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A Continuous Kinetic Assay for RecA-Mediated DNA Strand Exchange Using DNA-Based Fluorescent Signal

by

Xun Zhu

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Master of Arts

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August, 1999
ABSTRACT

A Continuous Kinetic Assay for RecA-Mediated DNA Strand Exchange Using DNA-Based Fluorescent Signal

by

Xun Zhu

A continuous kinetic assay for RecA-mediated DNA strand exchange using a DNA-based fluorescent signal has been successfully developed and employed. 2-Aminopurine deoxyribonucleoside phosphoradmitie was synthesized and incorporated into oligonucleotides. The ODNs were HPLC-purified and gave excellent time-dependent spectrophotometric data on DNA hybridization. Active RecA protein was overexpressed and purified. The activation of the RecA protein for ATP hydrolysis by single-stranded DNA was investigated using this assay. From our preliminary kinetic data, the assay provides a view of the discrete steps along the reaction pathway. The first observation of a direct isomerization pathway for RecA monomers associated with DNA was accomplished. A mechanistic model in which association of RecA-ATP to single-stranded DNA is turnover-limiting was established. Synthesis of fluorescent nucleoside triphosphate is underway and expected to give satisfactory results.
ACKNOWLEDGMENTS

The research presented in this thesis is the cumulative effort of many hearts and minds, and to each person who offered his or hers, I am grateful.

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Finally, I would like to thank the Department of Chemistry, William Marsh Rice University, and Robert A. Welch Foundation for the financial support of myself and my graduate research.
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<td>2</td>
<td>2-aminopurine ribonucleoside</td>
</tr>
<tr>
<td>2AP</td>
<td>2-aminopurine</td>
</tr>
<tr>
<td>AA</td>
<td>ammonium acetate</td>
</tr>
<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5’-diphosphate</td>
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<tr>
<td>AIBN</td>
<td>2,2’-azobisisobutyronitrile</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
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<td>ammonium sulfate</td>
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<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
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<td>BB</td>
<td>Bromophenol Blue</td>
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<td>polyoxyethylene 23 lauryl ether</td>
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<td>Bz</td>
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<td>°C</td>
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<tr>
<td>CBB</td>
<td>Coomassie Brilliant Blue R-250</td>
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<tr>
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<td>β-cyanoethyl-N,N-diisopropyl</td>
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<td>controlled pore glass</td>
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<td>diethyl azodicarboxylate</td>
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<td>DCM</td>
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<td>ddH₂O</td>
<td>milli-Q water</td>
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<td>diethylaminoethyl</td>
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DMAP  4-dimethylaminopyridine
DMF  $N,N$-dimethylformamide
DMSO  dimethyl sulfoxide
dimethoxytrityl
deoxyribonucleic acid
double-stranded DNA
dithiothreitol
EDTA  ethylenediaminetetraacetic acid
Et  ethyl
h  hour, hours
HPLC  high-performance liquid chromatography
HAP  hydroxyapatite
ibu  isobutyryl
IPTG  isopropyl $\beta$-D-thiogalactopyranosine
Kan  kanamycin
LB  luria broth
LDH  lactate dehydrogenase
Me  methyl
MeOH  methanol
min  minute, minutes
NAD$^+$  nicotinamide adenine dinucleotide, oxidized form
NADH  nicotinamide adenine dinucleotide, reduced form
NDP  nucleoside diphosphate
NMP  nucleoside 5'-monophosphate
NMR  nuclear magnetic resonance
<table>
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<td>NTP</td>
<td>nucleoside 5'-triphosphate</td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxyribonucleotide</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PC</td>
<td>polycarbonate</td>
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<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
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<tr>
<td>Ph</td>
<td>phenyl</td>
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<tr>
<td>PIPES</td>
<td>1,4-piperazinediethanesulfonic acid</td>
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<tr>
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<td>pyruvate kinase</td>
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<td>PPCO</td>
<td>polypropylene copolymer</td>
</tr>
<tr>
<td>PTC</td>
<td>phenoxythiocarbonyl</td>
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<tr>
<td>RBF</td>
<td>round bottom flask</td>
</tr>
<tr>
<td>RP HPLC</td>
<td>reverse phase HPLC</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>SVC</td>
<td>speed vacuum concentrator</td>
</tr>
<tr>
<td>TB</td>
<td>terrific broth</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetrabutylammonium fluoride</td>
</tr>
<tr>
<td>t-Bu</td>
<td>tert-butyl</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<tr>
<td>TEA</td>
<td>triethylamine</td>
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<tr>
<td>TEAA</td>
<td>triethylammonium acetate</td>
</tr>
<tr>
<td>TEMED</td>
<td>$N,N,N',N'$-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracycline</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
</tr>
<tr>
<td>TPDS</td>
<td>1,1,3,3-tetraisopropyl-1,3-disiloxanediyl</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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CHAPTER 1
INTRODUCTION

