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Transfer of recA Protein from One Polynucleotide to Another

KINETIC EVIDENCE FOR A TERNARY INTERMEDIATE DURING THE TRANSFER REACTION*

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We have analyzed the transfer kinetics of recA protein from one polynucleotide to another by monitoring the change in fluorescence of a modified single-stranded M13 DNA, referred to as etheno M13 DNA, that accompanies recA protein dissociation. The observed rate of transfer is dependent on the concentration of competitor polynucleotide, polythymidylic acid (poly(dT)); increasing the poly(dT) concentration increases the rate of transfer. These data are inconsistent with the rate-limiting step in the transfer mechanism occurring by a simple dissociation process. Under certain conditions, the apparent rate constant displays plateau behavior at high poly(dT) concentrations. This result is indicative of transfer occurring through a ternary intermediate including etheno M13 DNA and poly(dT). The transfer reaction was found to occur through two kinetically distinct species of transferring recA protein•DNA complexes. The relative amounts of these two species was affected by both the $MgCl_2$ and protein concentration, suggesting that the two kinetic components reflect different aggregation states of the recA protein•DNA complex. Because etheno M13 DNA and poly(dT) contain no complementary sequences, we have concluded that recA protein has the intrinsic ability to form a kinetic ternary intermediate with two separate single-stranded DNA molecules in the absence of homology.

General genetic recombination (Clark, 1973) and the SOS response to DNA damage (Little and Mount, 1982) in *Escherichia coli* have been found to be dependent on the recA gene product. recA protein has been purified and found to possess several *in vitro* activities that are likely to be related to recombination. This protein is an active ATPase in the presence of single-stranded DNA (Weinstock *et al.*, 1981a, 1981b). recA protein also catalyzes the renaturation of two complementary single-stranded DNA molecules, in a reaction which is stimulated by the hydrolysis of ATP (Weinstock *et al.*, 1979; Bryant and Lehman, 1985; McEntee, 1985). ATP hydrolysis has also been found to be essential during the insertion and resultant exchange of a complementary single-stranded DNA molecule into a duplex molecule (McEntee *et al.*, 1979; Shibata *et al.*, 1981; Cox and Lehman, 1982). The latter reaction, referring to as strand exchange, is thought to be related to the mechanism by which recA protein mediates recombination *in vivo*.

The strand exchange reaction has been divided into at least three kinetically distinct steps (Gonda and Radding, 1983). recA protein binds to single-stranded DNA during presynapsis. Next, the recA protein-single-stranded DNA complex associates with duplex DNA, conducts a search for homology, and melts into the duplex to form a displacement loop (D-loop) during the second phase of strand exchange (synapsis). Finally, the D-loop formed during synapsis is extended by branch migration. These three steps may be separated kinetically, although each is complex and involves several elementary molecular steps.

In catalyzing the renaturation of complementary single-stranded DNA molecules, recA protein must both aid in the search for homology and allow duplex DNA formation. Thus, this reaction may be viewed as a simplification of the strand exchange reaction because the processes involved in duplex melting and branch migration are not involved. Although the protein-free renaturation reaction shows a second-order dependence on DNA concentration, renaturation catalyzed by recA protein has been found to be first-order (Weinstock *et al.*, 1979; Bryant and Lehman, 1985). During renaturation, recA protein must allow the single-stranded DNA molecules to pair homologously. In addition, recA protein is thought to dissociate from the DNA molecules, either before or after the formation of double-stranded DNA product molecules. Since dissociation is a first-order process, it is a prime candidate for the rate-limiting step in recA protein-catalyzed DNA renaturation. Therefore, understanding the dissociation properties of the recA protein·DNA complex may be necessary to fully understand the renaturation reaction.

In studying the bacteriophage T4-coded gene 32 protein (gp32),[1] Kowalczykowski *et al.* (1980) and Lohman (1984a and 1984b) have shown that equilibrium DNA-binding properties of this protein are reflected in the rate of dissociation of the protein·DNA complexes. At low salt concentrations, dissociation was initiated by the addition of a competitor DNA to preformed protein·DNA complexes (Lohman, 1984b). The excess of high affinity competitor added, poly(dT), was shown to act as a passive sink that trapped free protein that had dissociated from the initial single-stranded DNA molecule. The observed rate measured by this DNA transfer method was found to be identical with the rate of dissociation of gene 32 protein from the initial single-stranded

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[1] The abbreviations used are: gp32, bacteriophage T4-coded gene 32 protein; etheno M13 DNA, fluorescently modified single-stranded M13 DNA containing 1,N^6-etheno adenosine, and 3,N^4-etheno cytosine; poly(dT), polythymidylic acid.

DNA. By analogy, it is expected that any changes in the recA protein DNA binding affinity will be witnessed in the rate of dissociation of the protein·DNA complex. The binding of the recA protein to single-stranded DNA has been shown to be influenced by various solution factors (Menetski and Kowalczykowski, 1985a); ADP and NaCl decrease, while ATP increases the affinity of recA protein for single-stranded DNA. Both increasing salt concentration and the presence of ADP have been found to have an effect on the strand exchange and renaturation reactions (Cox and Lehman, 1982; Wu *et al.*, 1982; Weinstock *et al.*, 1979; Bryant and Lehman, 1985; McEntee, 1985). Therefore, the use of this transfer method to investigate the dissociation properties of recA protein may add molecular details regarding the rate of recA protein dissociation and its involvement in more complex reactions such as DNA renaturation.

The transfer of recA protein from one polynucleotide to another has been reported by Silver and Fersht (1983) and Bryant *et al.* (1984, 1985). They found that the observed rate of transfer increased in the presence of ADP. Curiously, Bryant *et al.* (1984, 1985) found that the rate of transfer also increased with the concentration of the competitor DNA, although the molecular mechanism of their results was not discussed. The results described in this paper offer a more complete analysis of the transfer of recA protein from etheno M13 DNA to poly(dT). These substrates allow the reaction to be continuously monitored by recording the fluorescence decrease that accompanies the transfer of recA protein from etheno M13 DNA to the high affinity competitor, poly(dT). We have extensively studied the effect of competitor DNA concentration on the observed transfer rate constant and show kinetic evidence for a ternary intermediate in this reaction. The proposed intermediate must include recA protein, etheno M13 DNA, and poly(dT) and does not require any sequence complementarity in the DNA substrates. We have also shown that the observed rate of transfer increases in the presence of ADP and increasing $MgCl_2$ concentration. In addition, we show that $MgCl_2$ and recA protein concentration have an effect on the molecular characteristics of the protein-DNA molecules involved in the transfer. We have interpreted this data to suggest that the aggregation state of the protein·DNA complex influences the transfer reaction.

