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Stable Binding of recA Protein to Duplex DNA

UNRAVELING A PARADOX*

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recA protein binding to duplex DNA is a complicated, multistep process. The final product of this process is a stably bound complex of recA protein and extensively unwound double-stranded DNA. recA monomers within the complex hydrolyze ATP with an apparent k_{cat} of approximately 19–22 min^{-1}. Once the final binding state is achieved, binding and ATP hydrolysis by this complex becomes pH independent. The weak binding of recA protein to duplex DNA reported in previous studies does not, therefore, reflect an intrinsically unfavorable binding equilibrium. Instead, this apparent weak binding reflects a slow step in the association pathway. The rate-limiting step in this process involves the initiation rather than the propagation of DNA binding and unwinding. This step exhibits no dependence on recA protein concentration at pH 7.5. Extension or propagation of the recA filament is fast relative to the overall process. Initiation of binding is pH dependent and represents a prominent kinetic barrier at pH 7.5. ATP hydrolysis occurs only after the duplex DNA is unwound. The binding density of recA protein on double-stranded DNA is approximately one monomer/4 base pairs. A model for this process is presented. These results provide an explanation for several paradoxical observations about recA protein-promoted DNA strand exchange. In particular, they demonstrate that there is no thermodynamic requirement for dissociation of recA protein from the heteroduplex DNA product of strand exchange.

Homologous genetic recombination involves an exchange of genetic information between two homologous duplex DNA molecules. A key intermediate in all recombination models is a joint molecule equivalent to, or approximating, the crossover structure first proposed by Holliday (1). Studies *in vitro* with the recA protein of *Escherichia coli* and the rec1 protein of *Ustilago maydis* have indicated that regions of ssDNA[1] may play an important role in the initiation of recombination events (2, 3). Regions of ssDNA could be generated *in vivo* in the course of DNA repair, replication, and conjugation. The formation of heteroduplex DNA during recombination is not restricted to regions in which one of the recombining DNA molecules is single-stranded, however. The extension of heteroduplex DNA into regions in which the exchange must involve four DNA strands has been observed *in vitro* (4, 5) and is supported *in vivo* by evidence such as the occurrence of aberrant $4^+:4^-$ tetrad products of meiotic recombination in *Ascobolus* and other fungi (6, 7).

The recA protein carries out a set of DNA strand exchange reactions *in vitro* which mimic the key steps in the formation and extension of heteroduplex DNA during homologous recombination *in vivo* (8–10). In these reactions, recA protein initially forms a nucleoprotein filament on ssDNA (11, 12). These complexes then align the ssDNA with homologous sequences in a duplex DNA molecule (10, 13–15). If not topologically restricted, a region of heteroduplex DNA will then be formed and extended in a unidirectional branch migration reaction coupled to ATP hydrolysis (8, 16, 17). Whereas these reactions are mediated by the initial interaction of recA protein with ssDNA, an interaction with duplex DNA appears necessary at some stages of DNA strand exchange. Evidence has been published that recA protein filaments formed in ssDNA gaps can extend into the neighboring duplex DNA (18). In addition, recA protein appears to be bound to the heteroduplex product of DNA strand exchange reactions (19, 20, 56). At least some of these observations appear to conflict with reports that recA protein does not bind to duplex DNA under conditions (pH > 7.0, $[Mg^{2+}]$ = 10–15 mM, ATP) which are optimal for DNA strand exchange (2, 21–24). This establishes an apparent paradox in that recA protein appears to bind to heteroduplex DNA but not to unreacted duplex DNA.

The resolution of this paradox, and the understanding of the key steps in recombination both *in vivo* and *in vitro*, requires detailed information about the interaction between recA protein and duplex DNA. Published results reveal a number of properties of this interaction. Binding of recA protein to duplex DNA is highly pH dependent, optimal near pH 6.0, and almost undetectable at pH 7.5 (21–23). Unlike the binding of recA protein to ssDNA, binding to dsDNA appears to have an absolute requirement for a nucleoside triphosphate (2, 23, 25–27). Binding to duplex DNA generally results in unwinding of the DNA (25–30). At pH 6.0, binding and unwinding of duplex DNA occur in the presence of ATPγS, ATP, or UTPγS (23, 25). At pH 7.5, binding and unwinding of duplex DNA appears to require either ATPγS (ATP is apparently ineffective), homologous ssDNA, or low (1 mM) concentrations of Mg^{2+} (25–31). Unwinding of DNA in the presence of ATPγS is manifested as a 50% extension of recA/DNA nucleoprotein filaments relative to unbound DNA as observed in the electron microscope (32, 33). The

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); FI, supercoiled closed circular form of a DNA molecule as isolated from *E. coli* cells; FII, nicked circular form of the same DNA molecule; FIII, linear form of the same dsDNA molecule; FIV, closed circular but relaxed form of the DNA molecule; UTPγS, uridine 5′-*O*-(3-thiotriphosphate); kb, kilobase.

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extent of DNA unwinding has been characterized in the case of the experiments with ATPγS, where the 50% extension of the DNA was shown to correspond to a 50% unwinding of the duplex DNA (34). The more limited results obtained with ATP at low pH or with ssDNA and ATP at high pH suggest a more limited degree of unwinding (25, 29, 30). recA/dsDNA nucleoprotein filaments formed in the presence of ATP are not as extended as they are when ATPγS is used (32). Duplex DNA binding at low pH in the presence of ATP is also accompanied by ATP hydrolysis (22–25) and the suggestion has been made that this hydrolysis is coupled to unwinding (26, 27).

When an attempt is made to apply this information to a general consideration of recA protein-promoted DNA strand exchange, the aggregate body of available information on the recA·dsDNA interactions is found to be deficient in many respects. No comprehensive picture of the duplex DNA-binding process has emerged which can accommodate all of the disparate observations. The relationship between binding and unwinding of the DNA has not been explored and it is not clear if they are entirely coupled. The extent of unwinding has not been determined under most conditions. No data has appeared which establishes a relationship between ATP hydrolysis and binding or unwinding. Existing estimates of the binding density of recA protein on dsDNA are in the range of one recA monomer/2–3 base pairs (33, 35, 36). This represents a binding density 1.5–2-fold higher than that generally reported for ssDNA. The inability to detect significant binding to dsDNA under optimal strand exchange conditions (21–23) has generally been assumed to represent an unfavorable binding equilibrium, although it could also reflect a slow step in the association pathway. This study represents an attempt to address these problems. Our purpose is to provide information needed in the consideration of models for recA protein-promoted branch migration and to provide a theoretical framework for a more detailed analysis of the interaction between recA protein and duplex DNA. We report here that the apparent weak binding at pH 7.5 represents a kinetic barrier to association rather than a weak binding equilibrium and we outline steps in the association pathway.

MATERIALS AND METHODS

Enzymes and DNA—*E. coli* recA protein was purified as previously described (37). The concentration of recA protein in stock solutions was determined by absorbance at 280 nm, using an extinction coefficient of $\epsilon_{280} = 0.59\ A_{280}\ mg^{-1}$ ml (38). Single-stranded and duplex circular DNA derived from the bacteriophages M13mp8 and φX174 and plasmid DNA was prepared as described (39, 40). The concentrations of ssDNA and dsDNA stock solutions was determined by absorbance at 260 nm, using 36 and 50 $\mu g/ml/A_{260}$ as conversion factors, respectively. DNA concentrations are expressed in terms of total nucleotides. A variety of plasmids were used in this work. None were constructed specifically for this project and all represent nonspecific duplex DNA sequences for purposes of this study. The plasmids previously described include pUC8, 2.7 kb (41), and pJFS38, 2.8 kb (42). The plasmid pRCB1, 11.6 kb, is the plasmid pMC1403 (43) with a 1.7-kb S_1 nuclease-treated *Eco*RI fragment of pMMC6 (44) inserted into the *Sma*I site. The plasmid pMMC5, 3.1 kb, is the plasmid pXF3 (40) with a 306-base pair *Bam*HI-*Cla*I fragment from bacteriophage M13 replacing the short *Bam*HI-*Cla*I fragment of pXF3. The plasmid pBR322-2, 8.7 kb, is a perfect dimer of the plasmid pBR322 (45). ATPγS was purchased from Boehringer Mannheim. Restriction endonucleases were purchased from New England Biolabs or International Biotechnologies Inc. All other biochemicals and enzymes were purchased from Sigma.