1.1 Homologous Genetic Recombination

Genetic recombination is the physical rearrangement of DNA. This process is a cornerstone of genetics because it ensures that genomes are rearranged between generations and this results in genetic diversity between the individuals of a species.¹ Homologous recombination is defined by the use of DNA sequence homology for the recognition of recombining partners. There are many consequences of homologous recombination. The most important of these is the repair of DNA that has been damaged on both strands, but homologous recombination can also mediate programmed DNA rearrangement, and assist accurate chromosome segregation.²

Our long term goal is to reach a structural and mechanistic understanding of the common strategies for genetic rearrangements that maintain the dynamic flux of chromosomal DNA. The distinct feature of homologous recombination is that the enzymes responsible can use any pair of homologous sequences as substrates. We want to study this type of recombination by using the RecA protein for several reasons. First, the RecA protein has been studied for over 30 years and its importance in cellular processes is well established. Moreover, many technical aspects of preparing and handling the protein have been delineated. Furthermore, RecA-like recombinase proteins have been found in essentially every organism from bacteria³ to humans.⁴,⁵ It is likely that the mechanisms employed by RecA
have been conserved throughout evolution. Thus, models established from kinetics of reactions with RecA will likely prove to be relevant to understanding recombination and genetic rearrangements in general.\textsuperscript{6,7} In addition, the research tools developed during investigating the RecA protein will undoubtedly be applicable to the future study of other recombinases.

1.2 RecA-Mediated DNA Strand Exchange

The RecA protein of \textit{E. coli} (37.8 kD) serves as a recombinase prototype. Even though the biochemical activities of the RecA protein have been extensively studied, the molecular mechanisms of the genetic exchange events remain unclear. The goal of our research is the elucidation of the molecular mechanism by which RecA performs DNA recombination. DNA strand exchange mediated by the RecA protein \textit{in vitro} occurs in three stages: presynapsis, synapsis and branch migration.\textsuperscript{8,9} A summary of the action in recombination is shown in Figure 1-1.

RecA first polymerizes around single- and double-stranded DNA to form helical presynaptic filaments. The kinetics of binding to single-stranded DNA are more rapid than to double-stranded DNA and it is thought that \textit{in vivo} single-strands are the primary signal for the formation of presynaptic filaments. Filament formation is also a prerequisite for RecA to form an active filament with DNA and a stoichiometry of 1 RecA per 4 DNA bases was estimated for the ssDNA•RecA filament at this stage.\textsuperscript{10} The filament is in a "collapsed" conformation and inactive to DNA strand exchange in the absence of cofactor or presence of ADP. In the presence of ATP or ATPγS, RecA•ssDNA and ATP
Overall:

\[ E \cdot A + ATP + B \cdot C \rightleftharpoons E \cdot (A \cdot C) + B + ADP + P_i \]

Stepwise:

Figure 1-1. RecA Protein facilitates the pairing of homologous DNA sequences and exchange of strands. \( E = \) RecA; \( A = \) initial ssDNA; \( B = \) strand of dsDNA with sequence identical to that of \( A; C = \) strand of dsDNA with sequence complementary to that of \( A. \) "col" means the collapsed formation and "ext" means the extended formation. The scheme is described in detail in text.
undergo two-step reaction in which an initial rapid equilibrium binding of ATP to the RecA•ssDNA complex is followed by a first-order isomerization of the complex from the “collapsed” to an “extended” conformation which has a stoichiometry of 1 RecA per 3 DNA bases. The rate constant for the rate-limiting step is 0.3 s\(^{-1}\) and identical to the steady-state turnover number for ATP hydrolysis. However, the mechanism of isomerization is still unclear and could happen in several ways.

When RecA•ssDNA•ATP “extended” filaments are mixed with dsDNAs they pair at regions of homology, a process described as synapsis. For synapsis, a three-step reversible mechanism was proposed. First, a second DNA binds to the RecA•ssDNA•ATP complex. The association of the second DNA to “extended” filaments is fast and nonspecific. However, the dissociation of the second DNA depends strongly on sequence complementarity, which means the non-homologous DNA dissociates quickly. Second, the DNA strands pair at the regions of sequence homology, with all three strands remaining within the RecA filament and the DNA strands wound around each other in a three-stranded structure. This structure must be very different from normal DNA because it is stretched 1.5-fold. The incoming DNA strand, originally within the presynaptic filament may make some hydrogen-bonding interactions with its complementary strand but the outgoing strand remains closely associated to this newly formed duplex.