MATERIALS AND METHODS

recA Protein—recA protein was prepared using the procedure of Cox *et al.* (1981) as described in Menetski and Kowalczykowski (1985a). Protein concentration was determined using an extinction coefficient of 2.7×10^4 M^{-1} cm^{-1}.

Etheno M13 DNA and Poly(dT)—Etheno M13 DNA was prepared as described by Kryzosiak *et al.* (1981) and modified as described in Menetski and Kowalczykowski (1985a). However, the extinction coefficient reported in the previous paper was listed incorrectly and should have been 12,668 cm^{-1} M^{-1}. In this paper, we use an extinction coefficient for the etheno M13 DNA that has been determined by phosphate analysis (Ames, 1966; Lanzetta *et al.*, 1979) to be 7.0 ($\pm$0.3) $\times$ 10^3 cm^{-1} M^{-1}. This changes the previously reported site size of recA protein to 8 ($\pm$1) nucleotides per recA protein monomer (Menetski and Kowalczykowski, 1985b). This value for the site size is in good agreement with those described elsewhere after correction for differences in the recA protein extinction coefficients used (8.3, Silver and Fersht, 1982; 9.6 and 4.8 with adenosine 5′-*O*-3′-(thiotriphosphate) and ATP, respectively, Bryant *et al.*, 1984).

Poly(dT) was purchased from P-L Biochemicals and dissolved as a concentrated stock in TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The concentration of poly(dT) was determined using an extinction coefficient of 8540 cm^{-1} M^{-1} at 259 nm.

Chemicals and Buffers—All chemicals used were reagent grade, and all solutions were made in glass-distilled water. Transfer experiments were conducted in TD buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol) at NaCl and $MgCl_2$ concentrations indicated in the figure legends. ADP was purchased from P-L Biochemicals and was dissolved as a concentrated stock solution at pH 7.0.

Transfer Reaction—recA protein-etheno M13 DNA complexes were formed in TD buffer at 25 °C and the desired NaCl and magnesium concentration. At time zero, poly(dT) (generally a 5-fold excess relative to the etheno M13 DNA concentration) was added to induce transfer. The fluorescence intensity was observed on a Perkin-Elmer MPF-44E fluorescence spectrophotometer at excitation and emission wavelengths of 300 and 405 nm, respectively. The change in fluorescence was recorded by a Hewlett-Packard HP-85 computer interfaced to a HP-3421 data acquisition and control unit. The solution in the cuvette was stirred continuously to maintain homogeneity.

Data Analysis—The experimental data were analyzed by the nonlinear least squares routine in the *DA*ta *E*valuation *S*ystem *80* program (DAES80), obtained from R and L Software. This program could fit the data to either a single exponential decay plus a variable end point or a double exponential decay plus a variable end point. The equation that is used to describe a double exponential decay is:

$$F = A_{fast}e^{-k(fast)t} + A_{slow}e^{-k(slow)t} + B$$

where F is the fluorescence signal at a given time t; A_{fast} and A_{slow} are the amount of each transferring species at $t = 0$ and are called the amplitude of the component; and k_{fast} and k_{slow} are the observed first order rate constants for each component; B is the end point where $t = \infty$. In a single exponential decay A_{slow} is equal to zero. Error associated with values of k are typically 10%, unless stated otherwise. In some cases, the value of k_{fast} exceeds the rate of the mixing method used. Under these conditions the rate of this component cannot be determined. However, the amplitude of this component can be determined accurately by subtracting the amplitude of the slow component from the total fluorescence change. Errors on the determination of amplitude, either by the fitting procedure or by subtracting the slow component from the total fluorescence change, are generally within 10%.

RESULTS

Transfer of recA Protein from One Polynucleotide to Another—The binding of recA protein to etheno M13 DNA results in an increase in the fluorescence of this modified single-stranded DNA (Silver and Fersht, 1982; Menetski and Kowalczykowski, 1985a). Upon addition of a nonfluorescent competitor DNA, the transfer of recA protein from etheno M13 DNA can be followed continuously by monitoring the decrease in fluorescence that accompanies the dissociation of recA protein from etheno M13 DNA. The competitor used in the following experiments, poly(dT), has been shown to bind recA protein more tightly than any other polynucleotide (McEntee *et al.*, 1981; Cazanave *et al.*, 1984). Reactions were conducted and analyzed as described under "Materials and Methods" and a typical reaction time course is shown in Fig. 1. Under certain conditions (described fully below), the transfer reaction could not be fit by a single exponential decay; however, the data fit well to a double exponential decay (Fig. 1). A double exponential suggests that the transfer reaction is proceeding through at least two different kinetic pathways in which recA protein transfers with different apparent rates. When two components are observed, they will be referred to as the fast and slow components. The two components cannot be attributed to protein-free relaxation of the etheno M13 DNA substrate, because such structural changes in the DNA would be expected to occur rapidly under these conditions. During the time course of these experiments, we have not observed any fluorescence changes upon addition of either magnesium chloride or sodium chloride to protein-free etheno M13 DNA. Also, as described below, the relative amounts of the two components vary with ADP, ATP, and protein concentration; these variables can have an effect on the fluorescence properties only through the recA protein-etheno M13 DNA complex. Therefore, we believe the two components represent different kinetic pathways in the transfer reaction.

