UTP Is a Cofactor for the DNA Strand Exchange Reaction Performed by the RecA Protein of *Escherichia coli*†

Piero R. Bianco‡ and George M. Weinstock*

Department of Biochemistry and Molecular Biology, University of Texas Medical School, 6431 Fannin Street, P.O. Box 20708, Houston, Texas 77225

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ABSTRACT: The RecA protein of *Escherichia coli* is required for homologous genetic recombination and induction of the SOS regulon. In order for RecA protein to function in these two roles, a nucleoside triphosphate cofactor, usually ATP or dATP, is required. We have examined the ability of UTP to substitute for (r,d)ATP as nucleoside triphosphate cofactor. We have found that although UTP is hydrolyzed by RecA protein in the presence of long DNA molecules, it is not hydrolyzed in reactions in which the cofactors are oligodeoxyribonucleotides less than ∼50 nt in length. We show that UTP can efficiently substitute for ATP as nucleoside triphosphate cofactor for the DNA strand exchange reaction in vitro. The RecA1332 protein (Cys129 → Met), which was originally shown to be defective for homologous recombination in vivo, is able to perform DNA strand exchange in vitro with ATP, but is unable to do so with UTP. These results suggest that UTP may be a cofactor for DNA strand exchange in vivo. The inability of RecA protein to hydrolyze UTP with oligodeoxyribonucleotides as cofactor and the ability of RecA to utilize UTP as cofactor in DNA strand exchange suggest a separation of the functions of RecA protein into those that require exclusively ATP and those which can utilize additional nucleoside triphosphate cofactors.

The RecA protein of *Escherichia coli* can promote DNA strand annealing or DNA strand exchange in vitro. These are two key reactions thought to mimic the role of the enzyme in vivo (*33*, *39*, *41*). In order for RecA protein to function in these reactions, it must form a ternary complex known as the presynaptic filament. This filament consists of RecA protein monomers wrapped around the DNA with a stoichiometry of 3 nts/monomer and 6 monomers/turn (*8*, *9*), and can exist in two forms: an active and an inactive form. The inactive filament is formed in the absence of a nucleoside triphosphate cofactor and exists in a collapsed conformation with a helical pitch of 65 Å. In the presence of a nucleoside triphosphate cofactor, the filament exists in an extended conformation with a helical pitch of 95 Å (*10*); it is this extended conformation that is the active species in RecA protein-promoted reactions (*15*). Nucleoside triphosphate cofactors play a key role in the formation of this active species: it is the binding of the nucleoside triphosphate cofactor that is required to convert RecA protein into the high-affinity ssDNA binding state (*22*), which is the active conformation of RecA protein within the extended filament and a requirement for all the activities of the protein (*14*).

To carry out DNA strand exchange and DNA strand annealing in vitro, the presence of a nucleoside triphosphate cofactor is required; indeed, RecA protein is a DNA-dependent nucleoside triphosphatase (*43*). In addition to hydrolyzing ATP (the most widely used cofactor for RecA-promoted reactions), RecA protein is capable of hydrolyzing dATP,[1] (r,d)UTP, and (r,d)CTP (*43*), and under certain conditions, it is also able to hydrolyze (r)GTP (*23*). Menetski et al. demonstrated that all of the nucleoside triphosphate cofactors that are hydrolyzed by RecA protein are able to induce the high-affinity ssDNA binding state of the RecA, which is the active form of the protein (*22*). A more recent series of papers from the Bryant laboratory has shown that the ability of a nucleoside triphosphate cofactor to stabilize the active conformation of RecA protein in DNA strand exchange is directly related to the $S_{0.5}$ for that cofactor (where $S_{0.5}$ is the substrate concentration required for half-maximal velocity) (*18*, *24*, *36*, *37*). Only the nucleoside triphosphate cofactors whose $S_{0.5}$ value is 100−120 μM or lower are capable of stabilizing the strand exchange-active conformation of RecA protein. Of the nucleoside triphosphate cofactors tested, ATP ($S_{0.5}$ = 45 μM) and purine riboside

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* Corresponding author. Tel: (713) 500-6083. Fax: (713) 500-0652. E-mail: georgew@utmmg.med.uth.tmc.edu.

‡ Present address: Sections of Microbiology and of Molecular and Cellular Biology, University of California at Davis, Davis, CA, 95616. Tel: (530) 752-9027. Fax: (530) 752-9014. E-mail: prbianco@ucdavis.edu.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; dATP, deoxyadenosine 5′-triphosphate; UTP, uridine 5′-triphosphate; CTP, cytosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; PTP, purine riboside triphosphate; UTP[γ]S, uridine 5′-*O*-(γ-thiotriphosphate); ATP[γ]S, adenosine 5′-*O*-(γ-thiotriphosphate); ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RF, bacteriophage M13 replicative form DNA; SSB, single-stranded DNA binding protein of *Escherichia coli*; $S_{0.5}$, the substrate concentration required for half-maximal velocity.

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triphosphate (PTP; $S_{0.5}$ = 100 μM) satisfied this criterion (*24*).

ATP is the most widely used cofactor for studies of RecA protein-promoted reactions in vitro (*14*, *27*). In addition to binding, hydrolysis of this nucleoside triphosphate cofactor has also been shown to play a role in RecA protein-promoted activities. The hydrolysis of ATP is required to dissociate RecA protein from the heteroduplex products of DNA strand exchange once the reaction is complete (*20*, *29*, *35*), to facilitate the bypassing of structural barriers such as heterologous sequences (*11*, *30*), and to maintain polarity of the DNA strand exchange reaction (*12*, *32*).