After the strands have paired, a third step takes place where the outgoing strand of the DNA that was originally double-stranded is unpaired from the intermediate to generate the products: a heteroduplex with one strand from each substrate DNA molecule and a displaced ssDNA from the original
This reaction does not require the hydrolysis of ATP but is normally accompanied by ATP hydrolysis. The reaction proceeds in the 5'-3' direction with respect to the single-stranded region initially nucleating the binding of RecA protein.

1.3 The Homology Search

If strand exchange is to occur, the single strand must find its complement in the recipient double helix. A key question is: how does the RecA protein guide the search for homology between two reacting DNA species? This search for homology is a central problem in elucidating a mechanistic description of RecA-directed recombination. In principle, RecA mediated recognition between homologous DNA molecules is similar to sequence-specific DNA binding by proteins or triple-helix-forming ODNs. A key difference here is that RecA protein-DNA interactions must be sufficiently nonspecific such that RecA can interact with any DNA sequence and allow the DNA molecules to provide the interactions that determine homology or non-homology. In fact, it is the nucleofilament that searches the dsDNA for homology. However, the RecA complex must be specific enough so that only the homologous region is recognized. Furthermore, homology recognition directed by the RecA protein results in products that are nearly isoenergetic with substrate DNAs rather than a more stable complex. Thus, intermediates that are important for homology search are likely to be kinetically unstable, in contrast to the triple-helical complexes formed in the absence of RecA.\textsuperscript{19,20}
Although the detailed mechanism has not been accomplished, two alternative models, pairing after strand separation (also named the melting-annealing or base pair) model and pairing before strand separation (also named the triplex) model, have been proposed to explain key three-stranded intermediates during homology recognition (Figure 1-2). The distinguishing characteristic of the two models is the type of base-base interactions that control homology recognition. The first model speculates that proteins mediating homologous recognition first locally open the double helices of interacting duplexes, and then help to form classical Watson-Crick pairing between incoming and recipient strands. Thus DNA synapsis and exchange of DNA strands are mechanistically coupled, and the original base pairs have to be broken for this type of recognition to occur. The base pair model is supported by experimental evidences by several groups. In contrast to base pair model, The triplex model postulates that there is a triplex intermediate during recombination. Through specific hydrogen bonds, the non-Watson-Crick pairing connects an incoming ssDNA to the dsDNA which retains the original Watson-Crick base pairs. Proteins mediating homologous recombination would first help to form such three-stranded structures and would later resolve these structures into the classical products of strand exchange. If an intact A*T base pair in a dsDNA molecule aligns with an A residue of the incoming strand within the nucleoprotein complex, the interaction involving the exocyclic amino group at C6 of purine heterocycle would be important for homology search in a triplex structure, and the stability of such a structure would be decreased if this amino group is removed.
Figure 1-2. The two models of the homology search. The semi-circular line indicates the RecA protein. The hashed lines represent Watson-Crick-type hydrogen bonds. The "pairing after strand separation" model is shown in the upper scheme. It postulates that the RecA protein first locally opens the double helices of the interacting duplex, and then helps to form classical Watson-Crick hydrogen bonding of the single strand with its complement from the locally melted duplex. The driving structural force is the base pairing of newly formed heteroduplex. The "pairing before strand separation" is shown in lower scheme. It proposes the RecA protein first help to form a three-strand structure with the ssDNA and interacting duplex. The driving structural force is the formation of triplex.
1.4 The Continuous Kinetic Assay: Stopped-Flow Kinetic Study

In order to understand homologous genetic recombination, we need to understand the details of homology pairing. Such understanding would require knowing how intermediates are formed from substrates and result products. Because we expect the intermediates are to be transiently lived, it requires that we use rapid spectra techniques. More general need for studying rapid reactions in solution has been increasing in the field of biochemistry because most enzyme reactions are fast. In response to this trend, stopped-flow apparati of high performance have been produced and are commercially available.\textsuperscript{29} Some of them have dead times as short as 0.5 ms.\textsuperscript{30,31} Figure 1-3 illustrates the principle of stopped-flow apparatus. Two reagents are driven together from two syringes through a mixer. The mixed reagents immediately enter a viewport having displaced spent reagents downstream to a stopping syringe. The movement of the stopping-syringe plunger closes a microswitch which initiates the recording of signal changes from a detector. The technique directly detects the signal changes of reaction and therefore dynamic information can be obtained in real time. Because many of steps of the DNA strand exchange process occur with half-times of less than 1 second, the use of stopped-flow spectrometry will provide a view of the discrete steps along the reaction pathway. A continuous kinetic assay would provide more information to elucidate the mechanisms of rapid reactions than traditional kinetic assays.
Figure 1-3. The illustration of stopped-Flow apparatus. Two reagents are driven together from syringes L (flush) and R through a mixer. The mixed reagents immediately enter a viewpoint having displaced previous reagent downstream to a stopping syringe. The movement of the stopping-syringe plunger closes a microswitch which initiated the recording of signal changes from a detector. The dead time is the time between when the flow is stopped and when the record begins.
1.5 Conservatively Modified Fluorescent Nucleosides