^{3}H-Labeled DNA was prepared by a published procedure (14). Linear DNA was produced by digesting plasmid or bacteriophage DNA with a restriction endonuclease which had a single cut site in the DNA in question. Nicked circular duplex DNA was prepared from plasmid DNA by the method of Shibata *et al.* (46). Greater than 95% of the resulting DNA was present as nicked circles as determined from scanning densitometry of photographic negatives from ethidium bromide-stained agarose gels. Form X DNA is a highly unwound form of covalently closed circular DNA originally described by Shibata *et al.* (46). This DNA migrates faster than supercoiled FI DNA in a 0.8% agarose gel. Form X DNA was prepared from pBR322-2 plasmid DNA which had been nicked according to the above procedure. This DNA (30 μM) was incubated with recA protein (20 μM) in buffer A (20 mM Na maleate, 74% dianion (final pH = 6.35 at 25 °C), 10 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol) with 1 mM ATP and an ATP-regenerating system (described under "Reaction Conditions," below), for 30 min at 37 °C. T4 DNA ligase (1 mg/ml final) was then added and the incubation continued for 1 h. The reaction was stopped by adding 20 mM EDTA. The form X DNA was purified by centrifugation in a CsCl/ethidium bromide gradient, which separates form X from FI, FII, FIII, and FIV DNA, as described (40). Greater than 80% of the resulting material was present as form X, determined as described above.

Instrumentation—Light scattering measurements were carried out on an SLM Instruments series 8000 scanning fluorometer equipped with a thermojacketed cuvette holder attached to a constant temperature water circulator and with an externally controlled magnetic-stirring motor for the sample chamber. This instrument has a focal length of 2 inches (lens to sample) and mean beam path lengths of 128 cm (source to sample) and 105 cm (sample to photomultiplier). Signal acquisition was fixed at 5 s in all experiments; cell path length and bandpass were equal to 1 cm and 2 nm, respectively.

Absorbance measurements were obtained on a Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with two 6-position thermojacketed cuvette holders attached to a constant temperature water circulator. Cell path length and bandpass were equal to 1 cm and 2 nm, respectively, in all experiments.

Reaction Conditions—Except for pH titrations and variations in buffer A in which the Na maleate was maintained at 34% dianion (pH 5.66) or 64% dianion (pH 6.11), experiments were carried out in buffer A (described above) or buffer B (25 mM Tris-Cl (80% cation, pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 5% glycerol). The indicated pH is the pH under final conditions at 25 °C. Unless indicated otherwise, reactions included 1 mM ATP, an ATP-regenerating system (2.24 units/ml pyruvate kinase, 2.34 mM phosphoenolpyruvate, 0.44 mM KCl), 10 μM dsDNA (in the topological form indicated), and the indicated concentration of recA protein. All reactions were carried out at 37 °C. In reactions employing duplex DNA in the FI, FII, or form X conformations, the integrity of the DNA samples was checked before and after the experiment by agarose gel electrophoresis. Experiments in which detectable degradation of DNA or alteration of topological form occurred over the course of the experiment were discarded.

Nuclease Protection Assay—Aliquots (25 μl) of reaction mixtures were incubated with 5 μl (2 units, 0.75 μg) of pancreatic DNase I (Sigma) under standard reaction conditions for 30 s at 37 °C. The reaction was stopped by adding 30 μl of a solution containing 0.25 mg/ml calf thymus DNA (heat denatured) and 0.375 M EDTA; followed immediately by 0.9 ml of ice-cold 10% trichloroacetic acid. The samples remained on ice for 30 min and were then filtered through Whatman GF/C filter discs. The filters were washed three times with 1 ml of ice-cold trichloroacetic acid followed by 2 ml of 95% ethanol. Filters were then dried and assayed for radioactivity by liquid scintillation. In each experiment, 100% protection was defined by a separate control aliquot from which DNase I was omitted. An additional control in which a sample of the same DNA was treated with DNase I in the absence of recA protein was used to define background levels of protection. Data (% protection) were obtained by dividing radioactivity measurements for each sample by the value for 100% protection, after the background was subtracted from both. Background represented less than 10% of the 100% protection control unless noted.

The extent of protection afforded to DNA by recA protein differed for single-stranded and duplex DNA and also exhibited a dependence on the presence of nucleotides. Typical DNase I titration curves are illustrated in Fig. 1 for binding reactions carried out at pH 6.35. Where recA protein is present, it is in excess and DNase I is added only after binding (measured separately) has reached an end point. All DNase I incubations were carried out for 30 s. Protection of duplex DNA in the presence of ATP is only partial and exhibits a dependence on DNase I concentration. When the products of the DNase I digestion were examined by agarose gel electrophoresis both strands appeared to be equally susceptible to DNase I digestion.

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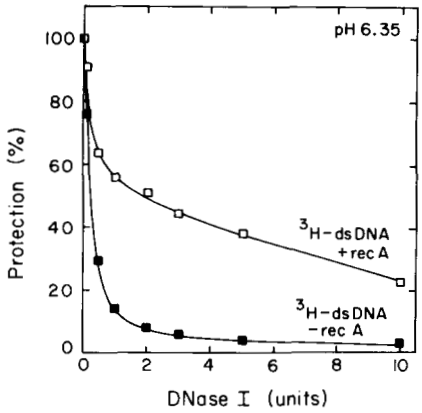


FIG. 1. **DNase I titration on recA·dsDNA complexes at pH 6.35.** Reactions were performed in buffer A as described under "Materials and Methods." Reactions contained 3.5 μM recA protein (when present) and 10 μM linear dsDNA (^{3}H-labeled pMMC5). These components were incubated at 37 °C for 30 min before addition of the DNase I, at the concentrations shown, to assay for protection.

Degradation also appeared to involve all DNA molecules uniformly, indicating that the partial protection of the DNA is a property of all the DNA molecules rather than total protection of a fraction of the molecules. Greater protection is observed when ATPγS is added, or when ^{3}H-ssDNA is employed as a substrate for binding. The possible implications of the partial protection of duplex DNA are presented under "Discussion." The level of DNase I employed in these studies generally maximizes the difference observed upon recA protein binding.

Ninety Degree Light Scattering—Experiments to measure recA protein binding to DNA were carried out essentially as described previously (47). Relative light scattering intensities were recorded as fluorometer output at an excitation and emission wavelength of 350 nm. Unless noted, DNA-binding reactions were initiated by the addition of recA protein. Identical reaction mixtures were generally run in parallel with and without DNA. In some experiments, a low level of light scattering was observed in the absence of DNA, representing, in part, DNA-independent aggregation of recA protein (47). Since the level of this aggregation is not necessarily equivalent when a large fraction of the recA protein is bound to DNA, no correction was made for this contribution to scattering unless noted. Under the binding conditions employed in this study, DNA-independent aggregation of recA protein was minimized, representing less than 10% of the light scattering observed in the "+" DNA experiment unless noted. The maximum signal recorded in the recA protein titrations at each recA protein concentration remained constant over at least 5 min. Correction of data for DNA-independent aggregation had no effect on the reported binding stoichiometries.

ATP Hydrolysis—The coupled assay for ATP hydrolysis is described elsewhere (48). The assay employs pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase, and NADH to couple ATP hydrolysis to the conversion of NADH to NAD. Concentrations of pyruvate kinase and phosphoenolpyruvate were equivalent to those used in the standard ATP-regenerating system. Lactate dehydrogenase and NADH were used at 2.24 units/ml (Sigma) and 1.5 mM, respectively. The oxidation of NADH was monitored spectrophotometrically by a decrease in absorption. All ATP hydrolytic reactions in this study employed this assay, with data obtained at 380 nm. NADH absorbance was measured at 380 nm instead of 340 nm, its absorbance maximum, in order to accommodate the high concentration of NADH needed to reach steady state while remaining in the linear absorbance range of the spectrophotometer. Absorbance values at 380 nm were highly reproducible despite the steep slope of the NADH absorbance spectrum in this region. Error from all sources in steady state rates of ATP hydrolysis was never greater than 10% in runs carried out on different days. Rates of ATP hydrolysis, expressed as micromolar ATP hydrolyzed to ADP/min, were calculated from $-\Delta A_{380}$ min^{-1} obtained at steady state using an extinction coefficient of $\epsilon_{380} = 1210$ M^{-1} cm^{-1} for NADH. Reactions were carried out in 1.0-ml quartz cuvettes with a total reaction volume of 0.91 ml and were initiated by addition of recA protein. Changes in absorption in the absence of recA protein were found to be negligible. Since ATP is constantly regenerated in this assay, the steady state conversion of NADH to NAD reflects the initial rate of ATP hydrolysis at all times. Increasing the concentration of ATP or any or all of the components in the coupling system in no case affected the observed rate of ATP hydrolysis in this assay, so that data reflects the maximum velocity of ATP hydrolysis at ATP saturation under all conditions used in this study. A short lag (up to 2 min) due to the coupling system is always observed in this assay before a steady state NADH to NAD conversion is achieved. The length of the coupling system lag was generally evaluated in experiments employing ssDNA as a cofactor for recA protein-promoted ATP hydrolysis. This ssDNA-mediated reaction exhibits no significant lag when ATP hydrolysis is measured with other assays. In experiments in which a lag in ATP hydrolysis is observed and measured with a dsDNA cofactor, the system lag measured in the presence of ssDNA was subtracted from all data.

