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# Interaction of the RecA protein of *Escherichia coli* with single-stranded oligodeoxyribonucleotides

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## ABSTRACT

**The RecA protein of *Escherichia coli* performs a number of ATP-dependent, *in vitro* reactions and is a DNA-dependent ATPase. Small oligodeoxyribonucleotides were used as DNA cofactors in a kinetic analysis of the ATPase reaction. Polymers of deoxythymidilic acid as well as oligonucleotides of mixed base composition stimulated the RecA ATPase activity in a length-dependent fashion. Both the initial rate and the extent of the reaction were affected by chain length. Full activity was seen with chain lengths ≥30 nt. Partial activity was seen with chain lengths of 15–30 nt. The lower activity of shorter oligonucleotides was not simply due to a reduced affinity for DNA, since effects of chain length on $K_m^{ATP}$ and the Hill coefficient for ATP hydrolysis were also observed. The results also suggested that single-stranded DNA secondary structure frequently affects the ATPase activity of RecA protein with oligodeoxyribonucleotides.**

## INTRODUCTION

DNA strand exchange catalyzed by *Escherichia coli* RecA protein *in vitro* has been divided into three distinct stages: presynapsis, synapsis and branch migration (1,2). ATP binding is required during all three stages of the reaction, but the role of hydrolysis is less clear. The ATPase activity of RecA protein is strongly stimulated by single-stranded (ss)DNA (3–6). Early experiments suggested that ATP hydrolysis was necessary to form stable heteroduplexes (7–8) and was required only for the branch migration stage of strand exchange (8–9). Menetski *et al.* reported that all three phases of the strand exchange reaction can occur in the absence of ATP hydrolysis, since the non-hydrolyzable analog ATP[γ]S can replace ATP (10). In addition, they found that plectonemic joint molecules up to 3.4 kb in length were formed. These studies utilized completely homologous DNA molecules as substrates and, under these reaction conditions, it was shown that the ATPase activity of RecA protein was required to dissociate the protein from the heteroduplex products once the reaction was complete (10–12). Recently Kim *et al.* (13) and Rosselli and Stasiak (14) showed that in addition to recycling RecA, ATP hydrolysis is required to facilitate the bypassing of structural barriers, such as heterologous sequences, that may exist in either one or both strands during strand exchange. In addition, Kim *et al.* showed that ATP hydrolysis is required for strand exchange involving four strands (15). Finally, Konforti and Davis showed that the displaced strand and ATP hydrolysis are factors that determine the polarity of strand exchange *in vitro* (16).

The majority of studies referenced above used genome length DNA, such as M13 and φX174, as the ssDNA cofactor and the replicative forms of these phages as homologous duplex DNA. If the DNA cofactor is a short oligonucleotide, simpler complexes of RecA, ATP and ssDNA can be formed. A number of investigators used single-stranded oligodeoxyribonucleotides of defined length and sequence as ssDNA cofactors to investigate the repressor cleavage activity of RecA (4,5,17,18), the ssDNA binding activity of the protein (19), the sequence dependence of the ATPase activity (20), the importance of ends in the ATPase activity of RecA protein (21), the ability of RecA to form D loops (22,23), the role of the ATPase activity in strand exchange (14), the synapsis event in the homologous pairing of DNA molecules by RecA (23), the role of RecA in blocking transcription (24), the base pairing and interactions involved in homologous recognition promoted by RecA protein (25–27), the interaction between RecA and fluorescent dye-labeled oligonucleotides (28) and the interactions between strands in RecA–DNA complexes (29).

Analysis of the binding of RecA to oligonucleotides was performed using non-stoichiometric amounts of RecA and the non-hydrolyzable ATP analog ATP[γ]S (19). Under these conditions, RecA protein can bind to oligonucleotides 16–20 nt in length, suggesting a complex of 5–7 RecA monomers. Furthermore, this binding required a 10- to 100-fold molar excess of RecA over nucleotide concentration and required ATP[γ]S. RecA was able to bind weakly to oligonucleotides 9–15 nt in length but was unable to bind to oligonucleotides 8 nt in length.

Amaratunga and Benight demonstrated that $(dA)_{16}$,$(dT)_{16}$ and$(dT{\cdot}dC)_8$ were able to stimulate the ATPase activity above that of the DNA-independent reaction, while oligo$(dT{\cdot}dC{\cdot}dA)_8$, $(dC{\cdot}dG)_{16}$ and $(dA{\cdot}dG)_8$ could not (20). Although RecA can bind to oligonucleotides 9–15 nt in length, polymers of (dT) or (dA) less than 20 nt in length were inefficient as cofactors in either the ATPase reaction or in the repressor cleavage reaction (17). For polymers of (dT) >25 nt in length, $K_D^{(app)}$ in the ATPase reaction was a function of DNA chain length (21). Oligo$(dT)_{50}$ was shown to be as effective as poly(dT) in stimulating the ATPase activity of RecA, oligo$(dT)_{40}$ and oligo$(dT)_{30}$ were found to be less active than poly(dT) and oligo$(dT)_{25}$ was marginally active. It was concluded from this study that efficient binding to oligonucleotides, with ATP as cofactor, required an oligonucleotide of 30–50 nt in length, suggesting 10–17 RecA monomers in the complex if the

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binding site is 3 nt/RecA. Thus it is apparent that although RecA can bind to oligonucleotides 20 nt in length, efficient binding (as determined by maximum stimulation of ATPase activity) occurs when the chain length is ≥25 nt; below this length, binding appears to be less efficient and ATPase activity is not stimulated significantly above background.