UTP is the only other nucleoside triphosphate cofactor hydrolyzed by RecA protein for which a biochemical analysis has been done (*44*). The hydrolysis of UTP by RecA protein occurs at the same or overlapping site and is comparable to that of ATP, with both ATP and UTP having similar effects on the binding of RecA protein to DNA (*17*). The ssDNA-dependent UTP hydrolysis of RecA protein differs from ATP hydrolysis in that K_m^{UTP}, V_{max}, and the dependence on RecA protein concentration are all sensitive to pH (*44*). In addition, the ssDNA-dependent UTPase activity of RecA protein has a pH optimum of 6.0 (*44*), while the ssDNA-dependent ATPase activity has a very broad pH optimum (*43*). Watanabe et al. found, using circular dichroism at pH 7.5 in the absence of DNA, that UTP and dUTP produced similar spectral changes in RecA protein to those of ATP or dATP, although the magnitude of the spectral change was not as large (*40*). They also found that although the K_m for UTP is higher than that for ATP (*44*), these nucleoside triphosphates have similar K_d's (17 $\pm$ 7 μM and 18 $\pm$ 3 μM, respectively). Similar values for K_d in the presence of DNA were reported by Kowalczykowski using competition experiments; the K_d measured for ATP was 15 $\pm$ 3 μM and for UTP it was determined to be 13 $\pm$ 5 μM (*13*).

In contrast to ATP, however, UTP is unable to efficiently function in a number of reactions performed by RecA protein. It is inactive in ssDNA annealing (*41*), inactive in D-loop formation in the absence of single-stranded DNA binding protein (SSB) (*16*, *33*, *34*), and 20-fold less active in promoting dsDNA binding by RecA protein than ATP, and both rUTP and dUTP are 5-fold less active in stimulating the phage λ repressor cleavage activity of RecA protein than ATP (*41*). UTP[γ]S, in contrast to UTP, is able to promote the formation of both RecA protein-dsDNA and RecA protein−ssDNA complexes as efficiently as ATP[γ]S (*17*). It is therefore evident that although UTP is hydrolyzed by RecA protein and is able to partially substitute for ATP in a limited number of activities of RecA protein, the significance of these activities is unclear. It is also unclear whether UTP plays a role as a regulator of the ATP-dependent reactions of the protein perhaps as a competitor for binding, or whether it is a cofactor for additional activities not yet studied, such as in the DNA strand exchange reaction.

In our efforts to characterize a mutant RecA protein, RecA1332, in vitro, we discovered that UTP is an efficient cofactor for the DNA strand exchange reaction promoted by wild-type RecA. RecA1332 was originally shown to be defective for homologous recombination in vivo (*46*). We have recently shown that although RecA1332 is completely defective for all activities assayed in vivo, it possesses considerable activity in vitro (*2*). This mutant protein is able to perform the DNA strand exchange reaction with ATP as cofactor, albeit it less efficiently than wild type. Surprisingly when we tested UTP (the only other cofactor which is hydrolyzed efficiently by RecA protein), we discovered that wild-type RecA protein was able to perform DNA strand exchange efficiently with this cofactor, whereas the mutant protein was unable to do so. This result prompted us to examine this reaction more closely.

Inasmuch as UTP is present at significant concentrations in the cell (well above its K_m for RecA-promoted reactions) and is distinguished from other nucleoside triphosphates in its ability to promote some ATP-dependent reactions, UTP may play a role in the physiology of RecA reactions. To further understand the role of UTP in the function of RecA protein, we examine the hydrolysis of UTP in the presence of DNA cofactors of varying length. We have previously shown that RecA protein is able to hydrolyze ATP efficiently in the presence of single-stranded cofactors 30 nt and greater in length (*1*). In contrast, hydrolysis of UTP requires ssDNA cofactors >200 nt in length. We demonstrate the ability of UTP to substitute for ATP in the DNA strand exchange reaction in vitro in the presence of SSB, and we further show that a mutant RecA protein, RecA 1332 (Cys129 → Met), while being recombination-deficient in vivo, is proficient in the DNA strand exchange reaction in vitro. It is, however, able to perform this reaction only with ATP and not with UTP. Since the nucleoside triphosphate cofactor UTP substitutes efficiently for ATP in DNA strand exchange in vitro, we propose that UTP may be one of the cofactor(s) for recombination promoted by RecA protein in vivo.

MATERIALS AND METHODS

Materials. Phosphocellulose P11 was obtained from Whatman; ATP, ADP, UTP, and UDP were purchased from Boehringer Mannheim (Indianapolis, IN), and Polymin P was from BRL. [2, 8-^{3}H]ATP (specific activity = 25 Ci/mmol) and [5,6-^{3}H]UTP (specific activity = 25 Ci/mmol) were from NEN DuPont; plastic-backed polyethyleneimine cellulose sheets were from Brinkmann and Ecolume was from ICN. *Bam*HI restriction endonuclease was from Boehringer Mannheim, and single-stranded DNA binding protein was from Stratagene (La Jolla, CA).

DNA. Oligo d(T)'s were purchased either from Pharmacia (poly d(T) and oligo $d(T)_{10}$) or from Bio-Synthesis, Inc., Denton, Texas. The oligonucleotides were stored in 1xTE buffer (Tris-HCl-EDTA buffer, pH 8.0), at −20 °C, and initial concentrations were determined by measuring the absorbance at 260 nm, using ϵ = 8520 M^{-1} cm^{-1} to determine concentration (*38*). All concentrations are reported as total nucleotides. In all experiments, unless otherwise stated, oligonucleotides were always used in a 200-fold excess over protein. M13 replicative form DNA was linearized using *Bam*HI restriction endonuclease. The DNA was then extracted with phenol, precipitated with 100% ethanol, and then resuspended in 1xTE buffer. Single-stranded M13 phage DNA (M13 ssDNA) was prepared by infecting the *E. coli* strain JM101 with wild-type M13, at a multiplicity of infection of 1. The procedure used to purify single-stranded phage DNA was that of Neuendorf et al. (*25*). The concentrations of ssM13 DNA and M13 dsDNA were determined using ϵ = 8784 and 6500 M^{-1} cm^{-1} at 260 nm,

respectively (*19*). The purified DNA was dispensed into aliquots and stored in 1xTE buffer (pH 8.0) at −20 °C. All concentrations are reported as total nucleotides.

