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Transfer of recA Protein from One Polynucleotide to Another

EFFECT OF ATP AND DETERMINATION OF THE PROCESSIVITY OF ATP HYDROLYSIS DURING TRANSFER*

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The transfer of recA protein from a fluorescently modified single-stranded DNA, containing 1,N^6-ethenoadenosine and 3,N^4-ethenocytosine, to polydeoxythymidylic acid (poly(dT)) was shown to occur by a complex mechanism in both the absence and presence of ADP (Menetski, J. P., and Kowalczykowski, S. C. (1987) *J. Biol. Chem.* 262, 2085–2092). A part of the mechanism involves the formation of a kinetic ternary intermediate. Since the binding and hydrolysis of ATP by recA protein is involved in many of the recA protein *in vitro* activities, we have analyzed the effect of ATP on the transfer reaction. In the presence of ATP, the transfer reaction is dependent on the concentration of the competitor single-stranded DNA, poly(dT). This result suggests that transfer does not occur by a simple dissociation mechanism. The reaction occurs via two kinetically distinct species of protein•DNA complexes with properties that are similar to those characterized for the transfer reaction in the absence of ATP. There is a complicated effect of nucleotide concentration on the rate of transfer. At low concentrations of ATP (<50 μM), increasing nucleotide concentration increases the rate of transfer; this is similar to the effect of ADP. However, at high concentrations of ATP (>50 μM), increasing ATP concentration decreases the rate of transfer. Finally, the processivity of ATP hydrolysis during transfer was found to increase with increases in ATP concentration. Less than one ATP molecule was hydrolyzed per transfer event at low ATP concentrations (<20 μM) while greater than 50 molecules were hydrolyzed at high ATP concentration (>250 μM). These data suggest that the rate of transfer is not directly coupled to the rate of hydrolysis.

In the accompanying paper, we have shown that the transfer of recA protein from one polynucleotide to another occurs via a complex kinetic mechanism. Transfer involves the formation of a ternary intermediate which includes recA protein, the initial DNA, and the final DNA. The formation of product molecules from this intermediate proceeds by an isomerization reaction or an exchange of the DNA molecules within the intermediate. Under certain conditions the transfer reaction can occur by two kinetically distinct species. The interconversion between these species of protein·DNA complexes is related through an apparent aggregation equilibrium constant and is greatly influenced by magnesium concentration.

The binding affinity of the recA protein for single-stranded DNA is influenced by the presence of NaCl, ADP, and ATP (Menetski and Kowalczykowski, 1985a). NaCl and ADP decrease, while ATP increases the affinity of recA protein for single-stranded DNA. In agreement with equilibrium results, the transfer of recA protein between polynucleotides was found to be influenced by NaCl and ADP concentration. An increase in either the salt or ADP concentration resulted in an increase in the observed rate of transfer.

recA protein can catalyze the hydrolysis of ATP in the presence of single-stranded DNA (Weinstock *et al.*, 1979, 1981a). Hydrolysis of ATP has also been shown to stimulate recA protein-mediated renaturation (Bryant and Lehman, 1985; McEntee, 1985) and is necessary to catalyze strand exchange (McEntee *et al.*, 1979; Cox and Lehman, 1981). Therefore, in this report, we focus on the effect of ATP on the transfer reaction and the manner in which this reaction may give insight to other recA protein-catalyzed reactions.

Silver and Fersht (1982) measured the affect of ATP on the transfer reaction and found that the transfer rate decreased in its presence. Bryant *et al.* (1985) report, however, that ATP increases the transfer rate. In this paper we report that the effect of ATP on the transfer of recA protein from etheno M13 DNA[1] to poly(dT) is dependent on the concentration of ATP. At low ATP concentrations, the rate is increased relative to the transfer rate observed in its absence. However, at high ATP concentrations, the rate is decreased relative to the rate observed in the absence of cofactor. In addition, the rate of transfer is influenced by the concentration of the competing DNA, poly(dT). These data suggest that, in the presence of ATP, transfer does not occur by a simple dissociation mechanism. Thus, the data can be interpreted to suggest that transfer, in the presence of ATP, may occur by a mechanism similar to that determined for transfer in the absence of cofactor. Finally, the processivity of hydrolysis during the transfer reaction was determined. The number of ATP molecules hydrolyzed per recA protein transferred varies from fewer than one, at low ATP concentrations, to over 50, at ATP concentrations above 100 μM.

MATERIALS AND METHODS

All materials and methods are identical to those described in the accompanying paper (Menetski and Kowalczykowski, 1987) except those listed below.

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[1] The abbreviations used are: etheno M13 DNA, modified single-stranded M13 DNA containing 1,N^6-ethenoadenosine, and 3,N^4-ethenocytosine; ATPγS, adenosine-5′-O′-(3-thiotriphosphate); poly(dT), polydeoxythymidylic acid.

Transfer Reaction—ATP was purchased from P-L Biochemicals and dissolved as a concentrated stock solution at pH 7.0. The transfer reactions were conducted by forming recA protein-etheno M13 DNA complexes in the presence of ATP at 25 °C. Reactions were then initiated by the addition of excess poly(dT) (generally 5-fold excess relative to the etheno M13 DNA concentration). Reaction buffer contained 20 mM Tris-HCl, 4 mM $MgCl_2$, 0.1 mM dithiothreitol, and 10 mM NaCl. Changes in the magnesium and NaCl concentrations are indicated where applicable. An ATP-regenerating system was used in all reactions containing ATP. The system consisted of 2 units/ml of pyruvate kinase and 2 mM phosphoenolpyruvate (Sigma), except at low ATP concentrations when 1 mM phosphoenolpyruvate was used. This concentration of phosphoenolpyruvate did not effect the rate of transfer when added in the absence of ATP. The regenerating system was sufficient to maintain a constant ATP concentration for approximately 1 h. ATPγS was purchased from Boehringer-Mannheim and was used either without further purification or after purification using a Pharmacia Mono-Q fast protein liquid chromatography column with no difference in results.

