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Dissociation Pathway for recA Nucleoprotein Filaments Formed on Linear Duplex DNA

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recA protein forms stable filaments on duplex DNA at low pH. When the pH is shifted above 6·8, recA protein remains stably bound to nicked circular DNA, but not to linear DNA. Dissociation of recA protein from linear duplex DNA proceeds to a non-zero endpoint. The kinetics and final extent of dissociation vary with several experimental parameters. The instability on linear DNA is most readily explained by a progressive unidirectional dissociation of recA protein from one end of the filament. Dissociation of recA protein from random points in the filament is eliminated as a possible mechanism by several observations: (1) the requirement for a free end; (2) the inverse and linear dependence of the rate of dissociation on DNA length (at constant DNA base-pair concentration); and (3) the kinetics of exposure of a restriction endonuclease site in the middle of the DNA. Evidence against another possible mechanism, ATP-mediated translocation of the filament along the DNA, is provided by a novel effect of the non-hydrolyzable ATP analog, ATPγS, which generally induces recA protein to bind any DNA tightly and completely inhibits ATP hydrolysis. We find that very low, sub-saturating levels of ATPγS completely stabilize the filament, while most of the ATP hydrolysis continues. If these levels of ATPγS are introduced after dissociation has commenced, further dissociation is blocked, but re-association does not occur. These observations are inconsistent with movement of recA protein along DNA that is tightly coupled to ATP hydrolysis. The recA nucleoprotein filament is polar and the protein binds the two strands asymmetrically, polymerizing mainly in the 5′ to 3′ direction on the initiating strand of a single-stranded DNA tailed duplex molecule. A model consistent with these results is presented.

1. Introduction

The recA protein of *Escherichia coli* promotes strand exchange reactions *in vitro* that mimic its *in vivo* homologous genetic recombination functions. Strand exchange reactions require stoichiometric amounts of recA protein, since the protein is active only in the form of a nucleoprotein filament (Cox & Lehman, 1987). A useful *in vitro* reaction system involves circular ssDNA‡ and homologous linearized duplex DNA derived from bacteriophage (Cox & Lehman, 1981*a*,*b*). In the first step of the strand exchange reaction, recA protein coats the ssDNA at a stoichiometry of one protein monomer per four nucleotides (West *et al.*, 1980; Bryant *et al.*, 1985; Morrical & Cox, 1985; Morrical *et al.*, 1986). The resulting nucleoprotein filament homologously aligns with the duplex DNA (Das Gupta *et al.*, 1980; Gonda & Radding, 1983; Julin *et al.*, 1986; Tsang *et al.*, 1985; Schutte & Cox, 1988). The strand of the duplex that is complementary to the ssDNA is transferred 3′ to 5′ to the ssDNA, at a rate of 5 to 6 bp s^{-1}, as ATP is hydrolyzed (West *et al.*, 1981; Kahn *et al.*, 1981; Cox & Lehman, 1981*a*,*b*; Cox *et*

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‡ Abbreviations used: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; FI DNA, supercoiled closed circular form of a DNA molecule as isolated from *E. coli* cells; FII DNA, the nicked circular form of the same molecule; FIII DNA, the linear form of the same molecule; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); Mes, 2-(*N*-morpholino)ethane sulfonic acid; Tris, tris-(hydroxymethal)aminomethane; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; kb, 10^3 base-pairs; bp, base-pairs; HAP, hydroxylapatite.

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al., 1983). ATP hydrolysis occurs throughout the nucleoprotein filament without any detectable enhancement of hydrolysis at the filament ends or at the branch point (Brenner *et al.*, 1987; Schutte & Cox, 1987; Kowalczykowski & Krupp, 1987). The recA protein remains bound to the nicked circular heteroduplex product (Pugh & Cox, 1987*b*). The stability of the initial recA nucleoprotein filament directly correlates with the rate and efficiency of the strand exchange reaction (Morrical & Cox, unpublished results). *In vivo*, the heteroduplex DNA product of strand exchange can extend for thousands of base-pairs (Broker, 1973; Tsujimoto & Ogawa, 1977; Sodergren & Fox, 1979). The stability of recA nucleoprotein filaments *in vivo* may affect the length of heteroduplex DNA formed and other features of the reaction.

Many of the unresolved questions with regard to the mechanism of recA-mediated DNA strand exchange concern the structure and dynamics of these nucleoprotein complexes. A detailed understanding of DNA–recA association and dissociation pathways is required. The interaction with duplex DNA is especially relevant to events that must occur *in vivo*. The association of recA protein with duplex DNA has been studied in some detail (Pugh & Cox, 1987*a*; Shaner *et al.*, 1987; Shaner & Radding, 1987; Kowalczykowski *et al.*, 1987; Pugh & Cox, 1988). The apparent weak binding of recA protein to duplex DNA at neutral pH is caused by a slow step in association, rather than an unfavourable binding equilibrium (Pugh & Cox, 1987*a*, 1988; Kowalczykowski *et al.*, 1987). The rate-limiting step to binding is nucleation, which requires the initiation of duplex DNA underwinding (Pugh & Cox, 1987*a*). Although a nucleotide triphosphate is required for the association of recA protein with duplex DNA, no hydrolysis occurs until after the DNA is bound and underwound (Pugh & Cox, 1987*a*). While nucleation is slow, resulting in an association lag averaging more than three hours at pH 7·5 for circular duplexes, propagation of recA protein along the DNA is rapid (Pugh & Cox, 1987*a*, 1988). The resultant nucleoprotein filament hydrolyzes ATP with a turnover number of 22 to 26 min^{-1} at the monomer level. It has one recA monomer for every 3 to 4 bp and the bound DNA is underwound by 39·6% (Pugh *et al.*, 1988). Electron micrographs of complexes formed in the presence of the non-hydrolyzable ATP analog ATPγS show that recA protein condenses onto DNA as a helical rod-like filament (DiCapua *et al.*, 1982; Stasiak & DiCapua, 1982; Stasiak *et al.*, 1981). There is evidence suggesting that the protein binds along the minor groove, interacting primarily with the sugar–phosphate backbone and not interrupting the hydrogen-bonding between base-pairs (Leahy & Radding, 1986; DiCapua & Müller, 1987). The DNA is held in the underwound configuration, close to the transition state for a strand exchange reaction.

The rate of nucleation of recA protein binding to duplex DNA can be increased by several methods. The association reaction proceeds faster near pH 6 (McEntee *et al.*, 1981; Pugh & Cox, 1987*a*). An increase in the DNA superhelicity or length, or an increase in reaction temperature decreases the average initiation time (Pugh & Cox, 1987*a*, 1988; Kowalczykowski *et al.*, 1987). Single-stranded gaps, *Z*-form DNA, mismatched base-pairs, thymidine dimers and the ends of linear DNA also increase the rate or extent of association (Shaner *et al.*, 1987; Shaner & Radding, 1987; Lu *et al.*, 1986; Blaho & Wells, 1987; this work). All of these effects can be accommodated by a single association pathway (Pugh & Cox, 1988).

The presence of a short single-stranded gap or tail in a duplex DNA molecule greatly stimulates recA protein association with the duplex DNA. The binding of recA protein to duplex DNA with 5′ single-stranded tails approaches binding saturation, as reflected by steady-state ATP hydrolysis, much more quickly than reactions with 3′ tailed duplex molecules or fully double-stranded molecules (Shaner & Radding, 1987; Shaner *et al.*, 1987; this work). This and other evidence suggest that the association of recA protein with duplex DNA is primarily in the 5′ to 3′ direction, relative to the extended strand. Within the nucleoprotein filament, this 5′ tailed strand exhibits significantly greater resistance to DNase digestion than the other strand, suggesting that recA protein binds the two strands of the duplex asymmetrically (Chow *et al.*, 1988). recA protein binding to ssDNA also occurs in the 5′ to 3′ direction (Register & Griffith, 1985).

The single-stranded tail can be separated from the linear duplex DNA by digestion with large amounts of a restriction enzyme after complete nucleoprotein filaments are formed. As a result, the rate of ATP hydrolysis decreases, indicating that the nucleoprotein filament on linear duplex DNA is not stable (Shaner & Radding, 1987; Lindsley & Cox, unpublished results). This result creates an apparent paradox, since recA nucleoprotein filaments formed on nicked circular duplex DNA at pH 6·2 remain intact when the pH is shifted to 7·5 (Pugh & Cox, 1987). There are at least three possible mechanisms for recA protein dissociation from linear duplex DNA, as illustrated by Figure 1. Two of these readily account for the stability differences between nucleoprotein filaments formed on linear and circular dsDNA. One possibility is that recA protein translocates unidirectionally along the DNA in a reaction coupled to ATP hydrolysis, continuing indefinitely on circular DNA molecules but moving off the ends of linear molecules (Shaner & Radding, 1987). In this case, a 5′ single-stranded tail provides an accessible loading point for recA protein reassociation. The protein subsequently moves into the duplex region. Another possibility is that recA protein progressively dissociates from one end of the nucleoprotein filament. Here, the 5′ tail would simply stabilize the end most likely to dissociate. A third model, in which recA protein dissociates randomly from the DNA, does not explain the apparent difference between the filaments formed on linear and circular

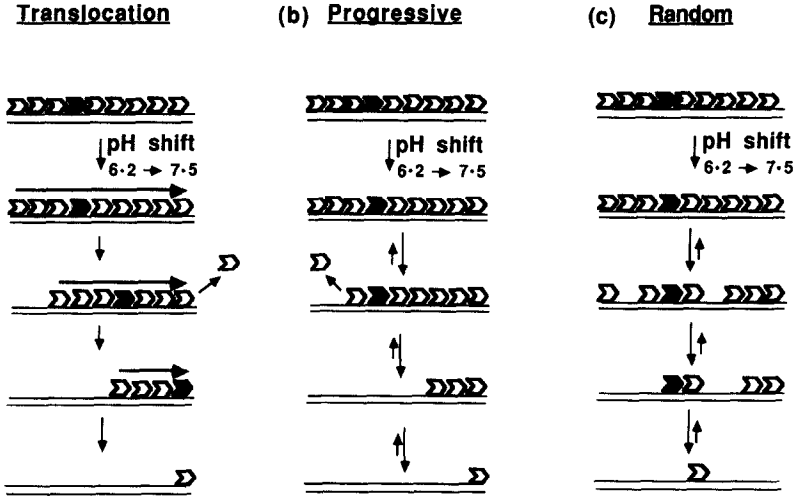


Figure 1. Three models for recA protein dissociation from linear duplex DNA. (a) Translocation involves an ATP-mediated movement of recA protein on the DNA, with recA monomers, or other units, dissociating from the head of the filament as it moves off the DNA. The filled symbols were used only as reference points in the filament. (b) Progressive dissociation involves dissociation of recA protein from the tail of the filament, without movement of the filament. Note that the same area of DNA is exposed with time in the case of (a) or (b). (c) recA protein dissociates randomly from the filament.