We are interested in using fluorescence as a spectroscopic signal for monitoring the DNA strand exchange. The principal advantages of fluorescence which encourage its use are its sensitivity and selectivity. Unfortunately, all natural nucleosides, oligonucleotides, and DNAs are not sufficiently fluorescent for quantitative kinetic studies. There are many fluorescent dye labels can be covalently to DNAs to make them fluorescent. We are interested in fluorescent non-natural nucleosides. 2-aminopurine deoxyribose nucleoside 1 and 2-aminopurine nucleoside 2 which possess high intrinsic fluorescence can be used in place of natural adenosine to provide intrinsic DNA or RNA label.

Fluorescent nucleoside 1 is our first intrinsic probe used to study DNA strand exchange because it is extremely sensitive to the surrounding environment in DNA structures,\textsuperscript{32} and it can be incorporated into ODNs by automated, solid-phase synthesis. Furthermore, the excitation and emission maxima for 1 (315 nm and 370 nm, respectively) are well separated from those of tryptophan and tyrosine residues in proteins (295 nm and 345 nm, respectively), increasing its usefulness for studying protein-DNA interactions. 1
has been used to study the Klenow fragment of DNA polymerase I,\textsuperscript{33} T4 DNA polymerase,\textsuperscript{34} and T7 RNA polymerase I.\textsuperscript{35}

One important feature of 1 is that it is a conservatively modified nucleoside. It forms a Watson-Crick type base pair with thymine and produces relatively little structural disturbance in dsDNA unlike nucleotide analogs containing bulky fluorescent moieties.\textsuperscript{36,37} However, it does result in changes in the hydrogen-bond donating and accepting functional groups on Watson-Crick pairing edge of the bases (Figure 1-4). The order of duplex stability is A\textbullet T > 1\textbullet T > 1\textbullet C > A\textbullet C.\textsuperscript{38,39} An increase or decrease in the strength of base pairs in initial dsDNA might be expected to decrease or increase, respectively, the rate of homology recognition if the rate-limiting step for the search is strand separation within pairing after strand separation model. Similarly, increasing the strength of the heteroduplex after strand exchange might increase the rate of joint molecule formation if such a mechanistic model applies. Modified substrates such as 1 will therefore allow the stabilities of the substrates, products, and intermediate species to be rationally controlled.

By employing 1, we also are able to distinguish the two homology search models easily. Because it is very difficult for 2-aminopurine to form triplex with A\textbullet T base pair, the ssDNA containing 1 should deter strand exchange if a triplex intermediate is required for homology search. As for pairing after separation model, 2-aminopurine does not change the number of hydrogen bonds and thus pairing occurs uninhibited (Figure 1-5). If we can establish the correct homology search model, we also are able to know how the incoming strand invades the dsDNA, i.e., if a triplex intermediate is required, we can also
Figure 1-4. (A) An adenine-thymine (A•T) base pair. (B) A base pair involving 2-aminopurine (1), a fluorescent analog of adenine, and thymine. Note the changes in the position of exocyclic amino functional group from the C6 position of the purine ring system in adenine to the C2 position in 2-aminopurine.
Figure 1-5. Influence of functional group change on homology recognition. The semi-circular line indicates the RecA protein. The hashed lines represent Watson-Crick hydrogen bonds. In the upper scheme, if the triplex intermediate is required, the exchange of an adenine in the incoming strand for an adenine in the A•T base pair of original duplex can happen. In the lower scheme, if the triplex intermediate is required, the exchange of a 2-aminopurine in the incoming strand for an adenine in the A•T base pair of original duplex shall deter because the three bases can not form a triplex intermediate.
determine if the incoming strand should invade from major groove of dsDNA or minor groove.

Recently, the stopped-flow fluorescence technique has been used in conjunction with 1 to study protein-DNA interactions.\textsuperscript{34,35,40} In addition, several rapid kinetic investigations involving the RecA protein have appeared recently.\textsuperscript{10-12} However, no one has used this fluorescence-based continuous kinetic assay using 1 to study RecA protein.

