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ATP-dependent renaturation of DNA catalyzed by the recA protein of *Escherichia coli*

(ATPase/single-stranded DNA/*lexB30* and *tif-1* mutants/cold-sensitive mutation/recombination intermediate)

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ABSTRACT **The product of the *recA* gene of *Escherichia coli* has been purified to near-homogeneity by a simple three-step procedure. Incubation of the recA protein with complementary single strands of DNA, Mg^{2+}, and ATP results in the rapid formation of large DNA aggregates containing many branched structures. As judged by resistance to S1 nuclease and by electron microscopy, these aggregates contain both duplex and single-stranded regions. The renaturation and aggregation of DNA catalyzed by the recA protein is coupled to the hydrolysis of ATP. The recA protein purified from a cold-sensitive *recA* mutant does not catalyze DNA renaturation or aggregation at 28°C, but does so at 37°C, a finding which correlates with the recombination defect observed *in vivo* and indicates that this activity is an intrinsic function of the recA protein. These results suggest that the recA protein plays a specific role in strand transfer during recombination and possibly in postreplication repair of damaged DNA.**

Mutation of the *recA* gene of *Escherichia coli* produces a complex, pleiotropic phenotype (1, 2), the most conspicuous manifestation of which is an inability to perform general recombination [as opposed to site-specific or illegitimate recombination, which are *recA*-independent (3)]. This process, involving transfer of homologous regions of DNA, depends upon the annealing of complementary strands to produce, at an early stage in recombination, a region of duplex DNA containing one strand from each parental DNA molecule (3, 4). Numerous studies have suggested that such heteroduplex overlaps are not formed in *recA* mutants, in contrast to other recombination-deficient mutants (e.g., *recB*, *recC*) (5–10). Thus, although a number of genes can affect recombination, only the *recA* function has been implicated in strand transfer.

Examination of the DNA of UV-irradiated *recA* mutants indicates that one function of the recA product is in postreplication repair. This process involves the repair of single-strand gaps that are believed to result from the inability of DNA polymerases to insert nucleotides opposite pyrimidine dimers (11, 12). In wild-type cells, these gaps are filled by a recombinational mechanism, whereas they are not repaired in *recA* mutants (13). A number of other processes that occur after DNA damage are absent in *recA* mutants, including mutability, induction of prophages, W-reactivation of damaged phages, and control of DNA degradation (2, 14–17). Many of these processes observed after damage to DNA are inducible ("SOS functions") (16, 18) and are altered by mutations at the *recA* locus [*lexB30* (19), and *tif-1* (20)] that do not significantly affect recombination. Thus, the role of the recA product in DNA repair is complex and may involve both structural and regulatory functions.

The product of the *recA* gene has recently been identified as a protein with a subunit molecular weight of about 40,000 (21). Although normally present in relatively low amounts, treatments that damage DNA lead to the accumulation of several thousand molecules of the recA protein per cell (22–25).

Thus far, two enzymatic activities have been associated with recA protein. Roberts *et al.* (26) have demonstrated that the recA protein cleaves the phage λ repressor *in vitro*, a reaction that probably reflects the role of *recA* in prophage induction; and Ogawa (27) and Roberts *et al.* (26) have reported that recA protein catalyzes hydrolysis of ATP in the presence of single-stranded DNA.

We have developed a simple purification procedure for the recA protein that serves equally well for the wild-type protein and several of its mutant forms. This procedure, which yields a nearly homogeneous protein, takes advantage of strains containing a regulatory mutation that allows overproduction of the various recA proteins. By using the purified wild-type and mutant proteins, we have found that the recA protein catalyzes the formation of duplex DNA from complementary single strands in a reaction coupled to hydrolysis of certain nucleoside triphosphates. This reaction is consistent with a direct role for the recA protein in the formation of heteroduplex overlaps during recombination and in the filling of single-strand gaps during postreplication repair.