DNA, but is consistent with most other published results.

The experiments described here were done to further probe the dynamics of the nucleoprotein–duplex DNA filament. In particular, the pathway and requirements for recA protein dissociation from duplex DNA were examined. Experiments showing the polarity and asymmetry of recA–duplex DNA filaments are described and correlated with a model for filament dissociation.

2. Materials and Methods

(a) *Reagents*

E. coli recA protein was purified and stored as described (Cox *et al.*, 1981). The recA protein concentration was determined by measuring absorbance at 280 nm, using an extinction coefficient of $\varepsilon_{280} = 0{\cdot}59\ A_{280}$ mg^{-1} ml (Craig & Roberts, 1981). Supercoiled circular duplex DNA (FI) derived from bacteriophages M13mp8 (7229 bp; Messing, 1983), M13Gori1 (8623 bp; Kaguni & Ray, 1979) and M13oriC26 (12,173 bp; Kaguni *et al.*, 1979) was prepared as described (Neuendorf & Cox, 1982). ϕX174 FI DNA was purchased from New England Biolabs. The plasmids pXF3 (Maniatis *et al.*, 1982), pBR322 (Bolivar *et al.*, 1977), pBCS2 (Schutte & Cox, 1988) and pUC119 (Vieira & Messing, 1987) were purified as described (Davis *et al.*, 1980). ^{3}H-labeled ssDNA and dsDNA were prepared by an established procedure (Julin *et al.*, 1986) and had a specific activity of 10 to 50 Ci mol^{-1}. The concentrations of dsDNA stock solutions were determined by measuring absorbance at 260 nm, using 50 μg ml^{-1} A_{260}^{-1} as a conversion factor. DNA concentrations are expressed as total nucleotides. FII DNA was prepared from FI DNA using DNaseI and ethidium bromide (Shibata *et al.*, 1981). This method produces FII DNA with a single random nick. FIII DNA was derived from FI stocks by complete digestion with *Eco*RI, or another enzyme where indicated. After digestion, residual protein was removed by extraction (1 : 1, v/v) sequentially with phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.) and chloroform/isoamyl alcohol (24 : 1, v/v), followed by precipitation with ethanol. The 322 bp fragment used in this study was generated by complete digestion of pUC119 FI DNA with *Pvu*II and purified from a 1·2% (w/v) agarose gel (Maniatis *et al.*, 1982). Restriction endonucleases were purchased from New England Biolabs. Pyruvate kinase, lactic dehydrogenase, NADH, DNase I, and ATP were purchased from Sigma. Mes buffer was from Research Organics Inc.; ATPγS and Tris buffer were purchased from Boehringer-Mannheim Biochemicals. The purity of the ATPγS was checked by thin-layer chromatography (Shibata *et al.*, 1981) and was shown to be at least 90% pure, with the major contaminant being ADP.

(b) *Preparation of gapped duplex DNA*

Circular duplex DNA with a precisely defined gap was prepared in the following way. First, circular ssDNA (the viral + strand), from M13mp8 with a non-homologous DNA insert, was prepared. This DNA will be referred to as M13mp8.*X*, where *X* corresponds to the length of the insert in nucleotides. The insert was from the *galT* gene of *E. coli* and ranged from 34 to 1041 nucleotides. These inserts were positioned at a unique *Bam*HI site in the M13mp8 duplex. Next, FI M13mp8 DNA was linearized with *Bam*HI. The linear duplex molecules were denatured with a final concentration of 100 mM-NaOH (pH 12·5). A 12 to 15-fold excess (in molecules) of ssDNA circles (M13mp8.*X*) at 65°C in neutralizing buffer (200 mM-Tris·HCl, 95% cation, pH ~6·8) was combined (1 : 1, v/v) with the denatured linear DNA. The mixture was maintained at 65°C for 30 min and then allowed to cool slowly to room temperature. The gapped molecules were purified away from the remaining ssDNA on a small hydroxylapatite (HAP) column. All phosphate buffers (P buffers) were 70% NaH_2PO_4/30% Na_2HPO_4 (approx. pH 7), with the number in parentheses representing the total phosphate concentration. The DNA, in 1 ml of P (0·1 M) buffer, was loaded directly onto the HAP column, equilibrated in the same buffer. Single-stranded molecules

were eluted with 10 to 20 column volumes of P (0·17 M) buffer. Gapped duplex DNA was eluted with 5 volumes of P (0·3 M) buffer. Fractions were collected and analyzed by gel electrophoresis. Fractions that contained the most highly purified gapped duplex DNA were pooled and dialyzed extensively against TE buffer (10 mM-Tris · HCl, 80% cation, 1 mM-EDTA). The preparations contained at least 85 to 90% gapped duplexes as determined by densitometric scanning of photographic negatives of agarose gels. The remaining DNA comigrated with linear duplex DNA markers. The gapped molecules were shown to be competent in 4 strand exchange reactions (data not shown). The strand of the gapped duplex molecules that was derived from the viral ssDNA, and is single-stranded in the gap, is designated the (+) strand. The complementary strand is the (−) strand.

Tailed dsDNA molecules were made by digesting gapped duplexes with restriction enzymes that cut uniquely near the gap. The *Eco*RI site is 12 bp to the 5′ side of the gap and the *Hin*dIII site is 20 bp to the 3′ side of the gap, both relative to the (+) strand. Cleavage by either enzyme effectively results in a duplex molecule with a tail of a defined length at one end or the other. The cut DNA was extracted and precipitated with ethanol, as described above. The DNA was fully cut as judged by agarose gel electrophoresis.

Radioactively labeled gapped duplex molecules were prepared using either ^{3}H-labeled ssDNA M13mp8.50 or ^{3}H-labeled FI M13mp8. Using the above procedure, gapped duplex molecules ^{3}H-labeled throughout the (+) or the (−) strand were made. The specific activities of the labeled gapped duplexes ranged from 2·0 to 5·7 Ci/mol.

(c) *Reaction conditions*

All reactions were carried out in 25 mM buffer, 10 mM-magnesium acetate, 2% (v/v) glycerol, 1 mM-ATP and an ATP regenerating system (4·5 units of pyruvate kinase/ml, 4·5 units of lacate dehydrogenase/ml, 3 mM-NADH, 3 mM-phosphoenolpyruvate, 1 mM-KCl). The buffer for reactions with gapped duplex DNA was Tris-acetate (80% cation, pH 7·5). Buffers used in other reactions are described for each experiment. DNA and recA protein concentrations are indicated for each experiment. Reactions were incubated at 37 °C for 10 min before ATP, or recA protein for filter binding reactions, was added to start the reactions. All reactions were carried out at 37 °C. The integrity of the DNA was compared before and after several of the ATPase experiments. No detectable degradation occurred.

(d) *pH shift reactions*

Reactions were started as described above in Mes/NaOH (56% anion, pH 6·27) buffer. The reactions were incubated for 20 min to reach a stable steady-state (the binding lag under these conditions is less than 5 min). A 200-μl portion of the reaction was then added to 200 μl of a buffer solution, also at 37 °C, containing 25 mM-Tris-acetate, 10 mM-magnesium acetate, 2% glycerol, 1 mM-ATP and ATP regenerating system. The Tris-acetate buffers used ranged in pH from 7·61 (75% cation) to 9·21 (10% cation). The final pH of the reactions, after the pH shift, ranged from 6·27 (for a reaction diluted 2-fold in Mes/NaOH (56% anion) buffer) to 8·28.

(e) *ATPase assay*

This assay was used as a measure of recA protein binding to duplex DNA. A Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with 2 thermojacketed cuvet holders, each capable of holding 6 cuvets, was used for absorbance measurements. The cell path-length and the band pass were 0·5 cm and 2 nm, respectively. The coupled assay used to measure ATP hydrolysis was as described (Morrical *et al.*, 1986). The regeneration of ATP from ADP and phosphosoenolpyruvate with the oxidation of NADH can be followed by the decrease of absorbance at 380 nm. Absorbances were measured at 380 nm, instead of 340 nm (the absorbance maximum for NADH) to remain within the linear range of the spectrophotometer. NADH was used at a high concentration (3 mM) to ensure that all reactions could reach a steady-state level of ATP hydrolysis before the NADH was oxidized. An extinction coefficient of $\varepsilon_{380} = 1{\cdot}21$ mM^{-1} cm^{-1} was used to calculate the rates of ATP hydrolysis. The lag time before a reaction reached steady-state ATP hydrolysis was calculated from the intersection of 2 lines drawn along the initial ATPase rate and the steady-state rate. This method was used for both association and dissociation lag times. The time at the point of intersection is defined as the lag time. See Fig. 2 or Fig. 11 for an example. An increase in the concentration of ATP or any of the coupling system components did not change the observed ATPase rate; the data obtained reflect the true initial velocity of ATP hydrolysis at all times.

(f) *Dissociation rate constants*

These constants (k_{diss}) reflect the net number of recA protein monomers dissociating per complex per minute, and have units min^{-1}. The constant k_{diss} is calculated by dividing the number of recA monomers present in each recA–dsDNA complex at saturation (assuming 1 recA monomer per 4 bp or 1807 recA monomers per M13mp8 duplex, unless otherwise indicated) by the observed dissociation lag time, in minutes. This constant was calculated only for reactions in which the final rate of ATP hydrolysis was less than 15% of that observed before dissociation commenced. This remnant rate was ignored in calculating k_{diss}. The resulting rate constant is meaningful only if the dissociation process is relatively synchronized between complexes after a pH shift. The rate constants are applicable to any of the mechanisms considered.

(g) *Restriction enzyme protection experiments*

The rate of ATP hydrolysis was followed in standard reactions of 600 μl with 20 μM-FIII M13 Gori1 DNA and 5 μM-recA protein. At various times, 20-μl samples were removed from the cuvet, mixed with 2·5 μl of *Bam*HI restriction enzyme (diluted 10-fold in high salt reaction buffer, which was supplied with the enzyme, to 2 units μl^{-1}) and digested for 2 min at 37 °C. A 10-μl portion of GED/SDS (25% glycerol, 5 mM-EDTA, 0·005% bromphenol blue, 5% SDS) was added to stop the reaction. Samples were electrophoresed overnight in a 0·8% agarose gel. The percentage of DNA that was cut by *Bam*HI was determined by scanning photographic negatives of the gels stained in ethidium bromide. The densitometric scans were performed on a Zeineh Soft Laser scanning densitometer, SL-504-XL, from Biomed Instruments, Inc.

