RecA and RecA423 protein-mediated, ssDNA-dependent ATPase rates in the presence and absence of linear duplex DNA

Protein	Duplex DNA	Rate of hydrolysis (μM^{-1} min^{-1})
RecA	–	29.9
RecA	+	21.3
RecA423	–	8.4
RecA423	+	7.9

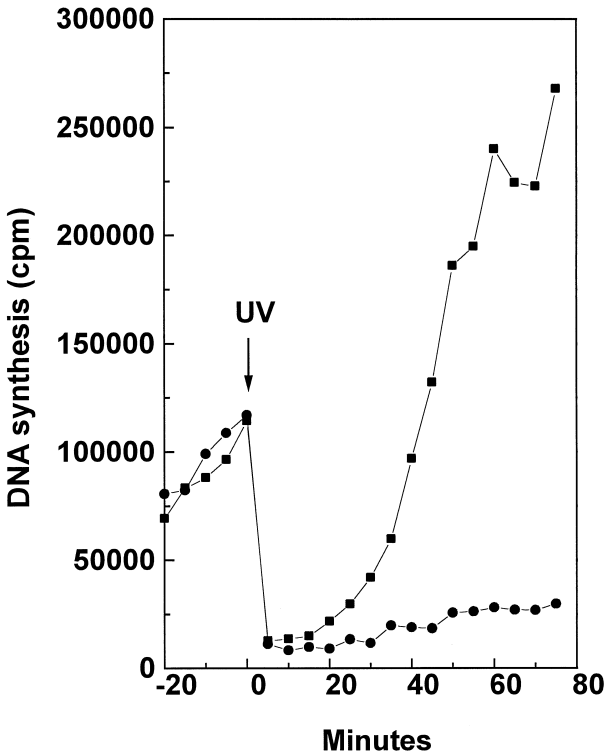

Figure 2. *recA423* bacteria are impaired in recovery of DNA replication after UV-irradiation. *recA*$^+$ (■) and *recA423* (●) bacteria exponentially growing in YM9C were exposed to a 10 J/m^2 UV dose. DNA synthesis was measured as described in Materials and Methods.

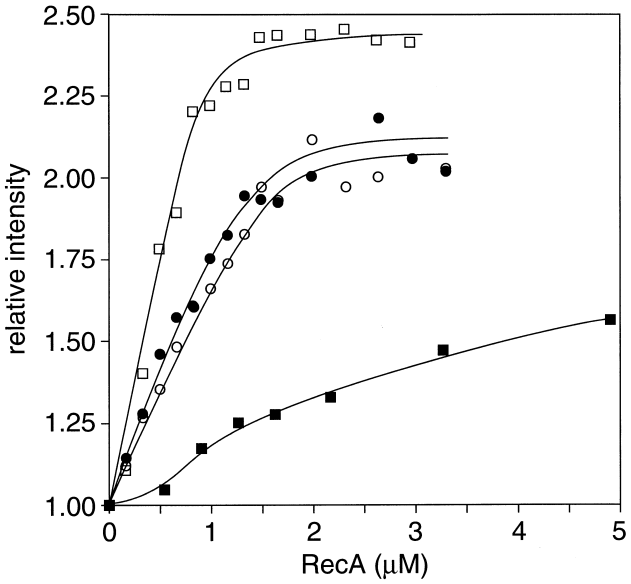

Figure 3. Titration of ϵM13 DNA by RecA protein in the presence and absence of ATP. Reactions were done under standard reaction conditions at 37°C as described in Materials and Methods. The concentration of ϵM13 ssDNA was 7 μM. Symbols are (□) wild-type RecA, or (■) RecA423 with 100 μM ATP and its regenerating system; (○) wild-type RecA, or (●) RecA423 with no cofactor.

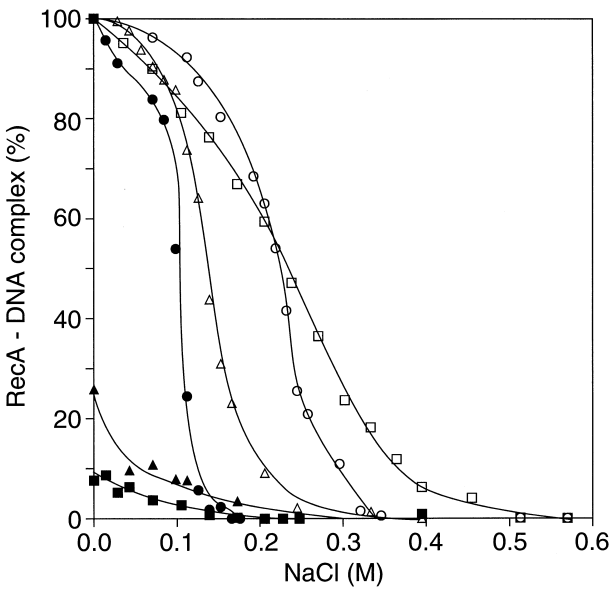

Figure 4. Effect of cofactors on the stability of RecA protein–ϵM13 DNA complexes to dissociation by NaCl. Reactions were done under standard reaction conditions at 37°C as described in Materials and Methods. The concentration of ϵM13 ssDNA and RecA protein was 5 μM and 1 μM, respectively. Symbols are: (□) wild-type RecA or (■) RecA423 with 100 μM ATP and its regenerating system; (△) wild-type RecA or (▲) RecA423 with 100 μM ADP; (○) wild-type RecA or (●) RecA423 with no cofactor.

impair recombination by reducing the stability of the RecA nucleoprotein filament.

In vitro characterization of RecA423 protein

DNA binding and ATP hydrolysis

DNA binding was measured directly with two different assays. The ATP hydrolytic activity was measured both to monitor that activity directly and to provide another method to monitor DNA binding under conditions where ATP hydrolysis is DNA-dependent.

Binding of wild-type and mutant RecA protein to ϵM13 single-stranded DNA

In order to get a quantitative understanding of the DNA binding properties of the wild-type and mutant RecA proteins, we used M13 single-stranded DNA chemically modified at the adenosine and cytidine bases with chloroacetaldehyde to produce 1,N^6-etheno-adenosine and 3,N^4-etheno cytidine. The fluorescent DNA product is etheno-M13 single-stranded DNA (ϵDNA: Barrio *et al*., 1972; Secrist *et al*., 1972). The result of adding wild-type RecA and RecA423 protein to ϵM13 DNA is shown in Figure 3. Each point represents a single reaction containing the indicated components. The titration curves for wild-type RecA protein were similar to those obtained by others (Menetski & Kowalczykowski, 1985; Zlotnick *et al*., 1993). In the absence of cofactors, RecA423 mutant protein exhibited almost the same titration curve as found for wild-type RecA protein. However, the titration curve in the presence of ATP and its regeneration system for the mutant was quite different from that for the wild-type protein. The increase in fluorescence observed with addition of the mutant protein was very small and the titration curve does not exhibit saturation. The wild-type protein exhibits saturation at RecA concentrations of about 1 to 1.5 μM in the presence of 7 μM ssDNA. The apparent increase in binding site size (one RecA monomer per six to seven nucleotides of ssDNA compared with the normal one monomer per three nucleotides) when the titration measures binding to ϵM13 has been shown to be a function of the capacity of the RecA filament to bind two separate strands of DNA (Zlotnick *et al*., 1993). The binding of the RecA423 mutant protein to ϵM13 DNA appears to be very weak in the presence of ATP.