MATERIALS AND METHODS

Bacterial Strains. The wild-type recA protein was purified from strain KM1842 which bears the *recA*Δ*21* deletion (28, 29) on the chromosome and a plasmid, pLC1842 (29, 30), containing a wild-type *recA* gene. This strain also carries the *spr51* mutation (31) at the *lexA* locus which allows high-level constitutive expression of *recA*. The tif protein was purified from strain KM444, which contains a *spr*-like mutation (K. McEntee, unpublished results) as well as the *tif-1* allele of the *recA* gene. The *tif-1* mutation allows constitutive expression of SOS functions at high temperature but has little or no effect on recombination (20). The cold-sensitive recA protein was purified from KM1629 which contains the *recA629* and *spr51* mutations. The *recA629* mutation was obtained by selecting revertants of *tif-1* that were unable to induce a λ prophage at high temperature. This revertant possesses a UV-sensitive, recombination-deficient phenotype at 30°c but not at 37°C (K. McEntee, unpublished results). The lexB protein was purified from KM1193, which contains the *lexB30* mutation at the *recA* locus and the *spr51* mutation. The *lexB30* mutation prevents induction of SOS functions but has little effect on recombination (19).

Materials. R buffer is 20 mM Tris·HCl, pH 7.5/10% (vol/vol) glycerol/1 mM dithiothreitol/0.1 mM EDTA. P buffer contained 20 mM potassium phosphate (pH 6.5), replacing the Tris·HCl in R buffer. ^{3}H- and ^{32}P-labeled nucleoside triphos-

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Abbreviations: ATP[γ-S], adenosine 5′-[γ-thio]triphosphate; NaDodSO$_4$, sodium dodecyl sulfate; φX174 RFII, nicked double-stranded φX174 DNA.

phates were purchased from Amersham/Searle or New England Nuclear. Adenosine 5'[γ-thio]triphosphate (ATP[γ-S]) was from Boehringer Mannheim. S1 nuclease of *Aspergillus oryzae* was from Sigma.

Purification of the recA Protein. The recA protein was purified from cells containing high levels of the protein as a consequence of the *spr* mutation and nalidixic acid treatment. The wild-type protein was further amplified by a multicopy plasmid carrying the *recA* gene. Throughout the purification, the recA protein was assayed by polyacrylamide gel electrophoresis.

Cells were grown at 37°C in 6 liters of L broth (1) to an OD_{595} of about 1, treated with nalidixic acid (40 μg/ml) for 90 min, and then harvested. The cells (10–15 g) were resuspended in 30 ml of cold 50 mM Tris·HCl, pH 8.1/25% (wt/vol) sucrose. All subsequent operations were performed at 4°C. The cells were lysed by a modification of the procedure of Clewell and Helinski (32). Lysozyme (6 ml of a 5 mg/ml solution in 0.25 M Tris·HCl, pH 8.1) was added to the cell suspension followed after 15 min by 13 ml of 25 mM EDTA (pH 8.0). After 10 min, 50 ml of a solution composed of 1% (wt/vol) Brij-58, 50 mM Tris·HCl (pH 8.1), and 2 mM dithiothreitol was added, and the suspension was mixed and incubated for 30 min with occasional stirring. The lysate was centrifuged for 60 min at 18,000 rpm in a Beckman JA-20 rotor. The viscous supernatant amounted to about 75% of the Brij lysate. Polymin P [10% (vol/vol), pH 7.9 (33)] was added to the supernatant to a final concentration of 0.5%. Mixing was continued until the viscosity was greatly reduced. Precipitated protein and nucleic acids were pelleted by low-speed centrifugation and the pellet was resuspended in one-half the volume of the original supernatant with R buffer containing 150 mM ammonium sulfate. After 10 min, the suspension was centrifuged and the resulting pellet was resuspended in one-fifth volume of R buffer containing 300 mM ammonium sulfate. After low-speed centrifugation, the recA protein was quantitatively recovered in the supernatant. Ammonium sulfate was added (0.25 g/ml of supernatant); the suspension was stirred at 4°C for 60 min and then centrifuged at 14,000 rpm for 60 min in a Beckman JA-20 rotor. The pellet was resuspended in 3 ml of R buffer and dialyzed overnight against 1 liter of P buffer. The dialyzed ammonium sulfate fraction (1.5 ml) was applied to a 24-ml phosphocellulose column that had been equilibrated with P buffer and eluted with a 250-ml gradient of KCl (0–250 mM in P buffer). Approximately 10 mg of nearly homogeneous recA protein was obtained from a 6-liter culture of KM1842.

The recA$^+$, tif, and lexB proteins were eluted from phosphocellulose columns at approximately 80 mM KCl, whereas the recA629 protein was eluted at about 120 mM KCl. Each of these proteins was greater than 90% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate ($NaDodSO_4$) (Fig. 1). The only contaminant noted in the wild-type recA protein had a molecular weight of approximately 85,000; it accounted for less than 5% of the total protein.