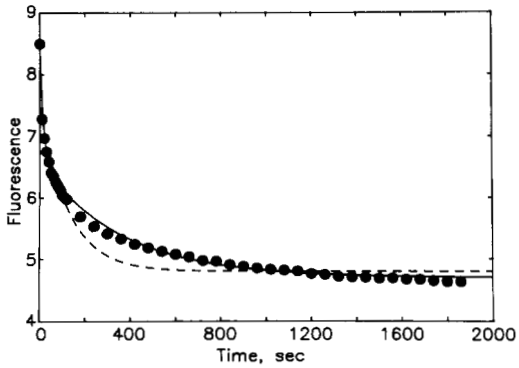

FIG. 1. **The time-dependent decrease in fluorescence after the addition of excess poly(dT) to recA protein-etheno M13 DNA complex.** At time zero, 25 μM poly(dT) was added to a preformed recA protein-etheno M13 DNA complex. Standard buffer was used containing 2 mM $MgCl_2$ and 10 mM NaCl. recA protein and etheno M13 DNA concentrations were 0.56 and 4.2 μM, respectively. A least squares fit (as described under "Materials and Methods") of the time course yielded the following parameters: double exponential function (*solid line*); k_f, 6.5×10^{-2} s^{-1}; A_f, 1.9×10^{-3}; k_s, 2.8×10^{-3} s^{-1}; A_s, 1.9×10^{-3}; and a single exponential (*dashed line*) k, 7.9×10^{-3} s^{-1}; A, 2.7×10^{-3}.

To understand the molecular nature of these transferring species, we have characterized this reaction kinetically and have determined the effects of nucleotide cofactors, magnesium concentration, and substrate concentration on this reaction.

Transfer Does Not Occur by a Simple Dissociation Mechanism—Dissociation of a protein from single-stranded DNA is a first-order process. Therefore, if the rate-limiting step in the transfer reaction is the dissociation of recA protein from etheno M13 DNA, then the concentration of the competing DNA should not influence the measured rate of transfer. However, the apparent rate of recA protein transfer was found to vary as a function of the concentration of competitor (Fig. 2). Under the conditions tested the reaction could be described adequately only by a double exponential decay. As the concentration of poly(dT) is increased, the apparent transfer rate for both the fast (Fig. 2*A*) and the slow (Fig. 2*B*) components is increased. This result is inconsistent with the rate of transfer being limited only by the rate of dissociation. In studies with gp32, the rate of dissociation was shown to be independent of the competitor concentration (Lohman, 1984b). In control experiments (not shown), we have also found that the rate of gp32 transfer from etheno M13 DNA to poly(dT) is not influenced by the concentration of poly(dT). Thus, the dependence of the recA protein transfer rate on competitor concentration is not an artifact of the system. The data described generate the two following questions: 1) what is the molecular nature of the two components and when are they observed? and 2) why is the rate dependent on the concentration of poly(dT) for both the components?

Effect of Magnesium Chloride Concentration on the Transfer Reaction—Many of the enzymatic and physical properties of the recA protein are sensitive to the concentration of $MgCl_2$ in solution (Cotterill and Fersht, 1983; McEntee, 1985; Tsang *et al.*, 1985; Roman and Kowalczykowski, 1986). Therfore, we determined the effect of $MgCl_2$ concentration on the transfer reaction. These experiments were conducted at conditions that show double exponential decay behavior, and the effect of $MgCl_2$ concentration on the amplitude of each rate component is shown in Fig. 3. It is apparent that as $MgCl_2$ concentration increases, so does the proportion of the fast component. The increase in the amount of the fast component is accompanied by a concomitant decrease in the amount of the slow component. The sum of the two components remains constant, suggesting that the slow form is being converted to the fast transferring form as the $MgCl_2$ concentration is increased. Since, increasing the $MgCl_2$ concentration increases the aggregation state of recA protein (Cotterill and Fersht, 1983; Tsang *et al.*, 1985), these data suggest that the fast component is an aggregated form of the slow component.

Increasing the magnesium chloride concentration also influenced the rate of the transfer reaction. The poly(dT) dependence was determined at various $MgCl_2$ concentrations below 6 mM and is shown in Fig. 4; above 6 mM $MgCl_2$, the rate of the fast component exceeded the rate of mixing and could not be analyzed accurately. Therefore, increasing the $MgCl_2$ concentration increased both the apparent rate of transfer as well as the dependence of the transfer rate on poly(dT) concentration.

Effect of recA Protein Concentration on Transfer—The aggregation state of recA protein should be influenced by the protein concentration. If the fast transferring species is an aggregated form of the slow species, then changes in the recA protein concentration will be expected to influence the proportions of the two components. Fig. 5 shows the effect of recA protein concentration on the amplitudes of the two kinetic components of transfer. The amplitude of the slow component increases with protein concentration up to approximately 0.3 μM, above which it does not change. As the slow component amplitude achieves its maximum, the amplitude of the fast component begins to increase and continues to be formed up to approximately 0.6 μM recA protein. These

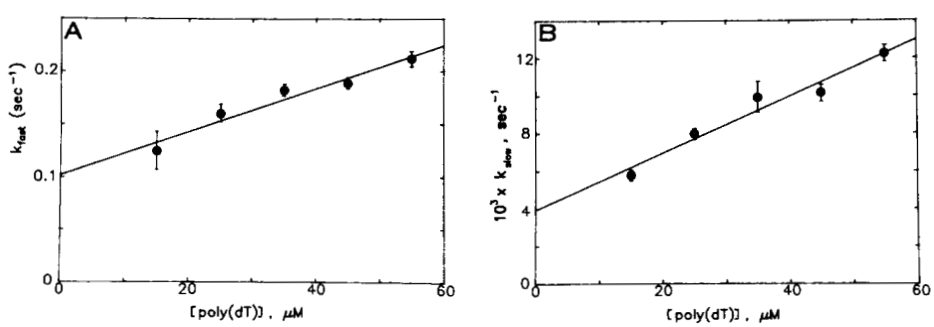

FIG. 2. **Dependence of the transfer rate on competitor concentration.** The fast component (*A*) and slow component (*B*) are shown. The apparent rate constants were obtained by the procedure described under "Materials and Methods." Experiments were conducted in standard assay buffer containing 5 mM $MgCl_2$ and 10 mM NaCl. recA protein concentration was 0.56 μM and etheno M13 DNA concentration was 4.2 μM. Values for the slope and intercept of these data are 2.0×10^{-3} s^{-1} μM^{-1} and 0.102 s^{-1} for the fast component and 1.5×10^{-4} s^{-1} μM^{-1} and 3.9×10^{-3} s^{-1} for the slow component, respectively.