ATPase Assay—recA protein ATPase activity was measured by the method described by Kreuzer and Jongeneel (1983) as adapted by Kowalczykowski and Krupp (1986). In this assay the steady-state oxidation of NADH to NAD^+ is coupled to the regeneration of ATP from ADP. The change in absorbance at 340 nm, due to the oxidation reaction, can be measured spectrophotometrically. Thus, the rate of ATP hydrolysis can be determined from the rate of NADH oxidation.

RESULTS

Comparison of Transfer in the Absence and Presence of Nucleotides—The transfer reaction was conducted in the presence of 250 μM ATP to determine the affect of this cofactor on the transer rate. recA protein-etheno M13 DNA complexes were formed, then excess poly(dT) was added to initiate the transfer reaction. In the presence of ATP, the transfer reaction is qualitatively similar to that observed in the absence of cofactor (not shown). The time course can be separated into two kinetically distinct exponential decay processes, or transferring species. As shown previously, the transfer reaction in the absence of cofactor also occurs by two species (Menetski and Kowalczykowski, 1987). The apparent rate constants obtained for the two components in the presence of ATP are shown in Table I. For comparison, the apparent rate constants obtained in the absence and presence of ADP are also shown. Under these conditions, the observed rate constant for transfer of the protein·DNA complex follows the trend, ADP > No Cofactor > ATP. Since the recA protein·DNA complex is known to be stabilized by ATP and destabilized by ADP (Menetski and Kowalczykowski, 1985a), these data suggest that some part of the transfer reaction must be influenced by the equilibrium-binding properties of the recA protein-nucleotide-DNA complex.

The nonhydrolyzable ATP analogue, ATPγS, has been shown to bind to the recA protein-single-stranded DNA complex and induce a high affinity state that is stable in 2.5 M NaCl (Menetski and Kowalczykowski, 1985a). We were interested in determining whether the transfer reaction would be inhibited by this analogue. In the presence of ATPγS, approximately 30% of the total transfer expected actually occurs. The relative proportions of the two components observed in that 30% are similar to those in the absence of cofactor. Also, the rate of the fast component is similar to that observed in the absence of cofactor. However, the rate of the slow component appears to be more similar to that in the presence of ATP. Thus, the data suggest that a portion of recA protein-etheno M13 DNA molecules are always available for transfer, even in the presence of ATPγS.

TABLE I

Effect of cofactors on the transfer reaction

All reactions were carried out in standard buffer containing 4 mM $MgCl_2$ and 10 mM NaCl. The reactions contained 0.56 μM recA protein, 4.3 μM etheno M13 DNA, and 25 μM poly(dT).

	Rate		Percentage of component	
	Fast	Slow	Fast	Slow
	s^{-1}			
No cofactor[a]	0.15	9.5×10^{-3}	64	36
250 μM ADP[a]	>0.4	3.0×10^{-2}	52	48
250 μM ATP	6.1×10^{-2}	4.3×10^{-3}	35	65
250 μM ATPγS[b]	0.13	3.2×10^{-3}	65	35

[a] From Menetski and Kowalczykowski, 1987.

[b] Total amplitude observed represents only 31% of the total expected transition.

Dependence of Transfer Rate on Competitor Concentration—Both in the absence and presence of ADP, we have shown that the rate of transfer is dependent on the concentration of competitor, indicating that the transfer mechanism involves a ternary intermediate (Menetski and Kowalczykowski, 1987). The observed rates of transfer for both the fast and slow components obtained in the presence of ATP are shown in Fig. 1. The rates of both the fast and slow components are influenced by changing the poly(dT) concentration; however, the effect is different than observed in the absence and presence of ADP. The rate of the fast component appears to decrease slightly as poly(dT) concentration increases from 15 to 55 μM (Fig. 1*A*). The rate of the slow component is relatively unaffected up to 35 μM poly(dT), but then decreases by almost 2-fold, as poly(dT) concentration is increased further (Fig. 1*B*). The observed decrease in rate for both the fast and slow components is slight but is within the experimental uncertainty and, therefore, can be considered significant. Although the rate change is opposite to what is expected, the fact that the competitor concentration has an affect on the rate of transfer implies that the transfer reaction in the presence of ATP cannot occur by a simple dissociation mechanism. Since, this behavior can not be explained by the bimolecular mechanism described previously (Menetski and Kowalczykowski, 1987), the mechanism of transfer in the presence of ATP must be more complex than that in the presence and absence of ADP. Yet, as it will be demonstrated more clearly below, the transfer reaction in the presence of ATP bears considerable similarities to the reaction in the absence of ATP.

Effect of recA Protein Concentration on Transfer in the Presence of ATP—The transfer reaction, in the presence and absence of ADP, possesses several characteristic properties with respect to protein concentration (Menetski and Kowalczykowski, 1987). Changes in the relative amounts of the two kinetic components are witnessed when the recA protein concentration is increased while the etheno M13 DNA and poly(dT) concentrations are held constant. Increasing the recA protein concentration first shows an increase in the slow kinetic component followed by an increase in the fast component. Thus, the fast transferring species appears to be an aggregate of the slow component. Fig. 2 shows the relative proportion of the two components as a function of protein concentration in the presence of ATP. These data show that the slow component is present at all protein concentrations and that the fast component appears only at protein concentrations greater than 0.3 μM. This trend is very similar to that seen in the absence of ATP (Menetski and Kowalczykowski, 1987). However, in the presence of ATP, the proportion of the fast component is less than that observed in the absence of this cofactor. This result suggests that ATP disrupts the formation of recA protein aggregates and is consistent with