Preparation of DNAs. Supercoiled ColE1 DNA was isolated from chloramphenicol-treated cells and purified by CsCl/ethidium bromide equilibrium centrifugation. G4, ϕX174, and nicked ColE1 DNAs were generously donated by members of this department. P22 DNA was prepared essentially as described by Botstein (34). Calf thymus DNA was purchased from Calbiochem.

S1 Nuclease Assay for Duplex DNA. DNA renaturation catalyzed by the recA protein was performed at 28 or 37°C in 25-μl reaction mixtures containing 20 mM Tris·HCl (pH 7.5), 1 mM dithiothreitol, 10 mM $MgCl_2$, bovine serum albumin (Pentex) at 50 μg/ml, 10 mM KCl, 5% (vol/vol) glycerol, heat denatured P22 [^{3}H]DNA (2 μg, 6 × 10^4 cpm), and recA protein. NTPs, when present, were at 800 μM. The reaction was stopped by the addition of 0.5 ml of S1 buffer (300 mM NaCl/50 mM sodium acetate, pH 4.6/1 mM zinc acetate), 10 μg of heat-denatured calf thymus DNA, and 400 units of S1 nuclease. Incubation was continued at 37°C for 30 min at which time 0.5 ml of 10% (wt/vol) trichloroacetic acid and carrier calf thymus DNA (40 μg/ml) were added. Acid-precipitable radioactivity was measured by liquid scintillation counting.

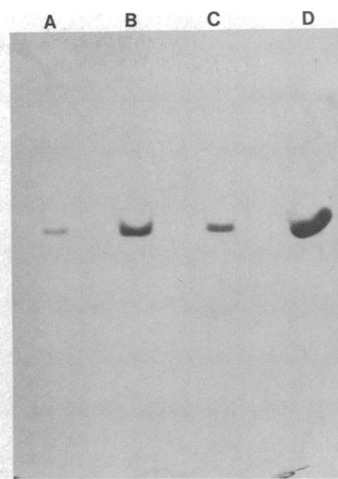


FIG. 1. Polyacrylamide gel electrophoresis of purified recA proteins. The recA proteins were purified by phosphocellulose chromatography. Active fractions were pooled, precipitated with $(NH_4)_2SO_4$ (0.36 g/ml), resuspended in R buffer, and dialyzed overnight against 500 vol of R buffer. An aliquot (10 μl) of each protein fraction was analyzed by electrophoresis in a polyacrylamide gel (11%) containing $NaDodSO_4$. Lanes: A, lexB30 protein (4 μg); B, recA629 protein (10 μg); C, tif-1 protein (7 μg); D, recA$^+$ protein (20 μg).

Assay for Hydrolysis of NTP. Formation of NDP was measured by using PEI-cellulose chromatography (35). The reaction mixtures (25 μl) were identical to those described for the renaturation reaction except that radioactive NTP (1 μCi) (1 Ci = 3.7 × 10^{10} becquerels) and unlabeled NTP (80 or 800 μM) were present and the DNA, when included, was heat-denatured calf thymus DNA (5 μg). Incubation was at 28 or 37°C for 30 min. With ATP and dUTP, the reaction was proportional to enzyme concentration up to 50% hydrolysis.

Electrophoresis. Agarose gel electrophoresis (36) was performed in 0.8% agarose, and gels were stained with ethidium bromide (0.5 μg/ml). Reaction mixtures were adjusted to 25 mM EDTA, 10% (vol/vol) glycerol, 0.01% (wt/vol) bromphenol blue, and 1% Sarkosyl prior to electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was as described (22).