Analysis of the D loop reaction using a mixture of ADP and ATP[γ]S (1.1 and 0.3 mM) showed that oligonucleotides with 26 bases of homology are able form D loops, whereas 20 bases are not sufficient (23). Maximum efficiency was seen with 38–56 bases of homology, in agreement with another study showing that oligonucleotides 30–50 nt in length (with ATP[γ]S) were required to form D loops (22). It was later shown that RecA protein was able to pair homologous oligonucleotides 15 bp long, with as few as 8 bp of homology being required to form synaptic complexes that were unstable in the absence of RecA (23). Using fluorescent dye-labeled oligonucleotides, DNA substrates to which RecA protein has an enhanced affinity, it was shown that using this artificial substrate in the presence of ATP[γ]S, RecA could bind an 18mer and use this chain length in strand exchange reactions (28). Thus, from these studies using oligodeoxyribonucleotides as ssDNA cofactor, it is clear that although RecA can bind to oligonucleotides 9–20 nt in length under non-stoichiometric conditions, longer chain lengths (i.e. >25 nt) are required for the repressor cleavage activity, the ability to form D loops and for maximum stimulation of ATPase activity of the enzyme.

To further investigate this length dependence of RecA protein, a steady-state kinetic analysis of the ATPase activity with oligodeoxyribonucleotides as ssDNA cofactor was performed. These experiments were designed to provide insight into the inability of short oligonucleotides (i.e. <25 nt) to function as cofactors for RecA and into the ability of longer chain lengths (≥30 nt) to function efficiently as ssDNA cofactor. Our results show that the ability of an oligodeoxyribonucleotide to function as an efficient cofactor in the ATPase reaction correlates with its ability to promote efficient ATP binding, as evidenced by a low $K_m^{ATP}$ and a Hill coefficient for ATP binding ≥3. In contrast, oligonucleotides which are not efficient cofactors for ATPase do not promote efficient ATP binding, i.e. they demonstrate an increase in $K_m^{ATP}$ and a Hill coefficient for ATP binding <3. The ability of an oligodeoxyribonucleotide to function as a cofactor for ATPase is further complicated by the effects of single-stranded DNA secondary structure.

## MATERIALS AND METHODS

### Materials

Phosphocellulose P11 was obtained from Whatman, ATP and ADP were purchased from Boehringer Mannheim and Polymin P was from BRL. [2,8-$^3$H]ATP (sp. act. 25 Ci/mmol) was from NEN DuPont, plastic backed polyethyleneimine (PEI)–cellulose sheets were from Brinkman and Ecolume was from ICN.

The sequences of the oligonucleotides used in this study are shown in Table 1. The nomenclature for the oligonucleotides is based on an index number in a laboratory collection. Oligo(dT)s were purchased from either Pharmacia [poly(dT) and oligo(dT)$_{10}$] or from Bio-Synthesis Inc. (Denton, TX). Oligodeoxyribonucleotides of mixed base composition were either purchased from Bio-Synthesis Inc. or synthesized in-house on an Applied Biosystems DNA Synthesizer (Applied Biosystems Inc., Foster City, CA). The oligonucleotides were stored in 1× TE buffer (Tris–HCl–EDTA buffer, pH 8.0) at –20°C and initial concentrations determined by measuring the absorbance at 260 nm. For oligo(dT)s an ε value of 8520 $M^{-1}cm^{-1}$ was used to determine concentration (30) and for the oligonucleotides of mixed base composition, nucleotide concentrations were determined by taking an $A_{260}$ of 1 to be 20 μg/ml (31). All concentrations are reported as total nucleotides. In all experiments, unless otherwise stated, oligonucleotides were always used in a 200-fold excess so that concentration differences due to variations in extinction coefficients due to base composition were not significant

Single-stranded M13 phage DNA (M13 ssDNA) was prepared by infecting *E.coli* strain JM101 with wild-type M13, at a multiplicity of infection of 10. The procedure used to purify single-stranded phage DNA was that of Neuendorf *et al.* (32). The concentration of phage DNA was determined by taking an $A_{260}$ of 1 to be 40 μg/ml (31). The purified DNA was aliquoted and stored in 1× TE buffer, pH 8.0, at –20°C. All concentrations are reported as total nucleotides.