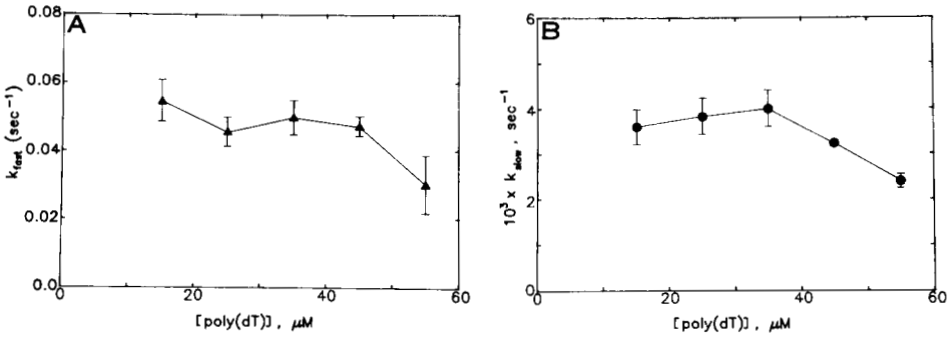


FIG. 1. **Poly(dT) concentration dependence of the observed rate of transfer in the presence of ATP.** Reactions were conducted in standard buffer which contained 250 μM ATP, 0.56 μM recA protein, and 4.2 μM etheno M13 DNA. The fast component (*A*) and the slow component (*B*) of the transfer reaction are shown.

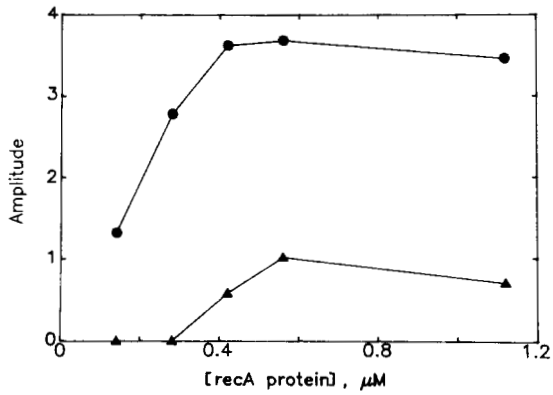


FIG. 2. **recA protein concentration dependence of the amplitude of the slow and fast component (arbitrary units).** Reactions were conducted in standard with 250 μM ATP, 4.2 μM etheno M13 DNA, and 25 μM poly(dT). recA protein was added to the concentration indicated from a concentrated stock solution. The amplitudes of the fast component (*triangles*) and slow component (*circles*) are shown.

the studies of Cotterill and Fersht (1983) and Roman and Kowalczykowski (1986).

Effect of Magnesium Chloride Concentration on Transfer in the Presence of ATP—The transfer reaction in the absence of nucleotide cofactor was shown to be greatly influenced by $MgCl_2$ concentration. The proportion of fast component increases with increasing $MgCl_2$ concentration. Also, the rates of both the fast and slow component increase as $MgCl_2$ concentration increases. The rate of transfer was determined in the presence of ATP at various $MgCl_2$ concentrations and is shown in Fig. 3. The observed rate constants appear to increase as $MgCl_2$ concentration is increased. However, this increase is much less pronounced in the presence of ATP than in the absence of cofactor. Thus, the qualitative effect of $MgCl_2$ concentration on the rate of transfer is similar in the presence of ATP, as well as in its absence; however, the extent of the $MgCl_2$ concentration effect is smaller.

The relative proportions of the two transferring species have also been shown to be influenced by $MgCl_2$ concentration in the absence of nucleotide cofactor. Fig. 4 shows the effect of $MgCl_2$ concentration on the proportion of the two components in the presence of ATP. The proportion of the two transferring species is not significantly affected by $MgCl_2$ concentration. Furthermore, the amount of fast component in these reactions is greatly reduced compared to reactions in the absence of ATP. Therefore, the data suggest that ATP inhibits the aggregation of the slow component to fast component.

Effect of NaCl Concentration on Transfer in the Presence of ATP—In both the absence and presence of ADP, salt concentration has been shown to affect the transfer rate of the recA protein (Menetski and Kowalczykowski, 1987). The rate was shown to be independent of NaCl concentration between 10 and 100 mM NaCl. Above 100 mM NaCl, the rate of transfer increased with salt concentration. The effect of increasing the salt concentration on the rate of transfer in the presence of ATP is shown in Fig. 5*A*. The data are presented as a log *k versus* log [NaCl] plot so that the slow and fast components data can be easily compared. A *decrease* in the observed rate constant for the transfer reaction is observed for both the fast and slow component. Note that the observed fast component rate constant decreases approximately 10-fold from 50 to 100 mM NaCl. Also, the observed slow component rate constant decreases approximately 5-fold over this range. This result is opposite to that found in the absence of ATP.

The effect of salt concentration on the relative amounts of the fast and slow components is shown in Fig. 5*B*. In the presence of ATP, increasing salt concentration increases the observed proportion of the fast component. This increase coincides exactly with a decrease in the relative proportion of the slow component. It is possible that, in the presence of ATP, increasing the salt concentration causes a change in the rate-determining step of the reaction. For example, since the apparent rates of transfer decrease as NaCl concentration is increased, it is possible that the rate of the fast and slow species approaches the rate of interconversion between the two species. The slow component could be converted to fast component, resulting in an increase in the amount of the fast transferring species observed. Note that the range of salt

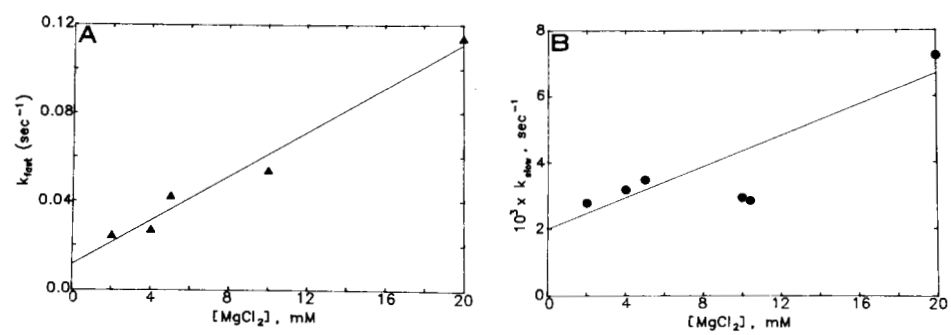


FIG. 3. **Magnesium chloride concentration dependence of the rate of transfer.** The reactions were conducted in standard buffer containing 250 μM ATP, 0.56 μM recA protein, 4.2 μM etheno M13 DNA, and 25 μM poly(dT). The rates of the fast component (*A*) and slow component (*B*) are shown.