(h) *Nitrocellulose filter binding*

Nitrocellulose filters from Schleicher & Schuell (BA 85, 0·45 μm; 24 mm circles) were soaked in 0·4 M-KOH for 10

min and rinsed extensively with double-distilled water (Lin & Riggs, 1972). The filters were soaked in reaction buffer (25 mM, at the pH of the reaction, 10 mM-magnesium acetate, 2% glycerol, 1 mM-ATP) for at least 10 min before filtering. Reaction conditions were the same as described earlier, except that [^{3}H]DNA (FII and FIII M13mp8) was used. Portions of 100 μl were filtered at a constant rate (1270 mm Hg below atmospheric pressure; ~ −67,966 Pa) on a 10-place filter holder equipped with individual Teflon valves (Hoeffer model FH 224V). Immediately after the sample was filtered, 1 ml of reaction buffer was added to wash through DNA not bound by protein. Background counts (no recA protein present) were always less than 2% of total counts (reaction sample spotted directly onto filter; no filtering). In reactions at pH 6·27, filtered 20 min after recA protein was added, 100% of either FII or FIII DNA was retained by the filters. The percentage of DNA bound to each filter was determined by liquid scintillation counting (Beckman LS 3801).

(i) *DNase protection of gapped duplex molecules*

Nuclease protection assays were done as described (Pugh & Cox, 1987*a*). Portions (20 μl) of reaction mixtures containing 20 μM-DNA and 10 μM-recA protein were incubated with 5 μl (3 units, 1·12 μg) of pancreatic DNase I 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 heat-denatured calf thymus DNA/ml and 0·375 M-EDTA. This was followed immediately by adding 0·9 ml of ice-cold 10% (w/v) trichloroacetic acid and 2 ml of 95% (v/v) ethanol. Filters were dried and assayed for radioactivity by liquid scintillation counting.

In each experiment, 100% protection was defined by a separate control portion of the reaction mixture not treated with DNase I. To determine background counts, a separate portion lacking recA protein was treated with DNase. Data, percentage protection, were obtained by dividing radioactivity measurements for each sample by the value for 100% protection, after the background was subtracted from both. The DNase I concentration was chosen such that the background counts were always less than 10% of the 100% protection control. All experiments were done in triplicate. The mean value shown in Table 1 for each reaction.

3. Results

(a) *recA protein dissociates from linear duplex DNA. Circular (FII)* versus *linear (FIII) DNA*

Potential differences in recA protein binding to linear *versus* circular dsDNA were examined first. The stable association of recA protein with FII DNA has been described extensively (Pugh & Cox, 1987*a*,*b*, 1988). The rate of DNA-dependent ATP hydrolysis catalyzed by recA protein is a quantitative measure of recA protein bound to DNA. ATP hydrolysis has been correlated to binding by DNase protection, restriction site protection, DNA unwinding, and light-scattering experiments (Pugh & Cox, 1987*a*,*b*; this work). Parallel measurements of recA protein association with FII and FIII DNA, as measured by increases

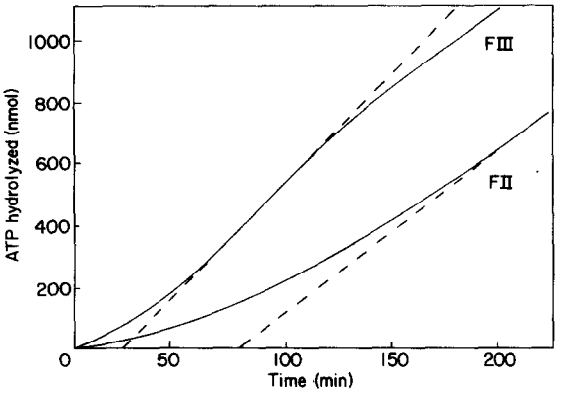


Figure 2. ATP hydrolysis as a function of time; FII *versus* FIII DNA as cofactors. Reactions were carried out as described in Materials and Methods at pH 7·67 (50% Mes/NaOH (56% anion), 50% Tris-acetate (40% cation)). The FII or FIII DNA derived from M13mp8, was at 15 μM. The recA protein concentration was 7·5 μM. The broken lines were drawn at points of maximum velocity achieved during the reaction.

in the rate of ATP hydrolysis, are shown in Figure 2 and 3. At pH 6·0, the time-courses for recA protein binding to FII or FIII DNA are identical; full binding is complete within minutes (data not shown). At pH 7·67, recA protein gradually binds to FII DNA through a slow initiation step, followed by rapid propagation of binding throughout the entire DNA molecule. Binding of recA protein to duplex DNA is characterized by an intrinsic k_{cat} (rate of ATP hydrolysis per bound monomer) of about 25 min^{-1}. This rate varies little over conditions used in this work (Pugh & Cox, 1987*a*; this work). The percentage of DNA bound by recA protein at a given time can be estimated by dividing the observed rate of ATP hydrolysis by the rate expected if the DNA were saturated and all bound recA monomers were hydrolyzing ATP at the intrinsic k_{cat}. After 3·5 hours, only about 30% of the DNA was stably associated with recA protein in a reaction with 15 μM-FII DNA (M13mp8, 7229 bp) and 7·5 μM-recA protein ($V_{obs} = 13$ μM min^{-1}, $V_{saturation} = 44$ μM min^{-1}). Even at this late time, the reaction had not reached a steady state. The results of ATP hydrolysis reactions were much different for recA protein associating with FIII DNA with the same reaction conditions, as shown in Figure 2. The initial rate of association was more rapid onto FIII DNA. However, instead of reaching a stable steady-state, the rate approaches a maximum and then declines with time. The broken line across the FIII curve in Figure 2 indicates a rate of 16 μM min^{-1}, the maximum ATPase rate in this reaction. This rate correlates to 34% of the DNA bound by recA protein. The lag time before reaching the maximum rate was only 20 minutes. Within two hours, the rate of ATP hydrolysis began to decline slowly, and a new steady-state rate was not reached during the experiment. The maximum rate observed increases when higher concentrations

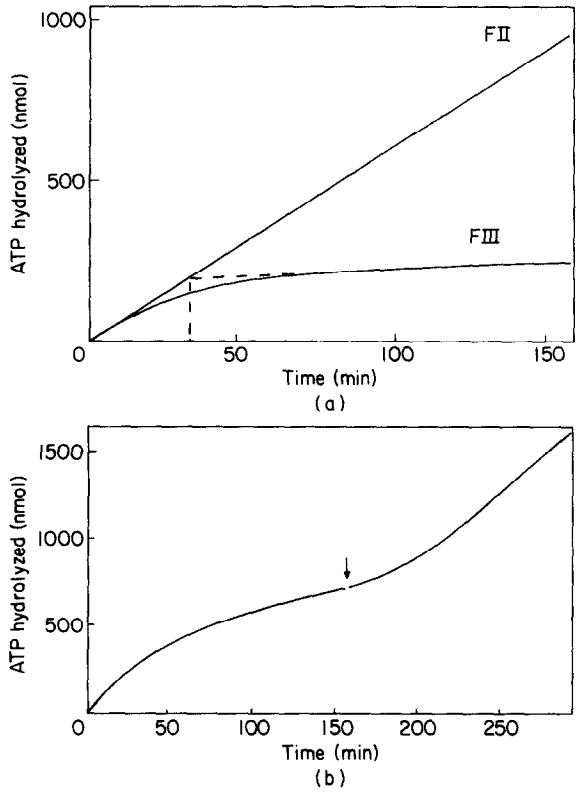


Figure 3. pH shift of ATPase reactions; FII *versus* FIII DNA as cofactors. (a) ATPase reactions were started at pH 6·27 (Mes/NaOH (56% anion)). After 20 min (time = 0 in the Figure), 200 μl of each was added to 200 μl of buffer (Tis-acetate (40% cation)) to give a final pH of 7·67. The final DNA concentration was 5 μM of FII or FIII DNA derived from M13mp8. The final recA protein concentration was 3 μM. The intersecting broken lines show how dissociation lag times were determined for pH shift reactions. The time at which the lines from the initial ATPase rate and the final steady-state rate intersect is defined as the lag time. (b) As in (a), ATP hydrolysis as a function of time for a reaction with FIII M13mp8 DNA, shifted from pH 6·27 to pH 7·67 (time = 0 in the Figure) is shown. At 160 min after the first pH shift, 300 μl of the reaction was added to 300 μl of buffer (Mes/NaOH (42% anion)) to give a final pH of 6·47. The final DNA and recA protein concentrations were each 5 μM. After a 20 min lag, the reaction reached a new steady-state ATPase rate of 16 μM min^{-1}, corresponding to an apparent $k_{cat} = 25{\cdot}6$ min^{-1} (assuming 1 recA monomer bound per 4 bp and hydrolysis by bound monomers only).

of recA protein are used (data not shown). All of the components in the reaction were still active for up to five hours, as demonstrated by the fact that a shift to more favorable dsDNA binding conditions (pH 6·47) restored optimal ATP hydrolysis (see below).

To further examine the stability differences between recA nucleoprotein filaments on linear *versus* circular dsDNA, ATPase reactions that had reached steady state at pH 6·27 were shifted to pH 7·67. The final reactions contained 3 μM-recA protein and 5 μM-M13mp8 DNA. As shown in Figure 3(a), and previously (Pugh & Cox, 1987*a*), the recA protein remains bound to FII DNA, and continues to hydrolyze ATP at the same steady-state rate (when corrected for dilution) as before the pH shift. However, the rate of ATP hydrolysis decreased after a pH shift in a reaction with FIII DNA. Changing the restriction enzyme used to linearize the dsDNA had no effect on this process. The final steady-state ATPase rate was only 10 to 15% of the rate calculated immediately after the pH shift. The lag time before reaching this reduced steady state was 32 minutes. With a k_{cat} of 25 min^{-1} and one recA monomer binding to every four base-pairs, 45,000 ATP molecules are hydrolyzed by each complete nucleoprotein filament per minute. Assuming complete and linear dissociation of the filament within 32 minutes, a total of 720,000 ATP molecules, or 400 per recA monomer, are hydrolyzed during each nucleoprotein filament dissociation.

To confirm that everything in the pH shift reaction was still active after reaching the final remnant steady-state ATPase rate, the pH was shifted back to pH 6·47. The data in Figure 3(b) show that after a 20 minute lag, the recA protein re-binds to the FIII DNA. This lag is approximately the same as the lag for *de novo* binding at the same pH (Pugh & Cox, 1988). Since the binding lag at pH 6·47 is not less than the binding lag for *de novo* association, any recA protein remaining bound to the DNA prior to the pH shift down is not affecting the re-binding of recA protein. The final steady-state ATPase rate at pH 6·47 gives an apparent k_{cat} of 25·6 min^{-1}. Similar results were obtained when enough DNA was present in the "shift down" experiment to permit binding of all of the recA protein (data not shown). This shows that all components of the reaction are fully active, even after five hours.