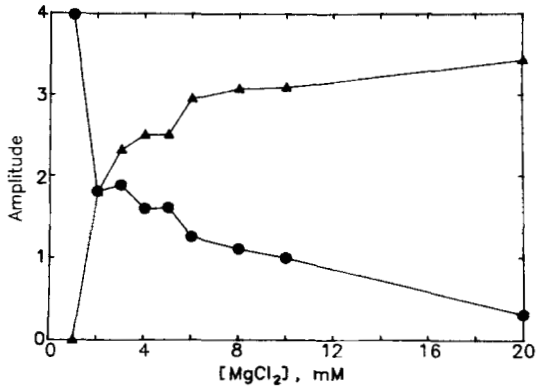

FIG. 3. **Magnesium chloride concentration dependence of the fast and slow component amplitudes of the transfer reaction (in arbitrary units).** Transfer reactions conducted in standard buffer containing 10 mM NaCl. The concentrations of recA protein, etheno M13 DNA, and poly(dT) were 0.56, 4.2, and 25 μM, respectively. Slow component (*circles*) and fast component (*triangles*) of the transfer reaction.

data are also consistent with the interpretation that the fast component is an aggregated form of the slow component.

The rate of transfer was also influenced by the protein concentration. The apparent rate constants at varying recA protein concentration are presented in Fig. 6. The rate of the fast component increases linearly as protein concentration is increased (Fig. 6*A*). The rate of the slow component also increases with protein concentration but appears to saturate at approximately 0.4 μM (Fig. 6*B*). It is interesting to note that recA protein concentrations in excess of that required to fully saturate the etheno M13 DNA (0.56 μM), continue to increase the apparent transfer rate constant of the fast component. The data may suggest that this additional protein is not free in solution but rather is associated with the recA protein-etheno M13 DNA complex in some way.

Effect of NaCl Concentration on the Transfer Reaction—The affinity of recA protein for single-stranded DNA is affected by the salt concentration (Menetski and Kowalczykowski, 1985a). Therefore, we conducted the transfer reaction at various concentrations of NaCl. The effect of NaCl concentration on the transfer reaction rate is shown in Fig. 7, in the form of a log [NaCl] *versus* log *k* plot so that the fast and slow component rates can be compared easily. Below 100 mM NaCl, the apparent rate constants for both the fast and slow components of the transfer reaction are relatively insensitive to salt concentration. Above 100 mM NaCl, both of the rates become more sensitive to changes in salt concentration. This change in the salt sensitivity of the rate at 100 mM NaCl could signify a switch in the reaction mechanism or in the rate-limiting step of the reaction. However, since the rate of transfer at high NaCl concentration is still influenced by the competitor concentration (not shown), this suggests that the mechanism at high NaCl concentrations is similar to, if not the same as, that at low NaCl concentrations.

The aggregation of recA protein has been shown to be influenced by the presence of NaCl (Cotterill and Fersht, 1983; Tsang *et al.*, 1985). Since the fast and slow component of transfer are thought to be related to the aggregation state of recA protein, it is interesting to consider the effect of NaCl concentration on the relative proportion of the two components. As described above, the fast component persists to high salt concentration, and the proportion of each component remains relatively constant (not shown). These data suggest that the fast component, or the aggregated transferring form, is relatively insensitive to NaCl concentration.

Effect of ADP on the Transfer Reaction—ADP has been shown to decrease the equilibrium binding affinity of recA protein to single-stranded DNA (Menetski and Kowalczykowski, 1985a). This cofactor has also been shown to influence the degree of aggregation of recA protein (Cotterill and Fersht, 1983; Tsang *et al.*, 1985) as well as inhibit many of the *in vitro* activities of the protein (Weinstock *et al.*, 1979; Cox and Lehman, 1981). Therefore, it was of interest to determine the affect of ADP on the transfer reaction.

The rate of transfer in the presence of ADP is also dependent on the concentration of competitor (Fig. 8). The dependence of observed rate constant on poly(dT) concentration, in the presence of 20, 100, and 250 μM ADP is shown; only the slow component in the presence of ADP is plotted because the fast component rate exceeds the rate of mixing and cannot be determined accurately. These data show that the apparent transfer rate constant saturates at high poly(dT) concentration for each ADP concentration. The data also show that the plateau rate achieved, at high poly(dT) concentration, increases as ADP concentration increases. Generally, the appearance of saturation behavior with respect to competitor concentration is indicative of a mechanism involving a rapid pre-equilibrium to an intermediate, followed by a rate-limiting isomerization to products (Bernasconi, 1976).

The complete analysis of the recA protein concentration dependence in the presence of ADP is hampered by the inability to determine the rate of the fast component. However, we can qualitatively determine the proportion of fast and slow component by subtracting the amplitude of the slow component from that of the total observed fluorescence change (as described under "Materials and Methods"). Fig. 9

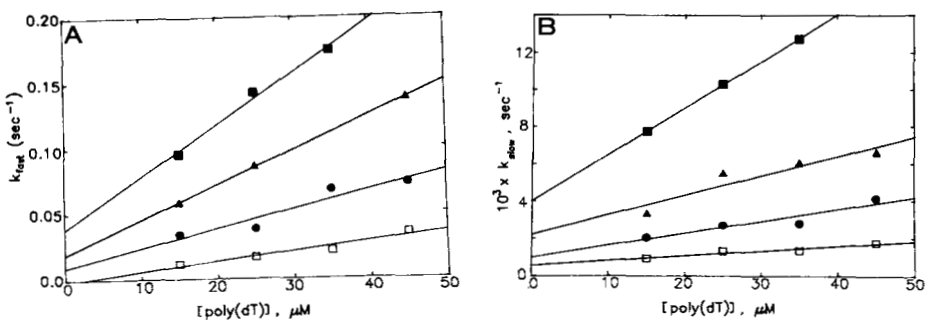

FIG. 4. **Poly(dT) concentration dependence of the apparent transfer rate constant at various $MgCl_2$ concentrations.** Reactions were conducted in standard buffer containing 10 mM NaCl, 0.56 μM recA protein, and 4.2 μM etheno M13 DNA. Data shown for fast (*A*) and slow (*B*) component for the following magnesium chloride concentrations; 2 mM (*open squares*); 3 mM (*closed circles*); 4 mM (*closed triangles*); 6 mM (*closed squares*).