1.6 Fluorescent Nucleoside Triphosphates

Progress in biochemical research has clearly pointed out the unique importance of ribo- and deoxyribo-5'-triphosphate in biological systems. ATP is well known as the primary chemical energy source in many systems, including RecA-mediated DNA strand exchange. In addition, ATP also serves as direct precursors of both RNA\textsuperscript{41} and DNA.\textsuperscript{42} Because of the multiple functions of ATP, it has been extremely useful in a wide variety of biochemical studies.\textsuperscript{43,44} ATP binding and hydrolysis energies are transduced during the whole DNA strand exchange process. Because of similarity of 1 and A as described above, we intend to use the 5'-triphosphate of 1 as another probe for our kinetic studies. Such experiments will provide mechanistic details for the role of ATP binding and hydrolysis in this important recombination process. Structural and positional changes in fluorescent 1TP can provide novel insights into the strand exchange process and how ATP binding and hydrolysis are coordinated with strand exchange.
CHAPTER 2
OBJECTIVES

In spite of intense study over 30 years, some deceptively simple questions have yet to be conclusively answered regarding the molecular mechanisms and the nature of intermediary structures of RecA-mediated DNA strand exchange. The objective of this thesis was to develop a continuous kinetic assay for DNA pairing and exchange using a DNA-based fluorescent signal and employ the assay to investigate the early events of RecA-mediated DNA strand exchange in vitro.

\[ \text{RecA} + \text{ssDNA}^* + \text{ATP} \rightleftharpoons [\text{RecA}\cdot\text{ssDNA}^*\cdot\text{ATP}] \]

The scheme above shows the activation of RecA protein for ATP hydrolysis and DNA strand exchange by ssDNA. We planned to employing the fluorescence labeled ssDNA (*) as a probe to characterize the kinetics of these processes using rapid-mixing stopped-flow spectrophotometry. Therefore, the active RecA protein was to be overexpressed and purified; 2-aminopurine deoxyribonucleoside phosphoramidite was to be synthesized and incorporated into oligodeoxynucleotides; fluorescence labeled oligonucleotides were to be HPLC-purified for kinetic studies. Once we have the necessary substrates, kinetic studies would be conducted to not only study the process of DNA strand exchange but also test the practicality of our designed assay.
CHAPTER 3
EXPRESSION AND PURIFICATION OF RecA PROTEIN

3.1 Introduction

A number of methods for expression and purification of RecA protein have been published.45-48 Two difficulties have plagued researchers in this field. First, the overall yield of protein is often low because RecA becomes toxic at moderate levels of expression. Second, nuclease contamination is an ever-present worry in RecA preparations. We needed to find a way to prepare large quantities of highly purified RecA suitable for quantitative kinetic investigations. We elected to use a tightly regulated T7 expression system.

3.2 Expression of RecA Protein

In order to find the best combination of plasmids and strains for RecA expression, we chose to do a comparison experiment. As described in Table 3-1, we tested eight different kinds of cells. The strains, plasmids, and properties of the cells are also listed in Table 3-1. One colony of the bacterial strain carrying the plasmids of interest was grown in LB media which contains appropriate antibiotics. After the OD$_{600}$ reading reached 0.6 ~ 0.8, lactose or IPTG was added to induce RecA protein expression. The expression results were monitored by protein SDS-PAGE and listed in Table 3-1. It is concluded that cell STL327/pAIR79/pT7POL26 is the best choice for RecA expression because the strain background is free of nucleases and the two-plasmid system
Table 3-1. RecA Expression Results

<table>
<thead>
<tr>
<th>Strain</th>
<th>Expression plasmid</th>
<th>Secondary plasmid</th>
<th>Host recA?</th>
<th>Nucleases?</th>
<th>Induction level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IPTG</td>
</tr>
<tr>
<td>GE645</td>
<td>pGE226</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>BL21</td>
<td>pAIR79</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>pAIR79</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BL21 (DE3) pLysS</td>
<td>pAIR79</td>
<td>pLysS</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BLR</td>
<td>pAIR79</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>BLR (DE3)</td>
<td>pAIR79</td>
<td>—</td>
<td>—</td>
<td>+</td>
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</tr>
<tr>
<td>BLR (DE3) pLysS</td>
<td>pAIR79</td>
<td>pLysS</td>
<td>—</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>STL327</td>
<td>pAIR79</td>
<td>pT7POL26</td>
<td>—</td>
<td>—</td>
<td>++</td>
</tr>
</tbody>
</table>

The results are rated where "++" indicates "excellent", "+" indicates "moderate" or "yes" and "-" indicates "poor" or "no".