Sensitivity of the RecA protein–DNA complex to salt

To get further insight into the binding properties of the mutant protein, we investigated the destabilization of the RecA protein–DNA complex by addition of salt. Since, in general, the stability (i.e. affinity) of most protein–nucleic acid complexes decreases with increasing salt concentration (Record *et al*., 1978), the stability of RecA protein–ϵM13 DNA complexes was determined as

a function of increasing NaCl concentrations. The results are displayed in Figure 4, and it is readily apparent that there are distinct differences in the stability of RecA protein–ϵM13 DNA complexes between wild-type RecA and RecA423 proteins. The approximate midpoint for dissociation of the wild-type RecA–ϵM13 DNA complex is 230 mM NaCl in the absence of cofactor and it decreases to 130 mM NaCl in the presence of 100 μM ADP. These results are in general agreement with published results obtained from fluorescence (Menetski & Kowalczykowski, 1985) and filter binding experiments (McEntee *et al*., 1981). In the presence of ATP, these data cannot be considered equilibrium results, owing to the continual hydrolysis of ATP. If we assume the data represent pseudo-equilibrium conditions, the apparent midpoint is around 240 mM NaCl.

As illustrated in Figure 4, the apparent level of RecA423 binding to ϵM13 DNA in the presence of ADP is less than 30% of that observed in the absence of cofactor, even before addition of NaCl. The stability of the complex is even lower in the presence of ATP than with ADP, which is quite different from what is observed with wild-type protein. In the absence of cofactor, the midpoint for the dissociation of RecA423 protein from the ϵM13 DNA was 100 mM NaCl, significantly lower than that observed for the wild-type protein.

Binding of three DNA strands to RecA423 protein

From the observation of variation of LD signals of the RecA–DNA complex, it was shown that each wild-type RecA filament can bind up to three DNA strands (Kubista *et al*., 1990; Takahashi *et al*., 1989b). In the presence of the ATP analog, ATPγS, wild-type RecA can bind either three ssDNA or one ssDNA and one dsDNA with a stoichiometry of three bases (or base-pairs for dsDNA) per RecA monomer for each DNA. This observation led to a proposal that RecA has three DNA binding sites (Kubista *et al*., 1990), an idea that is consistent with an array of data from a number of laboratories (Cox, 1995).

We examined, using the same technique, if the mutant is deficient in binding of DNA to any of the three binding sites identified in earlier studies and gained the conclusion that the mutant can bind up to three DNA strands in the presence of ATPγS in a similar way as wt RecA. The addition of first poly(dϵA) generates negative LD bands at approximately 260 and 320 nm and a positive band around 285 nm. The addition of second poly(dϵA) enhances the negative LD bands without increasing in intensity the positive LD band. Further addition of heat-denatured DNA as a third DNA to the RecA–poly(dϵA)$_2$ complex (six bases of poly(dϵA) per RecA monomer) leads to a decrease in the negative LD around 260 nm, probably due to a positive contribution of nucleo-bases from the third DNA. Similar changes were observed with wild-type RecA (Kubista *et al*., 1990). The mutant is thus similar to wild-type RecA in its capacity to bind three ssDNA molecules even in absence of sequence complementarity. The addition of dsDNA to RecA423–poly(dϵA) complex also modified the LD spectrum indicating that RecA423 can simultaneously bind one ssDNA and one dsDNA molecule. The changes are again similar to those observed with wild-type RecA. Since LD signal directly relates to the structure of the macromolecule, e.g. the orientation of DNA bases (Nordén *et al*., 1992), the data argue that there is no substantial change in the overall structure of RecA–DNA complexes elicited by the mutation in RecA423 protein.

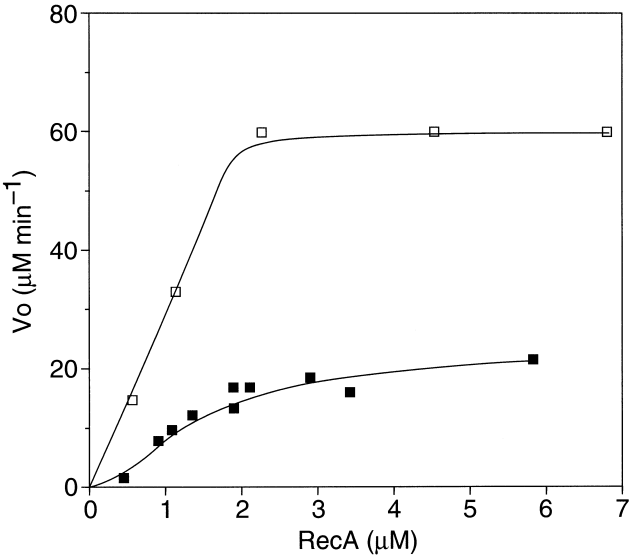

Figure 5. ATP hydrolysis by wild-type RecA and RecA423 protein: dependence on RecA protein concentration. ATP hydrolysis rates were determined by using the coupled-enzyme assay as described in Materials and Methods. The concentration of M13 ssDNA was 8 μM. Each RecA concentration point was a separate experiment. (□) wild-type RecA; (■) RecA423.

Slow binding to ssDNA revealed by ssDNA-dependent ATP hydrolysis

Figure 5 shows the rate of ATP hydrolysis catalyzed by the mutant and wild-type proteins as a function of protein concentration in the presence of SSB. The results provide an indirect measure of DNA binding (Pugh & Cox, 1988a). Each of the points on the binding isotherms was obtained by monitoring the ATPase of a single sample. As shown in Figure 5, the binding curve for wild-type RecA saturated at 2.3 μM, giving a stoichiometry of one RecA monomer per three nucleotides and a k_{cat} of 29.9 minute^{-1}, in agreement with values obtained by Bedale & Cox (1996) and by Morrical *et al*. (1986). In the RecA423 mutant, the titration curve has a sigmoidal character and the saturation point was not clear. The rate of ATP hydrolysis at a RecA423 concentration of 2.3 μM gives an apparent k_{cat} of 8 minute^{-1}. Although the real k_{cat} value for the mutant

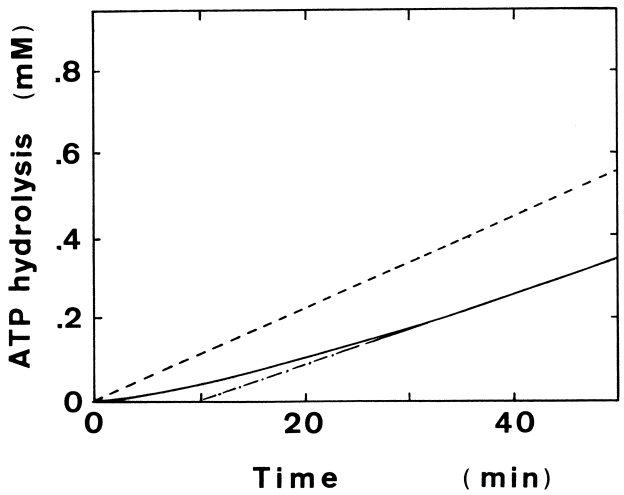

Figure 6. Slow association of RecA423 to single-stranded DNA in the presence of ATP. The association kinetics of 2 μM RecA423 (—) and wild-type RecA (– –) to 30 μM ϵDNA was monitored by enhancement of the ATPase activity of RecA protein at 30°C in a buffer containing 20 mM potassium phosphate (pH 6.4) and 4 mM $MgCl_2$. The second dot/dash line (– · –) indicates the rate of ATP hydrolysis observed for RecA423 protein at very late times. This line is drawn merely to emphasize the slow increase in the rate of ATP hydrolysis observed over this time course.