RESULTS

recA protein catalyzes renaturation of DNA

Denatured nicked ColE1 or P22 DNA, incubated with recA protein, Mg^{2+}, and ATP, was converted to a form that remained at the top of an agarose gel during electrophoresis (Fig. 2, lanes 11 and 24). The product was unaffected by Sarkosyl (1%) or $NaDodSO_4$ (1%) but was converted to a form that entered the gel by brief incubation in 0.1 M NaOH or by heating at 100°C for 1 min (data not shown). Treatment with the single-strand-specific S1 nuclease also converted it to a form that migrated into the agarose gel (data not shown). When examined in the electron microscope, DNA that had been incubated with the recA protein, Mg^{2+}, and ATP was found predominantly in the form of complex aggregates (Fig. 3*a*). Occasionally, less complex forms could be observed that contained branches, single-stranded bubbles, and regions of duplex DNA (Fig. 3*b*). The presence of double-stranded DNA in these products was confirmed by digestion with S1 nuclease (see below). Incubation of denatured ColE1 or P22 DNA in the absence of recA protein did not result in formation of DNA aggregates; instead, ex-

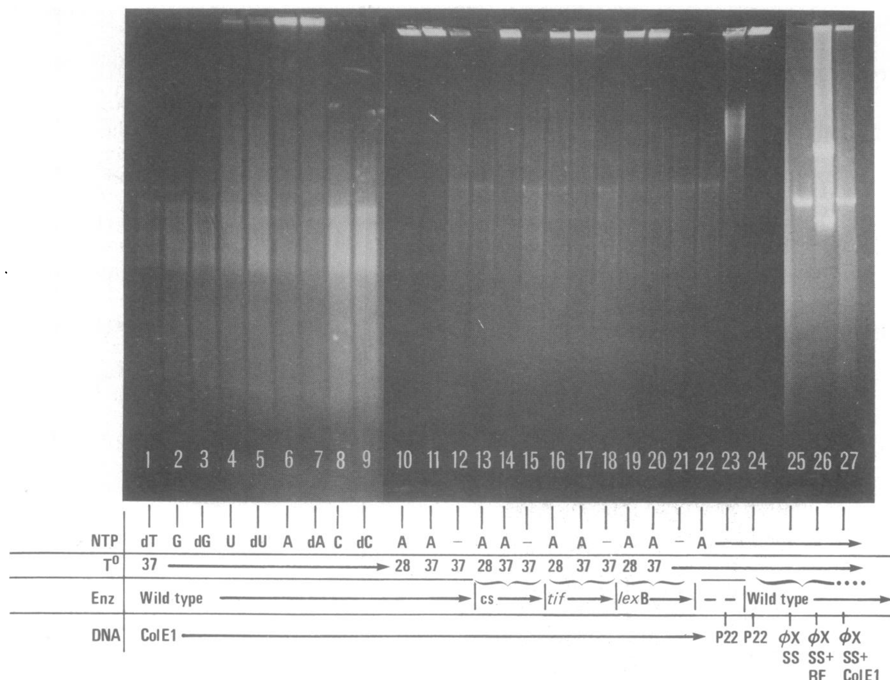
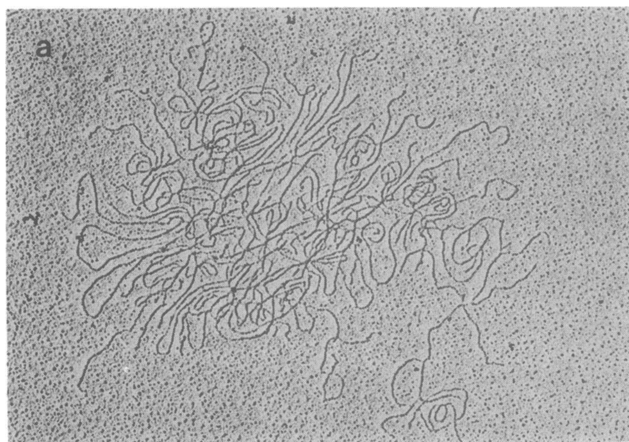
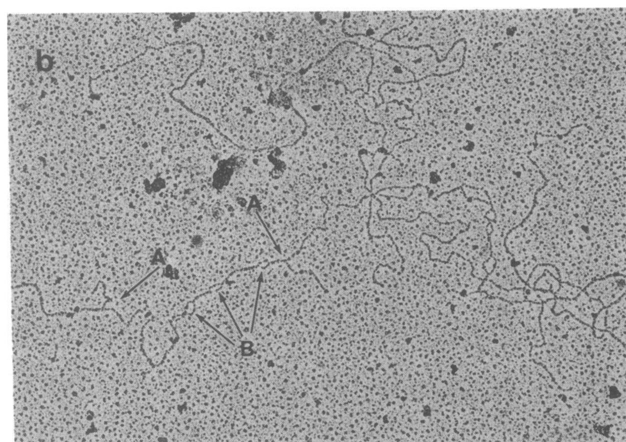