DNA Unwinding Assay—This assay measures recA protein binding to dsDNA by monitoring recA protein-induced unwinding of the DNA. When a nicked circular DNA substrate is employed for binding, the recA protein-induced unwinding can be topologically trapped by covalently sealing the nick with DNA ligase. When recA protein is removed after sealing the nick, the topology is maintained in the form of supercoils. This highly supercoiled DNA can be resolved from the relaxed DNA substrate by agarose gel electrophoresis.

Aliquots (25 μl) of reaction mixture containing nicked circular pBR322-2 or M13mp8 FII DNA (prepared as described above) were incubated with 1 μl of T4 DNA ligase (1 mg/ml final) for 2–5 min. The reactions were stopped with the addition of 12 mM EDTA, 1% sodium dodecyl sulfate, 0.05% bromphenol blue, and 5% glycerol. The samples were then electrophoresed in a 0.8% agarose gel containing the indicated concentrations of ethidium bromide. The tank buffer (0.04 M Tris acetate, 0.002 M EDTA) also contained the same concentration of ethidium bromide. After electrophoresis the gels were illuminated by ultraviolet light and photographed. The photographic negatives were scanned by a laser densitometer.

RESULTS

Four different assays were used to monitor recA protein binding to dsDNA: 1) 90° light scattering, 2) nuclease protection, 3) DNA unwinding, and 4) ATP hydrolysis. Ninety degree light scattering provides information on filament formation and has been previously employed to measure recA protein binding to ssDNA (47). Nuclease protection measures recA protein binding to ^{3}H-labeled DNA. Only protected DNA remains acid insoluble after treatment with high concentrations of DNase I, thus binding can be quantitated. This assay provides independent confirmation of results obtained in light scattering experiments. Duplex unwinding employs DNA ligation and agarose gel electrophoresis to monitor topological changes induced in nicked circular duplex DNA as a result of recA protein binding. A similar assay has been used to study the unwinding of duplex DNA by recA·ssDNA complexes (26, 46). ATP hydrolysis provides another measure of binding because this reaction is, to a first approximation, DNA dependent. These assays complement one another to provide information about molecular events in this process and also provide multiple mechanisms to verify any result.

A variety of duplex DNA molecules were employed in this study, differing primarily in size (2–12 kb). In a series of side by side experiments, the steady state rate of ATP hydrolysis obtained with recA protein present in excess at pH 6.35 was found to vary less than 10% with different DNA cofactors. This degree of error was similar to the error from all sources observed when the same DNA was used in experiments on different days. However, under these conditions a significant effect of DNA size was observed on the duration of the lag time before steady state ATP hydrolysis was achieved. The lag decreased as the length of the DNA increased (Table I). This effect is described in more detail below where appropriate. Unless noted, the effects and trends described in this study are characteristic of all duplex DNA molecules and serve to define a general pathway for recA protein binding to duplex DNA. The effects of DNA size and other factors on

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TABLE I

Effect of DNA length on the lag observed in dsDNA-dependent ATP hydrolysis

Reactions were performed in buffer A (pH 6.35) as described under "Materials and Methods." Reactions contained 10 μM FIII dsDNA (except pBR322-2, which was FII), 1 mM ATP, and 2.5 μM recA protein.

DNA	Length	τ_{obs}[a]
	kb	*min*
pUC8	2.7	19.5
pJFS38	2.8	22.0
φX174	5.4	14.5
M13mp8	7.2	15.5
pBR322-2	8.7	12.0
pRCB1	11.6	13.0

[a] The lag time (τ_{obs}) required to reach a steady state rate of ATP hydrolysis was determined by extrapolation from the linear portion of the ATPase curves at steady state back to 0 ATP hydrolysis.

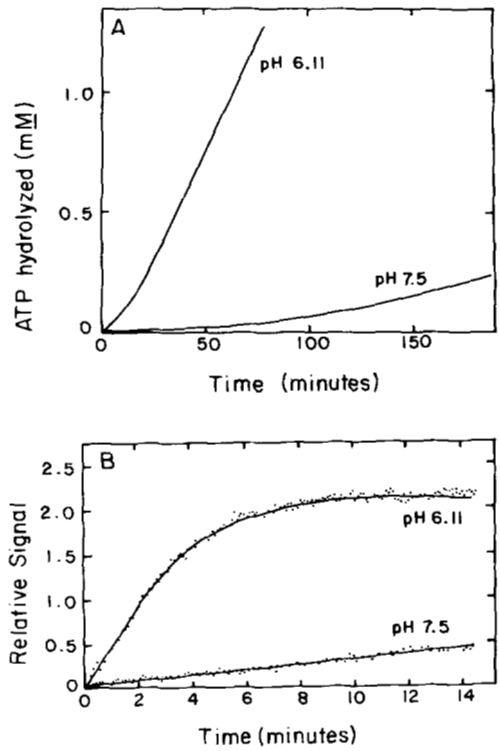


FIG. 2. **Binding of recA protein to dsDNA.** Reactions were carried out at pH 6.11 and pH 7.5 as described under "Materials and Methods." Reactions at pH 6.11 were performed in buffer A (except Na maleate was 64% dianion); buffer B was at pH 7.5. Reactions contained 2.5 μM recA protein, 1 mM ATP, and 10 μM linear dsDNA. Activities are: *panel A*, ATP hydrolysis with φX174 dsDNA; *panel B*, light scattering (DNA binding) employing pRCB1 dsDNA.

the rates of specific steps in this pathway will be described in detail elsewhere.

The initial interaction of recA protein with duplex DNA is very pH dependent, with efficient binding near pH 6.0 but no significant binding reported at pH 7.5 (21–23). Typical binding reactions at pH 6.11 and 7.5 are presented in Fig. 2, monitoring ATP hydrolysis and light scattering. recA protein is at a concentration equivalent to one monomer/2 base pairs in these experiments. At pH 6.11, binding is relatively efficient, but is slow enough for convenient measurement, reaching completion after approximately 10 min. At pH 7.5, binding is much slower but occurs at a detectable rate. The pH-dependent decrease in the rate of DNA binding is mirrored in the apparent rates of ATP hydrolysis observed at these pH values. The rates of ATP hydrolysis and DNA binding continue to increase at pH 7.5 over a period of hours, however, suggesting a slow rate of association rather than an unfavorable binding equilibrium. When monitored over a period of several hours, the extent of binding and rate of ATP hydrolysis is seen in some experiments to approach a final extent comparable to that observed at the lower pH. These results suggest that recA protein can be stably bound to dsDNA under both sets of conditions, but that a significant kinetic barrier to this binding exists which is at least partially pH dependent. When the same duplex DNA was employed in both assays, the increase in the rate of ATP hydrolysis with time paralleled DNA binding closely, and DNA length-dependent decreases in the lag in ATP hydrolysis with different DNAs were mirrored by increases in the rate of DNA binding (data not shown). This further suggests a correspondence between the two phenomena. Further experiments were carried out to attempt to define molecular events occurring during the lag in ATP hydrolysis.