The cells were grown in LB media after 4-h lactose or IPTG induction at 37 °C. The final concentration of lactose or IPTG is 0.2% (w/v) or 0.4 mM, respectively.
gives consistent results. We received the overexpressing RecA plasmid (pAIR79) from Alberto Roca and Michael Cox (Department of Biochemistry, University of Wisconsin at Madison).\(^4^9\) The plasmid has the recA gene cloned into a commercial expression vector (pET21d+, Novagen), placing it under regulation of the promoter for T7 RNA polymerase.\(^5^0\) Protein production from this plasmid is controlled by induction of the T7 RNA polymerase gene coded on a second plasmid, pT7POL26,\(^5^1\) which is regulated by a lac-type promoter. This plasmid provides tight transcriptional control in T7 promoter based plasmids by attenuating basal expression of the T7 RNA polymerase using a series of tandemly arranged transcription terminators. This expression system will prevent leaky expression of the RecA protein which is toxic when overproduced. The whole expression system is maintained in strain STL327, which is deficient in exonucleases I and III, endonuclease I, and recombinase J.\(^5^2\) The STL327 strain is used to ease purification by decreasing nuclease contamination. There is little difference of inductive effect between IPTG and lactose, so we chose lactose as our inducer.

3.3 100-mg Scale Overexpression and Purification of the RecA protein

STL327/pAIR79/pT7POL26 cells were grown in the rich media TB at 37 °C and were induced by the addition of lactose to a final concentration of 0.2% (w/v) of 3 L cultures. The cultures had reached $\text{OD}_{600} = 4 \sim 5$. 10 g of a wet cell pellet was collected from a 3-L culture. SDS-PAGE analysis revealed that the RecA protein was successfully overexpressed. A detailed protocol is described in Appendix I.
The *E. coli* RecA protein was purified and stored essentially as described by Cox and his coworkers previously. Dr. Alberta Roca generously provided a written protocol for performing purification. We have attempted to follow this exemplary text here with some modifications. An overview of the process of RecA purification is illustrated in Figure 3-1. *E. coli* cells harvested from 3 L of TB culture were lysed with lysozyme (0.1 mg/mL) and Brij-35 (5 mg/mL) followed by sonication. The supernatant was then precipitated with 10% polyethyleneimine (Polymin P) and the RecA protein was extracted from the pellet with R Buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT) plus 150 mM ammonium sulfate. The extracted supernatant was then pelleted with solid ammonium sulfate (0.28 g/mL). The pellet was resuspended in 50 mL R Buffer containing 50 mM KCl and dialyzed against 3 L of the same buffer prior to loading on a 75-mL DEAE-Sepharose column. A total of 50 mL of protein-containing solution was loaded on the column, and the loading flow-through containing the RecA protein (150 mL) was collected (Figures 3-2 and 3-3). The collected flow-through was dialyzed against R+50 buffer and loaded onto a hydroxyapatite column and eluted using a gradient from low-salt P Buffer (20 mM potassium phosphate, pH 6.9, 0.1 mM EDTA, 1 mM DTT) to P Buffer containing 350 mM potassium phosphate. The RecA-containing fractions (Figures 3-4 and 3-5) were collected and pooled, and the RecA was concentrated by the addition of ammonium sulfate (0.52 g/mL). The pellet was resuspended in 15 mL R Buffer and dialyzed against 2 x 3-L portions of R Buffer. The concentration of pure RecA protein was measured using the absorbance at 280 nm and the published extinction coefficient ($\epsilon_{280} = 0.59$ mL/mg). Following dialysis, the protein solution was separated into small
Figure 3-1. An overview of the RecA protein purification procedure. During the cell lysis step, lysozyme, Brij-35 and sonication were used to lyse the cells. Polyvinyl P was used to precipitate the free DNA and proteins. Then Ams was used to precipitate the cellular proteins. DEAE-Sepharose and HAP column were used to separate RecA protein from other protein contaminants. The final amount of RecA protein we purified from 3 L of culture was 127 mg.
Figure 3-2. Protein SDS-PAGE pictures of DEAE-Sepharose column fractions. M is Rainbow Protein Molecular Weight Marker (Amersham Life Science). The big bands of sample B1, B2 and 24 are in the middle of 46K-30K from molecular weight marker M. Since the molecular weight of RecA is 38K, the two gel pictures show B1, B2 and 24 have a lot of RecA protein. Since sample 21 and 27 have no sign of RecA protein, sample 24 could be contaminated by B1 when protein gel was prepared. Farther SDS-PAGE analysis (data not shown) shows that the RecA appearing in fraction 24 was due to a loading error which is cross-contamination from B1.
Running buffer: 25 mM Tris base; 14.4% Glycine (w/v); 1% SDS (w/v)
Lower Gel: 14.8% Acrylamide (w/v); 0.39% N,N-methylenebisacrylamide
Stacking Gel: 4.9% Acrylamide (w/v); 0.13% N,N-methylenebisacrylamide
Constant Current: 30 milli-amps (DC)
Gel Glass plates: 10 x 8 cm
Figure 3-3. Protein SDS-PAGE picture of DEAE-Sepharose column fraction 1-3 and beaker 1-4. M is Rainbow Protein Molecular Weight Marker (Amersham Life Science). There is no band between 46K-30K range. The big bands of sample B1, B2 and B3 are in the middle of 46K-30K from molecular weight marker M. Since the molecular weight of RecA is 38K, the gel picture indicates B1, B2 and B3 have a lot of RecA protein. Some RecA protein is still in the column (B3) even though most of it (B1 and B2) comes out quickly. Note that the sequence of DEAE fractions is B1, B2, fractions 1-48, B3 and B4.