### Purification of RecA protein

RecA protein was purified from *E.coli* strain GE1171 [F$^-$ Δ(*lac pro*)XIII *ara argE*(*Am*) *gyrA*(Nal$^r$) *rpoB*(Rif$^r$) *sfiA11 lexA*::Tn*5*(Kan$^r$) Δ*recA1398 srl*::Tn*10*(Tet$^r$)], which contains the plasmid pGE226, a pBR327-based plasmid with the *tet*$^r$ gene removed and replaced by a 3 kb fragment containing the *E.coli recA*$^+$ gene. The plasmid confers ampicillin resistance and is ~5 kb in size. The procedure used to purify RecA is based on the procedures of Weinstock *et al.* (33) and Griffith and Shores (34). The procedure involves a selective extraction from Polymin P followed by phosphocellulose chromatography. This is followed by spermidine acetate precipitation of RecA protein (pH 7.5, final concentration of spermidine acetate 7 mM). The precipitate is pelleted at 10 000 r.p.m. for 15 min at 4°C. The resulting pellet is resuspended in a buffer containing 20 mM Tris–HCl, pH 7.5, 1 mM DTT, 0.1 mM EDTA and 20% glycerol and stored aliquoted at –80°C. The concentration of purified RecA protein was determined at 280 nm using ε = 27 000 $M^{-1}cm^{-1}$ (2). Using an assay that followed the conversion of native [$^3$H]DNA to acid-soluble nucleotides, no detectable nuclease contaminant was found.

### Adenosine triphosphate assay

The hydrolysis of ATP by RecA protein was monitored using a thin layer chromatography assay employing [$^3$H]ATP. The concentrations of ATP and ADP were determined at 259 nm (pH 7.0) using ε = 15 400 $M^{-1}cm^{-1}$ (35). 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), DNA at a final concentration of 200 μM nucleotides and RecA protein as indicated. Reactions were conducted in 500 μl plastic Eppendorf tubes at 37°C. Aliquots (0.5 μl) were spotted onto PEI–cellulose strips (7 × 50 mm) containing ATP and ADP markers. The chromatography plates were developed in 1 M formic acid and 0.5 M LiCl. The ATP and ADP spots were identified with a UV lamp, cut out and counted in scintillation fluid (Ecolume, ICN).

**Table 1.** Sequences and numbering system for the oligodeoxyribonucleotides

| Oligonucleotide no. | Length (nt) | Oligonucleotide sequence |
|---|---|---|
| 44 | 25 | GGGCCTCTAGATTTAAAGCTAGCGC |
| 51 | 25 | GATCCATAGTGAGGGCAACTAAACC |
| 28 | 27 | TGGGTCCAGCGCCTATTCAGCATCGAT |
| 29 | 27 | CGCAAGGCCCATCTAAGAGTCGCCGAT |
| 30 | 27 | CGTCAGCGTGGTCTAACCGGAAGATTC |
| 31 | 27 | TTAAAACAATAAGCTTAAAAATAAATA |
| 32 | 27 | TGTCAAGACTGTAAGCTTCCATTTTTG |
| 50 | 27 | AAGATCATTCCTTTGATGGTTAGCTTC |
| 41 | 32 | GATCCTAAGTAAGTAAGGAGAAAAAAATGGCT |
| 42 | 32 | GATCAGCCATTTTTTTCTCCTTACTTACTTAG |
| 43 | 32 | GGCCGCGCTAGCTTTAAATCTAGAGGCCCCCC |
| 45 | 33 | AATTAGCCCGCCTAATGAGCGGGCTTTTTTTTG |
| 46 | 33 | AATTCAAAAAAAAGCCCGCTCATTAGGCGGGCT |
| 49 | 33 | GCAAAACGGGAAAGGATCCGTCCAGGACGCTCA |
| 156 | 40 | TCTGGACTTGTTTCATAAGGGCGGCCTTCATCGACATAAG |
| 157 | 40 | GAAGTAGCTGTATTCGAACCGTCTGAAGCTGAGATCTCTC |
| 158 | 40 | TTCGACTCTAGAGAGTGTGAGTCTTTCGAGACCTAGGACC |
| 149 | 41 | GATCGGTACCGAACATATTGACTATCCGGTATTACCCGGCA |
| 150 | 41 | AGCTTTCCGGGTAATACCGGATAGTCAATATGTTCGGTACC |

Nomenclature is based on the oligonucleotide number in a laboratory collection. Oligonucleotides were stored in 1× TE buffer (Tris–EDTA buffer, pH 8.0) at –20°C. Sequences are shown in the 5′→3′ orientation.

### Data analysis

All assays used to measure ATP hydrolysis were performed as described above. At least eight ATP concentrations were used to determine $K_m^{ATP}$ and at each concentration, the velocity of the reaction was determined from time points taken before 30% hydrolysis had occurred during the linear portion of the time course. At 40% hydrolysis, the time course became non-linear due to the accumulation of ADP, a competitive inhibitor of the ATPase activity of RecA (33), and as a result these data points were not included in rate calculations. The values for $K_m^{ATP}$ and $V_{max}$ were determined from Eadie–Hofstee plots (36). The Hill coefficients were calculated from the slope of Hill plots, where data were replotted by analogy with the Hill plot for ligand binding (37).