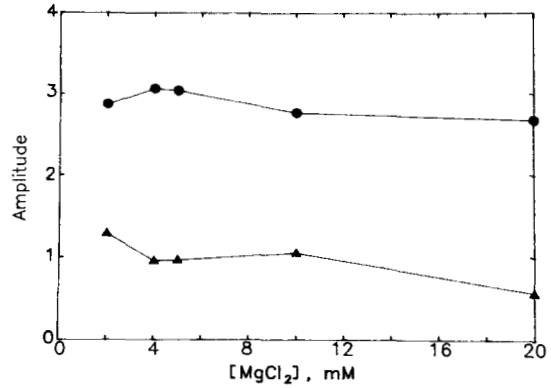


FIG. 4. **Magnesium concentration dependence of the amplitude of the fast and slow components.** Reactions were carried out in standard buffer containing 0.56 μM recA protein, 4.2 μM etheno M13, and 25 μM poly(dT), except that concentrated magnesium chloride stock was added to attain the final magnesium concentration. The amplitudes of the fast component (*triangles*) and the slow component (*circles*) are shown.

concentration that decreases the rate of transfer drastically, also coincides with the switching in the proportion of fast and slow component.

Effect of Nucleotide Concentration on Transfer Rate—The binding of ATP to recA protein has been shown to be noncooperative (Kowalczykowski, 1986). However, the rate of ATP hydrolysis catalyzed by recA protein has been shown to be cooperative with respect to ATP concentration (Weinstock *et al.*, 1981b). We have found that ATP concentration affects the rate of the transfer reaction in a complex manner. The observed rate of transfer as a function of ATP concentration is shown in Fig. 6. Surprisingly, at low concentrations of ATP (0–50 μM), the rate of transfer increases with increasing ATP concentration. This behavior is most easily seen in the apparent rate constant of the slow component. However, between 75 and 100 μM ATP, the rate of transfer decreases with ATP concentration. Above approximately 200 μM ATP, the rate of transfer is insensitive to ATP concentration.

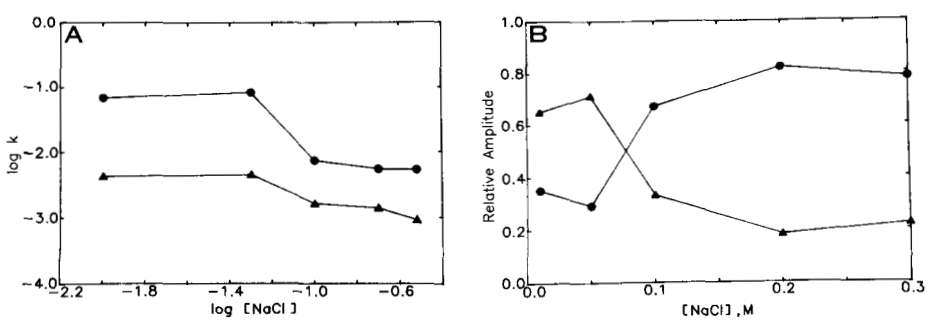
Experiments substituting ADP for ATP were conducted in an attempt to better understand the ATP concentration effect. Increasing ADP concentration increased the rate of transfer in an hyperbolic manner (Fig. 6*B*). For these data, only the observed rate constant for the slow component are presented because the rate of the fast component is too rapid to measure accurately. The ADP concentration at the half-maximal rate is about 130 μM; this is similar to the value of the binding constant of ADP to recA protein in the presence of single-stranded DNA (72–90 μM; Cotterill *et al.*, 1982). The kinetic data demonstrate that at low ATP concentrations (below 50 μM), ATP has a destabilizing effect on the lifetime of the recA protein-etheno M13 DNA complex that is qualitatively similar to the effect of ADP. However, ATP concentrations above 75 μM induce a state of recA protein that transfers more slowly.

Effect of Nucleotide Concentration on the Fluorescence of the recA Protein-etheno M13 DNA Complex—The binding of ATP and ATPγS to the recA protein-etheno M13 DNA complex results in an increased fluorescent intensity of this complex over that of the no cofactor complex (Silver and Fersht, 1982; Menetski and Kowalczykowski, 1985a). This is apparently due to a further disruption of the etheno M13 DNA base-stacking interactions through a change in conformation of the recA protein·DNA complex. To quantify the effect of ATP concentration on this structural change, the fluorescence of the recA protein-etheno M13 DNA complex measured at different ATP concentrations was divided by the fluorescence measured in the absence of ATP. The dependence of the magnitude of this relative fluorescence increase on ATP concentration is shown in Fig. 7. As the ATP concentration increases to approximately 150–200 μM ATP, the magnitude of the relative fluorescence becomes larger. Above approximately 200 μM ATP, the effect of ATP concentration on the relative fluorescence increase appears to saturate. As seen in Fig. 6, the rate of transfer decreases to its slowest rate at 200 μM ATP (this is most easily observed in the slow component). Thus, the ATP-induced conformation change of recA protein-etheno M13 DNA complex may be related to the induction of a high affinity state that causes a decrease in the rate of the fast and slow component Fig. 6. To investigate the relationship of this conformation change to ATPase activity, the rate of hydrolysis was determined at increasing ATP concentrations (Fig. 8). The data show that the rate of hydrolysis appears to plateau at about 75 μM ATP, well below the ATP concentration necessary to induce the full relative fluorescence increase. Therefore, the conformation change does not appear to be directly related to ATP hydrolysis activity.