pH Effects—The dependence of the ATP hydrolytic reaction on pH are presented in Fig. 3. Rates of ATP hydrolysis, obtained after the reactions had reached an apparent steady state, are plotted for a series of reactions employing φX174 FIII as a DNA substrate. This set of data extends previously published results (21) which did not examine the possibility that the initial and steady state rates of ATP hydrolysis may not be equivalent. As shown in Fig. 3, the observed pH dependence above pH 6.0 is in part a function of the lag described above rather than the intrinsic capacity for ATP hydrolysis. The long lag time (τ_{obs}) encountered at pH values above 6.5 permit only an estimate of the lower limit of the steady state rate of ATP hydrolysis. The fastest rate measured over a several hour time course at the higher pH values varied from one series of experiments to another, from the rates plotted in Fig. 3 to rates approaching those measured consistently at pH 6.0. Light scattering, nuclease protection, and

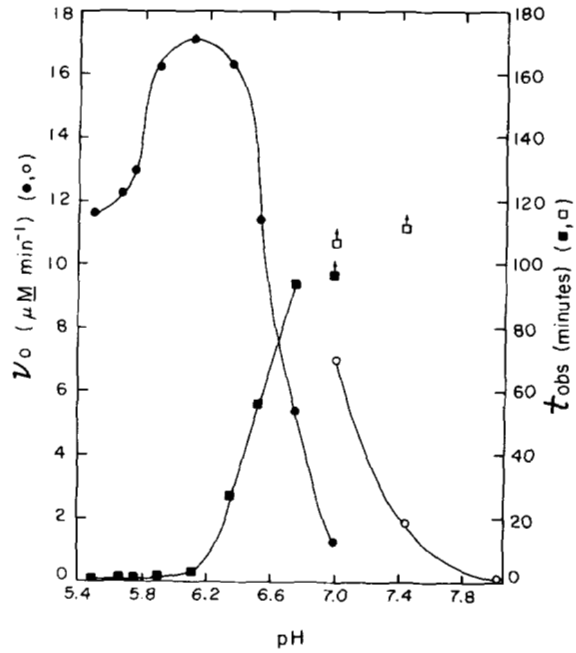


FIG. 3. **Dependence of dsDNA-dependent ATP hydrolysis on pH.** Reactions were adjusted to the indicated pH by varying the ionization state of Na maleate in buffer A (*closed symbols*) or Tris-Cl in buffer B (*open symbols*). Each reaction contained 10 μM linear dsDNA (φX174), 1 mM ATP, and 2.5 μM recA protein. v_0 is the velocity of ATP hydrolysis at an apparent steady state. τ_{obs} is the time lag before apparent steady state ATP hydrolysis is achieved.

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DNA unwinding data at pH 7.5 show that 5–6 h after the addition of recA protein, binding has not leveled off. Thus the rate of ATP hydrolysis measured at the limit of this assay (3–4 h) may not reflect a true steady state. These results, however, suggested that the rates of ATP hydrolysis observed once the lag has been completed are relatively pH independent. This possibility is addressed in the next section.

The reduced rate of ATP hydrolysis below pH 6 does not represent a decrease in the apparent binding density of recA protein (see titration data below), but reflects an inhibition of its intrinsic ATPase activity, possibly by titration of an ionizable group. This effect was not characterized further in this study. As noted previously by Weinstock *et al.* (21, 22), we have found that DNA-independent ATP hydrolysis was less than 5% of the observed DNA-dependent hydrolysis but exhibits the same magnitude increase in rate as the pH is decreased (data not shown). This is the principal argument that protonation of the protein, rather than the DNA, is responsible for these pH effects.

Stable Binding of recA Protein to dsDNA at pH 7.5 in the Presence of ATP—To further investigate the effects of pH on the duplex DNA-dependent ATPase reaction, a series of pH shift experiments were carried out. We wished to determine if complexes formed at low pH would maintain their stability when shifted to pH 7.5. recA protein was bound to dsDNA at pH 6.35 under standard conditions (buffer A). After ATP hydrolysis had reached steady state, the pH was abruptly shifted to pH 7.5. This was accomplished by 2-fold dilution into a Tris-Cl buffer set up to titrate all remaining maleic acid and produce the desired final pH while minimizing any change in ionic strength (Fig. 4, *upper curve*). A similar experiment in which the pH was maintained at pH 6.35 before and after the dilution yielded identical results. In a control experiment (Fig. 4, *lower curve*), the reaction was shifted to pH 7.5 before the addition of recA protein. The pH values were checked before and after each experiment. The steady state rate of ATP hydrolysis shown in the *upper curve* of Fig. 4 before the shift was 48 μM min^{-1} and after the shift was 24 μM min^{-1}. After correction for the 2-fold factor of dilution, no change was observed in the rate of ATP hydrolysis after the pH shift. This rate of ATP hydrolysis continued for at least 1 h following the shift. This result demonstrates that recA protein binds stably to dsDNA even at pH 7.5, once the binding process is complete. Further evidence for this based on DNA unwinding is presented in the next section.

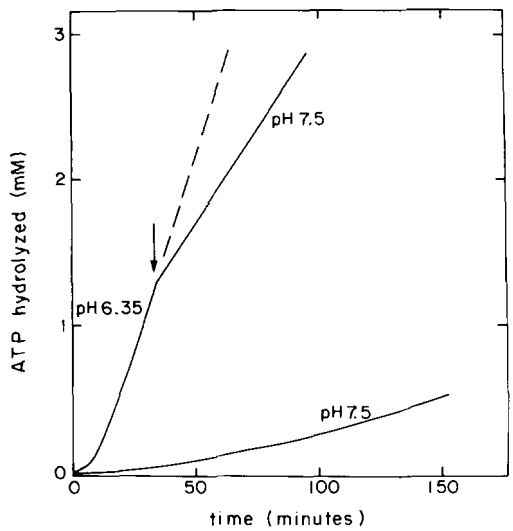


FIG. 4. **recA protein-promoted ATP hydrolysis is maintained after a shift from pH 6.35 to 7.5.** Reactions in buffer A (pH 6.35) contained 20 μM nicked circular dsDNA (pBR322-2), 1 mM ATP, and 20 μM recA protein. Reactions were started with the addition of recA protein. Reactions were shifted to pH 7.5 either before (*lower curve*) or 30 min after (*upper curve*) the addition of recA protein to start the reaction. The initial buffer (pH 6.35) contained 20 mM Na maleate at 74% dianion. The shift was accomplished by 2-fold dilution into a buffer containing 20 mM Tris-Cl at 50% cation, which titrated the remaining maleic acid and produced the desired pH. Reactions at pH 7.5 therefore contained 10 μM dsDNA and 10 μM recA protein. Concentrations of ATP and ATP-regenerating systems were maintained constant before and after the shift. An identical shift employing mixed Tris maleate buffers but maintaining the pH at 6.35 produced results identical to those presented in the *upper curve*. The *dashed line* represents the rate of ATP hydrolysis after correction for the 2-fold dilution.

recA Protein-mediated Unwinding of Duplex DNA—It has been well established that dsDNA is often highly unwound when it is bound by recA protein. Several experiments were carried out to determine the relationship of the unwinding to the overall process of binding to duplex DNA and the extent of unwinding in the final bound complex. To detect unwinding, nicked duplex DNA (FII), prepared as described under "Materials and Methods," was employed as a substrate for recA protein binding. At appropriate times, aliquots were removed and treated with a high concentration of DNA ligase for 5 min to trap any recA protein-induced topological changes in the DNA.

As shown in Fig. 5, this treatment results in the appearance of a DNA species which migrates faster than the supercoiled FI form of the same DNA. In the discussion to follow, this new species will be referred to as form X, as previously described by Shibata *et al.* (46). The time course of the appearance of this band exhibits the same pH dependence as dsDNA binding and ATP hydrolysis, occurring much slower at pH 7.5 than at pH 6.35. The apparent maximum yield of form X DNA at pH 6.35 in this experiment (>60%) is a function of the length of the incubation time with DNA ligase. Under these conditions (see "Materials and Methods"), but in the absence of recA protein, complete ligation is achieved within a few seconds. We attribute this difference to inhibition by recA protein binding at the nicked DNA binding site. The apparent extent of recA protein binding is therefore underestimated in this assay.