## RESULTS

### Effects of ssDNA chain length on $k_{cat}$ and extent of reaction

To ensure that ssDNA chain length was the principal variable, ATPase activity was measured under conditions where the concentration of ATP (900 µM) and ssDNA (200 µM) were in large excess over RecA protein (1 µM). The kinetics of the hydrolysis of ATP when either M13 ssDNA or other genome size DNAs are the DNA cofactor are well characterized (6,33,36,38). Consequently, M13 ssDNA was the standard with which we compared other polynucleotides. Under the conditions of our assay with M13 ssDNA, RecA protein was able to efficiently hydrolyze ATP with a $k_{cat}$ of between 16 and 24 $min^{-1}$ (Fig. 1 and Table 2). These numbers agree with the published turnover numbers for RecA protein (36,39). For the reaction with M13 ssDNA, the maximum extent of hydrolysis varied between 60 and 80% in different experiments (Fig. 2 and Table 2).

The first series of experiments to determine the effects of ssDNA chain length on $k_{cat}$ compared a series of homopolymers of deoxythymidilic acid with M13 ssDNA. These polymers were

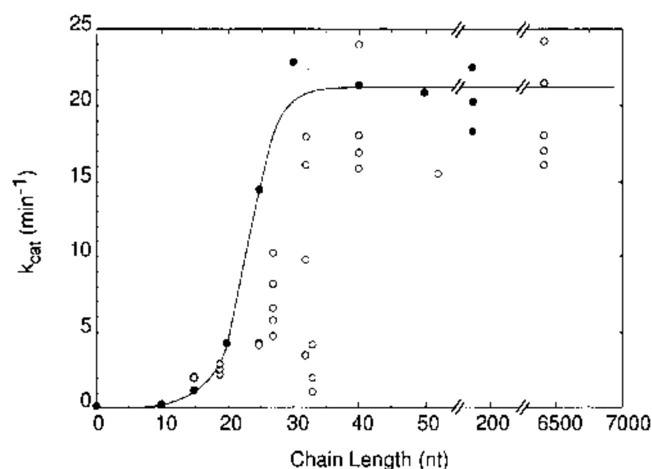


**Figure 1.** The effects of ssDNA chain length on $k_{cat}$. Reactions were carried out as described under Materials and Methods and contained 20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 200 µM ssDNA cofactor, 950 µM ATP and 1 µM RecA protein. Open circles represent M13 ssDNA (6407 nt) and the mixed base composition oligonucleotide series; closed circles represent the deoxythymidilic acid homopolymer series and the DNA-independent reaction. For the reaction with M13 ssDNA, the values from five separate experiments on different days are shown; for poly(dT), the values from three separate experiments on different days are shown. For the mixed base composition oligonucleotides and the homopolymer series the values shown are usually from a single experiment. In some cases duplicate experiments were performed.

chosen to minimize ssDNA secondary structure, which might affect the interaction of RecA protein with the DNA. The longest chain length in this series was poly(dT) (average length 167 nt). Turnover numbers for this oligonucleotide were found to vary between 18 and 30 $min^{-1}$ in different experiments, indicating that poly(dT) is able to function as efficiently as M13 ssDNA as DNA cofactor for the ATPase activity of RecA protein (Fig. 1 and Table 2).

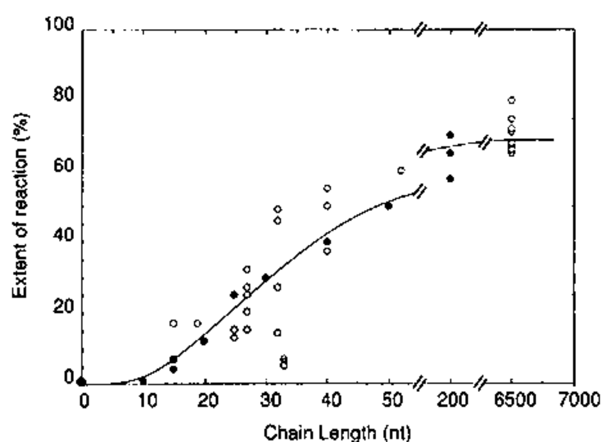


**Figure 2.** The effects of ssDNA chain length on the extent of reaction. Reactions were conducted as described under Materials and Methods and contained 20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 200 µM ssDNA cofactor, 950 µM ATP and 1 µM RecA protein. Open circles represent M13 ssDNA (6407 nt) and the mixed base composition oligonucleotide series; closed circles represent the deoxythymidilic acid homopolymer series and the DNA-independent reaction. For the reaction with M13 ssDNA, the values from eight separate experiments done on different days are shown; for poly(dT), the values from three separate experiments on different days are shown. For the mixed base composition oligonucleotides and the homopolymer series the values shown are usually from a single experiment. In some cases duplicate experiments were performed.