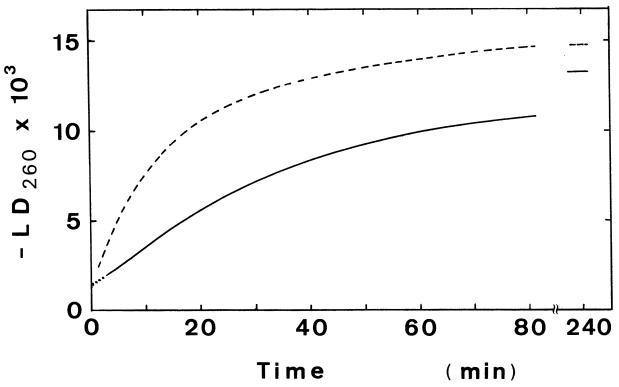

Figure 7. Association of RecA423 protein to double-stranded DNA. Experiments were carried out at pH 6.4 as described in Materials and Methods. The association kinetics of 1 μM RecA423 (—) and wild-type RecA (– –) to 3 μM calf thymus dsDNA was monitored at 25°C by the increase in intensity of LD signal at 260 nm.

is unclear due to lack of an evident saturation point, the ATPase activity of the mutant appears to be lower than that of wild-type RecA.

We investigated the possibility that the lower rates of ATP hydrolysis simply reflected an inability of the mutant RecA protein to displace SSB from the DNA, an effect that plays a prominent role in the activities of several other RecA mutants (Kowalczykowski, 1991). We compared the rates of ATP hydrolysis for RecA and RecA423 in the absence of SSB, using ϵDNA (which has no secondary structure to restrict RecA binding) as a DNA cofactor. The apparent k_{cat} values obtained in this experiment were 27 per minute for the wild-type protein and 8 per minute for the mutant protein (data not shown). This suggests that the decreased rate of ATP hydrolysis for RecA423 reflects an intrinsic decrease in activity.

An alternative possibility is that the intrinsic k_{cat} for ATP hydrolysis is unchanged in the mutant protein, but the mutant protein binds to ssDNA more slowly, than wild-type. The association kinetics of RecA423 to ssDNA in the presence of ATP is shown in Figure 6, again using the ATPase activity as a probe. The ATP hydrolysis rate of RecA423 in the presence of ϵDNA was slow at the beginning, increased with time and achieved a steady state only after a long incubation. The reaction with RecA423 thus exhibits a long lag time (about ten minutes) and suggests a slow association to ssDNA. In contrast, the ATP hydrolysis rate of wild-type RecA exhibits no significant lag time and suggests a very quick association to ϵDNA. The ATP hydrolysis rate of RecA423 at the steady state was slightly slower than that of wt RecA.

Binding to double-stranded DNA

The ATP hydrolysis of RecA423 in the presence of dsDNA also exhibits a longer lag time than that of wild-type RecA, although the difference appears smaller (Figure 7). The association kinetics in the presence of ATPγS was measured more directly and at higher salt and pH conditions by monitoring the change in intensity of LD at 260 nm. The initial association rate of RecA423 is about half of that of wild-type RecA. The mutation affects the association rate of RecA to DNA. In contrast, the final LD spectrum of RecA423–dsDNA complex is very similar to that of wild-type RecA–dsDNA complex (not shown).

Salt-induced ATPase activity

RecA exhibits an ATPase activity at very high salt conditions in the absence of DNA (Pugh & Cox, 1988b). We have measured this activity of RecA423 at various ATP concentrations. The results are compared with those of wild-type RecA (Figure 8). ATP hydrolysis of RecA423 is clearly reduced. The V_{max} of RecA423 is 3 μM/minute for 2 μM protein, about one-eighth of that exhibited by wild-type RecA (25 μM/minute). The K_m of RecA423 is 0.3 μM and one-sixth of wild-type RecA (2 μM) under the conditions of the experiments.

Strand exchange activity catalyzed by wild-type and mutant RecA proteins

Figure 9 presents the time course for the appearance of strand exchange intermediates and products in the presence of wild-type and mutant proteins. The results demonstrate that the RecA423

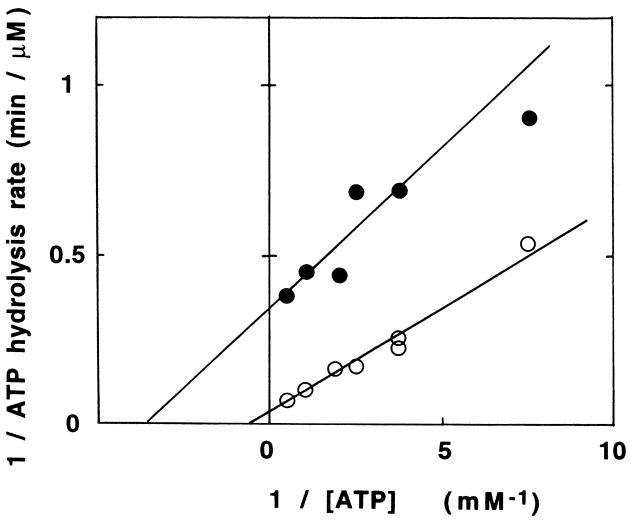

Figure 8. RecA423 mutation reduces salt-induced ATPase activity; Lineweaver–Burke plot. Salt-induced ATPase activity of 2 μM RecA423 (●) or wild-type RecA protein (○) was measured at 30°C in 2 M NaCl. K_m and V_{max} values determined from this plot are reported in the text.

protein was able to promote strand exchange between a linear duplex and a homologous circular single-stranded DNA, although this activity was reduced to less than 10% of the activity of the wild-type protein. In this experiment, there is no long preincubation to make up for a slower binding of RecA423 protein to single-stranded DNA. However, the reactions are followed over a much longer time course than is normal for RecA reactions.

RecA423 protein also promoted DNA strand exchange between the linear duplex M13mp8.52 and the circular single-stranded M13mp8 DNA. Strand exchange intermediates accumulated as illustrated in Figure 9. However, no strand exchange products were detected in reactions promoted by the mutant protein, indicating that the mutant is limited in its capacity to complete the strand exchange reaction when the substrate has a heterologous portion. This observation suggests that the mutant protein is deficient in the bypass of structural barriers during strand exchange

Hydrolysis of ATP during strand exchange

In Table 4, measurements of the ATP hydrolysis by wild-type and mutant RecA proteins before, and during, DNA strand exchange are presented. RecA–ssDNA complexes were formed initially in the absence of linear duplex DNA (Schutte & Cox, 1987). This permitted the establishment of a baseline rate of ATP hydrolysis by the RecA–ssDNA complex prior to initiation of strand exchange. This initial period also included the short lag in ATP hydrolysis attributable to the coupling system. Therefore, this system lag has no effect on the results described below. The control reaction represents ATP hydrolysis by wild-type RecA nucleoprotein filaments on M13mp8(+) ssDNA without addition of the duplex substrate. The rate of ATP hydrolysis remains essentially unchanged after addition of TE buffer. After addition of linear duplex DNA, the rate of ATP hydrolysis rapidly decreased by 33% as described by Schutte & Cox (1987). After this drop (<two minutes), the rate of ATP hydrolysis again becomes constant. The short time span required to complete this drop correlates well with the time required to complete the synapsis phase of DNA strand exchange (Cox *et al*., 1983; Kahn & Radding, 1984; Riddles & Lehman, 1985; Schutte & Cox, 1987).