The experiments described below were performed to understand why recA protein dissociates from linear but not from circular dsDNA. A kinetic characterization of the reaction is presented, followed by additional experiments that address the mechanism specifically. The final steady-state ATP hydrolysis rate observed after dissociation has reached an endpoint is characterized separately in each experiment. Rates of dissociation were estimated and compared as described in Materials and Methods only when this final ATP hydrolysis rate was less than 15% of the rate observed prior to dissociation. Experiments showing the asymmetry of recA–dsDNA filaments are described.

(b) *Characterization of recA–FIII DNA nucleoprotein filament dissociation*

(i) *Effect of pH*

It has been shown that the rate of association of recA protein with duplex DNA is pH-dependent (Pugh & Cox, 1987*a*, 1988). A net uptake of two protons per monomer accompanies the initiation of recA protein binding and concomitant underwind-

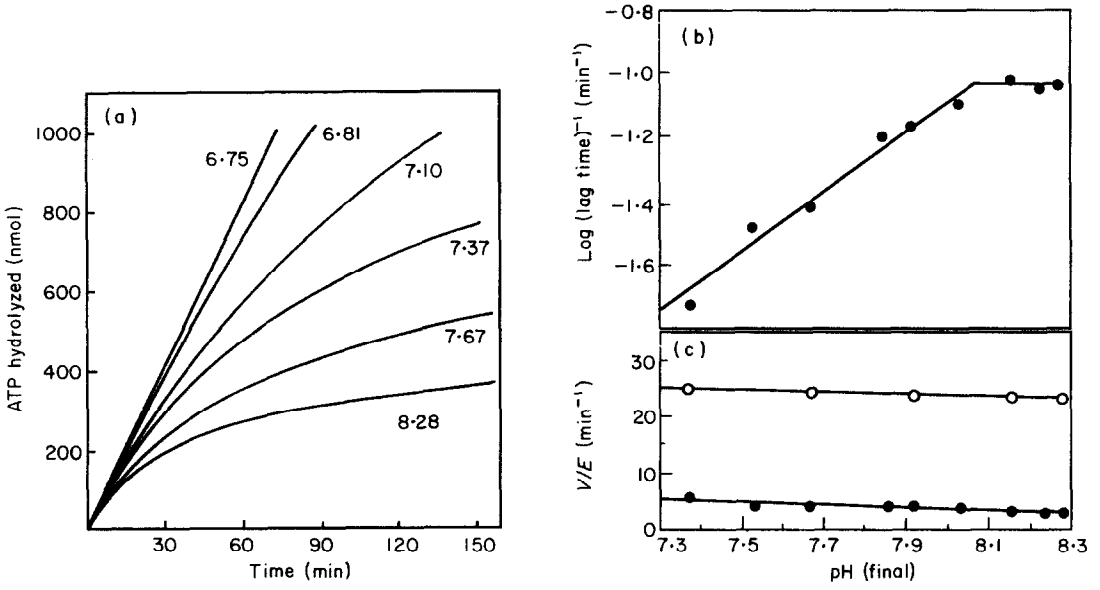


Figure 4. Effect of final pH on the dissociation of recA nucleoprotein filaments. pH shift reactions were performed as described in Materials and Methods. The final reactions contained 8 μM-recA protein and 10 μM-FIII DNA (M13mp8). The pH shift buffers contained Tris-acetate ranging from pH 7·61 to 9·21 (75% cation to 10% cation). (a) At time = 0 (20 min after the reaction was started), 200 μl of each reaction started at pH 6·27 was added to 200 μl of one of the pH shift buffers. The time-course of ATP hydrolysis is shown for 6 of these reactions. The final pH for each reaction is indicated beside the time-course. (b) The dissociation lag times for reactions were measured as described in Materials and Methods and demonstrated in Fig. 2. (c) The V/E of steady-state ATP hydrolysis was determined for pH shift reactions with FII (○) and FIII (●) DNA. In the case of FII DNA, the recA protein concentration used to calculate V/E is the concentration bound at DNA saturation, so V/E corresponds to k_{cat}. For FIII DNA, the same recA protein concentration is used to calculate V/E. In this case, V/E corresponds to an apparent k_{cat}, reflecting a sub-saturating level of actual recA protein binding.

ing of the DNA (Pugh & Cox, 1987*a*). The stability of recA–FIII DNA nucleoprotein filaments, pre-formed at pH 6·27, was studied by examining rates of dissociation at a variety of higher pH conditions. The reactions contained a final concentration of 8 μM-recA protein and 10 μM-M13mp8 FIII DNA. For at least an hour after a pH shift, nucleoprotein filaments on linear dsDNA remained stable at pH 6·75, as shown in Figure 4(a). When the reaction was shifted to pH 6·81 or to a higher pH, the complexes dissociated. The rate of net dissociation increased with the final pH up to about pH 8·1, as reflected by a decrease in dissociation lag time. A plot of log (dissociation lag time)$^{-1}$ *versus* final pH is shown in Figure 4(b). The slope of the line below pH 8·0 is −0·95. This value is consistent with a net release of one proton during the overall dissociation process (Record *et al.*, 1978). The highest pK_b of the functional groups on the DNA and/or the recA protein involved in the net dissociation appears to be at about pH 8·1. The intrinsic k_{cat} or V/E for ATP hydrolysis, determined from pH shift reactions with FII DNA, decreased only slightly as the pH was increased (Fig. 4(c)), so that the decreases in ATP hydrolysis observed with FIII DNA can be accounted for only by recA protein dissociation. The final steady-state rate of ATP hydrolysis for reactions with FIII DNA after dissociation also decreased only slightly with increased pH. Above pH 8·1, k_{diss} is approximately 180 min^{-1} for these complexes, assuming that one recA monomer is bound per four base-pairs.

(ii) *Effect of recA protein concentration*

The concentration of recA protein influences the rate of association of the protein to circular duplex DNA at pH values below 6·5, but not at pH 7·5 (Pugh & Cox, 1988). The effect of recA protein concentration on dissociation of pre-formed nucleoprotein filaments on linear dsDNA was studied. The average time for a recA protein–DNA complex to dissociate to its final state, or the "dissociation lag time" for the reaction, was determined for reactions with a range of recA protein concentrations, as shown in Figure 5(a). The final FIII DNA concentration was 10 μM; saturation of the DNA lattice should occur at a recA protein concentration of 1·25 μM, assuming a stoichiometry of one recA monomer per four base-pairs (Shaner & Radding, 1987; Pugh & Cox, 1987*a*). At recA protein concentrations too low to completely saturate the DNA, the dissociation lag increased sharply with increased recA protein. In the reactions with 6 μM to 12 μM-recA protein, the dissociation lag increased slightly, ranging from 27 to 31 minutes. This small increase, even at high concentrations of recA protein, was observed in several separate experiments. Assuming complete filament dissipation, these lag times correspond to dissociation rates of 67 to 58 recA protein monomers per

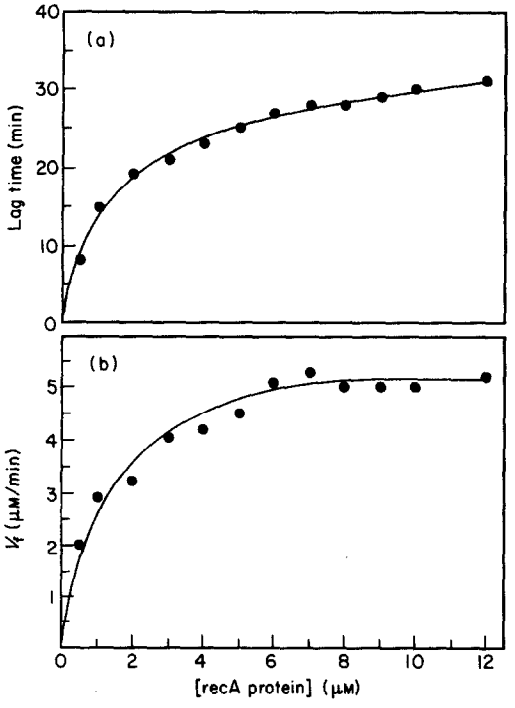


Figure 5. Effect of recA protein concentration on dissociation. ATP hydrolysis reactions with a final concentration of 10 μM-FIII M13mp8 DNA were performed with the indicated final concentrations of recA protein. The pH was shifted from 6·27 to 7·67, 20 min after the reactions were initiated. (a) The dissociation lag times before reaching steady-state ATPase rates after the pH shift were calculated as described in Materials and Methods for each reaction. (b) The final steady-state reaction rates (V_f) were calculated by linear regression. The ATPase rates were determined to be at steady state if they did not change for > 1 h.

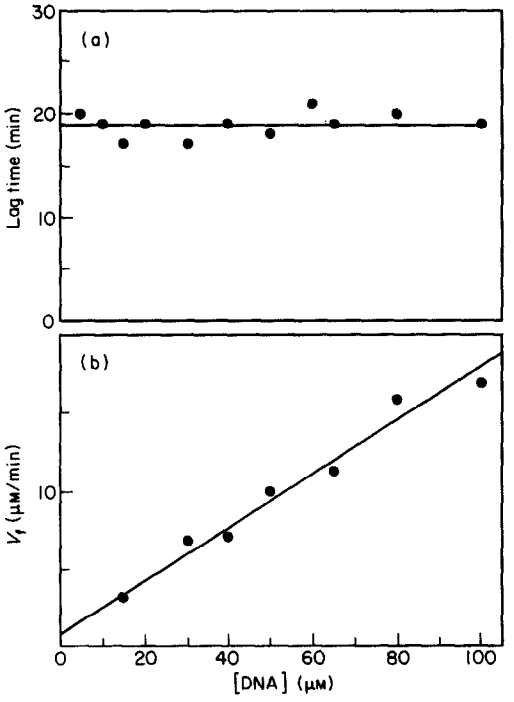


Figure 6. The effect of DNA concentration on dissociation. ATP hydrolysis reactions with a final concentration of 2 μM-recA protein and the indicated FIII M13mp8 concentrations (final) were carried out as described in Materials and Methods. The reactions were started at pH 6·27 and shifted to pH 7·67 after 20 min. (a) The lag time before reaching steady-state ATP hydrolysis was calculated for each reaction as described in Materials and Methods. (b) The final steady-state rates (V_f) of the reactions were calculated by linear regression for reactions in which the rates did not vary for > 1 h.

minute, respectively, for each complex. The low final steady-state ATPase rate exhibited a similar dependence on recA protein concentration, as shown in Figure 5(b). The final ATPase rate was independent of recA protein concentration above 6 μM. The maximum final steady-state rate was 15% of the initial rate determined immediately after the pH shift.