Homopolymers of (dT), varying in length from 10 to 50 nt, were assayed for their ability to stimulate the ATPase activity of RecA (Fig. 1 and Table 2). Oligonucleotides ≥ 30 nt were cofactors as efficient as poly(dT) as judged by $k_{cat}$ (Table 2). Oligo$(dT)_{25}$ gave an intermediate stimulation ($k_{cat}$ = 14 $min^{-1}$), while oligo$(dT)_{20}$ and oligo$(dT)_{15}$ were inefficient stimulators of the ATPase activity ($k_{cat}$ = 4–8 $min^{-1}$ and 1.1 $min^{-1}$ respectively). Little stimulation of the ATPase activity above that of the DNA-independent ATPase activity of RecA was seen with oligo$(dT)_{10}$, even at a DNA concentration of 2000 µM nucleotides (Fig. 1 and unpublished results).

Next, a series of oligonucleotides of mixed base composition were used to stimulate the ATPase activity of RecA (Fig. 1 and Tables 2 and 3). These experiments tested whether RecA was affected by short regions of either intra- or intermolecular base pairing in the DNA cofactor. In general the trend seen with these oligonucleotides was similar to that obtained with the homopolymer series, in that longer oligonucleotides were better cofactors. One 40mer (no. 156) was found to be as efficient as either M13 ssDNA or poly(dT) in stimulating the ATPase activity of RecA protein. It produced a $k_{cat}$ of 20–25 $min^{-1}$. However, a significant variation in activity between oligonucleotides of the same length was observed, which is attributed to cofactor secondary structure. This was most apparent in the group of oligonucleotides 32–33 nt in length. The three 32mers (nos 41–43) gave $k_{cat}$ values ranging from 3.4 to 18 $min^{-1}$, while the three 33mers (nos 45, 46 and 49) were ineffective in stimulating the ATPase activity of RecA protein, with $k_{cat}$ of from 1.4 to 4.0 $min^{-1}$ (Fig. 1 and Table 3). These 33mers are predicted to have significant secondary structure as judged by computer analysis (data not shown). The same effect was observed when comparing mixed base composition oligonucleotides 25–27 nt in length. These molecules produced $k_{cat}$ values of from 4 to 13 $min^{-1}$, while oligo$(dT)_{25}$ had a $k_{cat}$ of 15 $min^{-1}$. Mixed base composition oligonucleotides 15 and 20 nt in length produced lower values of $k_{cat}$ than the homopolymer of the same length (data not shown). The effects of chain length were independent of pH, since results identical to Figure 1 were obtained when ATPase assays were performed at pH 6.2, pH 7.0 and pH 8.0 (unpublished results). In addition, these effects were independent of protein concentration over a range of 1–3 µM RecA protein (unpublished results).

**Table 2.** Steady-state kinetic parameters for the ATPase activity of RecA protein

| DNA | Extent (%) | $k_{cat}$ (per min) | $K_m^{ATP}$ (µM) | $k_{cat}/K_m^{ATP}$ (min/µM) | Hill coefficient |
|---|---|---|---|---|---|
| No DNA | 0.9[a] | <0.2[a] | ND | ND | ND |
| oligo$(dT)_{10}$ | 1.4 [a] | 0.1–0.22[a] | ND | ND | ND |
| oligo$(dT)_{15}$ | 5.0[a] | 1.1[a] | ND | ND | ND |
| oligo$(dT)_{20}$ | 8.0 | 8.0 | >459 | 0.018 | 1.24 |
| oligo$(dT)_{25}$ | 22.0 | 14.0 | 300 | 0.049 | 2.30 |
| oligo$(dT)_{30}$ | 30.0 | 24.0 | 238 | 0.101 | 2.91 |
| oligo$(dT)_{40}$ | 40.0 | 21.0 | 140 | 0.150 | 3.05 |
| poly(dT) | 60–70 | 26.0 | 60 | 0.433 | 3.50 |
| 25mer (no. 51) | 15.0 | 5.0 | 246 | 0.022 | 2.00 |
| 40mer (no. 156) | 40.0 | 25.0 | 67 | 0.373 | 3.31 |
| ssM13 DNA | 60–80 | 23.0 | 49 | 0.469 | 3.30 |

ATPase assays were conducted as described in Materials and Methods. A minimum of eight concentrations were used to determine $K_m^{ATP}$ and at each concentration, the velocity of the reaction was determined from time points taken during the linear portion of time courses before 30% hydrolysis had occurred. The values shown for $K_m^{ATP}$ and $k_{cat}$ were determined by extrapolation from Eadie–Hofstee plots (40). Hill coefficients were determined from the slope of Hill plots (37).
[a]Determined from initial rates with initial [ATP] = 950 µM.
ND, not determined.