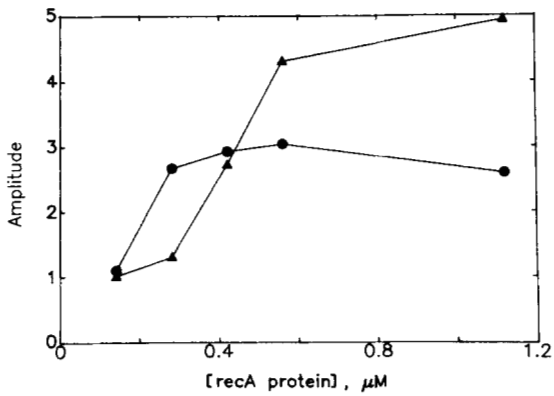

FIG. 5. **recA protein concentration dependence of the fast and slow component amplitudes (in arbitrary units).** Reactions were conducted in standard buffer containing 4 mM $MgCl_2$, 10 mM NaCl, 4.2 μM etheno M13 DNA and 25 μM poly(dT). Fast component amplitude (*triangles*) and slow component amplitude (*circles*) are shown.

shows the amplitudes of the fast and slow component in the presence of ADP as a function of recA protein concentration. Whereas the general trend is the same in the presence and absence of ADP (see Fig. 5), there is a more pronounced lag in the appearance of the fast component in the presence of ADP. Thus, higher concentrations of protein are required to form fast component. Since, ADP is known to disrupt recA protein aggregates (Cotterill and Fersht, 1983; Tsang *et al.*, 1985), this result is also consistent with the interpretation that the fast component is an aggregated form of the slow component.

The salt dependence of the transfer rate in the presence of ADP is plotted on Fig. 7. The data shown is for the slow component in the presence of 100 μM ADP. The rate of transfer, in the presence of ADP, is faster than in its absence at all NaCl concentrations tested. The sensitivity of the rate to NaCl concentration is qualitatively similar to that in the absence of ADP. Thus, the salt and protein concentration dependence data suggest that the mechanism in the presence of ADP is the same as that in its absence. Also, and most importantly, the appearance of saturation behavior with regard to the poly(dT) concentration, suggests that the transfer reaction occurs via a ternary intermediate involving recA protein, etheno M13 DNA, and poly(dT).

Viscosity Dependence of the Transfer Reaction—Because the observed transfer rate is dependent on the competitor concentration, the transfer mechanism must involve a bimolecular step. However, it is not clear whether this bimolecular collisional step is rate limiting in the transfer reaction. The rates of simple bimolecular reactions are known to be limited by the rate of substrate diffusion through the medium (Berg and von Hippel, 1985). For such simple diffusion-controlled reactions, an increase in the viscosity of the medium reduces the rate of diffusion and results in a decrease in the rate of these reactions. This phenomenon was shown to be true in studies of the rate of association of gp32 to single-stranded DNA; the rate of bimolecular association, under diffusion-controlled reaction conditions, decreased approximately 2-fold as sucrose concentration was increased from 0 to 20% (Lohman and Kowalczykowski, 1981). Thus, if the rate-limiting step in the recA protein transfer reaction occurred by a direct bimolecular collisional mechanism, then an increase in the solution viscosity should result in a decrease in the rate of transfer. To test this possibility for the transfer reaction, the viscosity was varied using both glycerol (0–40%) and sucrose (0–20%) because glycerol has been reported to affect the solution properties of the recA protein (Cotterill and Fersht, 1983). For both the fast and slow components, the rate of transfer, at 4 mM $MgCl_2$, was only slightly affected (increased by ≈10%) over this range of solution viscosity (not shown). Since, the rate of transfer did not decrease with increasing viscosity, this result suggests that the rate of transfer is not limited by diffusion. Therefore, these data are consistent with our proposal that transfer is occurring through a ternary intermediate.

Effect of Nucleation on the Transfer Reaction—It is possible that the rate-limiting step in the transfer reaction could be the nucleation of recA protein on the competitor DNA. Therefore, enough recA protein was added to poly(dT) so that 25% of the competitor DNA was saturated with protein. This prenucleated DNA substrate was then used as the competitor in a recA protein transfer reaction. Within experimental error, prenucleating the poly(dT) with recA protein had no effect on the rate of transfer (not shown). Therefore, the rate of recA protein nucleation on poly(dT) does not appear to be the rate-limiting step in the transfer reaction.

DISCUSSION

We have analyzed the kinetic mechanism of the transfer of recA protein from etheno M13 DNA to poly(dT) using a spectrofluorometric assay. The experiments were conducted by forming recA protein-etheno M13 DNA complexes, challenging them with poly(dT), and then following the reaction by recording the decrease in fluorescence upon dissociation of protein from etheno M13 DNA. This reaction exhibits some interesting properties that detail how recA protein interacts with single-stranded DNA substrates. First, the rate of the reaction is influenced by the concentration of the competitor. The rate increases as the concentration of poly(dT) is increased. This data is inconsistent with transfer occurring by a simple dissociation mechanism which would be independent of competitor concentration. Second, in the presence of ADP, the apparent transfer rate constant shows saturation behavior with respect to poly(dT) concentration, suggesting that a ternary intermediate is involved. Third, the rate of transfer is unaffected by changes in solution viscosity, demonstating

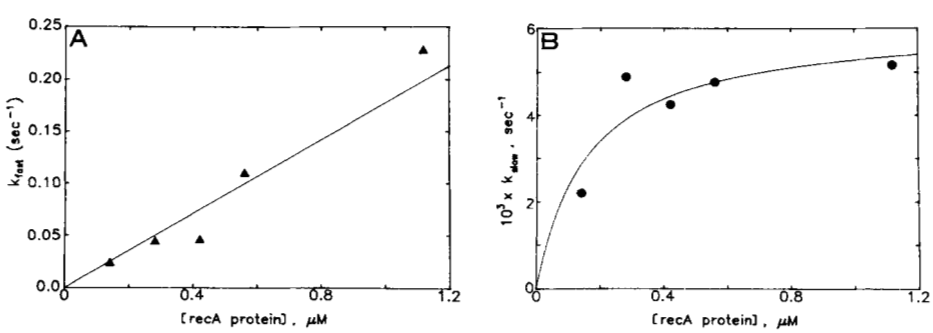

FIG. 6. **recA protein concentration dependence of the rate of transfer.** Reactions were conducted in standard buffer containing 4 mM $MgCl_2$, 10 mM NaCl, 4.2 μM etheno M13 DNA, and 25 μM poly(dT). The fast component (*A*) and slow component (*B*) rates are shown.