(iii) *Effects of DNA concentration*

Additional pH shift experiments were done to determine how the DNA concentration would affect the dissociation of nucleoprotein filaments on linear duplex DNA at pH 7·92. Figure 6(a) shows that the DNA concentration did not influence the lag time for filament dissociation. At a lower concentration (0·5 μM of recA protein) the dissociation lag time was shorter (10±1 min), but again did not change with DNA concentration (data not shown).

Because the final steady-state rate of ATP hydrolysis is significant in the pH shift reactions, we wished to determine how the total DNA concentration affected this rate. Figure 6(b) shows that the final steady-state ATPase rates increased linearly with the DNA concentration. At DNA concentrations above 16 μM, the recA protein could not saturate all of the binding sites. The initial ATPase rates were unaffected by the DNA concentrations above 16 μM. However the "excess" DNA used in these reactions does affect the final steady-state ATPase rates. At 100 μM-FIII DNA, the final steady-state ATPase rate is 35% of the initial rate. These results indicate that the final steady-state rate of ATP hydrolysis after a pH shift reaction varies with the DNA concentration, but not significantly with the recA protein concentration.

(iv) *Filter binding*

recA nucleoprotein filaments formed on linear dsDNA dissociate at pH values above 6·8. In the final state of the reaction, ATP is hydrolyzed at about 15% of the initial rate immediately after the pH shift. Reactions with high DNA concentrations have higher final ATPase rates. Does this mean that 15% of the nucleoprotein filaments remain intact and the remaining recA protein and DNA are not associated? Or are all of the DNA molecules bound by 15% of their original amount of recA protein? To try to distinguish between these possibilities, the

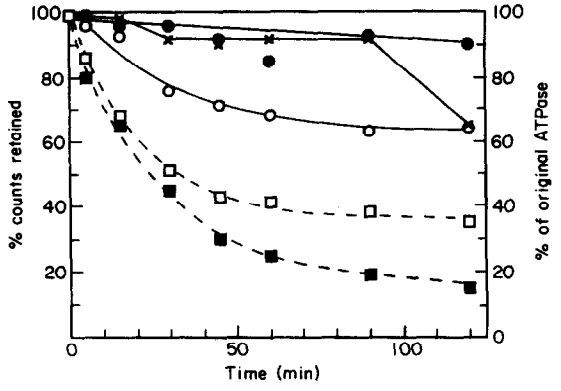


Figure 7. Comparison of DNA retention on nitrocellulose filters and ATPase rates after a pH shift. All reactions were started at pH 6·27. After 20 min (time = 0), the pH was shifted to 7·67. Filter binding was performed as described in Materials and Methods. All reactions had a final recA protein concentration (after the shift) of 2 μM. The symbols used for filter binding studies are: (●) 5 μM-FII DNA; (○) 5 μM-FIII DNA; (×) 40 μM-FIII DNA. Broken lines, (■) (5 μM-FIII DNA) and (□) (40 μM-FIII DNA) are used to represent ATPase results. All of the DNA used in these reactions was derived from M13mp8 and the concentrations indicated are final concentrations.

retention of [^{3}H]DNA on nitrocellulose filters was determined. Double-stranded DNA is not retained by the filters if it is not bound by protein. Although it is not known how many recA protein monomers must be bound to the DNA for filter retention under the conditions used, this technique can provide information about relative retention. Samples of reactions with FII or FIII DNA were filtered at various times after the pH was shifted from pH 6·27 to pH 7·67. When recA protein was left out of the reaction, less than 1% of the DNA was retained on the filters. Only 6% of the DNA was retained from a reaction with FII DNA started at pH 7·67 by adding recA protein and filtered one minute later. This control shows that recA protein retains the DNA only when bound to it. Twenty minutes after the recA protein was added, but before the pH shift, 100% of the DNA in each experiment was retained. Figure 7 shows that two hours after the pH shift, more than 90% of the FII DNA was retained on the filters. When 5 μM-FII DNA and 2 μM-recA protein were used, the retention of DNA dropped to 65% after the pH shift. When the FIII DNA concentration was increased to 40 μM, the retention again dropped to 65% after two hours. The drop in the rate of ATP hydrolysis for the two FIII reactions is shown for comparison. The percentage of DNA retained on the filters did not drop as much as the ATP rate, and the percentage of DNA retained in reactions with 40 μM-DNA dropped the same as reactions with 5 μM. This evidence indicates that the majority of the FIII DNA molecules have a subsaturating concentration of recA protein bound at steady state.

(c) *The general mechanism of dissociation*

(i) *Effects of DNA length*

The length of the linear DNA used in pH shift reactions should affect the rate of dissociation of the nucleoprotein filament in a mechanism-dependent fashion. If recA protein dissociates by translocation or progressive dissociation (Fig. 1(a) and (b)), and the rate of dissociation is constant, then complete filament dissipation should be slower from longer DNA molecules. If recA protein dissociates from the complex at random locations (Fig. 1(c)), the length of the DNA should have no effect. In these experiments, linear DNA 0·32 to 12·2 kb in length was used in pH shift reactions with a final concentration of 2 μM-recA protein. The final DNA concentration in nucleotides was held constant at 5 μM so that the total number of DNA molecules varied between experiments. The final pH was 7·67. The initial ATPase rates were the same (16 μM min^{-1}) regardless of the length of the DNA cofactor. The nucleoprotein filaments formed on shorter DNA fragments dissociated faster than filaments formed under the same conditions on longer DNA molecules. Figure 8(a) shows that the lag time for filament dissociation increased linearly with DNA length. The rate of recA protein dissociation under these conditions was approximately 100 monomers per nucleoprotein complex per minute for all DNAs.

At the endpoint of these reactions more than 90% of the recA protein had dissociated. The final steady-state ATPase rates were less than 10% of the initial rate just after the shift, and were not dependent on the DNA length, as demonstrated in Figure 8(b). These remnant rates were therefore also independent of the total number of DNA molecules.

(ii) *Restriction site protection*

To confirm that the decline in ATP hydrolysis correlates with the recA protein dissociating from the DNA and to further examine the progressive nature of the reaction, the shielding of a site from restriction enzyme digestion by recA protein was followed with time. There is a single *Bam*HI site near the middle of the 8·6 kb DNA molecule used (M13Gori1). Samples (20 μl) were removed from an ongoing ATPase reaction and digested for two minutes with *Bam*HI. The rate of ATP hydrolysis at a given time is compared in Figure 9 to the percentage of DNA cut by the restriction enzyme. As the rate of ATP hydrolysis dropped, the percentage of DNA cut by the restriction enzyme increased in a non-linear fashion. The *Bam*HI site was almost completely protected until the rate of ATP hydrolysis dropped by approximately 25%. The level of protection then dropped off sharply, with maximal digestion observed when ATP hydrolysis dropped by about 75%. This evidence suggests that there was a lag, after the pH shift, before a site in the center of the linear DNA molecule was exposed. All of the *Bam*HI sites were

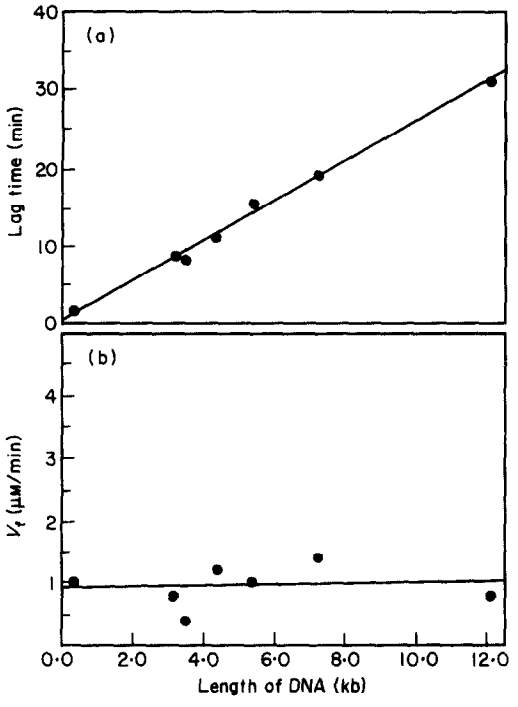


Figure 8. The effect of DNA length on the rate of dissociation. ATP hydrolysis reactions with final concentrations (after the pH shift) of 5 μM-DNA and 2 μM-recA protein were shifted from pH 6·27 to pH 7·67, 20 min after the reactions were started. DNA of different lengths was derived from several bacteriophages and plasmids: M13oriC26 (12·2 kb), M13mp8 (7·2 kb), φX174 (5·4 kb), pBR322 (4·4 kb), pBCS2 (3·5 kb), pXF3 (3·2 kb) and a fragment cut from pUC119 with *Pvu*II (0·3 kb). The DNA was linearized with *Eco*RI except when derived from φX174, when *Pst*I was used. (a) Lag times before reaching final steady-state rates of ATP hydrolysis were determined as described in Materials and Methods. (b) Final steady-state ATPase rates (V_f) were determined by linear regression.

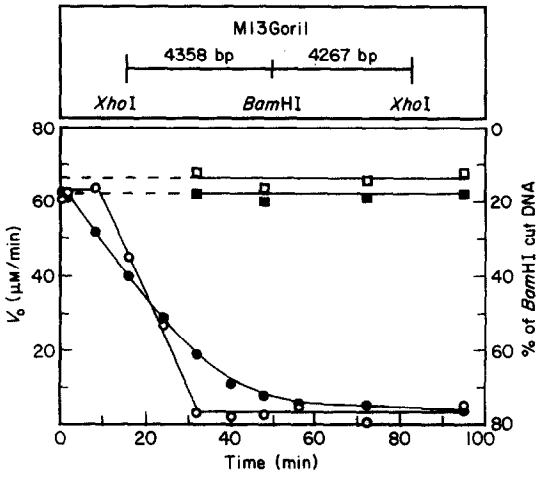


Figure 9. Protection of a restriction site during dissociation. Reactions were carried out as described in Materials and Methods. ATPase reactions (600 μl) with either *Xho*I-linearized M13Gori I DNA (8623 bp) or FII M13Gori I DNA were started at pH 6·27. After 20 min (time = 0) the reactions were shifted to pH 7·67, giving final DNA and recA protein concentrations of 20 μM and 5 μM, respectively. Rates of ATP hydrolysis (V_0) and the sensitivity of a *Bam*HI restriction site to *Bam*HI digestion were followed with time. A single *Bam*HI site is located approximately half-way between the ends of the FIII DNA. The percentage of DNA cut by *Bam*HI at various times after the pH shift are shown: FIII DNA (○); FII DNA (□). The rates of ATP hydrolysis at a given time are indicated by (●) for FIII DNA and (■) for FII DNA.

then quickly made susceptible to digestion before the ATPase rate completely decreased. The non-linear patterns of digestion provide another argument that random dissociation is not viable as a mechanism. If recA protein were randomly dissociating from the DNA, the restriction site protection curve would follow the ATPase rate curve more closely. This evidence further suggests that the recA protein is dissociating primarily from only one end of each nucleoprotein filament (or translocating in only one direction, as in the case of mechanism (a); Fig. 1). If the protein were dissociating from both ends, then the percentage of site protection would not begin to decrease until the ATPase rate had nearly dropped to the new steady-state rate. Finally, the results indicate a high degree of synchronization in the dissociation of different complexes following the pH shift.