In addition to affecting $k_{cat}$, the extent of the ATP hydrolysis reaction was also affected by chain length (Fig. 2 and Tables 2 and 3). The extent of reaction using either M13 ssDNA or poly(dT) as cofactor was 60–80% (Fig. 2 and Table 2), but decreased from 50% for oligo$(dT)_{50}$ (Fig. 2) to 1.4% for oligo$(dT)_{10}$ (Fig. 2 and Table 2). A similar trend was also observed for the set of oligonucleotides of mixed base composition (Fig. 2 and Table 3). The extent of reaction decreased from 40–45% for the 41mers to ~13% for the 25mers. In addition, a range of values for the extent of reaction was obtained for a single chain length. For example, for the group of oligonucleotides 25–27 nt in length, the extent of reaction ranged from 12.5% for no. 44 to 27% for no. 28. These effects on the values reported for both $k_{cat}$ and the extent of reaction may be due to single-stranded DNA secondary structure.

### Effects of ssDNA concentration on ATP dependence

A comparison of the titration of two mixed base composition oligonucleotides with M13 ssDNA is shown in Figure 3. It is evident that 40mer no. 156 and 25mer no. 51 are not as efficient as M13 ssDNA in stimulating the ATPase activity of RecA protein, even at 1000 µM, a concentration which is five times that used in our standard reaction. The half-maximal velocity was reached at DNA concentrations of ~100 µM for the 25mer, 75 µM for the 40mer and 10 µM for M13 ssDNA. The curves for the oligonucleotides showed a similar concentration dependence,

reaching a maximum $k_{cat}$ at 200–300 μM. This suggests that the affinity of RecA protein for the oligonucleotides is similar. Affinity for M13 ssDNA appears to be higher, however.

**Table 3**. $k_{cat}$ and extent of reaction for oligodeoxyribonucleotides of mixed base composition

| Oligonucleotide no. | Length (nt) | $k_{cat}$ ($min^{-1}$) | Extent (%) |
|---|---|---|---|
| Oligo(dT)$_{25}$ | 25 | 14.4 | 25.0 |
| 44 | 25 | 4.4 | 12.5 |
| 51 | 25 | 5.1 | 13 |
| 28 | 27 | 8–13 | 27.0 |
| 29 | 27 | 4.7 | 15.0 |
| 50 | 27 | 6.5 | 20.0 |
| 30 | 27 | 4.8 | 15.0 |
| 32 | 27 | 5.8 | 24.0 |
| 31 | 27 | 10.2 | 25.0 |
| Oligo(dT)$_{30}$ | 30 | 24 | 30.0 |
| 43 | 32 | 3.4 | 10.0 |
| 41 | 32 | 9.8 | 27.0 |
| 42 | 32 | 16–18 | 48.0 |
| 46 | 33 | 1.4 | 7.0 |
| 45 | 33 | 1.5 | 4.5 |
| 49 | 33 | 2.5–4.0 | 6.0 |
| Oligo(dT)$_{40}$ | 40 | 21 | 40.0 |
| 150 | 41 | 19–22 | 45.0 |
| 149 | 41 | 15–22 | 40.0 |
| 158 | 40 | 9–11 | 35.0 |
| 157 | 40 | 17–20 | 42.5 |
| 156 | 40 | 20–25 | 40.0 |

Values for $k_{cat}$ were determined from the initial rate of reaction with the initial [ATP] = 950 μM. A minimum of five time points were used to determine $k_{cat}$ for each oligonucleotide and only time points before 30% hydrolysis had ocurred were used in the calculation of $k_{cat}$. The values for extent were taken from the same time course, with time points taken over a 60 min time period.

## Effects of ATP concentration

Under the conditions of our assay, we determined a $K_m^{ATP}$ of 49 μM with M13 ssDNA as cofactor and a $K_m^{ATP}$ of 60 μM with poly(dT) as cofactor, indicating no significant difference between these two ssDNA cofactors. However, a significant effect of chain length on $K_m^{ATP}$ was observed for shorter molecules (Table 2). In Figure 4 examples of the ATP concentration dependence in the presence of shorter ssDNA cofactors are shown. For poly(dT), the curve was sigmoidal, with $V_{max}$ calculated to be 780 pmol/min ($k_{cat}$ = 26 $min^{-1}$), by extrapolation from the Eadie–Hofstee plot, and $K_m^{ATP}$ = 60 μM. When oligo(dT)$_{40}$ was present as cofactor, the velocity plot was less curved, with $V_{max}$ calculated to be 600 pmol/min ($k_{cat}$ = 21 $min^{-1}$) and $K_m^{ATP}$ = 140 μM. As chain length was decreased to 25 nt, the curves became straighter, showing a further decrease in $V_{max}$ to 380 pmol/min ($k_{cat}$ = 14 $min^{-1}$) and a further increase in $K_m^{ATP}$ to 300 μM. For oligo(dT)$_{20}$, the calculated $K_m^{ATP}$ was very high (>459 μM; Table 2) and the concentration range shown in Figure 4 is presumably at or below the $K_m^{ATP}$. As a result, saturation was not achieved and the curve appears as a straight line. A similar trend in $k_{cat}$ and $K_m^{ATP}$, complicated by secondary structure, was seen with the oligonucleotides of mixed base composition. $K_m^{ATP}$ was observed to increase 5-fold when chain length decreased from 6407 (M13 ssDNA) to 25 nt (oligonucleotide no. 51; Table 2). Thus, it is evident from these results that as ssDNA length changes, the ATP dependence also changes.