Processivity of ATP Hydrolysis during Transfer—The number of ATP hydrolytic events that occur during the transfer of recA protein from one polynucleotide to another may give insight into the function of ATP hydrolysis during recA protein-catalyzed reactions. This quantity will be referred to as the processivity of ATP hydrolysis during transfer (N_{trans}) and will be defined as the number of ATP molecules hydrolyzed during one transfer event. The value of N_{trans} can be obtained from the ratio of the number of ATP hydrolytic events that occur over a given time interval, the turnover number or k_{cat}, to the observed rate constant of transfer at the same ATP concentration, k_{trans}. The value of k_{cat} was determined from ATPase assays conducted at conditions identical to the transfer experiments. Although turnover number normally is used to describe the maximal ATP hydrolysis rate of the enzyme, the value can be determined at submaximal rates in order to compare k_{cat} to k_{trans}.

The value of k_{cat} for ATP hydrolysis as a function of ATP concentrations is shown in Fig. 8. The values for N_{trans} shown in Fig. 8 were calculated using the rate constant for the fast component as well as the slow component; thus, there are two

FIG. 5. **Salt concentration dependence of the transfer reaction in the presence of ATP.** Reactions were carried out in standard buffer containing 0.56 μM recA protein, 4.2 μM etheno M13 DNA, 25 μM poly(dT), and 100 μM ATP. *A*, the salt concentration dependence of the rate of transfer for the fast (*circles*) and slow (*triangles*) component of transfer. *B*, the salt concentration dependence of the amplitudes of the fast (*circles*) and slow (*triangles*) component of transfer.



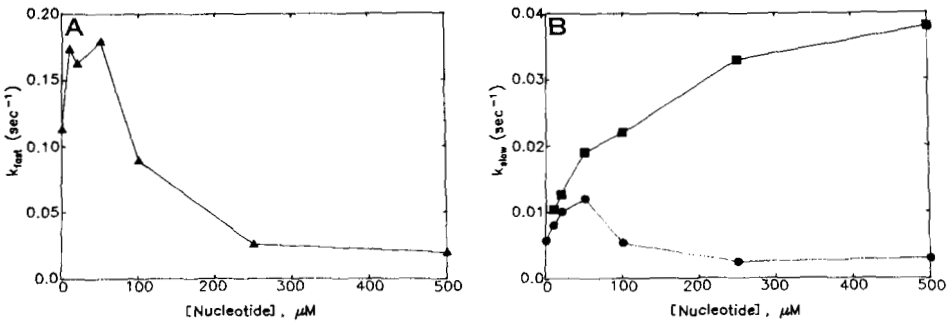


FIG. 6. **Nucleotide concentration dependence of the rate of transfer. Reactions were conducted in standard buffer containing 0.56 μM recA protein, 4.2 μM etheno M13, and 25 μM poly(dT).** *A*, the fast component in the presence of ATP (*triangles*). *B*, the slow component in the presence of ATP (*circles*) and ADP (*squares*).

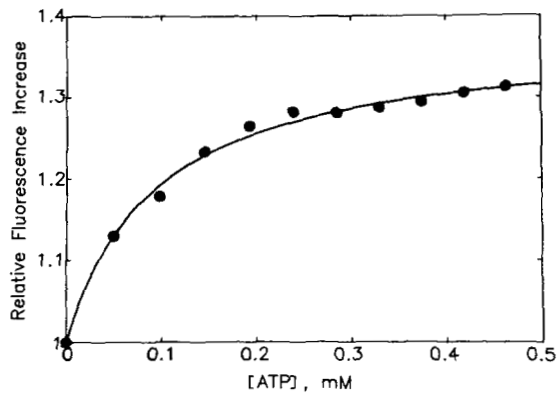


FIG. 7. **ATP concentration-dependent change in the fluorescence of the recA protein-etheno M13 DNA complex.** recA protein-etheno M13 DNA complexes (0.56 μM recA protein; 4.2 μM etheno M13 DNA) were formed in standard buffer containing 3 units/ml of pyruvate kinase and 1 mM phosphoenolpyruvate. A concentrated stock of ATP was then titrated into the complex and the increase in fluorescence was recorded after attaining a stable fluorescence value. The increase in fluorescence plotted is relative to the fluorescence of the recA protein-etheno M13 DNA complex observed in the absence of ATP.

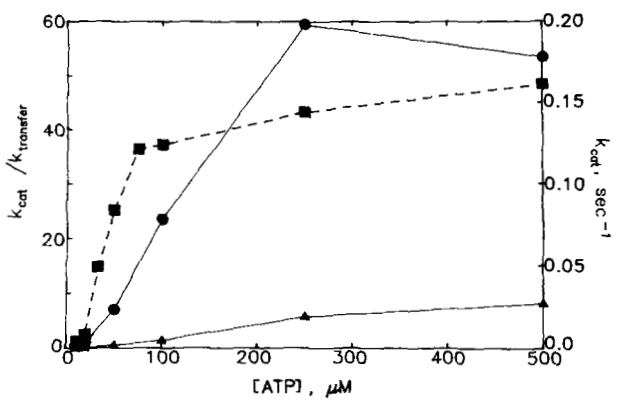


FIG. 8. **ATP concentration dependence of the processivity of ATP hydrolysis during the transfer reaction.** The reactions were carried out in standard buffer containing 0.56 μM recA protein, 4.2 μM etheno M13 DNA, and 25 μM poly(dT). The ATP hydrolysis rate, under identical conditions, is also plotted as a function of ATP concentration (*squares*). Values of N_{trans} were obtained from the ratio of k_{cat} to k_{trans} and were determined for the fast (*triangles*) and slow (*circles*) components of the transfer reaction.