During strand exchange by RecA423, little or no decrease in the rate of ATP hydrolysis was detected. The rate of ATP hydrolysis remains constant before, and after, addition of linear duplex DNA (Table 3). This observation suggests that, for the RecA423 mutant protein, the conformational changes that normally accompany synapsis are blocked or slowed. Alternatively, the conformational changes may simply not be reflected in the

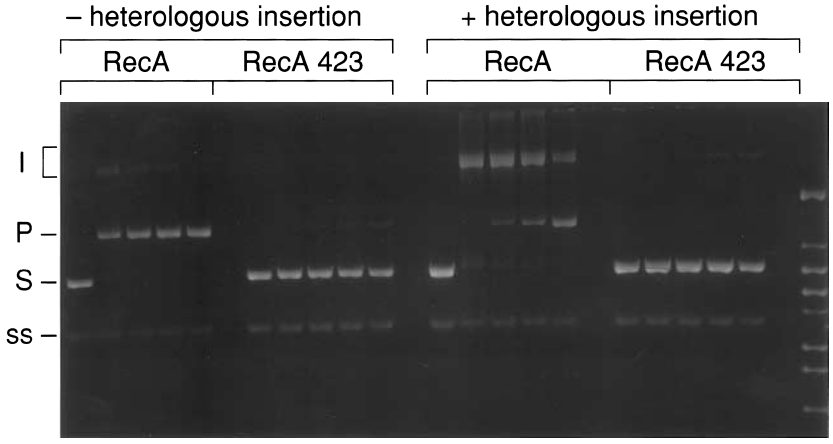

Figure 9. DNA strand exchange promoted by wild-type and RecA423 proteins. DNA strand exchange reactions were carried out under standard conditions described in Materials and Methods. Linear duplex DNA substrates (M13mp8 or M13mp8.52) were cleaved with *Alw*NI. The ssDNA substrate was M13mp8 ssDNA in all cases, so the reactions at the right involving a heterologous insertion utilize M13mp8.52 as the linear dsDNA substrate. In each set of reactions, the time points are zero, one, three, five, and eight hours from left to right. The RecA protein used is indicated at the top of each reaction. Symbols are: ss, circular M13mp8 ssDNA; S, linear dsDNA substrate; P, circular, nicked duplex DNA product of strand exchange; I, reaction intermediates.

Table 4. Bacterial strains

Strain	Relevant markers, prophage or plasmid	Source and origin
GY7066	*lacMS286* φ80*dIIlacBK1* Δ*recA306 sfiB114*	Dutreix *et al*. (1989)
GY7236	Hfr J2 *leu::Tn9*	Dutreix *et al*. (1989)
GY7313	Δ*lac sfiA211* Δ*recA306* (λcIind1 sfiA::lacZ$^+$)	Bailone *et al*. (1988)
GY7385	as GY7313 (λcIind1 sfiA::lacZ$^+$), (pMMB175)	Bailone *et al*. (1988)
GY4532	BL21 (DE3) (plysE)	Studier *et al*. (1990)
GY7742	as GY4532 but Δ*recA306*	This work
GY8379	as GY7742 but (pGY8239)	This work
GY8630	*recA13 uvrB501*, (F42-10)	Sommer *et al*. (1993)
GY9086	*recA938*::*cat lacZ*::kan Str^R (λind553)	This work
GY9091	*recA938*::*cat* Δ*lac* Nal^R	This work

ATPase activity of RecA423. As seen in Figure 9, the mutant protein can promote synapsis.

Discussion

The RecA423 mutant is altered at Arg_{169}. This arginine is one of the most conserved amino acid residues in the bacterial RecA protein (Roca & Cox, 1996). Our primary conclusion is that the mutation in RecA423 has a deleterious effect on the rate of ssDNA binding in the presence of ATP, resulting in lower rates of ATP hydrolysis, a slower rate of DNA strand exchange, and failure to bypass a heterologous insertion in the duplex DNA substrate during strand exchange. *In vivo*, the mutant strain is proficient for most types of homologous recombination, but deficient for UV repair. Recombination in Hfr crosses is therefore unaffected by a mutation that causes a quite general and substantial reduction in major RecA activities. In contrast, recombinational DNA repair is nearly eliminated by the same mutation. The sensitivity to UV radiation cannot be ascribed to the absence of some other repair function induced by the SOS response. The *recA423* mutation also confers a coprotease constitutive phenotype. The results indicate that the biochemical requirements for recombinational DNA repair are more exacting than those for recombination. They are also consistent with the idea that many of the biochemical features of RecA evolved as a response to the exigencies of DNA damage (Cox, 1993).

The *in vivo recA423* phenotype

RecA423 is proficient in three types of recombination events: (1) conjugational recombination, (2) transductional recombination, and (3) recombination of λred$^-$ *gam*$^-$ phage. These three processes have in common that (1) each involves, at some point, a linear DNA substrate, (2) each provides dsDNA ends that can serve as entry sites for RecBCD enzyme, and (3) in each, the recombining DNAs span large regions of perfect homology (see Kowalczykowski *et al*., 1994).

RecA423 is deficient in intrachromosomal recombination that occurs within a circular dsDNA molecule with no available ends (and is therefore independent of RecBCD enzyme activity (Zieg *et al*., 1978)). In this process, recombination takes place between DNAs having a relatively short region of homology interrupted by a region of heterology created by the *lacZBK1* deletion (Konrad & Lehman, 1975). Intrachromosomal recombination may be initiated by small regions of ssDNA created by normal DNA metabolic processes such as replication, transcription or repair of spontaneous DNA damage (Zieg *et al*., 1978). The reduced rate at which RecA423 protein binds to ssDNA may selectively impede binding to short ssDNA gaps. The evident link between intrachromosomal recombination and recombinational DNA repair seen in *recA423* reinforces a range of observations suggesting that DNA damage plays the major role in initiating intrachromosomal recombination (Roca & Cox, 1996). Another known similarity between recombinational DNA repair and intrachromosomal recombination that is not shared by conjugation is their common sensitivity to PsiB inhibition (Bailone *et al*., 1988).

RecA protein plays a crucial role in repair of DNA damage. RecA is required as a coprotease for enhanced synthesis of SOS repair proteins such as UvrAB, RuvAB, UmuDC and RecA itself and for processing UmuD protein to its active form UmuD'. RecA, as a recombinase, catalyzes early steps in recombinational repair such as homologous pairing and strand exchange. RecA is also part of the protein complex required for SOS mutagenesis which is thought to reflect trans-lesion DNA synthesis (Sommer *et al*., 1993). The UV-sensitivity of *recA423* bacteria, in spite of their ability to promote SOS induction and SOS mutagenesis, indicates that RecA423 protein is deficient in recombinational repair. As seen in Figure 1, *recA423* bacteria are somewhat more resistant to UV than those with a *recA* deletion. The residual resistance may reflect the SOS repair in *recA423* cells. The result suggests that most of the UV-sensitivity of a *recA* null mutant reflects the defect in recombinational repair rather than a defect in SOS induction.