As shown in Fig. 6*A*, this DNA species is unwound to a much greater extent than the corresponding FI DNA isolated from bacteria cells. The linking number of FI DNA isolated from *E. coli* cells is generally reduced 5–6% relative to relaxed B form DNA (49). The topoisomers present in FI DNA are relaxed in agarose gels run in the presence of 0.15 μM ethidium bromide (46). This concentration of ethidium bromide has no effect on the migration of the form X DNA. The migration of the form X DNA is relaxed only when ethidium bromide concentrations are raised about 10–100-fold. This suggests that form X is unwound to a much greater extent than FI. The diffuse band evident at the higher ethidium bromide concentrations suggests that all of the form X DNA is present as a limited number of topoisomers. This smearing may be due to differences in DNA secondary structure from one molecule to another. Elsewhere[2] we will show that this form X DNA is unwound at least 28–30% relative to covalently closed circular B form DNA which is relaxed.

In Fig. 6*B* we also demonstrate that the dsDNA to which recA protein is bound remains unwound after a shift in pH from 6.35 to 7.5. We have been unable to detect a difference between the form X DNA formed at pH 6 as a result of recA protein binding and the form X obtained 60 min after such a pH shift. This further confirms that the stability and structure

[2] B. F. Pugh, R. B. Inman, and M. M. Cox, unpublished data.

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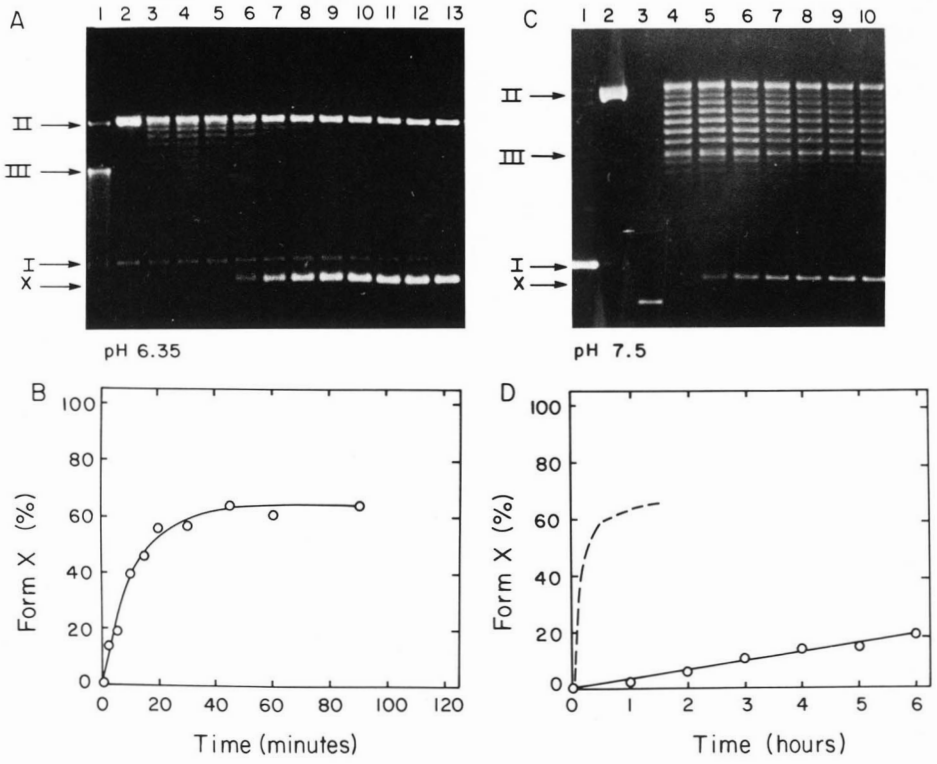


FIG. 5. **Unwinding of dsDNA by recA protein.** Reactions in buffer A (*panel A*) or buffer B (*panel C*) contained 10 μM nicked circular dsDNA (M13mp8 in *panel A* and pBR322-2 in *panel B*), 1 mM ATP, and 5 μM recA protein. At various times, samples were removed and treated with 1 mg/ml DNA ligase for 5 min, then electrophoresed in agarose gels in the absence of ethidium bromide. *Panel A*: *lane 1* contains FII and FIII markers; *lane 2*, DNA ligase was omitted; *lane 3*, recA protein was omitted. *Lanes 4–13* are 0, 2-, 5-, 10-, 15-, 20-, 30-, 45-, 60-, and 90-min time points. *Panel B*: *lanes 1–3* contain FI, FII, and 4.36-kb linear DNA markers, respectively. *Lanes 4–10* are 0, 1-, 2-, 3-, 4-, 5-, and 6-h time points. After staining with ethidium bromide the photographic negatives of *panels A* and *C* were scanned with a laser densitometer and the data plotted in *panels B* and *D*, respectively. The percent of total input DNA that was converted to form X is plotted as a function of time. In *panel D* the *dashed line* represents the curve in *panel B* drawn to scale.

of the recA·dsDNA complex is relatively unperturbed by this shift in pH. The complexes are not altogether equivalent at the two pH values, however. We have noted some differences in the level of nuclease protection of duplex DNA at the two pH values which might reflect a difference in the dynamic state of the complexes (see accompanying paper, Ref. 56).

Binding Stoichiometries and Dependence on ATP—recA protein titrations of dsDNA binding are presented in Fig. 7. Binding stoichiometries obtained from nuclease protection and light scattering were identical and the light scattering data is presented. Data in all cases reflects a stable maximum in the light scattering signal achieved at each recA protein concentration. Standard titrations in the presence of ATP at pH 5.66 and 6.11 are presented. At both pH values the apparent binding stoichiometry is one recA protein monomer/4 base pairs. This stoichiometry is consistent with the one recA monomer/4 nucleotides observed on ssDNA (25, 47, 48, 50). Inasmuch as data presented above demonstrate that the recA·dsDNA complex is not greatly altered by a shift to pH 7.5, this stoichiometry is probably pH independent. At pH 5.66, an increase in light scattering is observed above the point of apparent saturation by recA protein. This is an effect we have noted only below pH 6.0 and have not investigated it further. The very slow binding to dsDNA at higher pH values makes stoichiometry determinations impractical above pH 6.2. We also examined the ATP dependence of this binding. Measurement of binding to duplex DNA in the absence of ATP or other cofactors is complicated by DNA-independent recA protein aggregation (47). Within limits imposed by this problem,[3] we observed no binding of recA protein to duplex DNA without ATP as measured by light scattering and nuclease protection (data not shown). These results are consistent with earlier results which indicate that recA protein binding to dsDNA is largely an ATP-dependent reaction (2, 23, 25–27). This fact distinguishes the reaction from the binding of recA protein to ssDNA, which does not require ATP (47). The results described above indicate a strong correlation between rates of DNA binding and lags in ATP hydrolysis. This suggests that hydrolysis of ATP is not required for binding but occurs only after binding has been completed. The lack of a requirement for ATP hydrolysis for binding is examined below.

ATP Hydrolysis Occurs Only after dsDNA Is Bound and Unwound—The pH dependence of the unwinding reaction suggests that binding of recA protein to dsDNA and the extensive unwinding of the DNA occur, to a first approximation, simultaneously. Since a corresponding lag occurs in ATP hydrolysis, we infer that ATP hydrolysis does not occur until both dsDNA binding and unwinding has been completed. To explore this question further, we examined dsDNA binding in the presence of ATPγS. ATPγS has been shown to facilitate binding of recA protein to dsDNA (25–31), although comparative rates have not been reported. The kinetics of binding to duplex DNA at pH 7.5 in the presence of ATP or ATPγS, as measured by nuclease protection, is shown in Fig. 8. recA protein only partially protects dsDNA when bound in the presence of ATP, but provides full protection in the presence of ATPγS (see Fig. 1 and Ref. 56). Data obtained in the presence of ATP cannot, therefore, be compared directly with data obtained in the presence of ATPγS without correction. To minimize this problem, samples from the binding reaction in the presence of ATP were removed at various times and treated with ATPγS for 5 min before assaying for protection.

[3] DNA-independent aggregation of recA protein is a problem for two reasons. First, the light scattering signal for recA protein increases approximately 4-fold in the absence of ATP. This increase in background decreases the sensitivity of binding data obtained with this technique. If stoichiometric binding to DNA were observed, we estimate that the recA·dsDNA complexes would increase the light scattering signal by less than 2-fold at the equivalence point of a titration such as those in Fig. 7. Second, the aggregation reflected by this increase in light scattering has been shown to interfere with DNA binding (47). Inasmuch as the effects of aggregation have not been evaluated in previous studies, we believe that available data do not yet permit a definitive conclusion that *no* binding to duplex DNA occurs in the absence of ATP.