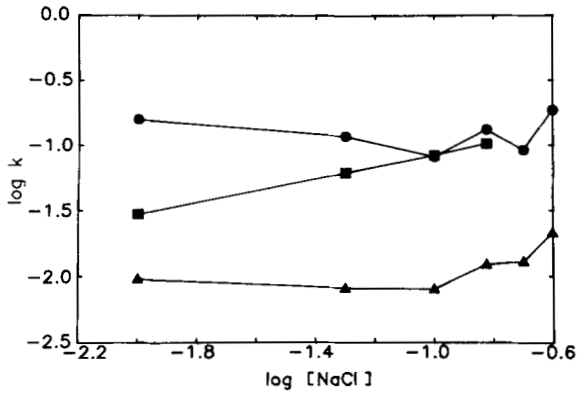

FIG. 7. **Dependence of the apparent rate constant of transfer on NaCl concentration.** Experiments were conducted in standard assay buffer containing 4 mM $MgCl_2$, 0.56 μM recA protein, 4.2 μM etheno M13 DNA, and 25 μM poly(dT). The fast component (*circles*) and slow component (*triangles*) of transfer in the absence of cofactor and the slow component (*squares*) rate in the presence of 100 μM ADP.

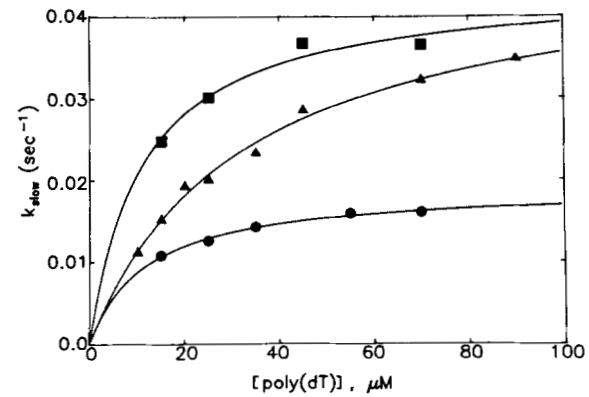

FIG. 8. **Poly(dT) concentration dependence of apparent transfer rate constant for the slow component in the presence of ADP.** Reactions were conducted in standard assay buffer containing 4 mM $MgCl_2$, 10 mM NaCl, 0.56 μM recA protein, and 4.2 μM etheno M13 DNA. The concentration of ADP are 20 μM (*circles*), 100 μM (*triangles*), and 250 μM (*squares*).

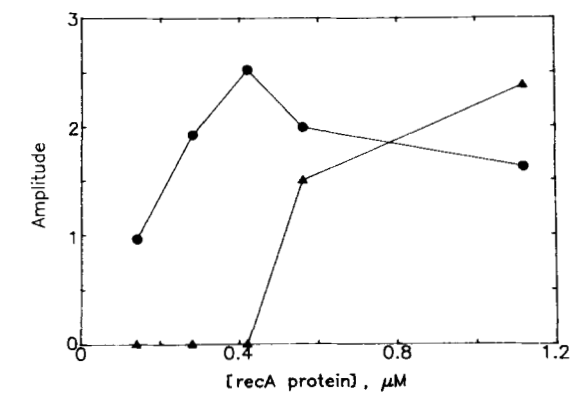

FIG. 9. **recA protein concentration dependence of the fast and slow component amplitude in the presence of ADP (arbitrary units).** Reactions were conducted in standard assay buffer 4 mM $MgCl_2$, 10 mM NaCl, 4.2 μM etheno M13 DNA, and 25 μM poly(dT). The fast component (*triangles*) and slow component (*circles*) amplitudes are shown.

that transfer is not occurring by a direct diffusion-limited bimolecular reaction. Finally, the transfer reaction proceeds through two different kinetic species that transfer at different rates. The relative amounts of the two components vary with experimental conditions and appear to be related to the aggregation properties of recA protein. Thus, the two major questions to be addressed in the interpretation of these results are: 1) what are the two components observed in the transfer reaction? and 2) why is the transfer rate dependent on competitor concentration?

The results that the rate can show saturation behavior with respect to competitor concentration and that it is not influenced by the solution viscosity suggest that this reaction follows a classical two-step mechanism involving a ternary intermediate (Bernasconi, 1976). This intermediate must consist of etheno M13 DNA, recA protein, and poly(dT):

$$\begin{array}{c} \text{recA·etheno M13 DNA} + \text{poly(dT)} \\ \rightleftarrows \quad K_1 \\ \text{poly(dT)·recA·etheno M13 DNA} \\ \downarrow \quad k_2 \\ \text{recA·poly(dT)} + \text{etheno M13 DNA} \end{array}$$

The first step of this mechanism is an initial rapid equilibrium, described by the binding constant K_1. The second step of the reaction is a slow isomerization to products defined by the first-order rate constant, k_2. The association of the recA protein-etheno M13 DNA complex with poly(dT) is assumed to be the rapid equilibrium step. Since a change in fluorescence is witnessed upon dissociation of protein from etheno M13 DNA, the isomerization to products is assumed to be the experimentally observed process. An experimental requirement of this mechanism is that the observed rate constant should plateau with respect to competitor concentration, as is observed in the presence of ADP (see Fig. 8). Also the rate should not be influenced by the viscosity of the buffer because the rate-limiting step is intramolecular. Thus, the above mechanism is the simplest mechanism that is consistent with all of the data.