FIG. 2. Requirements for the formation of DNA aggregates by the recA protein. Incubations were performed at the indicated temperatures and 25-μl samples were applied to wells of 0.8% agarose gels. Samples contained 0.7 μg of heat-denatured nicked ColE1 or P22 DNA and other DNAs (ColE1, φX174 viral, or φX174 RFII) as indicated, 1 μg of recA+ protein, and 20 nmol of NTP. cs, The cold-sensitive *recA629* mutant; T°, temperature (°C); enz, enzyme.

tended single-stranded molecules were seen by electron microscopy (Fig. 3*c*). These results indicate that the recA protein catalyzes renaturation of single-stranded DNA, resulting in the formation of aggregate structures.

In contrast to the results obtained with denatured duplex DNA, neither intact duplex P22 DNA nor ColE1 DNA (supercoiled or nicked) was altered in its electrophoretic mobility after incubation with recA protein (data not shown). Furthermore, single-stranded DNA from φX174 (or G4) phage lacking a complementary strand did not undergo the recA protein-promoted aggregation (Fig. 2, lane 25). However, incubation of viral strand DNA with recA protein and denatured φX174





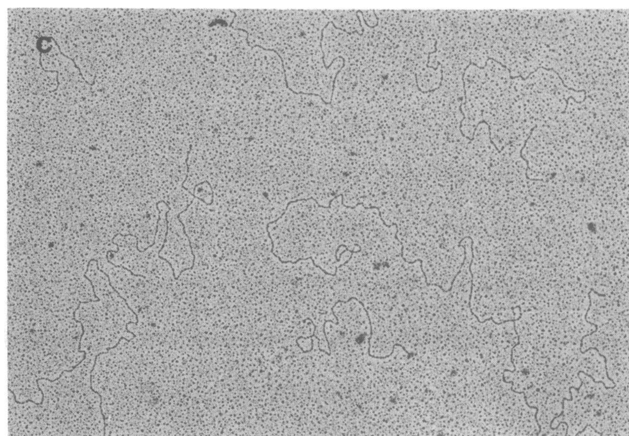


FIG. 3. Electron microscopy of DNA aggregates produced by the recA protein. One microgram of heat-denatured P22 or ColE1 DNA was incubated with recA+ protein (1 μg), ATP, and Mg^{2+} for 20 min at 37°C. The reaction products were spread by the formamide method of Davis *et al.* (37). (*a*) ColE1 DNA incubated with recA protein; (*b*) P22 DNA incubated with recA protein; (*c*) P22 DNA incubated without recA protein. In *b*, branches (A) and single-stranded bubbles (B) are indicated by the arrows.

RFII DNA (containing both complementary strands), but not heterologous denatured ColE1 DNA, resulted in a loss of material migrating at the position of viral strand DNA (Fig. 2, lanes 26 and 27). Therefore, the aggregation of DNA by recA protein requires that complementary single strands be present. We conclude that aggregation is not due to bound recA protein, but results from the annealing of complementary regions of the single-stranded DNAs.

Formation of the DNA aggregates was greatly stimulated by ATP (or dATP) (Fig. 2; compare lanes 11 and 12, 14 and 15, 17 and 18, 20 and 21), although some aggregation occurred in its absence. Of the other NTPs, only dUTP (or UTP) could substitute for ATP (or dATP), but much less efficiently (Fig. 2, lanes 1–9). The reaction was inhibited by the analog ATP[γ-S] (80 μM, data not shown), suggesting that ATP hydrolysis is required for aggregate formation. The recA protein purified from the cold-sensitive *recA629* strain was active in the aggregation reaction at 37°C but was inactive at 28°C (Fig. 2, lanes 13 and 14). In contrast, the wild-type, tif-1, and lexB30 products were active at both low and high temperatures (Fig. 2, lanes 16–21). These findings demonstrate clearly that the aggregation of single-stranded DNA is a function of the recA protein.

The extent of duplex DNA formed during the aggregation reaction was determined by conversion of the single-stranded P22 DNA to a form insusceptible to S1 nuclease digestion (Fig. 4 *left*). Incubation of denatured DNA with recA protein produced S1 nuclease-resistant material, and the reaction was strongly stimulated by ATP. In the absence of ATP, some duplex DNA was formed; however, it was only slightly greater than the amount that underwent spontaneous renaturation. At least 50% of the denatured P22 DNA could be converted to S1 nuclease-resistant material (data not shown). In contrast, when single-stranded DNA from phage ϕX174 was incubated with the recA protein and ATP, no detectable S1 nuclease-resistant material was formed (less than 2%, data not shown).