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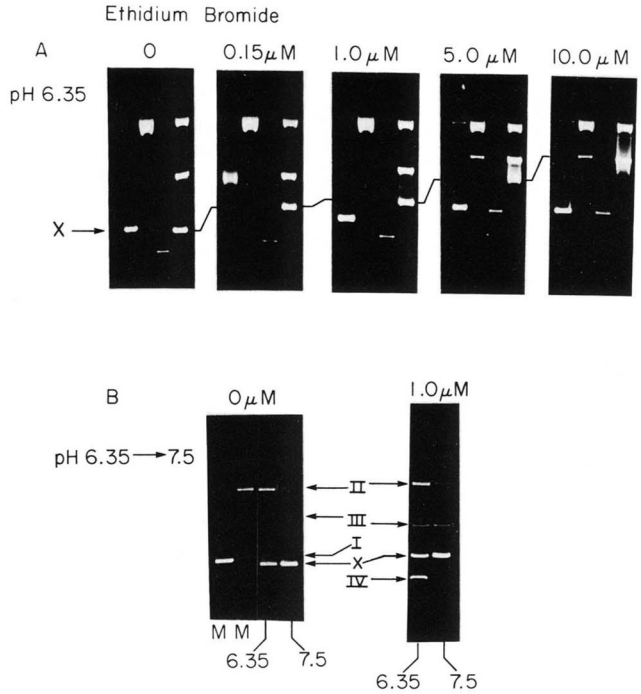


FIG. 6. **Form X DNA is highly unwound.** Samples were electrophoresed in agarose gels containing the indicated ethidium bromide concentrations. *A*, in each panel, *lanes* from *left* to *right* are pBR322-2 FI, pBR322-2 FII, a 4.36-kb linear DNA marker, and pBR322-2 form X prepared as described under "Materials and Methods." FII and FIII DNA is included in the form X lane for a direct comparison of mobility. *B*, form X made at pH 7.5 is as unwound as form X made at pH 6.35. Markers (*M*) from *left* to *right* are FI and FII. FIV co-migrates with FII in the absence of ethidium bromide. Lanes marked with *6.35* contain form X DNA made at pH 6.35 as described in the legend to Fig. 9. Lanes marked with *7.5* contain form X obtained at pH 7.5. This was achieved by binding recA protein to FII dsDNA (pBR322-2) at pH 6.35 then shifting it to pH 7.5 as described in the legend to Fig. 4. One hour after the shift to pH 7.5 excess DNA ligase was added for 5 min to produce form X DNA.

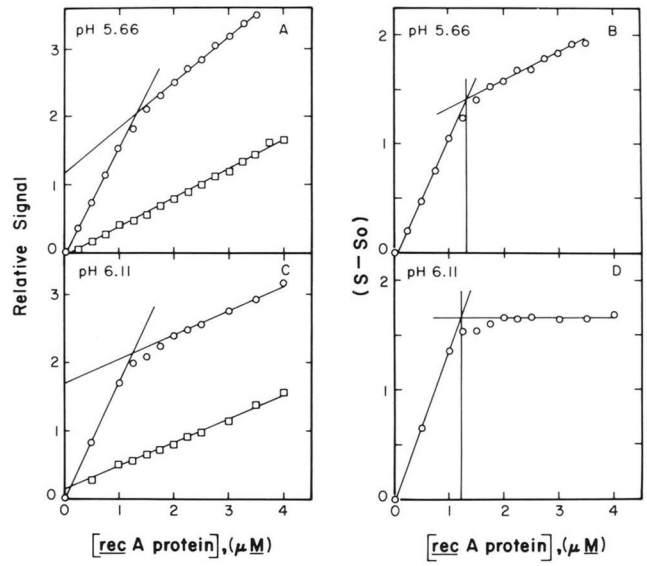


FIG. 7. **Stoichiometry of binding to dsDNA.** Reactions were monitored by light scattering as described under "Materials and Methods." Reactions in buffer A, adjusted to (*A*, *B*) pH 5.66 or (*C*, *D*) 6.11, contained 10 μM linear dsDNA (pRCB1), 1 mM ATP, and the indicated recA protein concentration. *Panels A* and *C*, contain the uncorrected data: (○) "+" DNA, (□) "−" DNA. In *panels B* and *D* the "−" DNA relative signal (S_0) has been subtracted from the "+" DNA relative signal (S) of *panels A* and *C*, respectively. The titration end points or equivalence points are defined by the intersection of asymptotic lines drawn on the plots. These are marked by *vertical lines* on the horizontal axis. The horizontal asymptotes at pH 6.11 mark the maximum light scattering intensity for saturated recA·dsDNA complexes. The titration end point is reached at 1.25 μM recA protein. At 10 μM dsDNA, this corresponds to a stoichiometry of one recA monomer/8 bases or 4 base pairs.

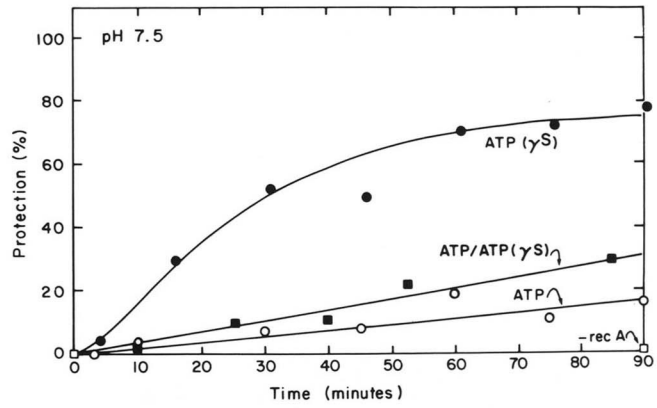


FIG. 8. **ATPγS stimulation of binding to dsDNA.** Reactions in buffer B contained 10 μM linear dsDNA (^{3}H-pMMC5) and either 1 mM ATPγS (●) or ATP (○). □, recA protein omitted. In the reaction containing ATP, aliquots were also removed at various times and incubated with 0.5 mM ATPγS for 5 min (■). The time points reflect this additional 5 min.

This permits a direct comparison of binding. The increased protection observed by this treatment may be due to an increased stabilization of the recA/dsDNA filaments. A small contribution of this increased protection is probably also due to ATPγS-induced binding over the 5-min incubation time. This data therefore should be considered an upper limit for binding in the presence of ATP alone. ATPγS alone increased the rate of stable binding to duplex DNA at pH 7.5 at least 5-fold relative to ATP. Evidence from electron microscopy (32–34) has shown that the dsDNA bound by recA protein is highly unwound in the presence of ATPγS. How ATPγS stimulates the rate of stable binding is unclear. Since the ATPγS is not hydrolyzed (50), the published experiments provide complementary evidence that DNA unwinding does not require ATP hydrolysis.

Propagation or Growth of a recA Filament Is Not Rate-limiting—We wished to determine if unwinding of the DNA was cooperative and occurred simultaneously throughout the DNA molecule, or if less unwound intermediates could be detected at early times. A wide range of highly unwound topoisomers migrate in the same position as form X in agarose gels, and such intermediates might not be detected in the experiments in Fig. 5. Intermediates of this kind might indicate a kinetically significant propagation or extension of the recA filament along the DNA. We assume that unwinding occurs only within the DNA to which recA protein is directly bound. Incomplete binding of recA protein to a DNA molecule should therefore result in unwinding to a lesser degree than that observed in form X DNA. These intermediate topoisomers should be present at early times in the reaction and the average superhelical density should increase with time as more recA protein is bound. To detect these intermediates, a DNA-binding time course was set up at pH 6.35 and timed aliquots treated with DNA ligase as described above. Each timed aliquot was subdivided four times and DNA from each time point was electrophoresed in the presence of 0, 0.1, 1.0, or 10.0 μM ethidium bromide. This should permit resolution over a wide range of DNA topoisomers. Representative results are

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presented in Fig. 9. No DNA species was observed, at any concentration of ethidium bromide, which was unwound to a lesser extent than form X DNA at any point in this time course (except the FIV DNA which represents ligated duplex DNA which was not bound by recA protein). Propagation or extension of the recA filament is therefore fast on this time scale. The slow DNA binding and the lag in ATP hydrolysis cannot be explained by a slow extension of the recA filament on the DNA. This is consistent with the observation noted in Table I that the lag in ATP hydrolysis is shortened rather than lengthened when the length of the duplex DNA is increased. If propagation were rate-limiting, a longer DNA molecule would lengthen the time required to complete the binding process.