Purification of Wild-Type and RecA protein1332 Proteins. Wild-type RecA protein was purified from *E. coli* strain GE1171 containing the plasmid pGE226, a pBR327 based plasmid with the *tet* gene removed and replaced by a 3 kb fragment containing the *E. coli recA*$^+$ gene (*46*). RecA1332 protein was purified from strain GE1171 containing the plasmid pGE556, which is the same as pGE226, but carries the cysteine to methionine mutation at position 129. The procedure used to purify both wild-type and mutant RecA proteins was identical to that used previously for wild-type RecA protein (*1*). Proteins were stored in a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.1 mM EDTA, and 20% glycerol 1-mL aliquots at −80 °C. For RecA1332 protein, two separate preparations were made and these produced essentially identical results in all assays. Protein concentration was determined using amino acid analysis of hydrolyzed protein. For amino acid analysis, proteins were precipitated in 20% TCA, resuspended in distilled water, and then hydrolyzed in 6 N HCl at 110 °C for 20 h. The resulting hydrolysates were analyzed on an LKB Alpha Acid Analyzer (Pharmacia LKB).

Nucleotide Triphosphatase Assay. The hydrolysis of nucleotide triphosphates (NTP) by RecA protein was monitored using a thin-layer chromatography assay employing ^{3}H-nucleoside triphosphates as described by Weinstock et al. (*43*). The concentrations of NTP and nucleoside diphosphate (NDP) were determined at 259 nm, (pH 7.0) using ϵ = 15 400 M^{-1} cm^{-1} for ATP and ϵ = 10 000 M^{-1} cm^{-1} for UTP (*7*). Unless otherwise specified, reaction conditions were as follows: reaction mixtures (30 μL) contained 20 mM buffer (Tris-HCl, pH 8.0), 10 mM $MgCl_2$, 30 mM NaCl, 1 mM DTT, [2, 8-^{3}H]ATP (67 μCi/mL) or [5, 6-^{3}H]UTP (67 μCi/mL), and DNA at a final concentration of 200 μM. Reactions were conducted in 500-μL plastic eppendorf tubes at 37 °C. Aliquots (0.5 μL) were spotted onto PEI-cellulose strips (7 mm × 50 mm) containing NTP and NDP markers. The chromatography plates were developed in 1 M formic acid and 0.5 M LiCl. The NTP and NDP spots were identified with an ultraviolet lamp, cut out, and counted in scintillation fluid (Ecolume, ICN).

DNA Strand Exchange Assays and Gel Analysis. The method used to measure joint molecules and product molecule formation was that of Cox and Lehman (*6*), except that a Tris-acetate buffer system was employed as this buffer system has been shown to increase the reaction rate (*28*). Unless otherwise specified, reaction conditions were as follows: reaction mixtures (40 μL) contained 25 mM Tris-acetate, (pH 8.0), 10 mM magnesium acetate, 5% glycerol, 3.05 mM ATP, or 0.43 mM ATP[γ]S or 3.0 mM UTP, 1 mM DTT, 3.0 μM wild-type RecA or 3.0 μM RecA1332 protein, 8 μM M13 ssDNA, 15.8 μM M13 dsDNA, and SSB at 1 μM. Reactions were conducted in 500-μL plastic eppendorf tubes at 37 °C and initiated by the simultaneous addition of NTP and SSB, following a 10 min preincubation of all other components at 37 °C. Reactions were quenched by the addition of EDTA (15 mM final) and SDS (1.15% final) at indicated time points. Ficoll gel loading dye (5 μL) (*31*) was added, and the entire sample was loaded onto a 0.8% agarose gel and subjected to electrophoresis in a TAE buffer system (40 mM Tris-acetate and 2mM EDTA) at 0.96 V/cm for 14 h. Gels were then washed in 50 mM EDTA for 1 h, rinsed for 1 h in dH_2O with three changes of water, and then stained with ethidium bromide (0.5 μg/mL in dH_2O) for 1 h.

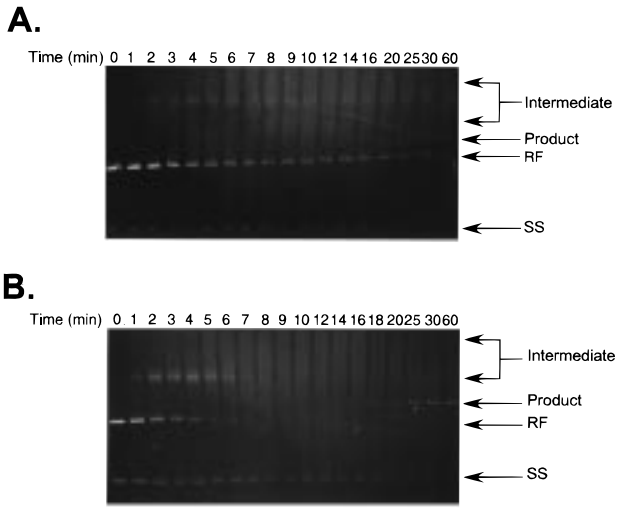

FIGURE 1: DNA Strand exchange reactions with ATP or UTP as cofactors. DNA strand exchange reactions with wild-type RecA protein using either ATP or UTP as cofactor were conducted as described in Materials and Methods: A, 3.0 mM ATP (initial concentration); B, 3.0 mM UTP (initial concentration); SS = M13 ssDNA; RF = M13 replicative form linearized with *Bam*HI restriction endonuclease.

The stained gels were photographed and the negatives scanned using a UMAX630 24-bit scanner interfaced with a Macintosh computer. The images were analyzed using the "Gel Analysis Macro" of the program NIH Image v1.49, which is public domain software. The amount of each species present was calculated as a percent of the amount of dsDNA of the lane in which the species is present.