The formation of duplex DNA by recA protein as measured by resistance to S1 nuclease demonstrated the same requirements as aggregate formation determined by agarose gel electrophoresis. Single-strand annealing was partially stimulated by dUTP (or UTP), but not by the other NTPs, and was inhibited by the analog ATP[γ-S]. Similarly, both reactions were inhibited by *N*-ethylmaleimide (data not shown). Furthermore, the annealing reaction catalyzed by the cold-labile recA629 protein was stimulated by ATP at 37°C but not at 28°C (Fig. 4 *right*). Thus, the recA protein catalyzes the annealing of complementary single strands of DNA.

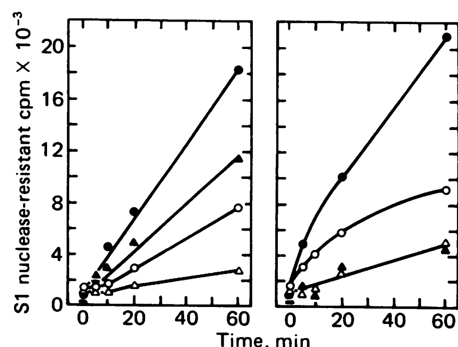


FIG. 4. Kinetics of annealing by the recA+ and recA629 proteins at 37 and 28°C. recA+ (*Left*) or recA629 (*Right*) proteins were incubated with 2 μg of ^{3}H-labeled heat-denatured P22 DNA (6 × 10^4 cpm total), and the amount of S1 nuclease-resistant material was determined. Reactions were performed with 1 μg of recA629 protein and with 0.4 μg of recA+ protein. ○, 37°C (no ATP); △, 28°C (no ATP); ●, 37°C (plus 800 μM ATP); ▲, 28°C (plus 800 μM ATP).

Table 1. Hydrolysis of nucleoside triphosphates by recA protein

NTP	% NDP formed
ATP	33 (60)
dATP	32 (66)
UTP	41
dUTP	40 (36)
CTP	27
dCTP	14 (8)
GTP	4
dGTP	6
dTTP	3

The recA+ protein (0.4 μg) was incubated for 30 min at 37°C with 800 μM NTP, and formation of NDP was measured. The values in parentheses are those obtained upon incubation with 80 μM NTP. In the absence of DNA, <2% hydrolysis was observed. All values have been corrected for NDP initially present in the NTP (usually less than 5%).

recA protein catalyzes DNA-dependent hydrolysis of nucleoside triphosphates

Purified recA protein catalyzed hydrolysis of some NTPs to NDPs (Table 1) as follows. The enzyme was most active on ATP, dATP, UTP, and dUTP, although some hydrolysis of CTP and dCTP could be detected at high concentrations (800 μM). GTP, dGTP, and dTTP were inactive as substrates. The hydrolysis reaction has an absolute requirement for single-stranded DNA which was satisfied by denatured linear DNAs (P22, calf thymus, or ColE1), poly(dT), and circular viral DNAs (ϕX174, G4) (data not shown). Thus, single-stranded DNAs that are inactive in the renaturation reaction may nevertheless participate in NTP hydrolysis. On the other hand, duplex DNAs (supercoiled or nicked ColE1, or P22 DNA) were not active in stimulating hydrolysis. The reaction required a divalent cation and was inhibited by *N*-ethylmaleimide (5 mM) and ATP[γ-S] (80 μM). Under optimal conditions, the recA protein catalyzes the hydrolysis of approximately 30 mol of ATP per mol of protein per min at 37°C.

Both ATPase and dUTPase activities eluted together with the recA protein during phosphocellulose chromatography (data not shown). These activities cochromatographed with the tif-1, lexB30 and recA629 mutant proteins (data not shown) despite the fact that the recA629 protein was eluted at higher ionic strength. The ATPase activity of the recA629 protein was more cold-labile than that of the other three recA proteins examined (Table 2). Thus, DNA-dependent nucleoside triphosphatase activity, like the aggregation reaction and formation of duplex DNA, is clearly an intrinsic property of the recA protein. These results confirm and extend the observations of Ogawa (27) and Roberts *et al.* (26).