The Rate-limiting Step—Additional experiments were carried out in an attempt to determine the nature of the rate-limiting step in the overall pathway leading to binding and dsDNA-dependent ATP hydrolysis. We observed that the rate of binding is independent of the order of addition of ATP, recA protein, and DNA. The rate-limiting step is therefore not a slow preliminary binding of recA protein to either ATP or DNA in an ordered pathway.

As shown in Fig. 10, the rate of DNA binding at pH 7.5, as measured by nuclease protection or ATP hydrolysis, is independent of the concentration of recA protein at concentrations above that required to saturate the DNA. As the pH is decreased, another step which exhibits a dependence on recA protein concentration becomes partially rate-limiting. This is evident in the rates of ATP hydrolysis at pH 6.35 plotted in Fig. 10*C*. A marked dependence on recA protein concentration is observed, with the lag decreasing with increasing recA protein, while the steady state velocity of ATP hydrolysis is unchanged. In preliminary experiments, extrapolation of the length of the lag to infinite recA protein concentration in a τ plot (58) indicates that the step which is first order still makes a significant contribution to the reaction rate under these conditions (not shown).

The slow step at pH 7.5 (and to a lesser degree at pH 6.35) must involve DNA unwinding, as illustrated in Fig. 11. The long lag in ATP hydrolysis at pH 7.5 is again evident. When the DNA was unwound prior to addition of recA protein, however, the lag was greatly reduced. This was accomplished

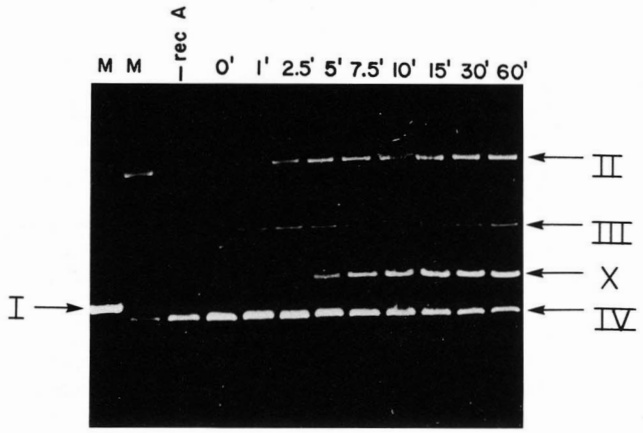


FIG. 9. **Extension of recA protein filaments is not rate-limiting.** Reactions in buffer A contained 10 μM nicked circular dsDNA (pBR322-2) and 10 μM recA protein. At the indicated times samples were removed, treated with excess DNA ligase for 5 min, then electrophoresed in an agarose gel containing 1 μM ethidium bromide. The first *three lanes* represent FI DNA marker, FII, and FIV DNA markers, and a reaction with recA protein omitted, respectively. *I*, *II*, *III*, *IV*, and *X* denote FI, FII, FIII, FIV, and form X DNAs, respectively.

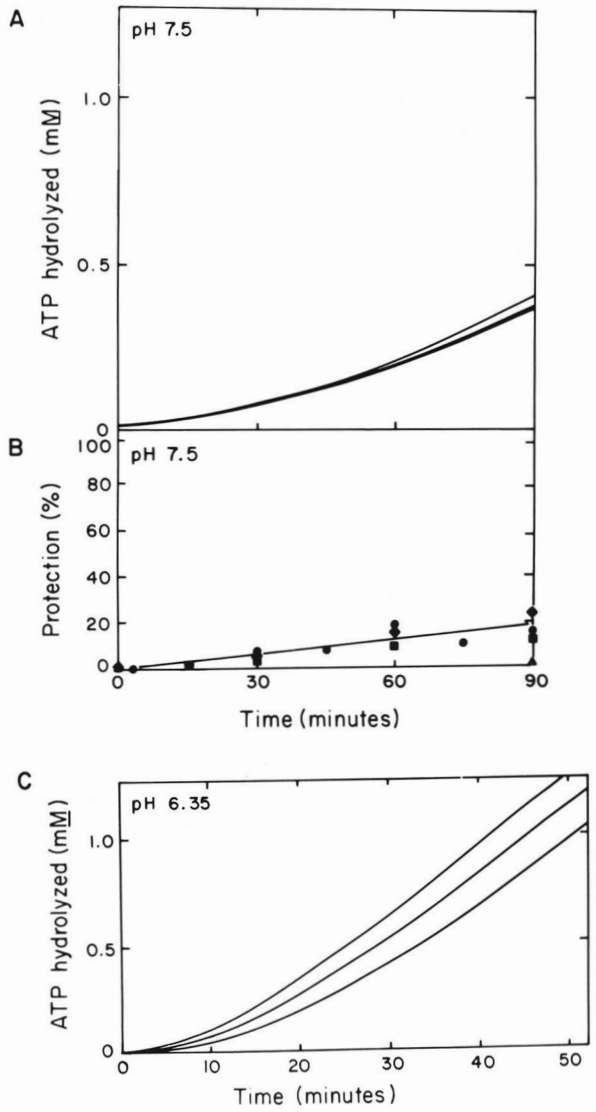


FIG. 10. **pH-dependent change of rate-determining step of recA protein binding to dsDNA.** Reactions were performed in buffer A (*A*, *B*) or buffer B (*C*) as described under "Materials and Methods." *A*, duplex DNA binding as measured by ATP hydrolysis. Reactions contained 10 μM FII dsDNA (pBR322-2), 1 mM ATP, and 40, 10, or 5 μM recA protein. The slight differences in rate in the three experiments reflect random error and no dependence on recA protein concentration is observed. *B*, duplex DNA binding as measured by nuclease protection. Reactions contained 10 μM FIII dsDNA (^{3}H-pMMC5), 1 mM ATP, and (▲) 0, (●) 5, (■) 10, or (◆) 20 μM recA protein. *C*, binding as measured by ATP hydrolysis. Reactions contained 10 μM FIII dsDNA (pBR322), 1 mM ATP, and 20, 10, or 7.5 μM recA protein (*curves top* to *bottom*).

by employing form X DNA, prepared as described under "Materials and Methods," as a cofactor for ATP hydrolysis at pH 7.5. The topological integrity of all DNA substrates was examined before and after this experiment. In the form X experiment, approximately 80% of the DNA was present as form X and no change was observed during the experiment. Extensive unwinding of the DNA prior to recA protein binding therefore largely bypasses the slow step in the binding process. Since extension of a recA filament appears not to be slow, the rate-limiting process may involve the initiation of unwinding or a nucleation step in binding. A similar reduction in the lag in ATP hydrolysis when form X was employed as a cofactor was also observed at pH 6.35. The fact that the lag is not totally abolished at either pH in this experiment may

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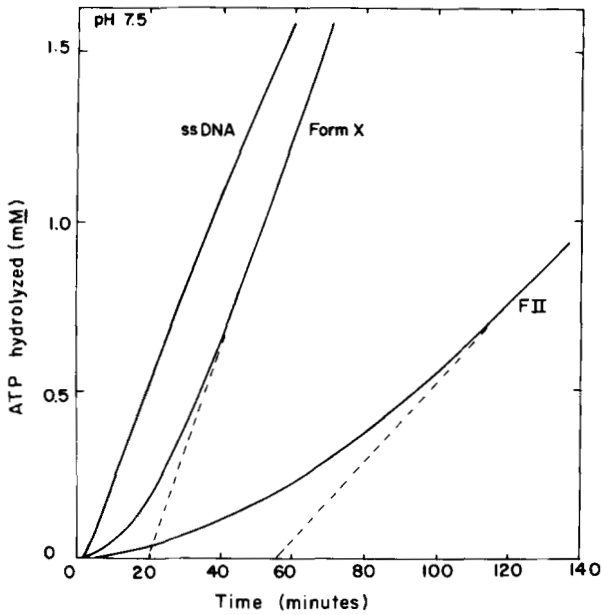


FIG. 11. **Rapid binding of recA protein to unwound dsDNA at pH 7.5.** Reactions were carried out in buffer B as described under "Materials and Methods" and contained 10 μM form X or FII DNA (pBR322-2) or 5 μM ssDNA (ϕX174) and 10 μM recA protein. ATP hydrolysis is employed as an indicator of DNA binding in this experiment. *Dashed lines* represent extrapolations used to estimate τ_{obs}.