Recombinational repair can restore the continuity of damaged DNA and fill the gap opposite a lesion by RecA-mediated strand exchange with the intact homologous sister duplex (Figure 10(A)). RecA recombinase activity can also be needed to rescue a stalled replication fork, in particular when the replication-blocking lesion is located on the leading

strand. The pol III holoenzyme becomes stalled at the lesion while the associated DnaB helicase continues separating the two strands. Uncoupling of leading strand synthesis from helicase movement at the fork generates a stretch of ssDNA on which RecA protein polymerizes. RecA-mediated strand exchange and reverse branch migration of the Holliday junction by RecG protein (Whitby *et al*., 1993) restore a normal configuration of the replication fork allowing the pol III holoenzyme to continue DNA replication (Figure 10(B)). RecA recombinase function is also required for stable DNA replication, a particular mode of DNA replication induced by DNA damage, independent of DnaA protein and maintained in the cells after arrest of protein synthesis contrary to normal chromosomal replication from *oriC*. Stable DNA replication involves activation of secondary replication origins (Asai *et al*., 1993; Asai & Kogoma, 1994). All these recovery mechanisms might be impaired in *recA423* bacteria, accounting for their failure to recover a normal rate of DNA synthesis after UV irradiation. The defect may again reflect a limited capacity to bind short stretches of ssDNA.

The coprotease constitutive phenotype is more difficult to explain. Only a few RecA coprotease-constitutive mutant proteins, such as RecA441 and RecA730, have been biochemically characterized *in vitro* (Lavery & Kowalczykowski, 1992). Each of these proteins exhibits an enhanced ability to compete with SSB protein for DNA-binding sites and an increased rate of association with ssDNA. It

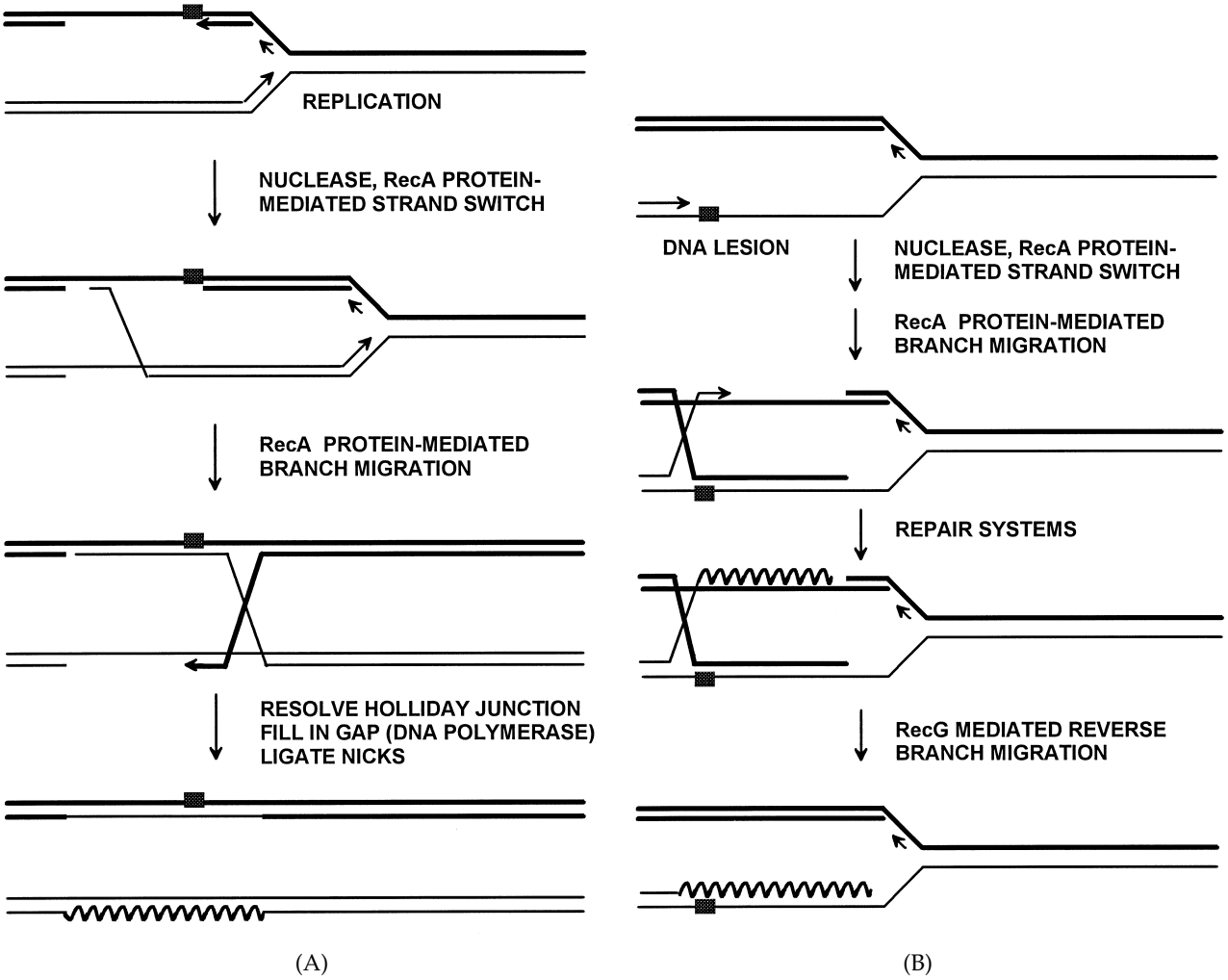

Figure 10. Models. (A) Postulated role for RecA protein in post-replication repair. A replication fork containing a lesion on a lagging strand template is shown. The pol III holoenzyme stops at the lesion and is recycled to the next primed site. The lesion is left behind in ssDNA after the replication has passed. Repair requires that this DNA be made duplex. This is accomplished by recruiting a complementary strand from the opposite side of the replication fork and pairing it with the lesion-containing strand *via* RecA-mediated strand exchange. (B) Postulated role for RecA protein in rescue of a stalled replication fork. A replication fork containing a lesion on the leading strand template is shown. The pol III holoenzyme becomes stalled at the lesion, while the associated DnaB helicase continues separating the two strands. Uncoupling of leading strand synthesis from helicase movement at the fork generates a stretch of ssDNA on which RecA protein polymerizes. RecA-mediated strand exchange and reverse branch migration of the Holliday junction by RecG protein (Whitby *et al*., 1993) restore a normal configuration of the replication fork, allowing the pol III holoenzyme to continue DNA replication.

has been proposed that these proteins are constitutively activated for repressor cleavage *in vivo* because they can compete with, and displace, SSB from ssDNA that exists transiently in the bacterial cell, such as at the replication fork (Lavery & Kowalczykowski, 1990; Phizicky & Roberts, 1981). RecA423 protein has a constitutive coprotease phenotype but possesses reduced, rather than enhanced, ssDNA-binding properties. This might be explained in at least two ways. First, RecA423 might bind ssDNA and displace some SSB quickly to form short filaments capable of promoting LexA cleavage. Extension of the nucleated filaments might be very slow, however, impeding the formation of longer filaments needed for recombinational repair. Alternatively, the RecA423 protein might possess a conformational or structural feature that allows it to promote LexA cleavage even when it is not bound to DNA. RecA423 might be activated by other endogeneous molecules such as RNA. If PsiB protein competes with RecA for DNA binding, a capacity to promote LexA cleavage without DNA would explain the capacity to bypass the PsiB inhibition. A third explanation for the constitutive coprotease activity of RecA423 protein can be proposed: the major defect in recombinational repair could increase the quantity of single-stranded DNA present in non-treated cells, resulting from a defect of repair of spontaneous DNA damage.