RESULTS

Effects of UTP on DNA Strand Exchange. Strand exchange with ATP as cofactor in the presence of the single-stranded DNA binding protein has been well characterized (*5*, *28*). These reactions are routinely done in an acetate-based buffer system, with an ATP regenerating system. For the experiments presented here, we did not include a regenerating system since we are not aware of one to regenerate UTP and, furthermore, all experiments were done in the presence of the SSB protein of *E. coli*. In Figure 1, gels comparing the ability of UTP and ATP to function as cofactors in strand exchange in the presence of the single-stranded DNA binding protein of *E. coli* are shown, and the analysis of these two reactions is presented in Figure 2. There are two immediately obvious differences between the two reactions. The first is that the amount of joint molecule species formed is higher in the UTP reaction and the second is the reduced decline in the ATP reaction in the intensity of the band corresponding to RF, the linear duplex substrate. In both reactions, joint molecule formation was observed to begin occurring within the first minute of the reaction (Figure 2). For the ATP reaction, joint molecules formed at a rate of 2%/min, reached a maximum of 25−30% in 6 min, in different experiments, and then decreased at a rate of 2.2%/min to a level that was undetectable in the gel in 16 min

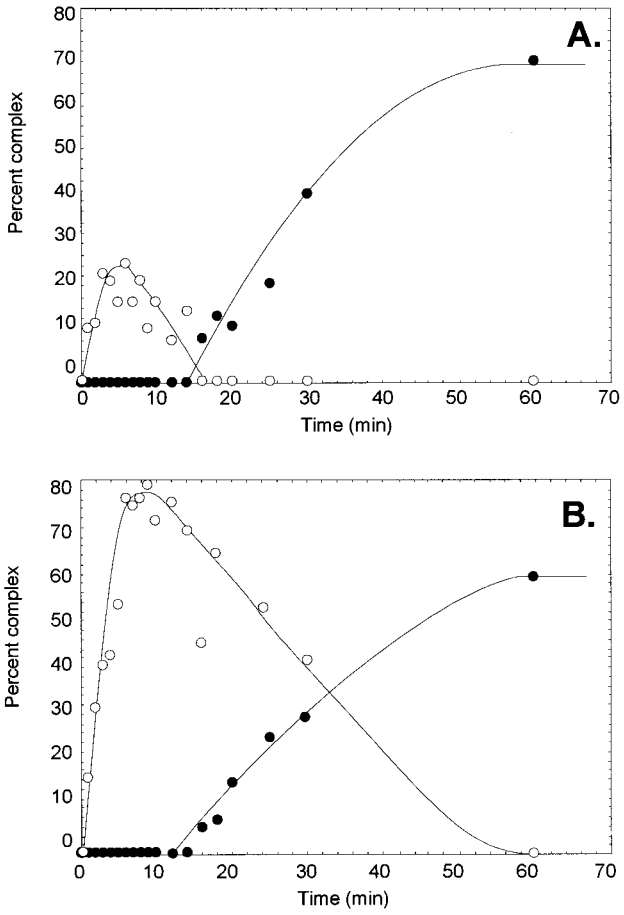

FIGURE 2: Analysis of the DNA strand exchange gels shown in Figure 1. Gels were analyzed using the program NIH Image 1.49. The amount of each species present is expressed as a percent of the total dsDNA species present in the lane: A, reaction with ATP as cofactor; B, reaction with UTP as cofactor; ○, joint molecule intermediates; ●, product (nicked circle).

(Figure 2A). Product formation began to appear at 8−14 min, increased at a rate of 1%/min, and reached a maximum at 60 min of 50−70% in different experiments (Figure 2A). For reactions containing UTP as cofactor, intermediates formed at a rate of 13%/min and reached a maximum of 70−90% (in different reactions) in 6 min. These species decreased at a rate of 2%/min, from the maximum to a low level that was undetectable in the gel (Figure 2B). Product began to appear at 14 min, increased at a rate of 2%/min, and reached a maximum of 40−60% in different experiments (Figure 2B). Thus UTP is able to substitute for ATP in strand exchange. Using these conditions, UTP is more efficient than ATP in the accumulation of joint molecule intermediates and as efficient as ATP in the formation the nicked circle product.

There was a significant difference between the rate of utilization of the dsDNA substrate in the ATP and UTP reactions, however. This is shown in Figure 3. In the ATP reaction, the amount of RF decreased from 100%, at a rate of 7%/min, to a low level in about 20 min of between 20% and 30% (Figure 3). In contrast, with UTP as cofactor, RF decreased from an initial level of 100% to a level that was undetectable in the gels within 10 min, corresponding to a rate of decrease of 10%/min. Thus the rate of utilization or disappearance of the double-stranded substrate in both the ATP and UTP reactions was similar, but the extent was

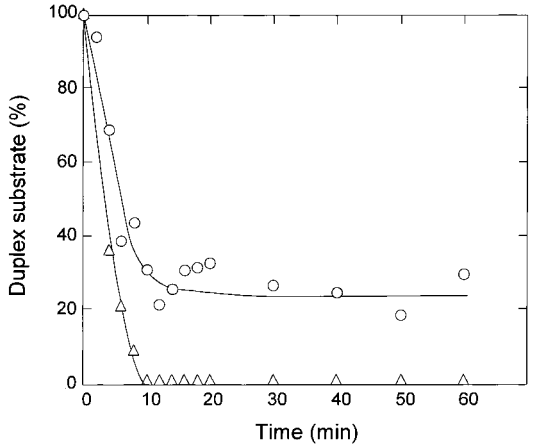

FIGURE 3: Rate of disappearance of the linear, duplex substrate in DNA strand exchange. Gels were analyzed as described in Materials and Methods, except that the percent duplex substrate is expressed as a percent of the amount of duplex substrate present at time 0: ○, strand exchange reaction in the presence of ATP; △, strand exchange in the presence of UTP. Initial nucleoside triphosphate concentrations were 3 mM.

greater for UTP than for ATP. This corresponds with an overall increase in the amount of joint molecule intermediates formed in reactions where UTP is present instead of ATP.