values of N_{trans} for each ATP concentration. The data show that the number of ATP molecules hydrolyzed for each transfer event changes with ATP concentration. At low ATP concentration (below 20 μM), the ratio of k_{cat} to k_{trans} is very low (less than or equal to one) for both the fast and slow component. This is because little ATP hydrolysis occurs at low ATP concentrations, whereas transfer can proceed in the absence of ATP hydrolysis. The processivity of ATP hydrolysis during transfer for the slow component increases up to a maximum of approximately 50,[2] between 30 and 250 μM; above 250 μM, N_{trans} for the slow component remains relatively constant. The value of processivity for the fast component increases from 0.1 ATP per transfer at 10 μM ATP to approximately 8 ATP molecules per transfer at 500 μM ATP. The actual values for the observed processivity can differ by as much as 10-fold for the fast and slow component. This difference is solely due to the differences in the transfer rate constant for each of the two components. If the rate of transfer were directly coupled to the rate of hydrolysis, then we would expect the ratio of k_{cat} to k_{trans} to be constant. However, since N_{trans} varies with ATP concentration, these data suggest that the transfer process is not directly coupled to the rate of ATP hydrolysis.

DISCUSSION

It has been shown that recA protein transfers from etheno M13 DNA to poly(dT) via a complex kinetic mechanism (Menetski and Kowalczykowski, 1987). The proposed mechanism involves the formation of a ternary intermediate that includes etheno M13 DNA, recA protein, and poly(dT). This reaction was also shown to occur by two kinetically distinct components that are thought to be influenced by the aggregation state of recA protein. Because ATP binding and hydrolysis stimulates the renaturation reaction (McEntee, 1985) and are required during the strand exchange reaction (Cox and Lehman, 1981), we have analyzed the effect of ATP on the transfer reaction. In this study we have found that the rate of transfer is influenced by poly(dT), $MgCl_2$, NaCl, and nucleotide concentrations.

Previously it was found that, in the absence of ATP, the transfer rate increased with increasing competitor concentration. This result suggested that the mechanism of transfer did not occur by a simple dissociation process (Menetski and Kowalczykowski, 1987). Here we have shown that, in the presence of ATP, the apparent rate constant of transfer is dependent on poly(dT) concentration. These data, obtained in the presence of ATP, are also inconsistent with transfer occurring by a simple dissociation mechanism. However, in contrast to the result in the absence of ATP, high concentrations of poly(dT) have a slight but experimentally significant inhibitory effect on the rate of transfer (see Fig. 1). This observation does not fit the two-step mechanism that we have proposed for the transfer reaction in the absence of ATP. Thus, transfer in the presence of ATP must occur by a mechanism that is more complex than either a simple disso-

[2] The processivity of ATP hydrolysis by recA protein has been determined by an alternative method in this laboratory (S. C. Kowalczykowski and R. Krupp, manuscript in preparation). The processivity measured by that method may be more directly related to the number of ATP molecules hydrolyzed per dissociation event. Although, the values for processivity, as determined by that method, are approximately 5-fold less than those described here, the general effects of ATP concentration appear to be consistent with the effect of ATP concentration during the transfer reaction.

ciation or the two-step mechanism previously described. The reason for inhibition of the rate at high poly(dT) concentration and the details of the kinetic mechanism are unknown at this point.

Despite the fact that the transfer reaction is more complicated in the presence of ATP, many similarities to the transfer reaction in the absence of ATP can be observed. In the absence of cofactor, the fast and slow components have been shown to be affected by recA protein concentration (Menetski and Kowalczykowski, 1987). Increasing the concentration of recA protein first increased the amount of the slow transferring component then, as the formation of the slow transferring species saturated, the amount of the fast transferring component increased. In the presence of ATP, the proportion of fast and slow component was also affected by recA protein concentration in the same qualitative manner as in the absence and presence of ADP (see Fig. 2). Thus, the fast and slow components in the presence of ATP appear to represent kinetic species similar to those found in the absence of ATP.

In the absence of ATP, increasing $MgCl_2$ concentration was shown to increase the proportion of the fast component at the expense of the slow component (Menetski and Kowalczykowski, 1987). The amount of fast component is smaller, in the presence of ATP, than that observed in the absence of ATP (see Table I). Also, increasing $MgCl_2$ concentration had little affect on the relative amounts of the two components in the presence of ATP. Various studies on the aggregation properties of recA protein have demonstrated that ATP has an inhibitory effect on the formation of recA protein, or recA protein·DNA aggregates (Cotterill and Fersht, 1983; Tsang *et al.*, 1985; Roman and Kowalczykowski, 1986). Therefore, the results for the transfer reaction are in qualitative agreement with other aggregation processes and are consistent with our previous conclusion that the fast component is an aggregated form of the slow component (Menetski and Kowalczykowski, 1987).

Another similarity of the transfer reactions in either the presence or the absence of ATP is observed in the effect of magnesium concentration on the rate of transfer. In the absence of ATP, the rate of transfer for both the fast and slow component was shown to increase as magnesium concentration increased. This is the same qualitative behavior observed in the presence of ATP. However, the observed rate constant in the presence of ATP is affected less by magnesium concentration than it is in the absence of ATP.