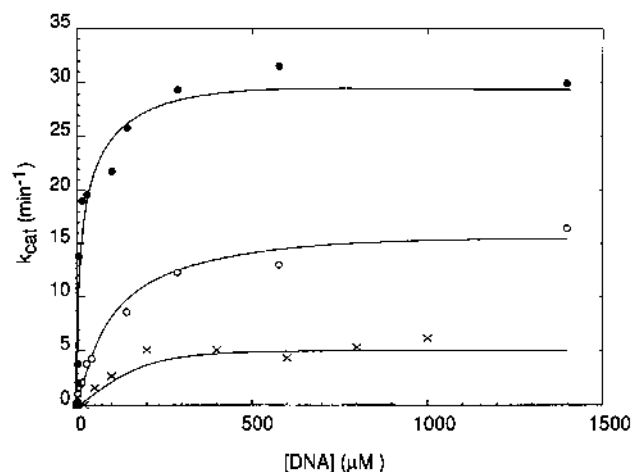


**Figure 3.** The effects of ssDNA concentration on ATPase activity. Reactions were carried out as described under Materials and Methods and contained 20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, ssDNA cofactor concentration as indicated, 950 μM ATP and 1 μM RecA protein for the 25mer (×) and M13 ssDNA (●) and 2.0 μM RecA protein for the 40mer (○).

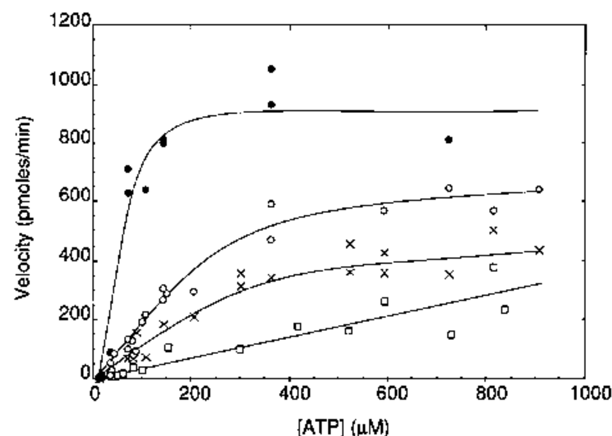


**Figure 4.** The effects of ATP concentration on length-dependent ATPase activity. Reactions were carried out as described under Materials and Methods. Reaction mixtures contained 20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 200 μM ssDNA cofactor, ATP concentration as indicated and 1 μM RecA protein. ssDNA cofactors: ●, poly(sdT); ○, oligo(dT)$_{40}$; ×, oligo(dT)$_{25}$; □, oligo(dT)$_{20}$.

The effects of chain length on ATPase activity were further evidenced by the effects on the ratio $k_{cat}/K_m^{ATP}$. This ratio is an indication of the efficiency with which RecA hydrolyzes ATP with different ssDNA cofactors (40). As the chain length of the mixed base composition oligonucleotides was decreased from 6407 to 25 nt, the efficiency of the ATPase activity of RecA decreased 20-fold (Table 2). The same effect was observed for the polymers of deoxythymidilic acid. Decreasing chain length from 167 to 20 produced a decrease in $k_{cat}/K_m^{ATP}$ of >20-fold (Table 2).

ssDNA chain length also affected the Hill coefficient for the ATP hydrolysis reaction. Previous studies (6,33) found that the ATPase activity of RecA protein with either ϕX174 DNA or

heat-denatured calf thymus DNA as cofactor exhibited a complex dependence on ATP concentration, with Hill coefficients of 3.3. We found the same complex dependence on ATP concentration for homopolymers of chain length ≥ 30 nt. The Hill coefficient with these oligonucleotides was ≥ 3 (Table 2). At chain lengths <30 nt, the Hill coefficient decreased to 2.3 for oligo$(dT)_{25}$ and 1.2 for oligo$(dT)_{20}$ (Table 2). This same change in the Hill coefficient was seen with oligomers of mixed base composition (Table 2) with a Hill coefficient of 3.31 for a 40mer, while for 25mer no. 51 the Hill coefficient was 2 (Table 2). This further emphasizes that chain length affects the interaction of RecA protein with ATP.

## DISCUSSION

The ATPase activity of RecA protein is affected by ssDNA in a length-dependent fashion. Our primary conclusion is that the steady-state kinetic parameters, $K_m^{ATP}$, $V_{max}$, Hill coefficient for ATP binding, the extent of the ATPase reaction and $k_{cat}$, are all a function of ssDNA chain length. These effects are likely to be interrelated. In addition to chain length, ssDNA secondary structure affects the efficiency of ssDNA in stimulating the ATPase activity of RecA protein.