Strains containing the *RecA protein 1332* allele have been shown to be defective for recombination using both conjugation and transduction assays (*2*, *46*). In vitro, the purified RecA 1332 protein possessed DNA-dependent ATPase activity that was reduced compared to wild type, and surprisingly, the mutant was able to perform the DNA strand exchange reaction with ATP as cofactor (Figure 4). It was as efficient as the purified wild-type protein in forming joint molecules (data not shown), but was reduced 3−4-fold compared to wild type in its ability to convert these to product (Figure 4A). In contrast, while the wild type is able to perform DNA strand exchange with UTP, RecA1332 is completely defective in this reaction using UTP as cofactor (Figure 4B). This may partially explain the deficiency of this mutant in recombination in vivo, and provides further evidence for the role of UTP as a cofactor for DNA strand exchange.

Effects of Chain Length on UTPase Activity. In Figure 5, the effects of ssDNA chain length on k_{cat} for the UTPase activity of RecA protein are shown. For comparison, the effects of chain length on the ATPase activity of RecA protein are also shown (●). The standard to which all chain lengths were compared was M13 ssDNA. Under the conditions of our assays with M13 ssDNA, RecA protein was able to efficiently hydrolyze both ATP and UTP with a k_{cat} of between 16 and 24 min^{-1} (Figure 5). These numbers are similar to published turnover numbers for RecA protein (*1*, *13*, *43*, *45*).

Previously, we demonstrated that ssDNA length affects the turnover number for hydrolysis of ATP in a length-dependent fashion (*1*). Surprisingly, a different length dependence for the UTPase activity than for the ATPase activity was found (■; Figure 5). Longer chain lengths were required for UTPase activity than for ATPase activity. Oligo $d(T)_{40}$ and oligo $d(T)_{30}$ were reduced 7-fold compared to the ATPase reaction; oligo $d(T)_{25}$ was reduced 5-fold and oligo $d(T)_{20}$ was reduced 15-fold. This effect was independent of

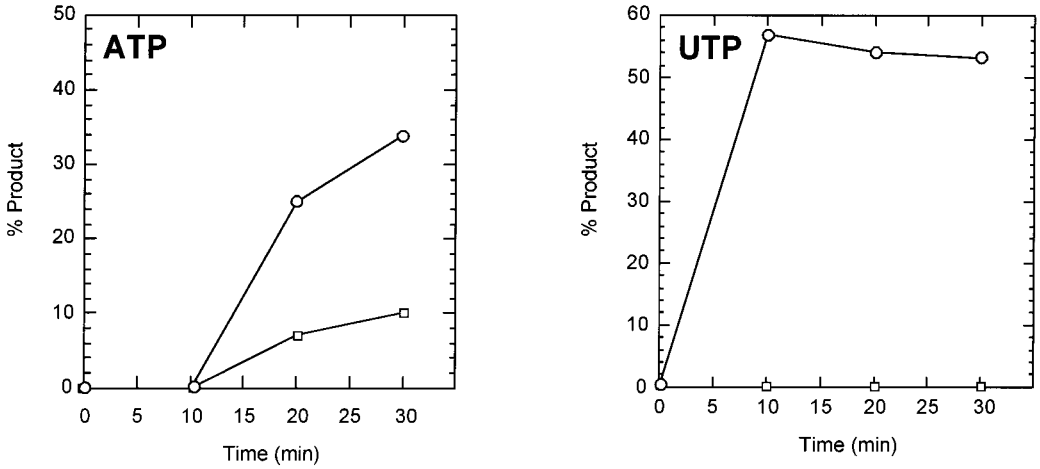

FIGURE 4: Comparison of the effects of ATP and UTP on product formation in DNA strand exchange performed by wild-type and mutant RecA proteins. Reactions were carried out as described in the legend to Figure 1: A, ATP (2.6 mM initial); B, UTP (3.9 mM initial); ○, wild-type RecA protein (2.99 μM); □, RecA1332 protein (2.99 μM).

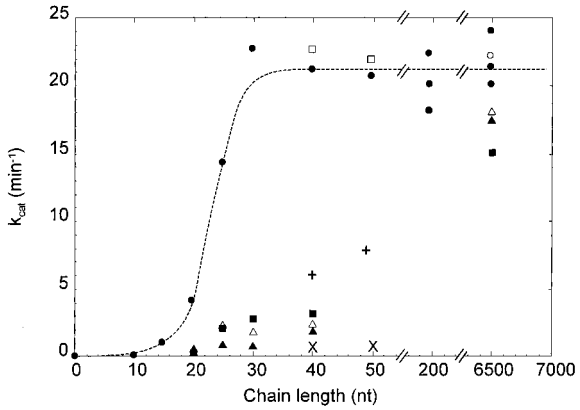

FIGURE 5: Effects of ssDNA chain length on k_{cat} for nucleoside triphosphatase activity. Assays were conducted for 60 min and k_{cat} was determined from the early, linear portion of time courses, with initial NTP concentration = 1067 μM. Dashed line and ●, hydrolysis of ATP with polymers of oligodeoxythymidilic acid and M13 ssDNA (length 6500 nt) done at pH 8.0 (Tris-HCl buffer). Hydrolysis of UTP with polymers of d(T) and M13 ssDNA at pH 8.0 (Tris-HCl buffer, ■); pH 7.0 (potassium phosphate buffer, ▲); and pH 6.2 (sodium maleate buffer, △). Two other symbols used were □, hydrolysis of ATP with mixed composition oligodeoxyribonucleotides as ssDNA cofactor at pH 8.0 (Tris-HCl buffer); and +, hydrolysis of UTP with mixed composition oligodeoxyribonucleotides as ssDNA cofactor at pH 6.2 (sodium maleate buffer) and pH 8.0 (×; Tris-HCl buffer).

pH, since these oligonucleotides showed low activity when assayed at pH 6.2, 7.5, and 8.0 (△, ▲, and ■ respectively; Figure 5). The inability of short chain length ssDNA cofactors to stimulate the UTPase of RecA protein is not unique to polymers of d(T), since random sequence oligonucleotides were also unable to simulate the UTPase of RecA protein to the levels seen in the ATPase reaction (ATP, □ vs UTP, + and ×; Figure 5). A larger effect of pH was observed with these DNA substrates, however.