Theoretically, the values of K_1 and k_2 can be derived from the data shown in Figs. 2, 4, and 8. The initial, linear region of these curves is equal to the product of K_1 and k_2, and the final rate at the plateau is proportional to k_2 (Bernasconi, 1976). However, the detailed analysis of the transfer mechanism has been hampered by experimental limitations. The concentration of poly(dT) needed to show saturation, in most cases, is greater than can be experimentally obtained. Therefore, in cases where no plateau is observed, extrapolation of the data is unreliable. Also, in those cases where a plateau is observed, the requirement for a 5-fold excess of poly(dT) over the etheno M13 DNA concentration imposes a lower limit to experimentally valid data. However, with these limitations in mind, two features of the transfer mechanism become apparent. First, the data in Figs. 2 and 4 show that as magnesium concentration increases the initial slope value increases. Based on our proposed mechanism, this result requires that the product, K_1k_2, increases as $MgCl_2$ concentration increases. Second, the data in Fig. 8 show that the plateau value of the apparent rate constant appears to increase as ADP concentration increases. Thus, increases in ADP concentration appear to increase the rate of isomerization, k_2. Because we are unable to separate K_1 and k_2 for the data in the absence of ADP, it is impossible to determine which parameter $MgCl_2$ is affecting. However, the result that k_2 is increased in the presence of ADP is consistent with the destabilizing effect of ADP on the equilibrium affinity of recA protein for etheno M13 DNA (Menetski and Kowalczykowski, 1985a).

RecA protein aggregation has been studied by two different methods. Cotterill and Fersht (1983) and Roman and Kowalczykowski (1986) have shown that the light scattering of recA protein in solution increases with increasing magnesium concentration. Using a sedimentation technique, Tsang *et al.* (1985) have shown that recA protein aggregates can also

contain both single- and double-stranded DNA. Both of the aggregation processes above require magnesium; however, they can differ with respect to NaCl concentration and ADP sensitivity. The aggregation of free recA protein and of both single- and double-stranded DNA are disrupted by low NaCl concentrations (<100 mM) and by the presence of ADP. However, the aggregation of recA protein and single-stranded DNA, as defined by the sedimentation assay, is insensitive to both NaCl concentration and the presence of ADP. We have found that the relative proportion of the fast and slow components in the transfer reaction are influenced by $MgCl_2$, NaCl, and recA protein concentration and by the presence of ADP. As $MgCl_2$ concentration is increased, the amount of fast component increases in direct proportion to the loss of the amount of slow component. Also, the data show that the amount of fast component is decreased, although not completely, in the presence of ADP. Finally, the fast component persists to high (200 mM) NaCl concentrations. The effect of magnesium chloride and the presence of ADP on the amount of the fast component suggest that the fast component is an aggregated form of the slow component and that at least a portion of the aggregation process is related to the aggregation of free recA protein. However, unlike the aggregation of free protein the amount of the fast component is relatively insensitive to NaCl. This result suggests that part of the aggregation process, in the transfer reaction, is related to the aggregation of single-stranded DNA by recA protein. Thus, the aggregation of the slow component into the fast component appears to share characteristics of both aggregation processes previously described.

In addition, we have found that concentrations of recA protein in excess of that necessary to saturate the single-stranded DNA, greatly influences the properties of the fast component in the transfer reaction. The rate of the fast component continues to increase as the protein concentration is increased. These data suggest that the excess protein may not be free in solution but instead may be associated with the protein·DNA complex. Thus, the fast transferring kinetic species may represent a multimeric complex of recA protein and single-stranded DNA.

Using the data described above, we have devised a model that schematically illustrates the transfer reaction and is consistent with our data (Fig. 10). In addition to the reaction parameters K_1 and k_2 described previously, it is necesary to introduce a third parameter to describe the equilibrium between the fast (*A*) and slow (*B*) transferring species of recA protein-etheno M13 DNA complexes. This equilibrium constant, K_{agg}, is influenced by magnesium concentration and ADP and seems to be related to the aggregation state of the protein·DNA complexes. Increasing magnesium concentration favors the more aggregated species (the fast component) while the addition of ADP shifts the equilibrium toward the nonaggregated species (the slow component). Both species of recA protein·DNA complex described can participate in a transfer reaction with another single-stranded DNA molecule by the pre-equilibrium mechanism outlined above. We envision the transfer reaction occurring as follows. Prior to the addition of poly(dT), the relative amount of the fast and slow transferring species is determined by the equilibrium constant, K_{agg}. Upon the addition of competitor, both the fast and slow components participate in the formation of a ternary intermediate that includes recA protein, etheno M13 DNA, and poly(dT). The amount of intermediate formed for the fast and slow transferring species is characterized by K_1^f and K_1^s, respectively. Next, the two single-stranded DNA molecules are exchanged within the ternary intermediate in a slow step characterized by k_2^f and k_2^s. In this illustration the slow and fast components are represented as monomeric and dimeric aggregates of a recA protein·DNA complex for simplicity. However, these species could be more highly aggregated forms (*e.g.* the slow component could be a dimeric complex, and the fast component could be a higher multimeric form). Our data cannot determine the exact molecular nature of the two transferring species, only that the fast component appears to be an aggregated form of the slow.

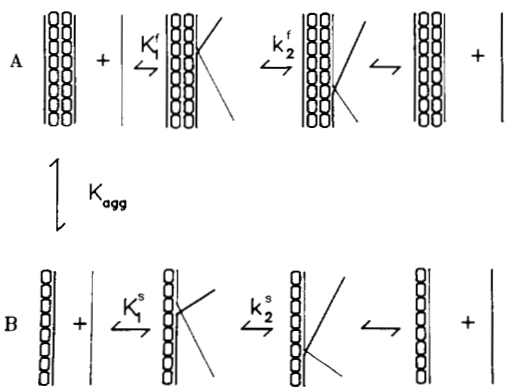

FIG. 10. **Proposed schematic model of transfer as deduced from kinetic analysis.** *Pathway A* represents the fast component and *pathway B* the slow component. K_{agg} is an apparent equilibrium constant between *pathway A* and *B*. K_1^f and k_2^f represent the equilibrium constant for intermediate formation and the isomerization rate of strand exchange within the intermediate for the fast component. K_1^s and k_2^s represent the equilibrium constant for intermediate formation and the isomerization rate of strand exchange within the intermediate for the slow component.