The effects of circular *versus* linear DNA, the length dependence, and the restriction site protection indicate that recA protein does not dissociate randomly from these complexes. Two models, ATP-mediated translocation and progressive dissociation (Fig. 1(a) and (b)), are still compatible with these data. The next set of experiments distinguishes between these two models.

(iii) *Stabilization of complexes by ATPγS*

When 1 mM-ATP is replaced with the same concentration of its non-hydrolyzable analog ATPγS, recA protein–duplex DNA filaments form more quickly and are more stable (DiCapua & Müller, 1987; Pugh & Cox, 1987*a*). ATPγS is also a potent inhibitor of recA protein-catalyzed ATP hydrolysis. We wanted to determine if the linear nucleoprotein filaments could be stabilized after a pH shift if a small concentration of ATPγS were included in the reaction. Figure 10(a) shows the results of these experiments. ATP (1 mM) and ATPγS were added simultaneously to start the reaction. The final recA protein and DNA concentrations were 2 μM and 5 μM, respectively. After 20 minutes (time = 0 in Fig. 10(a)) the pH was shifted up to pH 7·67. ATPγS concentrations as low as 6 μM were enough to largely inhibit ATP hydrolysis, although the nucleoprotein filament remained intact as shown by nearly complete restriction enzyme site protection (data not shown). Complete inhibition of ATP hydrolysis occurred at 10 μM-ATPγS (data not shown). In the reaction

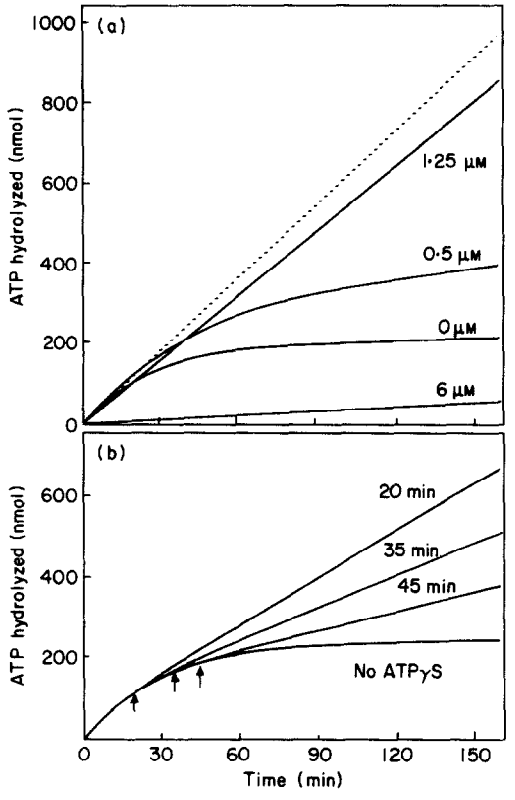


Figure 10. ATPγS can stabilize linear nucleoprotein filaments after a pH shift. (a) ATPase reactions with final concentrations of 5 μM-FIII M13mp8 DNA and 2 μM-recA protein were set up as described in Materials and Methods. Twice the ATPγS concentration indicated in the Figure was added simultaneously with 1 mM-ATP to start the reactions. After 20 min (time = 0 in the Figure) the pH was shifted from pH 6·27 to pH 7·67, giving the final ATPγS concentrations indicated. The broken line represents the time-course of a reaction with 5 μM-FII M13mp8 DNA and 2 μM-recA protein, also shifted in pH from 7·27 to 7·67, with no ATPγS. (b) A single 900-μl ATPase reaction was started at pH 6·27. After 20 min (time = 0 in the Figure), 200-μl portions were added to each of 4 200-μl portions of Tris-acetate (40% cation) buffer to give a pH of 7·67 and final concentrations of 5 μM-FIII M13mp8 DNA, 2 μM-recA protein and 1 mM-ATP. At 20 min, 35 min, 45 min or not at all, ATPγS was added to give a final concentration of 1·25 μM, as indicated by the arrows.

without any ATPγS, the ATPase rate dropped to the lower final steady-state rate, due to recA protein dissociation, within 30 minutes. When a final concentration of 0·5 μM-ATPγS was included, the initial ATPase rate was unaffected, but the lag time before the complex dissociated was significantly longer. A concentration of 1·25 μM-ATPγS was enough to decrease the initial ATPase rate by 10%, but that rate remained constant for more than 2·5 hours. In other words, low levels of ATPγS stabilized the nucleoprotein filament after the pH shift, without eliminating ATP hydrolysis. The recA protein or DNA concentration, or the DNA length did not change the effect of ATPγS shown in Figure 10(a). However, if 2 mM-ATP was used instead of 1 mM, somewhat more ATPγS was required to stabilize the nucleoprotein filament (data not shown).

The reaction with 1·25 μM-ATPγS (Fig. 10(a)) showed that the protein–DNA filament was stabilized, even though presumably only a small percentage of the recA monomers were bound to ATPγS. This is inferred from the remaining 90% of ATP hydrolysis. ATPγS may act to stabilize the filaments in several ways. It may (1) increase the rate of recA protein re-binding, (2) promote the formation of a stable nucleation site, or (3) simply block dissociation of recA protein. To differentiate between these possibilities, 1·25 μM-ATPγS was added to ongoing reactions (5 μM-FIII DNA, 2 μM-recA protein, 1 mM-ATP) at various times after the pH shift. Figure 10(b) shows that the rate of ATP hydrolysis slowed slightly after the ATPγS was added, but then continued at that same steady-state rate. In similar reactions started at pH 7·67, the inclusion of 1·25 μM-ATPγS, and 1 mM-ATP to initiate the reaction, did not increase the rate at which recA protein bound to the DNA (data not shown). These two observations eliminate (1) and (2) as plausible explanations. The ATPγS prevented further dissociation of recA protein from the nucleoprotein filament even though most recA monomers continued to hydrolyze ATP. The continued ATP hydrolysis in these complexes cannot be coupled to translocation of recA protein on the DNA.

(d) *The asymmetry of recA–duplex DNA filaments*

(i) *Binding of recA protein to gapped duplex DNA molecules*

At neutral pH, recA protein binds to duplex DNA with single-stranded tails rapidly (West *et al.*, 1980; Shaner *et al.*, 1987; Shaner & Radding, 1987). The resulting filament has been shown to be polar and to bind the two DNA strands asymmetrically (Shaner & Radding, 1987; Shaner *et al.*, 1987; Chow *et al.*, 1986, 1988). These results are confirmed and extended here. Circular duplex DNA molecules with single-stranded gaps of precise length and position were constructed. For simplicity, the gapped duplex molecules will be referred to as GD_x, where x is the length of the single-stranded region in nucleotides. For example, GD_{1041} refers to a circular molecule that is 7229 bp duplex DNA (the length of M13mp8) and 1041 bp ssDNA. GD_0, nicked circular (FII) DNA made by the above procedure, was used as a control in some experiments. The binding of recA protein to different gapped duplex DNA molecules was first studied. In order to have the same number of potential recA protein binding sites in each reaction, 15 μM-GD_{1041} (total nucleotide) was compared to 16 μM of the DNA molecules with much smaller gaps. recA protein was added to a final concentration of 4 μM. The time-courses of ATP hydrolysis at pH 7·5 for reactions involving duplex DNA molecules with several different length

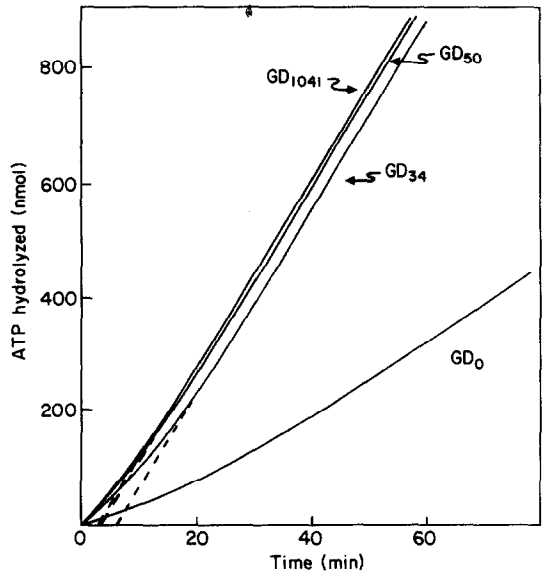


Figure 11. recA protein binding to gapped duplex DNA, pH 7·5. ATPase reactions were used as a measure of recA protein binding to DNA and were carried out as described in Materials and Methods in Tris-acetate (80% cation, pH 7·5) buffer. The gapped duplex concentrations were 15 μM (for GD_{1041}) and 16 μM (for GD_{50}, GD_{34} and GD_0). (The subscript refers to the length of the single-stranded gap, in nucleotides.) The recA protein concentration was 4 μM. The broken lines represent extrapolations used to estimate lag times. The reaction with GD_0 did not reach a steady state during the time shown.