In vitro properties of RecA423 protein

Our studies show that none of the three DNA binding sites is completely abolished by this mutation. Furthermore, the structural characteristics of RecA-DNA complexes that are monitored by linear dichroism are not affected by the mutation. However, the mutation strongly reduces the association rate of RecA to ssDNA, the first step of recombination, especially in the presence of ATP. This results in an associated decline in ATP hydrolytic activity. In contrast, the mutation has a minimal effect on the binding to ssDNA in absence of the ATP cofactor.

The *recA423* mutation may affect DNA binding directly and/or the conformational change that occurs in RecA upon binding to ATP. A few investigations suggested that the disordered L1 loop of RecA is exposed upon ATP binding (Kobayashi *et al*., 1987; Takahashi & Nordén, 1993). The proximity of the L1 loop to the mutation site of RecA423 supports a role for position 160 in a conformational change. The reduction of the salt-induced ATPase by the mutation is also consistent with this hypothesis. However, a number of results suggest a more direct role in DNA binding. Many of the mutants which have their mutation sites around the L1 region exhibit abnormal DNA binding. The RecA1 mutation ($Gly_{160} \rightarrow$ Asp: Kawashima *et al*., 1984) is completely inactive *in vivo* (Clark & Margulies, 1965), and *in vitro* binds single-stranded DNA very weakly and is unable to hydrolyze ATP (West *et al*., 1980). Notably, the RecA1 protein exhibits its major DNA binding defects mainly when ATP is present (Wabiko *et al*., 1983). Studies on an Asn_{160} (Bryant, 1988) and a His_{163} (Muench & Bryant, 1991) mutation also indicate that these mutations prevent an ATP-induced conformational change necessary for strand exchange.

Story & Steitz (1992) suggested that residues in, or around, loop L1 may be involved in the secondary binding of homologous duplex DNA necessary for recombination, since the RecA1 mutant protein does not bind a second strand of DNA in the presence of ATPγS (Bryant & Lehman, 1986). However, the data show that binding of RecA423 mutant protein to single-stranded DNA is also affected. This suggests that the L1 region or some part of it may also play a role in primary binding to ssDNA. Interaction of the L1 region with the primary ssDNA was recently indicated by a photocross-link technique (Wang & Adzuma, 1996; Malkov & Camerini-Otero, 1995).

These results bring us to a consideration of factors that distinguish recombinational DNA repair and homologous genetic recombination. The properties of RecA-mediated DNA strand exchange that can be attributed to ATP hydrolysis are those likely to be most important in repair. It should not be surprising therefore, that the weak ATPase activity of RecA423 protein might be enough to promote a strand exchange reaction adequate for homologous genetic recombination. If two DNAs can be paired and strand exchange facilitated by binding energy within a RecA nucleoprotein filament, RecA's essential role in recombination might be satisfied. The requirements of recombinational DNA repair appear to be more exacting, requiring more efficient DNA binding and ATP hydrolysis.

Materials and Methods

Bacterial strains, plasmids, phages, and cultures

Bacterial strains are listed in Table 4. Plasmid miniF-*recA*$^+$ was described by Dutreix *et al*. (1989). This plasmid is in numerical parity with the host chromosome and is stably maintained in the host cell. MiniF-*recA423* carries an hydroxylamine-induced *recA* mutation at nucleotide 558, changing Arg_{169} to His. The *recA730* mutation was transferred on the miniF-*recA* plasmid by homogenotization *in vivo*.

Plasmid pMMB175 contains the PsiB gene expressed under the control of the *tac* promoter (Bailone *et al*., 1988). Plasmid pGY7671 carries the *recA* gene placed under the control of the ϕ10 promoter of phage T7 (Dutreix *et al*., 1992). Plasmid pGY8235 is a derivative of pGY7671 containing a unique *Bam*HI site located downstream of the *recA* gene. Plasmid pGY8239, used for overproducing RecA423 protein, carries *recA423* under the control of the ϕ10 T7 phage promoter. This plasmid was derived from pGY8235 by replacing the *Nco*I-*Bam*HI fragment encoding the last two-thirds of the terminal part of *recA*$^+$ by the corresponding *recA423* fragment carried by miniF.

Plasmids pOU61-*recA*$^+$ and pOU61-*recA423* were

constructed by inserting the *Bam*HI *recA* fragment of miniF-*recA*$^+$ or miniF-*recA423* into the unique *Bam*HI site of pOU61, a runaway vector whose replication is under λ repressor control (Larsen *et al*., 1984). The recombinant plasmids were introduced into GY9086, a *recA*$^-$ λ immune host, in which they were stably maintained at one copy per chromosome.

F42-10 is an F'*lac* plasmid carrying 55 kb of the bacterial chromosome and a Tn*10* inserted into a dispensable part of the F plasmid (Sommer *et al*., 1993).

T7 RNA polymerase is expressed under *lac* promoter control in prophage DE3 (Studier *et al*., 1990). Plasmid *plysE* expressing T7 lysozyme, a natural inhibitor of T7 RNA polymerase, is used to reduce T7 polymerase activity (Studier *et al*., 1990). Phage λbio10 used in recombination assays carries a large deletion substitution removing *red* and *gam* phage genes (Lindahl *et al*., 1970).

Bacteria were grown in LBT medium or YM9C and colonies were scored on LAT medium (Devoret *et al*., 1983; Moreau *et al*., 1980). Lac$^+$ papillae were observed on colonies grown on plates containing 4% (w/v) MacConkey agar base (Difco) supplemented with 1% (v/v) lactose. GT plates used for plating of λbio were as described (Devoret *et al*., 1983). The concentration of ampicillin, kanamycin, chloramphenicol and streptomycin in liquid and solid media were 50 μg/ml, 50 μg/ml, 10 μg/ml and 200 μg/ml, respectively. All incubations were at 37°C.

Isolation of *recA423*

The mutation *recA423* was isolated in the course of a search for coprotease constitutive *recA* mutants insensitive to PsiB action. A miniF-*recA* plasmid was treated with hydroxylamine as described by Eichenlaub (1979) and introduced into a host expressing PsiB polypeptide from a *tac* promoter and carrying a *sfiA*::*lacZ* fusion as an indicator of SOS induction. Coprotease constitutive mutants insensitive to PsiB were detected as dark blue colonies on Xgal + 1 mM IPTG plates. Ten dark blue colonies were found out of 20,000 transformants. The dideoxy-chain termination procedure (Sanger, 1981) was used to sequence the *recA423* gene carried on the plasmid pGY8239. Plasmid DNA was prepared for sequencing by a published method (Hattori & Sakaki, 1986).

Recombination assays

Hfr × F$^-$ recombination: derivatives of GY7066 were mated with GY7236: HfrJ2 *leu*::Tn*9* Str^S donor (1 Hfr for 5 F$^-$) for 30 minutes at 37°C. Appropriate dilutions were plated on LAT + Cam + Str to select for *leu*::Tn*9* Str^R recombinants.