Standard UTPase reactions utilized 200 μM nucleotides of DNA. To determine whether ssDNA affinity was rate-limiting, experiments were conducted comparing the effects of oligodeoxyribonucleotides on the ATPase and UTPase activities of RecA protein at 200 and 2000 μM ssDNA. These data are shown in Table 1. For oligo $d(T)_{40}$ and M13 ssDNA, increasing the DNA concentration 10-fold, in either the ATPase or the UTPase reactions, did not significantly

Table 1: Effects of DNA Concentration on the UTPase Activity of RecA Protein

	k_{cat} (min^{-1})[a]			
	200 μM DNA		2000 μM DNA	
DNA	ATP[b]	UTP[c]	ATP	UTP
oligo $d(T)_{25}$	14.4	0.27	13.3	2.7
oligo $d(T)_{40}$	22	1.7	19.5	2.01
M13 ssDNA	25	15.0	24.0	13.0

[a] NTPase assays were conducted as described in Materials and Methods. Reactions were conducted for 50 min. The values for k_{cat} were determined from the slope of time courses with the initial [NTP] as indicated. [b] Initial [ATP] = 907 μM. [c] Initial [UTP] = 1000 μM.

affect k_{cat}. Thus, the reduced rate of the UTPase reaction with these chain lengths is not due to reduced affinity. The same increase in oligo $d(T)_{25}$ concentration produced a 10-fold increase in k_{cat} for the hydrolysis of UTP. No increase in k_{cat} for hydrolysis of ATP was observed, however which is consistent with our previous findings (*1*). Thus, for oligo $d(T)_{25}$ (a DNA length which does not fully stimulate the ATPase of RecA protein), the affinity of RecA protein for this DNA is a contributing factor to the ability of this oligonucleotide substrate to stimulate the UTPase activity of the protein but the reaction at higher DNA concentration still does not occur at the rate of the ATPase.

In addition to affecting k_{cat}, ssDNA chain length also affects the extent of ATP hydrolysis (*1*). As for the hydrolysis of ATP, a length dependence on extent of UTP hydrolysis was also observed (data not shown). There was less of a reduction due to chain length on the extent of the UTPase than there was on k_{cat}, however. This reduction of extent of reaction was also independent of pH. Although there is a pH optimum for the UTPase reaction with large chain lengths (*44*), the effects of shorter ssDNA chain lengths were independent of pH as they were for k_{cat}.

Purified RecA1332 protein has been shown to have ATPase activity that is reduced 2−4-fold compared to wild type (*2*). The pH and temperature profiles for this activity were also similar to that of wild type, except that they were reduced 2−4-fold. We examined the effects of pH on the hydrolysis of UTP by the mutant protein, with single- and double-stranded DNA as cofactor. With both DNAs, wild-type RecA protein was able to hydrolyze UTP efficiently at

Table 2: Effects of pH on the Hydrolysis of UTP by Wild-Type and Mutant Proteins

		k_{cat} (min^{-1})[a]		
protein	DNA	pH 6.2	pH 7.0	pH 8.0
RecA protein$^+$	ssM13	24	17	19
	linear RF[b]	27	13	12
RecA1332 protein	ssM13	5	1	0.04
	linear RF	3	2	ND[c]

[a] UTPase assays were conducted for 20 min. The values for k_{cat} were determined from the slope of the linear portion of the time course with initial [UTP] = 1067 μM. [b] RF = replicative form of phage M13 linearized with *Bam*HI. [c] ND = hydrolysis of UTP by the mutant protein under these conditions was not detectable.

all pH's tested, with k_{cat} for the ssDNA-dependent UTPase ranging from 17 to 24 min^{-1}, and 12−27 min^{-1} for the dsDNA-dependent reaction (Table 2). Higher k_{cat} values were determined for the dsDNA-dependent UTPase activity at pH 6.2, which is consistent with previously reported data showing a pH optimum for this activity of RecA protein of 6.2 (*44*). In contrast to this, RecA1332 protein was unable to efficiently hydrolyze UTP using either ssDNA or dsDNA cofactors (Table 2). For the reactions with ssDNA as cofactor, k_{cat} was reduced 5-fold at pH 6.2 and 475-fold at pH 8.0. A similar effect was observed with dsDNA, with k_{cat} reduced 9-fold at pH 6.2, and was undetectable at pH 8.0 (Table 2). Previously, we tested the ability of RecA1332 protein to utilize short oligonucleotides as cofactor for the ATPase reaction (*2*). In contrast to wild type (*1*), RecA1332 protein was unable to utilize oligonucleotides 50 nt and less in length as ssDNA cofactor for the hydrolysis of ATP. These data are consistent with a defect in monomer-monomer interactions within the nucleoprotein filament formed by this mutant protein (*2*).

DISCUSSION

The experiments presented here show that UTP is able to substitute for ATP in the DNA strand exchange reaction promoted by RecA protein in vitro and, further, UTP is not appreciably hydrolyzed by RecA when the DNA cofactor is less than 200 nt in length. These data demonstrate that although a nucleoside triphosphate cofactor must have an $S_{0.5}$ less than ∼120 μM in order to stabilize the strand exchange-competent form of RecA protein which UTP is capable of doing, the presynaptic complex formed in the presence of UTP, although similar, may not be identical to that formed in the presence of ATP.

It was previously shown that RecA protein was capable of hydrolyzing (r,d)UTP in addition to (r,d)ATP (*43*). In addition, the K_m for UTP was similar to that of ATP (33−147 μM vs 10−103 μM, respectively), and the Hill coefficient for UTP binding with ssDNA at pH 8.0 was greater than 3, as was the case for ATP (*44*, *45*). Menetski et al. showed that all of the nucleoside triphosphate cofactors hydrolyzed by RecA protein were capable of inducing the formation of the high-affinity ssDNA binding state of RecA protein, which is the active form of the protein (*22*). These cofactors included UTP, (r,d)CTP, and (r,d)ATP. Kowalczykowski (*13*), and Watanabe et al. (*40*) have shown that the K_d values for both ATP and UTP are essentially the same (15 μM and 13 μM, respectively). These data suggest that UTP is able to interact efficiently with RecA protein in a manner analogous to that of ATP and, by so doing, is able to convert the enzyme into the high-affinity ssDNA-binding state, which is a requirement for activity of the enzyme.