Increasing the salt concentration was found to have two unexpected effects on the transfer reaction in the presence of ATP. First, in the presence of ATP the rate of the transfer reaction decreased by 5- and 10-fold for the fast and slow component, respectively, as the concentration of NaCl increased from 50 to 100 mM (see Fig. 5*A*). This result was surprising because the rate of transfer was observed to increase with increasing salt concentration in the absence or presence of ADP (Menetski and Kowalczykowski, 1987). The data in the absence of cofactor are consistent with the general expectation that the rate of dissociation of a protein from a nucleic acid would increase as salt concentration is increased (Lohman *et al.*, 1978). This behavior is observed for the dissociation of gene 32 protein from polynucleotides (Kowalczykowski *et al.*, 1980; Lohman, 1984a and 1984b). Thus, the observed decrease in the apparent transfer rate in the presence of ATP with increasing NaCl concentration seems anomalous. However, it is known that the rate of ATP hydrolysis decreases as salt concentration increases (Roman and Kowalczykowski, 1986; Kowalczykowski and Krupp, 1986). Since, the rate of transfer is not directly coupled to the rate of hydrolysis (see below), the decrease in transfer rate is probably not a direct consequence of the decrease in ATP hydrolysis rate. However, it may be due to the following indirect effect. A decrease in hydrolysis rate may result in an increase in the lifetime of the ATP·recA protein·DNA complex. Since the ATP bound form of recA protein has a higher affinity for single-stranded DNA (Menetski and Kowalczykowski, 1985a), then the observed transfer rate would actually decrease due to the expected greater kinetic stability of the high DNA-binding affinity state of recA protein. Thus, the decrease in the rate of transfer as NaCl concentration increases may arise indirectly from a decrease in the rate of hydrolysis.

The second effect of increasing NaCl concentration is that the relative proportion of fast component increases. We had anticipated the amount of fast component to decrease, due to disruption of recA protein aggregation. However, if the rate-limiting step for the transfer reaction in the presence of ATP changes as salt concentration increases, then the change in the amount of the fast component could be related to the change in the rate-limiting step. One possible explanation for this behavior is that at high salt concentations, the rate of the slow component is decreased to such an extent that it is similar to the rate of interconversion between the fast and slow transferring component (see Fig. 10, Menetski and Kowalczykowski, 1987). Therefore, as the transfer reaction proceeded, the slow component would be converted to the fast component at a similar rate to the transfer of the slow component. The interconversion equilibrium would be pulled toward the formation of the fast component because this kinetic species is more quickly depleted than the slow component. Thus, under these conditions, transfer would occur primarily through the fast transferring pathway. Since in the presence of ATP the apparent rates of the slow and fast components decrease significantly as salt concentration increases, it is likely that more interconversion would occur at higher salt concentration. Note that the increase in the fast component correlates exactly with the decrease in the rate of transfer between 50 and 100 mM NaCl.

The concentration of ATP has been shown to have a complicated effect on the transfer reaction (see Fig. 6). Increasing ATP concentration was shown to increase then decrease the rate of transfer. Below 50 μM ATP, an increase in ATP concentration increased the transfer rate (most easily observed in Fig. 6*B*). This is similar to the effect observed when ADP concentration is increased. However, above 75 μM ATP, increasing the ATP concentration decreases the rate of transfer. The reason for this biphasic influence of ATP concentration on the apparent rate of transfer is unknown. When the ATP dependence of the transfer reaction is compared to other ATP-dependent properties of recA protein, it appears that the effect of low ATP concentrations (below 75 μM ATP) on the rate of transfer is similar to their effect on the rate of ATP hydrolysis. At these concentrations, both the rate of transfer and the rate of hydrolysis increase with ATP concentration. However, above 75 μM ATP, there seems to be no relationship between the rates of transfer and hydrolysis, since the hydrolysis rate does not change while the transfer rate decreases.

At concentrations of ATP above 75 μM, we have shown that ATP affects the fluorescence properties of the recA protein-etheno M13 DNA complex. The fluorescence of the etheno M13 DNA molecule is enhanced upon binding of recA protein and is further enhanced by the addition of ATP to this complex. This additional fluorescence increase has been interpreted to suggest that ATP induces a conformation change in recA protein-etheno M13 DNA complex (Silver and

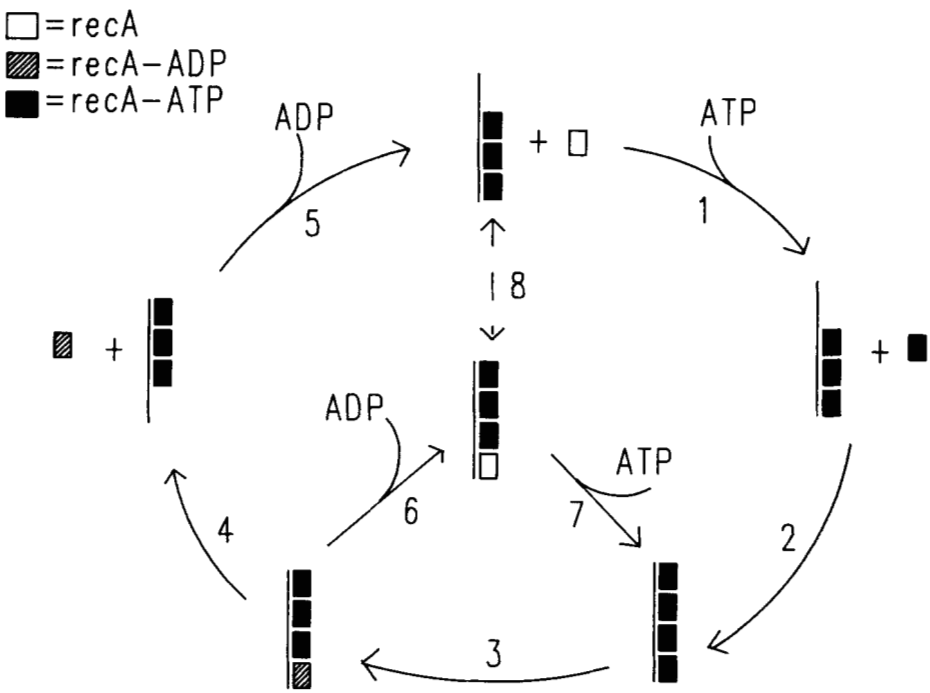


FIG. 9. **Extended single-stranded DNA-binding cycle of recA protein.** This cycle incorporates the possibility for the processive hydrolysis of ATP by recA protein. The outside pathway on this cycle is essentially the same as that described previously. *1*, recA protein binds ATP. The recA protein·ATP complex has a high affinity single-stranded DNA. *2*, this high affinity complex then binds to single-stranded DNA. *3*, ATP is hydrolyzed to ADP. The resultant recA protein·ADP complex has a low affinity for single-stranded DNA, and *4*, dissociates from the DNA. Finally, *5*, ADP dissociates from recA protein, and the cycle can be repeated. However, under some conditions, the recA protein·ADP complex can remain bound to single-stranded DNA, and *6*, ADP can dissociate from the recA protein. *7*, then another ATP can bind and undergo another round of hydrolysis, or *8*, the recA protein, with no cofactor bound, can dissociate from the DNA.