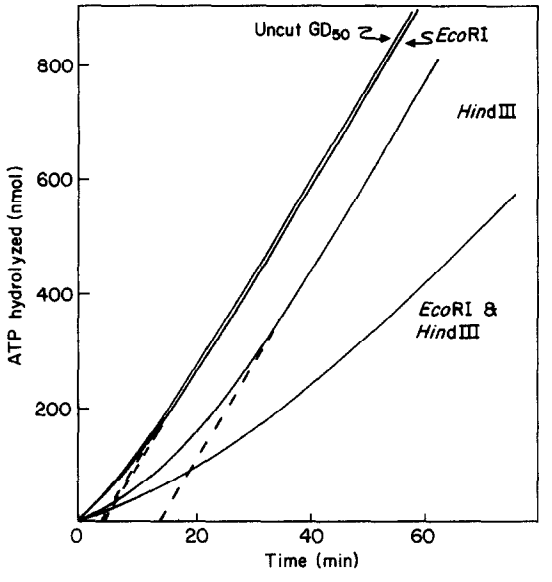


Figure 12. Polarity in single-stranded gap stimulation of recA protein binding. ATP hydrolysis was monitored using conditions and procedures described in Materials and Methods. Gapped, tailed or fully duplex DNA was present at a concentration of 16 μM. The recA protein concentration was 4 μM. Portions of GD_{50} were linearized with *Eco*RI, *Hin*dIII or both enzymes (to produce 5′ tailed, 3′ tailed and fully duplex DNA, respectively) prior to its use in this experiment.

gaps is shown in Figure 11. The association lag time was three to five minutes for GD_{1041}, GD_{50} and GD_{34}. A slight but reproducible increase in the association lag time was observed in reactions with GD_{34} relative to GD_{50} and GD_{1041}, although the same steady-state rate was eventually achieved in all three reactions. By contrast, reactions with GD_0 had a much longer association lag time; the ATPase rate did not reach steady state during the reaction time. The rates observed for binding to GD_0 are consistent with rates obtained for reactions with FII DNA prepared in the usual way. These results indicate that optimal stimulation of binding requires a gap of 34 to 50 nucleotides. The observed lag times provide a lower limit, 500 monomers per complex per minute, for the rate of polymerization of recA filaments on dsDNA under these conditions. If the polymerization phase of the reaction is not strictly rate-limiting (as is certainly the case when single-strand tails or gaps are not present; Pugh & Cox, 1987*a*), then the true rate of polymerization may be much faster.

(ii) *Polarity of binding to duplex DNA*

Duplex DNA molecules with 3′ or 5′ ssDNA tails were constructed by cleaving GD_{50} on one side or the other of the gap. *Eco*RI cleaves GD_{50} 12 bp to the 5′ side of the gap (with respect to the (+) strand) creating a 5′ tailed linear duplex molecule. *Hin*dIII cleaves 20 bp to the 3′ side of the gap, creating a 3′ tailed duplex molecule. When both *Eco*RI and *Hin*dIII cleave GD_{50} a long (7197 bp) linear duplex and a short fragment (82 nucleotides) are generated. Both molecules were incorporated in the reactions together. recA protein-dependent ATP hydrolysis stimulated by these cut gapped duplexes was compared to reactions with uncut GD_{50}, at pH 7·5, in Figure 12. recA protein bound to the 5′ tailed duplexes as quickly as it bound to uncut GD_{50}. In contrast, a much lower rate of ATP hydrolysis was initially observed with 3′ tailed duplexes. After a lag of 15 minutes, the ATPase rate increased to that exhibited in the presence of uncut or *Eco*RI-cut GD_{50}. When GD_{50} had been cut with both *Eco*RI and *Hin*dIII, the association reaction did not reach steady state within two hours. These data say that the ssDNA must be attached to the duplex for it to stimulate the association of recA protein with the duplex. The data also suggest that there is a polarity to the formation of a nucleoprotein filament on duplex DNA; binding occurs more quickly from a 5′ ssDNA tail than from a 3′ tail. These results are similar to those reported by Shaner & Radding (1987), who used duplex DNA molecules with tails of various lengths. Although the 3′ ssDNA tail does significantly stimulate the formation and stability of the nucleoprotein filament, the data presented in the next section suggest that it does so in a way distinct from the stabilizing effect of the 5′ tail.

(iii) *Binding of recA protein to duplex DNA as measured by DNase protection*

To examine and compare the assembly process on both 3′ tailed *versus* 5′ tailed molecules, DNase digestion experiments were performed. These reactions were similar to those done by Chow *et al.* (1988) with 5′ tailed molecules before and during strand exchange reactions. Pancreatic DNase I cleaves DNA that is not adequately protected by bound protein into mononucleotides and small oligonucleotides. GD_{50} was constructed with either the plus strand ^{3}H-labeled (GD_{50} $^3H(+)$) or the minus strand ^{3}H-labeled (GD_{50} $^3H(-)$). Portions of GD_{50} $^3H(+)$ or $GD_{50}{}^3H(-)$ were cut either with *Eco*RI (producing 5′ ssDNA tails) or *Hin*dIII (producing 3′ ssDNA tails), or with both restriction enzymes to separate the gap from the majority of the duplex DNA. Under standard reaction conditions, 20 μM-DNA and 10 μM-recA protein were incubated for either five or 30 minutes before DNase digestion. The complexes examined at the 30 minute time-points should be at or near saturation with respect to recA binding for all of the gapped or tailed DNAs (Fig. 12). The extent of protection from DNase digestion for these reactions is detailed in Table 1. recA protein significantly protects the circular gapped duplex DNA from digestion. Protection is not complete in any case for complexes examined in the presence of ATP, as observed previously (Pugh & Cox, 1987*a*; Shaner *et al.*, 1987; Chow *et al.*, 1988), even when recA protein binding is at saturation (Pugh & Cox, 1987*a*; Pugh *et al.*, 1988). Circular gapped duplexes and gapped duplexes linearized with *Eco*RI (5′ ssDNA tailed duplexes) are equally shielded from digestion after both five and 30 minutes of incubation with recA protein. The plus strand is protected about twofold more than the minus strand for both GD_{50} and the 5′ ssDNA tailed duplex. This suggests that recA protein is more closely associated with the strand that is single-stranded in the gap than its complementary strand. recA protein may initially bind to the ssDNA gap or tail and propagate along that strand, into the duplex. A similar difference in nuclease protection between the two strands of 5′ tailed molecules was noted by Chow *et al.* (1988).

Table 1

DNase I protection by recA protein on gapped duplex DNA molecules

	Uncut	*Eco*RI	*Hin*dIII	*Eco*RI and *Hin*dIII
GD_{50} $^3H(+)$				
5 min	40	40	17	7
30 min	57	53	31	20
GD_{50} $^3H(-)$				
5 min	22	23	16	5
30 min	34	35	31	19
FIII DNA				
5 min	0·2			
30 min	8			

DNase protection experiments were carried out as described in Materials and Methods. The concentrations of recA protein and DNA were 10 μM and 20 μM, respectively. GD_{50} $^3H(+)$ represents a circular gapped duplex molecule in which the (+) strand is ^{3}H-labeled throughout. GD_{50} $^3H(-)$ has the (−) strand labeled. Portions of the circular gapped duplex molecules were linearized with *Eco*RI or *Hin*dIII, or both enzymes, effectively producing 5′ ssDNA tailed, 3′ ssDNA tailed or purely duplex DNA. The FIII DNA, used as a control, was *Eco*RI-cut M13mp8 DNA. Reactions with one of the DNA forms was incubated 5 or 30 min, after ATP was added to initiate recA protein binding to the DNA, before the DNase I was added. The percentages shown are the percentage protection values obtained by dividing the acid-precipitable counts from each reaction by a 100% value (no DNase), after the background (no recA protein) is subtracted from both. All reactions were done in triplicate; the mean of the values is listed. The deviation from the mean was never greater than 5%.

These results indicate an inherent asymmetry in the recA–duplex DNA complex, with one of the two strands (the initiating strand) serving to orient the complex during assembly. Rapid assembly occurs 5′ to 3′ along the initiating strand. An extension of this experiment permits an examination of the mechanism of filament assembly on 3′ tailed DNA molecules. It has been suggested (Shaner & Radding, 1987) that the complexes formed on dsDNA with 3′ tails result from a slower association pathway, in which recA monomers bind to the unfavored end of the complex. The final structure of this complex should be the same, and exhibit the same strand protection asymmetry as complexes formed on 5′ tailed molecules. When the gapped duplex is cut with *Hin*dIII, leaving a 3′ ssDNA tail, the level of observed DNase protection is lower, especially at the early time-point, reflecting the slower rate of recA protein association with these molecules. More important, however, there is no difference in protection between the two strands of the 3′ tailed molecule, even when binding has reached completion. The tailed strand is no better protected than the strand that is fully base-paired. This indicates that the mechanisms of recA protein association onto 5′ *versus* 3′ ssDNA tailed duplex molecules are fundamentally different, since the final complexes are clearly different. These data demonstrate that the observed formation of complexes on the 3′ tailed linear duplexes is not in itself evidence for a slow association process occurring on the unfavored end of a recA nucleoprotein filament, although they do not rule out such a process.

4. Discussion

The slow dissipation of recA nucleoprotein filaments formed on linear duplex DNA at neutral pH is most readily explained by a model in which the filaments progressively dissociate from one end. Similar filaments formed on relaxed circular DNA are stable. At pH 6·2, the filaments formed on linear and circular DNA have identical properties, as shown by ATP hydrolysis, restriction endonuclease protection and filter binding. The differences between the two are evident only above pH 6·8, where the nucleoprotein filaments formed

on linear dsDNA dissociate. This dissociation does not occur randomly within the filament, but only from one end. Unidirectional movement or translocation of the recA protein along the DNA tightly coupled to ATP hydrolysis does not explain the filament dissipation, since low levels of ATPγS can stabilize the filament while allowing ATP hydrolysis to continue. We cannot completely eliminate the possibility that translocation occurs and is coupled minimally to ATP hydrolysis. The unidirectional nature of the dissipation process, however, implies some role for ATP. The kinetic patterns provide no suggestion of any relationship between dissipation and ATP hydrolysis that can be rationalized in terms of translocation.

The nucleoprotein complex exhibits an inherent polarity. Shaner & Radding (1987) have shown that a single-stranded DNA tail contiguous with duplex DNA stimulates the binding of recA protein to the duplex. The rate of recA protein association is five to ten times quicker onto duplex DNA with 5′ tails, as opposed to 3′ tails. This suggests that the binding reaction is polar, proceeding 5′ to 3′ along the initiating strand. Our data for recA protein binding to gapped or tailed duplex DNA agree with their results. We also show that ssDNA gaps or tails as small as 34 nucleotides are sufficient to stimulate binding, although a slight decrease in efficiency is observed for tails smaller than 50 nucleotides. The optimal rate of polymerization on dsDNA is at least 500 recA monomers per minute per complex at pH 7·5. If the tail is removed by nuclease digestion, the recA protein dissociates. This is the same phenomenon that we observe in pH shift experiments. The present study provides a probable general mechanism for this process.

The polymerization of recA protein in the 5′ to 3′ direction on ssDNA (Register & Griffith, 1985) is reflected in the inherent polarity of the filament structure (Stasiak *et al.*, 1988). The recA filaments formed on dsDNA also have a clear axial polarity (Egelman & Stasiak, 1986). Since recA nucleoprotein filaments formed on ssDNA and dsDNA, in the presence of ATPγS, share many of the same structural characteristics (Griffith & Harris, 1988), it is not surprising that recA protein polymerizes in a polar manner on double-stranded as well as single-stranded DNA.