Recently, several reports from the Bryant laboratory have shown that binding of a nucleoside triphosphate cofactor to RecA protein bound to ssDNA causes an isomerization of the protein from the inactive to the active form (*18*, *24*, *36*, *37*). They further showed that the ability of a nucleoside triphosphate cofactor to induce an isomerization in RecA protein to the active form is dependent on the $S_{0.5}$ value for that cofactor: the nucleoside triphosphate cofactors whose $S_{0.5}$ value is 100−120 μM or lower are capable of stabilizing the strand exchange-active conformation of RecA protein. These studies used ATP, PTP ($S_{0.5}$ values less than 120 μM), and ITP and GTP ($S_{0.5}$ values greater than 120 μM). ATP and PTP were capable of functioning as cofactors in DNA strand exchange, whereas ITP and GTP were not (*24*). In the present study, we show that UTP, which is able to induce the formation of the high-affinity ssDNA binding state of RecA protein (*22*), is also capable of functioning as a cofactor for DNA strand exchange in vitro. This implies that UTP is able to stabilize the strand exchange-active conformation of RecA protein, even though the affinity of RecA protein for this nucleoside triphosphate is slightly higher than 120 μM (at pH 8.0, the pH of the reactions performed here).

RecA protein is able to efficiently hydrolyze UTP and ATP with large DNA cofactors (*43*, *44*). The ATPase activity of RecA protein is affected by ssDNA cofactor in a length-dependent manner (*1*, *4*). Cooperative interactions between RecA protein monomers, bound contiguously along the DNA backbone, are essential to ATPase activity (*1*, *21*, *45*). Further, a RecA protein monomer only becomes a fully active ATPase when it is part of a cluster of monomers which has exceeded a certain size, bound contiguously along the ssDNA backbone (*13*). Full ATPase activity was achieved with a ssDNA chain length of 30 nt, suggesting that this cluster has a minimum size of 10 RecA protein monomers (*1*). In the present study, it was found that UTP is not appreciably hydrolyzed when the ssDNA cofactor is less than ∼200 nt in length. This means that the complex of RecA monomers bound to DNA that is required for full UTPase activity is significantly larger than the minimum size of 10 required for full ATPase activity. In addition, the affinity of RecA protein for these ssDNA substrates is also a factor that contributes to the effects of ssDNA chain length on UTPase activity, since raising the ssDNA concentration 10-fold produced an increase in k_{cat} for UTP hydrolysis. This is in contrast to the ATP hydrolysis reaction where the affinity of RecA protein for oligonucleotides less than 50 nt in length is not a factor contributing to full ATPase activity (*1*). Finally, an additional factor contributing to the inability of short oligonucleotides to function as DNA cofactor for UTP hydrolysis is the reduction in affinity of RecA protein for ssDNA induced by UDP, one of the products of the hydrolysis reaction. The reduction in affinity induced by UDP has been shown to be 2-fold greater than that caused by ADP as determined by the salt titration midpoint (*22*). A simple hypothesis to account for these observations is that RecA oligomerization is important for stable binding to oligonucleotides and for NTP hydrolysis. ATP binding is proposed to facilitate or stabilize these protein−protein interactions, while UTP does not. However, a sufficiently

stable structure can be formed in the presence of UTP on a long DNA molecule, presumably because of the larger number of RecA molecules involved. The accumulation of strand exchange intermediates in the UTP reaction could also be related to a deficient protein−protein interaction, needed for example to move from the initial strand exchange structure to a branch migration mode.

The differential effects of DNA chain length may have biological significance. Short oligonucleotides, or short ssDNA gaps may be one of the signals for SOS induction in vivo. UTP is not hydrolyzed with short chain lengths, and in addition, it is not utilized by RecA protein as cofactor in the phage λ repressor cleavage reaction in vitro; in contrast, ATP is hydrolyzed efficiently with DNA chain lengths as short as 30 nt and does support repressor cleavage in vitro (*1*, *42*). These in vitro findings suggest that ATP, and not UTP, may be the cofactor for these reactions in vivo.

The finding that UTP substitutes efficiently for ATP in strand exchange suggests that UTP may be a cofactor for recombination processes in vivo. This proposal is further supported by the finding that RecA1332 protein, while being completely defective for homologous genetic recombination in vivo, is proficient for strand exchange in vitro in the presence of ATP but not with UTP. Analysis of the values for the K_d for binding of nucleoside triphosphates reveals that, with the exception of CTP, all of these nucleoside triphosphates have the capacity to form a complex with RecA protein (*13*, *40*). Furthermore, all of the nucleoside triphosphates that are hydrolyzed by RecA are able to promote the formation of the high-affinity ssDNA binding state of the enzyme which is a prerequisite for activity; this includes both ATP and UTP (*22*). It is logical to suggest, then, that some fraction of RecA protein may in fact be associated with these nucleoside triphosphates in vivo, as was suggested by Kowalczykowski (*13*). On the basis of the assumption that the intracellular concentrations of nucleoside triphosphates in *E. coli* are similar to those reported for *Salmonella typhimurium* by Bochner and Ames (*3*), Kowalczykowski proposed that up to 22% of the RecA protein present in a cell may be complexed with UTP. The in vivo concentration of ATP is approximately 3000 μM and that of UTP is 894 μM (*26*); these concentrations are in sufficient excess of the K_m's of both ATP (10−103 μM) and UTP (33−147 μM) to allow for binding and activity of the enzyme in vivo (*44*, *45*). These calculations, taken together with our results demonstrating that UTP is an efficient nucleoside triphosphate cofactor for DNA strand exchange, suggest a role for UTP as a cofactor for some, but not all, RecA-promoted reactions in vivo.