The effects we have observed of ssDNA chain length on ATPase activity are not inconsistent with previous reports (19–21). Brenner *et al.*, however, concluded that RecA had different affinities for the various oligonucleotides and $V_{max}$ for ATP hydrolysis remained the same (21). Their experiments used DNA concentrations of 100 μM or lower, while our experiments used DNA concentrations up to 1000 μM and, in some cases, up to 2000 μM. At these high concentrations, the binding of RecA to ssDNA appears not to be the predominant factor.

The extent of the ATP hydrolysis reaction was also affected by ssDNA chain length. RecA protein is unable to hydrolyze 100% of the ATP in an *in vitro* reaction that does not contain an ATP regenerating system due to competitive inhibition of ATP binding by ADP (6,33). As chain length decreased, the maximum extent of ATP hydrolysis decreased from 60–70% for poly(dT) to 1.4% for oligo$(dT)_{10}$. Since the $K_m^{ATP}$ also increased with shorter chain lengths, it is possible that this results in greater sensitivity to inhibition by ADP, resulting in a lower extent of hydrolysis.

It was previously demonstrated (33,41), that cooperative interactions between RecA monomers along the DNA backbone are important for ATPase activity. It was further suggested (39) that ATPase activity requires the formation of clusters of 15–20 ATP–RecA molecules bound contiguously along the ssDNA backbone and that an individual RecA molecule becomes a fully active ATPase only when it is part of a cluster that has exceeded this size. Our data demonstrate that oligonucleotides <30 nt in length are unable to fully stimulate the ATPase activity of RecA protein. This suggests that short DNA molecules are unable to provide sufficient lattice length along which a sufficient number of RecA monomers can be contiguously bound to give full ATPase activity. The minimum length of 30 nt, assuming a stoichiometry of 3 nt/monomer (42), produces a minimum cluster size of 10 RecA monomers. Although the agreement between the two studies in terms of the size of the active complex is only approximate, it is clear that a large complex of RecA monomers is required for full ATPase activity.

What can we infer about the structure of the clusters? The Hill coefficient of >3 may mean that a RecA monomer contacts three or more other monomers, which facilitates ATP binding to the monomer. Structural studies (12,43,44) indicate that RecA forms a filament with six monomers per turn and each monomer contacts the two adjacent neighboring monomers. How can a structure where each monomer contacts only two other subunits give rise to a Hill coefficient of >3? One possibility is that filaments are also paired side by side, so that each monomer makes both inter- and intra-filament contacts. Such a structure has been observed (44). Alternatively, RecA monomers may not only contact their immediate neighbors in a filament, but also subunits located one turn up- or downstream, positioned on top of and below the monomer. Although such contacts were not observed in the RecA crystal structure (44), this structure did not include DNA and thus the monomers are likely to be in a different conformation than in the ATPase reaction.

These models also account for the increase in $K_m^{ATP}$ and decrease in the Hill coefficient as chain lengths get shorter. Although there will always be interaction with immediate neighbors, there will be fewer inter-filament or up- or downstream interactions as the chain length decreases. Hence, the Hill coefficient will decrease and affinity for ATP will be poorer due to this loss of cooperativity. Note that there need be no change in the affinity of RecA for DNA as chain length decreases in these models.

The fact that chain length affects ATP binding does not necessarily explain the effect of chain length on $k_{cat}$. What is the trigger for hydrolysis of ATP and why should it be affected by oligonucleotide chain length? A possible explanation is that hydrolysis requires, and perhaps is triggered by, the formation of the multisubunit complex. Thus, as chain length decreases the complexes formed are smaller, resulting in a reduced maximum velocity for hydrolysis. The idea that a multisubunit complex must form for hydrolysis is consistent with the model that hydrolysis of ATP may propagate longitudinally through the nucleoprotein filament in the form of coordinated waves (45). This requires that one monomer affect the activity of its neighbors separated by five or six RecA monomers (46), as proposed here.

The biological significance of the 30 nt length may be related to the minimum size required for homologous pairing. It has been demonstrated *in vivo* that there is a minimal DNA length (23–40 bp) below which RecA-dependent homologous recombination becomes inefficient (47,48). There is also a sharp cut-off between 30 and 151 bp of homology for the pairing reaction promoted by RecA protein *in vitro* (1) and it has also been reported that oligonucleotides with 26 bases of homology are able to form D loops, whereas 20 bases are not sufficient (23). Maximum efficiency required 38–56 bases of homology, in agreement with another report that oligonucleotides 30–50 nt in length (with ATP[γ]S) were required to efficiently form D loops (22). These observations suggest that the requirement for a multisubunit complex including 30 nt of ssDNA ensures that the region involved in homologous pairing is long enough to ensure unique recognition of sequences.

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