Fersht, 1982; Menetski and Kowalczykowski, 1985). In this paper we have shown that the magnitude of the ATP-induced fluorescence change increases with ATP concentration in a hyperbolic manner. The effect of ATP on the fluorescence appears to saturate at approximately 200 μM ATP. The specific nature of the ATP-induced conformation on the recA protein·DNA complex is unknown; however, the saturation point of the relative fluorescence increase corresponds closely to the ATP concentration necessary to fully induce the slow transferring form observed in Fig. 6.

There have been conflicting reports on the effect of ATP on the rate of transfer from one polynucleotide to another. Silver and Fersht (1982) reported that ATP decreased the rate of transfer relative to the rate in the absence of cofactor. However, Bryant *et al.* (1985) have shown that the rate of transfer is increased in the presence of ATP. A possible explanation for these disparate results is found in the ATP dependence of the transfer rate. Silver and Fersht (1982) used 770 μM ATP in their experiments. This concentration of ATP is sufficiently high to result in a decrease in the rate of transfer and, therefore, is consistent with our results. Bryant *et al.* (1985) used 200 μM ATP in their studies and, by direct comparison to our data, should have resulted in a decrease in the rate of transfer also. However, since they studied transfer between native φX174 single-stranded DNA molecules and not between etheno M13 and poly(dT) as we have, a direct comparison of the data may be inappropriate. Kowalczykowski (1986) has shown that recA protein binds ATP with a higher affinity in the presence of single-stranded DNA than in its absence. The ATP-binding data suggest that the binding affinities of recA protein for ATP and single-stranded DNA are linked. Since the affinity of recA protein for native single-stranded DNA is less than that for etheno M13, the binding affinity of ATP in the presence of these DNA molecules would be expected to also be lower. Therefore, since the affinity of recA protein for ATP is expected to be lower in the experiments of Bryant *et al.* (1985), it is likely that the effects of ATP that we have described above would have occurred at higher ATP concentrations. Thus, 200 μM ATP may be in a region of the curve (in Fig. 6) that would cause an increase in the rate of transfer relative to that in the absence of ATP when φX174 DNA substrates are used. Therefore, our data are consistent with that of Silver and Fersht (1982) and are likely to be consistent with that of Bryant *et al.* (1985) as well.

We have also determined the processivity of ATP hydrolysis during the transfer reaction (N_{trans}), or the number of ATP molecules hydrolyzed per transfer event, and have found that N_{trans} varies with ATP concentration. The value of N_{trans} appears to change from as little as 0.1 ATP molecules per transfer event at 10 μM ATP for the fast component, to as high as 50 ATP molecules per transfer event at 500 μM ATP for the slow component. Neuendorf and Cox (1986) have recently reported that the number of ATP molecules hydrolyzed during the exchange of recA protein between recA protein-single-stranded DNA complexes at 4 mM ATP and in the presence of *Escherichia coli* single-stranded DNA-binding protein, is approximately 200. They state, however, that due to uncertainties associated with their experimental procedure their value could be in error by a factor of two or three. Therefore, the data of Neuendorf and Cox (1986) appear to be in good agreement with that presented here. If the rate of transfer was directly coupled to the rate of ATP hydrolysis, then the value of N_{trans} should remain constant and equal to one. Since this is clearly not the experimental result, these data suggest that the rate of transfer is not directly coupled to the rate of ATP hydrolysis.

The processivity data, described above, allow us to further detail the binding cycle proposed in an earlier publication (Menetski and Kowalczykowski, 1985a). A more comprehensive version of the recA protein-binding cycle is shown in Fig. 9. Based on equilibrium data, we suggested that recA protein bound ATP and then bound single-stranded DNA (Menetski and Kowalczykowski, 1987). Upon binding to DNA, ATP was hydrolyzed. The ADP·recA protein complex could then dissociate from single-stranded DNA, release ADP and start the cycle again (*outer cycle* in Fig. 9). However, the equilibrium data were unable to determine whether only one ATP molecule was hydrolyzed per DNA-binding event or whether many ATP molecules were hydrolyzed before recA protein disso-

ciated from the DNA. The kinetic data described above allow us to further detail the relationship between the single-stranded DNA-binding cycle and the recA protein-dependent ATP hydrolytic cycle (although many kinetic details still remain to be elucidated). Since recA protein binds to single-stranded DNA in a polar manner (Register and Griffith, 1985), we have included this polar binding in the cycle shown in Fig. 9. The kinetic data described in this analysis suggest that the extent of processive ATP hydrolysis can vary significantly under different conditions. At low ATP concentrations, the hydrolysis cycle appears to follow the outside pathway on Fig. 9. ATP is bound, hydrolyzed, and then the ADP·recA protein complex dissociates from the DNA after the hydrolytic event. However, at high ATP concentrations, a new cycle must be added to the outer pathway. Under these conditions, it appears that ADP can dissociate from DNA-bound recA protein without dissociation of the recA protein. ATP can then bind to the recA protein·DNA complex. This effectively results in an exchange of ATP for ADP while recA protein is bound to DNA. The ADP·ATP exchange portion of the pathway allows the protein to hydrolyze many ATP molecules before it dissociates from the DNA molecule. Because the processivity of hydrolysis is influenced by ATP concentration (see Fig. 8), the data suggest that the rate-limiting step of this reaction which determines processivity involves the binding of ATP to recA protein.

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