Nuclease protection results presented here and the results of Chow *et al.* (1986, 1988) indicate that recA nucleoprotein filaments form on duplex DNA by binding the two strands asymmetrically. When challenged with DNase, recA protein protects the strand to which it initiated binding more than it protects the complementary strand. For 5′ ssDNA tailed duplex molecules, the initiating strand is the longer strand. recA protein could initiate binding on either strand of a fully duplex molecule. The filament would polymerize along the chosen strand, primarily in the 5′ to 3′ direction. The observed slower binding to 3′ tailed dsDNA molecules could similarly occur by a slower 3′ to 5′ association pathway, implying that binding is possible at both

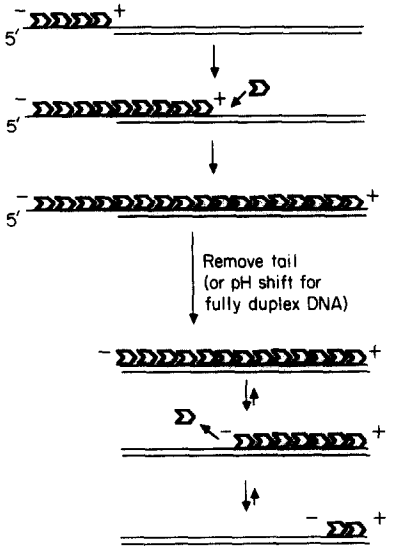


Figure 13. Model for recA protein association and dissociation with linear duplex DNA. recA protein is represented by the symbol (ᗤ) and linear duplex DNA is represented by the parallel lines. recA bound to DNA hydrolyzes ATP. Nucleation of the recA nucleoprotein filament occurs on the 5′ ssDNA tail, or at pH 6 for fully duplex DNA. recA protein preferentially adds to the (+) end of the nucleoprotein filament. Net filament dissociation is initiated by separating ssDNA tails from the duplex, or shifting the reaction from pH 6 to pH 7·5 for fully duplex DNA. Filament dissociation occurs at the (−) end. In the final steady state (not shown), recA protein binds and dissociates along the DNA with about 15% of the potential binding sites occupied at any instant. The top strand is the initiating strand as shown. When initiation is on the bottom strand, filaments have the opposite orientation.

ends of the filament (Shaner & Radding, 1987). Whereas binding is more rapid in the 5′ to 3′ direction, the same polar filament should result in either case. The lack of asymmetry in the nuclease protection patterns of the two strands in complexes formed on 3′ tailed dsDNA, however, indicates that complexes are oriented randomly on this DNA. The assembly process here must be more complicated. The observation that association is stimulated by 3′ ssDNA tails does not in itself constitute evidence for assembly at the unfavored end of the filament.

Figure 13 shows a model of how recA protein may associate and then dissociate from duplex DNA. The association of recA protein onto tailed duplexes is shown. Measurable dissociation occurs either when the ssDNA tail is separated from the duplex or when the reaction with fully duplex DNA is shifted from pH 6 to neutral pH. The (+) end of the filament is the preferential end for recA protein association; it is oriented toward the 3′ end of the initiating strand. Under the conditions described here, recA protein always polymerizes rapidly onto dsDNA at the (+) end. A net dissociation process from the same end is therefore inconsistent with our results. Instead, we expect that the measurable

recA protein dissociation occurs at the opposite (−) end, where reassociation is slow. This end can be stabilized, and dissociation prevented, by a 5′ single-stranded tail.

Both association and dissociation are non-random and interdependent. Once the filaments have largely dissociated, reassociation occurs much more slowly than *de novo* binding at neutral pH. Since *de novo* binding occurs more rapidly on duplex DNA with ends, reassociation may be partially blocked by residual recA protein bound at the ends. In addition, rates of reassociation will be affected by the fraction of DNA available for binding. The low steady-state rate of ATP hydrolysis observed after the reaction is shifted to neutral pH is probably the result of a complicated dynamic process of continued association and dissociation of recA protein.

Many properties of the interaction of recA protein with linear dsDNA were studied. While ssDNA tails provide optimal stimulation of binding to duplex DNA, blunt ends (or 4 nucleotide overhangs) also provide significant stimulation. recA protein can initiate binding much more rapidly on linear dsDNA than on dsDNA molecules without ends, independent of the restriction enzyme used to linearize the DNA. This is in agreement with other data, indicating that recA protein initiates binding more rapidly to unwound or easily unwindable segments of DNA (Pugh & Cox, 1987*a*; Kowalczykowski *et al.*, 1987). *De novo* association with linear dsDNA, at neutral pH, follows unusual kinetics. This can be rationalized in terms of the interdependence of the association and dissociation pathways presented in Figure 13. After about 30% of the potential recA protein binding sites are occupied, dissociation of the nucleoprotein filament begins to dominate the reaction. This dissociation is examined more directly when a full recA nucleoprotein filament is allowed to form at pH 6·2 before the pH is shifted to 7·5. Longer DNA molecules and buffers of lower pH decrease the net monomer dissociation rate of linear nucleoprotein filaments. Dissociation depends upon recA protein concentration, but exhibits a minimal dependence on total DNA concentration. The lower rate of dissociation seen with higher recA protein concentrations may reflect reassociation on the ends of the filaments that is overlooked by measuring net dissociation rates. This aspect of the reaction has not been investigated in detail. Once binding reactions reach a steady state, the amount of recA protein remaining bound to the DNA at any moment depends on the DNA concentration and the final pH. At pH 7·5, 10 to 15% of the potential recA protein binding sites are occupied at steady state. If the DNA concentration is increased, or the final pH is lowered, more of the recA protein will interact with DNA at steady state.

ATP hydrolysis modulates the affinity of recA protein for DNA (Menetski & Kowalczykowski, 1985; Cotterill *et al.*, 1982). Although it is reasonable to expect that dissociation and ATP hydrolysis would be coupled, this is not evident on first inspection of the data, as already noted. There is a net hydrolysis of 300 to 400 ATP molecules for the net dissociation of each monomer. The rate of dissociation varies with pH and recA protein concentration, and dissociation occurs only at filament ends. ATP hydrolysis occurs throughout the filament. The k_{cat} for hydrolysis varies only slightly with pH over the range in question and does not vary with recA protein concentration. These facts argue against coupling. However, if coupling does not occur, then the model shown in Figure 13 (where at neutral pH the filament can grow at one end while shortening at the other, if it is not stabilized) appears to defy the principle of microscopic reversibility. The equilibrium constants for subunit association and dissociation at the two ends of a filament must be identical, unless there is an input of energy. Wegner (1976) first proposed that filament assembly coupled to ATP or GTP hydrolysis could overcome this restraint. The observations that argue against coupling can potentially be reconciled by a model in which ATP hydrolysis results in measurable dissociation only when it involves recA monomers or units at the (−) end of the filament. Maximum coupling, even at the (−) end, may occur only above pH 8. This maximum rate of dissociation corresponds to about 200 recA monomers per minute per complex, or one recA monomer every 0·3 second. Each recA monomer ($k_{cat} = 25$ min^{-1}) hydrolyzes an ATP every 2·4 seconds. If recA protein dissociation and ATP hydrolysis are coupled at the (−) end, ATP hydrolysis in adjacent monomers must be co-ordinated to occur 0·3 second apart, with the entire hydrolytic cycle taking 2·4 seconds for each monomer. This admittedly speculative exercise suggests that ATP hydrolysis may be co-ordinated in traveling "waves" separated by eight or nine recA monomers and moving along the filament at 200 monomers per minute. Below pH 8, the rate of dissociation might decrease because of an effect on protein–protein interactions that decreases the probability that an ATP hydrolytic event will result in dissociation. Net dissociation is not observed below pH 6·8.

In the interior of the recA nucleoprotein filament formed on linear dsDNA or on relaxed circular DNA, ATP hydrolysis occurs without net recA protein dissociation. This evidence fits three possible models. (1) No dissociation occurs in the continuous filaments because there are protein–protein contacts on both sides of each monomer that prevent dissociation, irrespective of ATP hydrolysis. (2) Microscopic dissociation occurs within the filament, but the reassociation at the same site is rapid. No reduction in the total ATP hydrolysis is detected. This model predicts that there is no change in position of the subunits in the filament. (3) recA monomers, or larger units, dissociate completely from the complex, but again reassociation to available (+) ends is rapid. One would expect rearrangement of the protein subunits

in the last case. We cannot distinguish between these possibilities at present for recA–duplex DNA complexes. However, there is no evidence for recA protein dissociating from the interior of these complexes as long as ATP is regenerated. Neuendorf & Cox (1986) found that there was little detectable subunit exchange between recA complexes formed on ssDNA filaments and free, labeled recA protein.

We have noted a novel effect of the ATP analog ATPγS on the stability of recA nucleoprotein filaments on linear duplex DNA. High concentrations of ATPγS have been shown to stabilize recA–duplex DNA filaments (DiCapau & Müller, 1987; Pugh & Cox, 1988) and to completely inhibit ATP hydrolysis. Small concentrations of ATPγS, added with the ATP, stabilize the filament and allow ATP hydrolysis throughout the filament to continue at 85 to 90% of the full rate. When ATPγS is added after the pH shift, dissociation from the remaining filaments is halted. ATPγS at these levels does not promote re-binding of the recA protein that had dissociated earlier. The simplest explanation is that these low concentrations of ATPγS cause the tight binding of a small fraction of recA monomers that directly stabilize the filament. A recA protein subunit, with ATPγS bound, binds tightly to the DNA. It will prevent recA protein subunits on its (+) side from dissociating (see Fig. 13). All of the recA subunits not bound by ATPγS continue to hydrolyze ATP. The recA–ATPγS subunit does not provide increased re-binding to the (−) end of the filament.

Is recA protein dissociation coupled to strand exchange? The time required for net dissociation of a recA filament from 12·2 kb linear dsDNA is 31·5 minutes at pH 7·5. This corresponds to the exposure of 387 bp of DNA per minute. Interestingly, this is very close to the rate of branch migration (300 to 360 bp min^{-1}) in recA protein-mediated strand exchange at the same pH. The rate of net dissociation here, however, increases with pH between pH 7 and 8, whereas the rate of strand exchange does not (Lindsley, unpublished results). recA protein remains bound to the circular heteroduplex product after strand exchange (Pugh & Cox, 1987*b*), indicating that net dissociation is not required for strand exchange. The dissociation process presented here does not appear to be part of the mechanism of strand exchange. This process may instead facilitate recycling of recA protein after strand exchange has occurred *in vivo*.

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