Running buffer: 25 mM Tris base; 14.4% Glycine (w/v); 1% SDS (w/v)
Lower Gel: 14.8% Acrylamide (w/v); 0.39% N,N-methylenebisacrylamide
Stacking Gel: 4.9% Acrylamide (w/v); 0.13% N,N-methylenebisacrylamide
Constant Current: 30 milli-amps (DC)
Gel Glass plates: 10 x 8 cm
Figure 3-4. The protein gel pictures of HAP column fractions. M is Rainbow Protein Molecular Weight Marker (Amersham Life Science). 1', 2' and 3' are the samples in 50-mL falcon tubes. The big bands of samples 50-80 are in the middle of 46K-30K from molecular weight marker M. Since the molecular weight of RecA is 38K, the two gel pictures show fractions 50-80 have a lot of RecA protein. Note that the sequence of HAP fractions is B, 1'-3', and fractions 1-155.

Running buffer: 25 mM Tris base; 14.4% Glycine (w/v); 1% SDS (w/v)
Lower Gel: 14.8% Acrylamide (w/v); 0.39% N,N-methylenebisacrylamide
Stacking Gel: 4.9% Acrylamide (w/v); 0.13% N,N-methylenebisacrylamide
Constant Current: 30 milli-amps (DC)
Gel Glass plates: 10 x 8 cm
Figure 3-5. The protein gel pictures of HAP column fractions 41-49 and 81-89. It shows fractions 41-49 and 81-89 have some RecA protein but also have some impurities.

Running buffer: 25 mM Tris base; 14.4% Glycine (w/v); 1% SDS (w/v)
Lower Gel: 14.8% Acrylamide (w/v); 0.39% N,N-methylenebisacrylamide
Stacking Gel: 4.9% Acrylamide (w/v); 0.13% N,N-methylenebisacrylamide
Constant Current: 30 milli-amps (DC)
Gel Glass plates: 10 x 8 cm
aliquots, snap-frozen in liquid $N_2$, and stored at $-80$ °C. The protocol for RecA purification is described in Appendix II. RecA is > 95% pure based on the SDS-PAGE analysis of HAP. The final amount of RecA we purified from 3 L of culture was 127 mg.

3.4 Purity and Activity Tests

Nuclease tests

Hot and cold nuclease tests are utilized *in vitro* to detect the presence of contaminating exonucleases and endonucleases, respectively, within RecA preparation. The hot nuclease test monitors $^{32}$P-end-labeled single-stranded and double-stranded oligonucleotides degradation in the presence of purified RecA through PAGE. Depletion of a band signal or a shift in band size over time would indicate exonuclease activity. The cold nuclease test utilizes larger, circular ssDNA and dsDNA from phage to monitor endonucleolytion cleavage. Degradation of the ssDNA or dsDNA by purified RecA is observed through agarose gel electrophoresis. In the absence of nuclease activity, the unmodified supercoiled substrate DNA migrate in a single band. Nicking, or cleavage of the DNA can be observed as a shift in the position of this band.