Intrachromosomal recombination: derivatives of GY7066 carrying two copies of the *lac* operon, each with a non-overlapping deletion, were used. Recombination between the two defective *lac* genes produces Lac$^+$ recombinants, which form red papillae on white Lac$^-$ colonies on MacConkey lactose indicator plates. Lac$^+$ papillae per colony on ~100 plated cells were counted after two (*recA*$^+$ and *recA730*) or three (*recA423* and Δ*recA306*) days of incubation at 37°C.

P1 transduction: derivatives of GY7313:*leuB6* were infected at an m.o.i. = 0.2 with P1 *vir* phage grown on *leu*$^+$ bacteria. After 30 minutes at 37°C, the infected cells were centrifuged, washed and plated on appropriate minimal plates to select for Leu$^+$ transductants.

Plating of λbio: the indicator bacteria were derivatives of GY7066. Sequential phage λbio dilutions were spotted on a lawn of indicator bacteria on GT plates. Plaques were counted after overnight incubation.

Recombinational repair of a UV-damaged F'*lac* plasmid: GY8630: *recA13 uvrB501* (F42-10: *lac*$^+$) donors exposed to 0, 10 or 20 J/m^2 of UV light were mated with *recA*$^+$ and *recA423* derivatives of GY9086: F$^-$ *lacZ::kan*. The exconjugants were directly plated on LA + Str + Tet plates to determine the survival of the transferred F'. The exconjugants were mated with a second recipient GY9091: *recA938*::cam Δ*lac* to determine the *lac* genotype of the surviving F'*lac* plasmids (Sommer *et al*., 1993). The sexductants resulting from this second cross were plated on appropriate selective plates containing X-gal to measure the frequency of *lacZ::kan* recombinants.

Measurement of DNA synthesis after UV-irradiation

Bacteria exponentially growing in YM9C were exposed to a 10 J/m^2 UV dose. The bacteria were irradiated in their growth medium instead of in saline solution to minimize interruption of exponential growth (Witkin *et al*., 1987). Samples of the culture, 0.5 ml, were removed at five minute intervals and pulse-labeled with [^{3}H]thymidine (10 μCi, specific activity 85 Ci/mmol) for two minutes, and trichloroacetic acid-precipitable radioactivity was counted (Khidhir *et al*., 1985).

Enzymes and reagents

Escherichia coli RecA protein (wild-type) was purified to homogeneity and stored as previously described (Cox *et al*., 1981). *E. coli* single-stranded DNA binding protein (SSB) was purified as described (Lohman *et al*., 1986), except that an additional step utilizing DEAE-Sepharose chromatography was included to ensure removal of single-strand exonucleases. The RecA protein and SSB concentrations were determined by absorbance at 280 nm, using extinction coefficients of $\epsilon_{280\,nm} = 2.17 \times 10^4\ M^{-1}\ cm^{-1}$ (Craig & Roberts, 1981), and $\epsilon_{280\,nm} = 5.6 \times 10^4\ M^{-1}\ cm^{-1}$ (Lohman & Overman, 1985), respectively. ATP and ATPγS were purchased from Boehringer Mannheim Biochemicals. Concentrations of ATP and ATPγS were determined spectrophotometrically, using $\epsilon_{260\,nm} = 1.54 \times 10^4\ M^{-1}\ cm^{-1}$. DEAE-Sepharose was purchased from Pharmacia. Pyruvate kinase, phospho(enol)pyruvate, lactic dehydrogenase, ATP, and Tris were purchased from Sigma.

Purification of RecA423 protein

A six liter culture of *E. coli* strain, GY8379 bacteria which overexpresses RecA423 protein was grown at 37°C in LB supplemented with 0.2% (w/v) glucose, 25 μg/ml chloramphenicol and 100 μg/ml ampicillin. At A_{650} = 0.75, the culture was induced with 1 mM IPTG (final concentration). After two hours of incubation, the cells were collected by centrifugation. The cell pellet (21 g) was frozen in liquid nitrogen, and stored at −20°C.

Two different purification procedures were followed. In the first, RecA423 protein was purified as described (Cox *et al*., 1981), except that the single-stranded DNA column chromatography was omitted. Instead, a heparin agarose column (Biorad) was included in the purification scheme after the phosphocellulose chromatography step. The column was run in 100 mM NaCl in R buffer (Cox *et al*., 1981). Flow-through fractions were pooled and

applied to a 1 ml MonoQ FPLC column equilibrated to the same buffer. The mutant RecA protein was eluted from the MonoQ column with a linear gradient of 100 mM to 500 mM NaCl in R buffer, with the protein appearing at 180 to 190 mM NaCl. The final yield of RecA423 protein from 21 g cells was 2.8 mg.

In the second procedure, RecA423 protein was purified from crude extracts of GY8379 bacteria by the procedure as described by Kuramitsu *et al*. (1981) except that the DEAE-cellulose chromatography step was replaced with a DEAE 5PW (Tosoh, Japan) high performance ion-exchange liquid chromatography (Takahashi & Hagmar, 1991). The mutant protein was eluted from the phosphocellulose column at a lower salt concentration (0.12 M KCl) than wild-type RecA (0.18 M KCl), but eluted from the DEAE 5PW column at about the same salt concentration (0.35 M NaCl) as the wild-type RecA.

The concentration of RecA423 protein was determined spectrophotometrically using the extinction coefficient of wild-type RecA described above. In both cases, the RecA423 protein was at least 90% pure as estimated by SDS-PAGE, and was free of detectable endo- or exonuclease activities.

DNA

Calf thymus DNA was purchased from Sigma. Supercoiled circular duplex DNA, circular single-stranded DNA and their derivatives from M13mp8 (7229 bp) and M13mp8.52 (7281 bp) were prepared using methods described by Davis *et al*. (1980), Messing (1983) and Neuendorf & Cox (1986). The bacteriophage M13mp8.52 is the bacteriophage M13mp8 with a 52 bp sequence (*Eco*RV fragment from the *E. coli galT* gene inserted into the *Sma*I site (Bedale *et al*., 1993)). The concentrations of ssDNA and dsDNA stock solutions were determined by absorbance at 260 nm, using $\epsilon_{260\,nm} = 8.9 \times 10^3\ M^{-1}\ cm^{-1}$ and $\epsilon_{260\,nm} = 6.7 \times 10^3\ M^{-1}\ cm^{-1}$, respectively. DNA concentrations are reported in terms of total nucleotides. Complete digestion of supercoiled M13mp8.52 with *Alw*NI resulted in full-length linear duplex DNA substrates that, when paired with M13mp8 ssDNA, contain a 52 bp heterologous insertion, located internally. After digestion, residual protein was removed by 1:1 extraction with phenol/chloroform/isoamyl alcohol (25:21:1, by vol.) and chloroform/isoamyl alcohol (24:1, v/v) followed by precipitation in ethanol.

Poly(dεA) was prepared from poly(dA) (Pharmacia) as described (Cazenave *et al*., 1983). The degree of modification was 96% according to the formula of Ledneva *et al*. (1978). Concentrations were determined spectrophotometrically using the extinction coefficient: $\epsilon_{257\,nm} = 3.8 \times x10^3\ M^{-1}\ cm^{-1}$.