Table 2. Cold lability of ATPase activity of wild-type and mutant recA proteins

Source of recA protein	Protein added, μg	ATPase, % hydrolyzed 28°C	37°C	Ratio 37°C/28°C
recA+	0.4	8	31	3.9
tif-1	0.7	8	28	3.5
lexB30	0.9	17	38	2.2
recA629	1.0	3	32	11

Reactions containing the indicated amount of mutant or wild-type protein were performed as described in *Materials and Methods*. A 1-μl aliquot of the reaction mixture (25 μl) was applied to a PEI-cellulose strip and the fraction of ADP was determined. Each reaction contained 800 μM ATP.

DISCUSSION

The purified recA protein of *E. coli* catalyzes the renaturation of single-stranded DNA. Under our conditions, complex branched DNA structures with duplex regions are generated. The formation of these aggregates by the recA protein requires the participation of complementary single strands and involves the formation of double-stranded regions.

The renaturation reaction is markedly stimulated by ATP, although some duplex material is formed by the recA protein in its absence, perhaps by a mechanism analogous to the helix destabilizing proteins of *E. coli* and phage T4 (38, 39). Several lines of evidence suggest a close coupling between ATP hydrolysis and renaturation of DNA. Both reactions require divalent cations, are sensitive to *N*-ethylmaleimide, and are inhibited by the ATP analog ATP[γ-S] when it is present in the reaction at 10% of the level of ATP. Other nucleoside triphosphates (dCTP, dGTP, dTTP) are not efficiently hydrolyzed by the *rec*A protein and do not stimulate renaturation. dUTP (or UTP), which is efficiently hydrolyzed by the recA protein, is less effective than ATP in stimulating the annealing reaction. This result may reflect kinetic differences in hydrolysis compared to renaturation, or it might indicate multiple activities associated with the recA protein.

ATP hydrolysis by the recA protein can be uncoupled from reannealing by using noncomplementary single-stranded DNA, for example, ϕX174 viral DNA. In such a reaction, ATP hydrolysis proceeds without formation of duplex DNA and without DNA aggregation.

The annealing of single-stranded DNA catalyzed *in vitro* by the recA protein strongly suggests that this protein catalyzes the reannealing of single-stranded regions of DNA *in vivo* during recombination and the repair of postreplication gaps. Additional evidence for this *in vivo* role has come from the analysis of the recA protein purified from a cold-sensitive recA mutant. The *recA629* mutant strain is deficient in recombination and is extremely UV-sensitive at 28°C, whereas at 37°C it is recombination-proficient and only slightly more UV-sensitive than a wild-type strain (K. McEntee, unpublished results). The properties of the purified recA629 protein *in vitro* correlate well with the phenotype of the *recA629* strain *in vivo*. The recA629 protein is cold-labile for both ATP hydrolysis and the ATP-stimulated annealing of single-stranded DNA. We therefore suggest that *in vivo* the recA protein catalyzes the annealing of a single-stranded region from one DNA duplex to a complementary single-stranded region of another DNA duplex. This reaction, analogous to the reannealing of single-stranded DNA observed *in vitro*, would produce the single-strand crossover structure of the type that is observed early in recombination. Similarly, the recA protein-mediated reaction could be used to anneal an undamaged single strand to a single-stranded region of DNA containing a pyrimidine dimer as has been suggested for the *recA*-dependent repair of daughter-strand gaps—i.e., postreplication repair (11, 12).

The recA proteins purified from strains containing either the *tif-1* or *lexB30* mutation in the *recA* gene also efficiently catalyze DNA reannealing *in vitro*. These mutant strains are recombination-proficient *in vivo*, although *lexB30* mutant strains are more sensitive to killing by UV than wild-type cells (19). Recent evidence indicates that the UV sensitivity associated with the *lexB30* mutation reflects a regulatory defect in *recA*. After UV damage, the recA protein is not induced in the *lexB30* mutant (29). The high levels of recA protein that are normally produced in damaged cells may reflect an increased amount of single-stranded DNA and a corresponding increased requirement for *recA* function in repair. Consistent with this idea is the observation that, when the lexB30 protein is overproduced by introduction of other regulatory mutations (*spr* or *tsl*), the radiation sensitivity is suppressed (29).

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