The fact that UTP substitutes for ATP in some activities of RecA protein but not others suggests a separation of the functions of RecA protein into activities that are exclusively ATP-requiring and those which can utilize both nucleoside triphosphate cofactors. The activities which are exclusively ATP-dependent are the induction of the SOS response, which involves cleavage of repressors such as LexA and prophage λ, UV-induced DNA repair, and perhaps D-loop formation (*16*, *17*, *33*, *34*, *41*). These activities may all require short stretches of ssDNA, and our data showing that nucleoprotein filament formation on short oligonucleotides is not supported by UTP would be consistent. Those activities where a substitute such as UTP can be used as cofactor would be homologous genetic recombination where the DNA substrate is of sufficient length (i.e., >200 nt) to allow for efficient presynaptic filament formation.

REFERENCES

1. Bianco, P. R., and Weinstock, G. M. (1996) *Nucleic Acids Res. 24*, 4933−9.
2. Bianco, P. R., and Weinstock, G. M. (1997) *Genes to Cells* (in press).
3. Bochner, B. R., and Ames, B. N. (1982) *J. Biol. Chem. 257*, 9759−69.
4. Brenner, S. L., Mitchell, R. S., Morrical, S. W., Neuendorf, S. K., Schutte, B. C., and Cox, M. M. (1987) *J. Biol. Chem. 262*, 4011−6.
5. Cox, M. M., Soltis, D. A., Livneh, Z., and Lehman, I. R. (1983) *Cold Spring Harbor Symp. Quant. Biol. 2*, 803−10.
6. Cox, M. M., and Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3433−7.
7. Dawson, R., Elliott, D., Elliott, W., and Jones, K. (1968) *Data for Biochemical Research, 2nd ed., Data for Biochemical Research*, Oxford Press, Oxford.
8. Di Capua, E., Engel, A., Stasiak, A., and Koller, T. (1982) *J. Mol. Biol. 157*, 87−103.
9. Dombroski, D. F., Scraba, D. G., Bradley, R. D., and Morgan, A. R. (1983) *Nucleic Acids Res. 11*, 7487−504.
10. Egelman, E. H., and Stasiak, A. (1986) *J. Mol. Biol. 191*, 677−97.
11. Kim, J. I., Cox, M. M., and Inman, R. B. (1992) *J. Biol. Chem. 267*, 16438−43.
12. Konforti, B. B., and Davis, R. W. (1992) *J. Mol. Biol. 227*, 38−53.
13. Kowalczykowski, S. C. (1986) *Biochemistry 25*, 5872−81.
14. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D., and Rehrauer, W. M. (1994) *Microbiol. Rev. 58*, 401−65.
15. Kowalczykowski, S. C., and Krupp, R. A. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3478−82.
16. McEntee, K., Weinstock, G. M., and Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2615−9.
17. McEntee, K., Weinstock, G. M., and Lehman, I. R. (1981) *J. Biol. Chem. 256*, 8835−44.
18. Meah, Y. S., and Bryant, F. R. (1993) *J. Biol. Chem. 268*, 23991−6.
19. Menetski, J. P., Varghese, A., and Kowalczykowski, S. C. (1992) *J. Biol. Chem. 267*, 10400−4.
20. Menetski, J. P., Bear, D. G., and Kowalczykowski, S. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 21−5.
21. Menetski, J. P., and Kowalczykowski, S. C. (1985) *J. Mol. Biol. 181*, 281−95.
22. Menetski, J. P., Varghese, A., and Kowalczykowski, S. C. (1988) *Biochemistry 27*, 1205−12.
23. Menge, K. L., and Bryant, F. R. (1988) *Biochemistry 27*, 2635−40.
24. Menge, K. L., and Bryant, F. R. (1992) *Biochemistry 31*, 5151−7.
25. Neuendorf, S. K., and Cox, M. M. (1986) *J. Biol. Chem. 261*, 8276−82.
26. Neuhard, J., and Nygaard, P. (1987) in *Escherichia coli and Salmonella typhimurium. Cellular and Molecular Biology* (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., Eds.), pp 445−73, American Society for Microbiology, Washington, D.C.
27. Roca, A. I., and Cox, M. M. (1997) *Prog. Nucleic Acid Res. Mol. Biol. 56*, 129−223.
28. Roman, L. J., and Kowalczykowski, S. C. (1986) *Biochemistry 25*, 7375−85.
29. Rosselli, W., and Stasiak, A. (1990) *J. Mol. Biol. 216*, 335−52.
30. Rosselli, W., and Stasiak, A. (1991) *EMBO J. 10*, 4391−6.
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

32. Shan, Q., Cox, M. M., and Inman, R. B. (1996) *J. Biol. Chem. 271*, 5712−24.
33. Shibata, T., Cunningham, R. P., DasGupta, C., and Radding, C. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5100−4.
34. Shibata, T., DasGupta, C., Cunningham, R. P., and Radding, C. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1638−42.
35. Stasiak, A. Z., Rosselli, W., and Stasiak, A. (1991) *Biochimie 73*, 199−208.
36. Stole, E., and Bryant, F. R. (1994) *J. Biol. Chem. 269*, 7919−25.
37. Stole, E., and Bryant, F. R. (1995) *J. Biol. Chem. 270*, 20322−8.
38. Ts'o, P. O., Rapaport, S. A., and Bollum, F. J. (1966) *Biochemistry 5*, 4153−61.
39. Walker, G. C. (1987) in *Escherichia coli and Salmonella typhimurium*. *Cellular and Molecular Biology* (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., Eds.) pp 1034−43, American Society for Microbiology, Washington, D.C.
40. Watanabe, R., Masui, R., Mikawa, T., Takamatsu, S., Kato, R., and Kuramitsu, S. (1994) *J. Biochem. (Tokyo) 116*, 960−6.
41. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 126−30.
42. Weinstock, G. M., and McEntee, K. (1981) *J. Biol. Chem. 256*, 10883−8.
43. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem. 256*, 8829−34.
44. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem. 256*, 8856−8.
45. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem. 256*, 8845−9.
46. Weisemann, J. M., and Weinstock, G. M. (1988) *DNA 7*, 389−98.

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