Etheno M13 DNA (εM13 DNA) was made as described by Zlotnick *et al*. (1993). We incubated 500 μM single-stranded M13mp8 DNA, 50 mM NaOAc (pH 5.0), 1 mM EDTA, with one-sixth volume freshly distilled chloroacetaldehyde at 45°C for 90 minutes. The reaction was quenched with one-third volume of cold 1.5 M Tris-HCl (pH 8.8), then the sample was concentrated by centrifugation in a Centricon 30 (Amicon). The sample was washed with 20 mM Tris-HCl (pH 7.0), four times in a Centricon to remove all of the unreacted chloroacetaldehyde. The concentration of etheno-DNA was determined by using an extinction coefficient of $\epsilon_{260\,nm} = 7.0 \times 10^3\ M^{-1}\ cm^{-1}$. The product had an absorption maximum at 264 nm and a minimum at 245 nm with $A_{260/245} = 1.3$, $A_{260/270} = 1.0$, $A_{260/280} = 1.2$, and $A_{260/290} = 2.0$ (Menetski & Kowalczykowski, 1985; Zlotnick *et al*., 1993). The integrity of the resultant modified DNA was assessed by electrophoresis on 1% alkaline agarose gels followed by visualization using Kodak Stains-All (Zlotnick *et al*., 1993).

Reaction conditions

Unless stated elsewhere (e.g. see linear dichroism procedures), all reactions were carried out at 37°C in solutions containing 60 mM Tris-acetate (80% cation (pH 7.5)), 1 mM dithiothreitol, 5% (w/v) glycerol, 3 mM potassium glutamate, 10 mM magnesium acetate. The ATP regenerating system includes two units/ml pyruvate kinase and 3 mM phospho(enol)pyruvate.

DNA-dependent ATPase assay

The coupled spectrophotomeric assay used to measure ATP hydrolysis was as described (Iype *et al*., 1994; Morrical *et al*., 1986). The regeneration of ATP from ADP and phospho(enol)pyruvate with the oxidation of NADH can be followed by the decrease in absorbance at 380 nm. M13mp8 circular single-stranded DNA (6 to 8 μM as indicated) was preincubated with the indicated proteins and all other reaction components except ATP and SSB at 37°C for ten minutes. The assay was initiated by the addition of SSB (to 0.5 μM, final) and ATP (to 3 mM, final). Under these conditions, rates of ATP hydrolysis are essentially a linear function of wild-type RecA protein concentration until stoichiometric (one RecA monomer per three nucleotides of ssDNA) concentrations are reached, at which point the rate levels off. ATP hydrolysis during DNA strand exchange was measured as described by Bedale & Cox (1996) and by Schutte & Cox (1987). Linear duplex DNA was added after addition of ATP and SSB. The total change in reaction volume resulting from dsDNA addition was about l%; therefore, no correction was made for dilution. In control reactions to which no duplex DNA was added, an equivalent volume of TE buffer was added instead.

A few DNA-dependent ATPase experiments, noted in Results, were carried out at 30°C in a buffer containing 20 mM potassium phosphate and 4 mM $MgCl_2$ (pH 6.4). Salt-induced ATPase activity was measured at 30°C in a buffer containing 2 M NaCl in addition to the potassium phosphate and $MgCl_2$.

Agarose gel assay for DNA strand exchange

The formation of the nicked circular heteroduplex (FII) product of strand exchange was monitored by the agarose gel electrophoresis assay of Cox & Lehman (1981). Since the DNA strand exchange rate of RecA423 mutant protein was very slow, we used higher concentrations of each component to increase the sensitivity of the gel assay. The reactions contained 25 μM circular ssDNA, 25 μM linear duplex DNA, 8 μM RecA protein, 25 units/ml pyruvate kinase, 23 mM phospho-(enol)pyruvate, 24 mM ATP and 3 μM SSB. All components except SSB and duplex DNA were mixed and incubated at 37°C for five minutes. SSB was added and incubation was continued for another five minutes before addition of duplex DNA. Aliquots (10 μl) of the reaction mixture, taken at the indicated times, were added to a gel loading buffer containing 12 mM EDTA, 1% (w/v) SDS, 0.05% (w/v) bromophenol blue, and 5% (w/v) glycerol. Samples were loaded on a 0.8% (w/v) agarose gel and electrophoresed in buffer containing 0.04 M Tris-borate (pH 8.0) and 1 mM EDTA.

Fluorescence

The fluorescence of ϵDNA was monitored at excitation and emission wavelengths of 305 and 410 nm, respectively (Zlotnick *et al*., 1993). Titration data were obtained by using a SLM Series 8000 fluorometer. The sample chamber was temperature controlled at 37°C with a Brinkmann Instruments Lauda K-2/RD water circulator. Titrations in the presence of cofactors were done at 100 μM for ADP and ATP and 50 μM for ATPγS. In experiments with ATP, which is hydrolyzed rapidly in the presence of RecA protein and single-stranded DNA, an ATP regenerating system consisting of 2.6 units pyruvate kinase/ml and 2 mM phospho(enol)pyruvate was added to the cuvette (Menetski & Kowalczykowski, 1985). Since sequential additions of protein aliquots would alter the volume of the reaction mixture significantly, each point in the protein titrations represents a separate reaction mixture carried out in a volume of 100 μl. Salt titrations were carried out by sequential addition of a concentrated NaCl solution to a single 700 μl reaction mixture, since all additions resulted in a less than 6% change in the total volume.

Linear dichroism measurements

LD experiments were performed at 25°C in a buffer containing 20 mM potassium phosphate, 50 mM NaCl and 4 mM $MgCl_2$ (pH 6.8). LD was measured in a modified Jasco J-500A spectropolarimeter and expressed in absorbance units (Nordén & Seth, 1985). An inner rotating Couette cell (Wada & Kozawa, 1964) was used to align the samples. The shear force was 600 second^{-1}. RecA–poly(dϵA) complex was formed by adding 100 μM ATPγS to a mixture of 1 μM RecA and 3 μM (in bases) poly(dϵA). The solution was incubated for 30 minutes at 25°C before the measurement. The complex with two molecules of poly(dϵA), (RecA–poly(dϵA)$_2$ complex) was formed by the addition of 3 μM poly(dϵA) to the ATPγS–RecA–poly(dϵA) complex. To this RecA–poly(dϵA)$_2$ complex, heat-denatured calf thymus DNA (15 μM) was added to form the complex with three ssDNAs (ATPγS–RecA–poly(dϵA)–poly(dϵA)–ssDNA complex). After each addition of DNA the mixture was incubated for 30 minutes at 25°C. The complex with dsDNA was formed in a similar manner except with a longer incubation.

RecA–DNA complex formation is accompanied by an increase in the LD around 260 nm due to the stiffening of the DNA as a result of the firm support from the surrounding helical RecA filament (Kim *et al*., 1993; Takahashi *et al*., 1989a). The kinetics of complex formation thus could be followed by monitoring the LD intensity at 260 nm. For the kinetic studies, LD was observed with a mild shear gradient of 600 second^{-1} with which the signal from uncomplexed DNA is very small.

Acknowledgements

This work was supported by grants from the EEC: FI3P-CT92-0028, the Association pour la Recherche sur le Cancer no 6093, and the Fondation pour la Recherche Médicale (to R.D.), as well as from the US National Institutes of Health, GM32335 (to M.M.C.). G. Coste and D. Bouillon are thanked for their expert technical assistance. M Pierre is thanked for her help with the manuscript. M. T. thanks Professor B. Norden (Chalmers University of Technology, Sweden) for use of his LD equipment and encouragement and Professor T. Horii (Osaka University, Japan) for valuable discussions.

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Edited by M. Yaniv

(Received 22 April 1996; received in revised form 18 September 1996; accepted 23 September 1996)