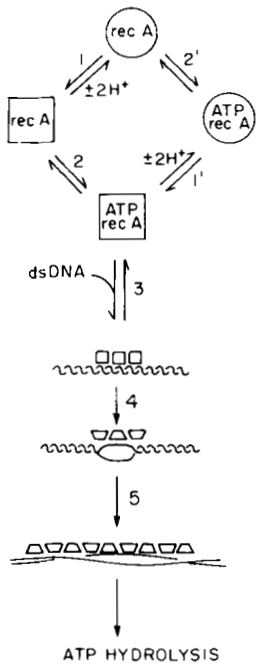


FIG. 12. **An outline of the binding steps for the association of recA protein to dsDNA.** See text for details.

reflect the presence of a small amount of relaxed DNA in the sample or indicate that additional steps are present which are also partially rate-limiting. It is possible that the reduction in lag observed with form X DNA reflects binding to ssDNA in regions which have been locally denatured. We prefer the interpretation that binding involves DNA which is unwound but still base paired, since denatured regions in form X DNA are not evident in the electron microscope unless conditions are employed for DNA spreading which promote denaturation.[4] If binding is initiated at regions of ssDNA, the renaturation activity of recA protein (59, 60) should lead to a final complex in which strands are paired.

DISCUSSION

recA protein has been widely reported to bind very weakly to dsDNA at pH 7.5 in the presence of ATP. Our principal conclusion is that this apparent weak binding reflects a slow step in the association pathway rather than an unfavorable binding equilibrium. Several related conclusions can be derived from this work which augment observations published elsewhere. We show here that recA protein (*a*) causes extensive unwinding (much greater than that found in FI DNA) of duplex DNA in the presence of ATP without a need for ssDNA or low Mg^{2+} conditions, (*b*) can bind stably to duplex DNA at pH 7.5 in the presence of ATP, and (*c*) binds at a density corresponding to approximately one monomer/4 base pairs. All of these results reflect the state of binding in solution. They were obtained by kinetic methods which should avoid any artifacts which might be associated with fixation for electron microscopy or other potentially disruptive procedures.

A possible pathway for recA protein binding to dsDNA is presented in Fig. 12. We can at present provide no more than an outline of the process and parts of the model are still speculative, including the order in which some of the steps occur. The model is useful to illustrate the types of steps that are likely to occur in the pathway, as inferred from results reported here and elsewhere. The steps we include are a pH-mediated conformation change, ATP binding, nucleation of binding by the recA·ATP complex, and a rapid cooperative extension of the nucleated complex to form a complete nucleoprotein filament on unwound dsDNA. Only the final complex is capable of ATP hydrolysis. A pH-dependent conformation change occurring prior to binding is based on the observation that the recA protein-mediated ATPase shows the same magnitude of stimulation with decreasing pH in the presence or absence of dsDNA. We infer from the ATP dependence of binding that ATP is bound at an early step.

The rate-limiting step in binding is not affected by the order of addition of ATP, DNA, or recA protein, suggesting that all three are present at this step. At pH 7.5 this slow step exhibits no dependence on recA protein concentrations above that required to saturate the DNA lattice. In addition, this step is bypassed by extensively unwinding the duplex DNA before binding. The slow step is not the extension or propagation of the recA filament (step *5* as drawn in Fig. 12). The topological DNA intermediates which would be expected if propagation were slow are not observed, and increasing the length of the DNA increases rather than decreases the rate of binding. We hypothesize that the rate-limiting process is the nucleation of binding and/or unwinding (step *4* in Fig. 12). A similar suggestion was made by Volodin *et al.* (36) based on the kinetics of dsDNA binding at pH 7.5 in the presence of ATPγS. We note that the decrease in the length of the lag in ATP hydrolysis as the DNA length is increased (Table I) may reflect an increase in potential nucleation sites in the larger DNAs. The nucleation step may be complex in itself and the actual rate-limiting event on the molecular level could be a conformational change in the recA protein, DNA, or both: a rearrangement of mono or divalent ions, etc. This step increases in rate as the pH is decreased. Preliminary results suggest that this involves the net addition of two protons to each recA monomer.[4] At pH near 6.0 another step, which exhibits a dependence on recA protein concentration, is partially rate-limiting. More work is required to define the mo-

[4] B. F. Pugh, unpublished results.

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lecular details of the binding pathway.

DNA binding and unwinding are inseparably linked in this process. No set of conditions were found in this study in which binding to duplex DNA did not result in the extensive unwinding manifested by form X DNA. This should result in a lower level of recA protein binding when the DNA is covalently closed and therefore topologically restricted. Such an effect has been noted by Dombrowski *et al.* (35) in the presence of ATPγS. When FI DNA is employed as a binding substrate at pH 6.1 in the presence of ATP, the level of observed recA protein binding and ATP hydrolysis is reduced approximately 30%.[4] This demonstrates a link between binding and unwinding even under conditions previously defined as optimal for duplex DNA binding.

Binding and unwinding are also ATP dependent. We stress, however, that ATP hydrolysis occurs only after binding and unwinding are complete, *i.e.* ATP hydrolysis is not coupled to unwinding. Once ATP hydrolysis begins, binding is apparently stable at pH 7.5. The hydrolysis of ATP only in the final binding state indicates that this process cannot be treated as a simple binding equilibrium. The investment of chemical energy may open up new pathways for dissociation without violating the principle of microscopic reversibility. It is not clear whether this ATP hydrolysis results in a cycle of association and dissociation by recA protein. When recA protein complexes are formed on ssDNA in the presence of *E. coli* single-stranded DNA-binding protein (SSB), no coupling between ATP hydrolysis and association/dissociation of recA protein is observed (51). Other studies carried out in the absence of SSB suggest that such a cycle may exist under some conditions (52). The partial protection of dsDNA from nuclease digestion illustrated in Fig. 1 may reflect such a cycle.

The stoichiometry for binding to duplex DNA reported here, one recA monomer/4 base pairs, is significantly different from the one per 2–3 base pairs previously reported (33, 35, 36). The earlier estimates were based largely on results from electron microscopy. Our estimate is based on methods which should accurately reflect the binding state of the protein in solution. This estimate also agrees well with the one recA monomer/3.5–5 nucleotides commonly reported as a binding stoichiometry on ssDNA (47, 51–54). If a binding site size of 4 base pairs is assumed, and if only bound recA monomers hydrolyze ATP, then the apparent k_{cat}, or turnover number, for ATP hydrolysis is approximately 19–22 min^{-1} with dsDNA, based on data in this study.

These results have several implications for the DNA strand exchange reaction. recA protein begins the reaction bound to ssDNA and should become associated with duplex DNA after the migrating DNA branch passes. The weak binding of recA protein to duplex DNA described in earlier studies has been believed to imply a requirement for recA protein dissociation at the branch point and this has been incorporated into models for the reaction (55). We have shown, however, that the apparent weak binding to dsDNA at pH 7.5 does not reflect an unfavorable equilibrium for recA protein binding to dsDNA. Instead, the system exhibits a considerable degree of hysteresis. The apparent weak binding is the result of a major kinetic barrier to the association reaction. Once this barrier is overcome, recA protein binds stably to the unwound dsDNA. As long as recA protein holds the heteroduplex DNA product of strand exchange in this unwound configuration, there is no requirement for dissociation of recA protein. Indeed, we show in the accompanying paper (56) that recA protein remains bound to the heteroduplex DNA after strand exchange is complete.

Assembly of recA protein on ssDNA is unidirectional (57). Since duplex DNA is antiparallel, this fact suggests that the interaction of recA protein with the two strands of DNA in the final complex in Fig. 12 may be very asymmetric. Evidence for this has recently been provided by Chow *et al.* (20). Cumulatively, these results indicate that recA protein exhibits one of the primary characteristics one might expect of a protein which catalyzes the exchange of DNA strands. When recA protein is bound to duplex DNA, it maintains the DNA at or near the transition state (unwound) for the strand exchange reaction. The data presented here should be useful in guiding continued efforts to study this interaction.

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