In related studies the rate of transfer in the presence and absence of ADP was found to follow qualitatively similar trends (Bryant *et al.*, 1985a and 1985b). However, the rates we observe are quantitatively different than those of Bryant *et al.* (1985a, 1985b); the rate of transfer from etheno M13 DNA to poly(dT) is always greater than the rate of transfer from one ϕX174 molecule to another. Since, the affinity of recA protein is greater for etheno M13 DNA than it is for native single-stranded DNA (Menetski and Kowalczykowski, 1985a), it might have been expected that the rate of transfer from etheno M13 DNA would have been slower than that from native single-stranded DNA. However, we have shown that one step involved in determining the rate of transfer is the formation of a ternary intermediate. Since affinity of recA protein for poly(dT) is also greater than it is for single-stranded DNA, it is likely that the rapid equilibrium constant, K_1, is sensitive to the intrinsic affinity of recA protein for the competitor DNA molecule. Thus, using this higher affinity competitor may cause an increase in the amount of intermediate formed, resulting in an observed rate of transfer that is faster than it is when native single-stranded DNA molecules are used.

The transfer model presented contains kinetic steps that may have similar roles in the recA protein catalyzed renaturation reaction. Thus, it is possible to apply results from this study to the understanding of renaturation. In renaturation, complementary single-stranded DNA molecules must be brought together by recA protein. The pairing reaction must occur rapidly, on the time scale of the renaturation mechanism, in order to limit the rate by a first-order process. McEntee (1985) has proposed a mechanism whereby recA protein catalyzes renaturation through a ternary complex of recA protein and both complementary molecules of single-stranded DNA. We have presented kinetic evidence that implies that recA protein has the intrinsic ability to form a

ternary intermediate that includes recA protein and two separate single-stranded DNA molecules, a step that is presumably necessary during the renaturation reaction. Another similarity is that the rate-limiting step in renaturation is a first-order process (Weinstock *et al.*, 1979). We have shown that the rate-limiting process in the transfer reaction is also a first-order process (the isomerization reaction). Therefore, since the transfer reaction mechanism does not contain a rate component involved in finding complementarity, this reaction may offer a means by which the base-pairing process of renaturation can be separated from the initial ternary complex formation process. Work in this laboratory is currently in progress to explore this possibility.

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REFERENCES

Ames, B. N. (1966) *Methods Enzymol.* **8,** 115–118
Berg, O. G., and von Hippel, P. H. (1985) *Ann. Rev. Biophys. Biophys. Chem.* **14,** 131–160
Bernasconi, C. (1976) *Relaxation Kinetics*, pp. 20–39, Academic Press, New York
Bryant, F. R., Riddles, P. W., and Lehman, I. R. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49,** 535–539
Bryant, F. R., Taylor, A. R., and Lehman, I. R. (1985) *J. Biol. Chem.* **260,** 1196–1202
Bryant, F. R., and Lehman, I. R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 297–301
Cazenave, C., Chabbert, M., Toulme, J-J., and Helene, C. (1984) *Biochim. Biophys. Acta* **781,** 7–13
Clark, A. J. (1973) *Annu. Rev. Genet.* **7,** 67–85
Cotterill, S. M., and Fersht, A. R. (1983) *Biochemistry* **22,** 3525–3531
Cox, M. M., and Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 3433–3437
Cox, M. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256,** 4676–4678
Cox, M. M., and Lehman, I. R. (1982) *J. Biol. Chem.* **257,** 8523–8532
Gonda, D. K., and Radding, C. M. (1983) *Cell* **34,** 647–654
Kowalczykowski, S. K., Lonberg, N., Newport, J. W., Paul, L. S., and von Hippel, P. H. (1980) *Biophys. J.* **32,** 405–418
Kryzosiak, W. J., Biernat, J., Ciesiolka, B., Gulewicz, K., and Wiewiovowski, M. (1981) *Nucleic Acids Res.* **9,** 2841–2851
Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) *Anal. Biochem.* **100,** 95–97
Little, J. W., and Mount, D. W (1982) *Cell* **29,** 11–22
Lohman, T. M., and Kowalczykowski, S. C. (1981) *J. Mol. Biol.* **152,** 67–109
Lohman, T. M. (1984a) *Biochemistry* **23,** 4656–4665
Lohman, T. M. (1984b) *Biochemistry* **23,** 4665–4675
McEntee, K., Weinstock, G. M., and Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 2615–2619
McEntee, K., Weinstock, G. M., and Lehman, I. R. (1981) *Prog. Nucleic Acid Res. Mol. Biol.* **26,** 265–279
McEntee, K. (1985) *Biochemistry* **24,** 4345–4351
Menetski, J. P., and Kowalczykowski, S. C. (1985a) *J. Mol. Biol.* **181,** 281–295
Menetski, J. P., and Kowalczykowski, S. C. (1985b) *Biochemistry* **24,** 3375
Menetski, J. P., and Kowalczykowski, S. C. (1986a) *J. Cell. Biochem.* **10,** 199 (abstr.)
Roman, L., and Kowalczykowski, S. C. (1986) *Biochemistry* **25,** 7375–7385
Shibata, T., DasGupta, C., Cunningham, R. P., Williams, J. G. K., Osber, L., and Radding, C. M. (1981) *J. Biol. Chem.* **256,** 7565–7572
Silver, M., and Fersht, A. R. (1982) *Biochemistry* **21,** 6066–6072
Silver, M., and Fersht, A. R. (1983) *Biochemistry* **22,** 2860–2866
Tsang, S. S., Chow, S. A., and Radding, C. M. (1985) *Biochemistry* **24,** 3226–3232
Weinstock, G. M., McEntee, K., and Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 126–130
Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981a) *J. Biol. Chem.* **256,** 8829–8834
Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981b) *J. Biol. Chem.* **256,** 8845–8849
Wu, A. M., Kahn, R., DasGupta, C., and Radding, C. M. (1982) *Cell* **30,** 37–44