Dr. Alberto Roca performed the nuclease tests at UW-Madison. The result of the hot nuclease test is shown on Figure 3-6. The picture shows that our RecA protein is free of detectable endo- and exonucleases which are the most contaminants of RecA purification.
Figure 3-6. Hot nuclease test for RecA protein. The test was done by Dr. Roca at University of Wisconsin at Madison. The "oligo" line represents the original oligonucleotide; the "exol" line represents the degradation of the oligonucleotide treated with exonucleases (please note the depletion of the band signal and the shift of band size). The "Cox 30" line represents the result of the oligonucleotide treated with RecA protein prepared by Cox Lab at University of Wisconsin at Madison after 30 min. The "Cox 90" line represents the result of the oligonucleotide treated with RecA protein prepared by Cox Lab after 90 min. The "XZ 30" line represents the result of the oligonucleotide treated with RecA protein we prepared after 30 min. The "XZ 90" line represents the result of the oligonucleotide treated with RecA protein we prepared after 30 min.
**ATPase Assay**

RecA protein is a DNA-dependent ATPase, hydrolyzing ATP with a high activity. The specific activity of RecA protein can therefore be measured by the rate of ATP hydrolysis. This measurement can be reliably using an indirect spectrophotometric ATPase assay with an ATP regeneration system.\(^{54,55}\) The ATP regeneration system contains a series of coupled enzyme reactions. In the presence of ADP, which is produced upon the hydrolysis of ATP by RecA, PK converts 1 eq of PEP to pyruvate and regenerates ATP from the ADP. Pyruvate is then reduced to lactate by LDH with the conversion of 1 eq of NADH to NAD\(^+\). The decrease in [NADH] results in the decrease in the absorbance by NADH as observed at 340 nm. Jie Xiao and Hansi Singh used my RecA to perform the ATPase assay one year after RecA had been prepared. Steady-state ATPase rates were measured as a function of [RecA] by monitoring the absorbance of NADH off-peak at 380 nm using a Perkin-Elmer Lambda 20 spectrophotometer equipped with an automated 6-cell changer and a PTP-6 programmable Peltier temperature controller. The reactions were carried out in 0.2 x 1.0 cm quartz cuvettes placed in thermojacketed cuvette holders with circulating cold water. The reaction temperature was maintained at 37.0 ± 0.1 °C using the external temperature control unit. The components of the enzyme coupling system (3 mM PE, 5 U/mL PK, 5 U/mL LDH, and 2 mM NADH), poly(dT) (20 μM-nts), and RecA protein were equilibrated at 37 °C in ATPase Buffer (25 mM Tris-HCl, pH 7.5, 3 mM KCl, 1 mM DTT, and 5% glycerol). After 10 min, the reactions were initiated by the addition of ATP and Mg(OAc)\(_2\) to final concentrations of 3 mM and 10 mM, respectively. The ATP hydrolysis rate (\(V_{obs}\)) at each [RecA] was obtained from the slope of the time-dependent NADH absorbance change using
Δε_{280} = 1.21 mM⁻¹cm⁻¹ (Figure 3-7). The slopes were measured by least-squares fitting of the linear portion of the absorbance vs. time plots using the Kinlab software supplied with spectrophotometer. For each reaction, the linear range was selected manually to include the most data points while maintaining the maximum correlation coefficient for the linear fit. The ([RecA], V_{obs}) data were analyzed using both standard titration binding isotherm analysis and double-reciprocal analysis. The former yielded $k_{cat} = 29 \pm 2$ min⁻¹ and a stoichiometry of $3.6 \pm 0.5$ nts/RecA monomer, while the latter yielded $k_{cat} = 32 \pm 2$ min⁻¹ and an apparent $K_d = 4.6 \pm 1.0$ μM. These results are internally consistent and agree well with literature values.56,57 Moreover, they demonstrate that the protein is highly purified (concentrations determined using the spectrophotometric and ATPase titration methods are in good agreement) and is > 99% active.

3.5 Conclusion

We have overexpressed and purified 127 mg of pure and active RecA protein which is suitable for our kinetic studies of DNA strand exchange from 10 g wet cell cultures.
Figure 3-7. ATPase assay of RecA protein. "XZ RecA" is the protein we made (O) and "Promega RecA" is the protein we purchased from Promega (●). Jie Xiao and Hansi Singh performed the ATPase assay one year after RecA had been prepared. Steady-state ATPase rates were measured as a function of [RecA] by monitoring the absorbance of NADH off-peak at 380 nm using a Perkin-Elmer Lambda 20 spectrophotometer equipped with an automated 6-cell changer and a PTP-6 programmable Peltier temperature controller. The reactions were carried out in 0.2 x 1.0 cm quartz cuvettes placed in thermojacketed cuvette holders with circulating cold water. The reaction temperature was maintained at 37.0 ± 0.1 °C using the external temperature control unit. The components of the enzyme coupling system (3 mM PE, 5 U/mL PK, 5 U/mL LDH, and 2 mM NADH), poly(dT) (20 µM-nts), and RecA protein were equilibrated at 37 °C in ATPase Buffer (25 mM Tris-HCl, pH 7.5, 3 mM KCl, 1 mM DTT, and 5% glycerol). After 10 min, the reactions were initiated by the addition of ATP and Mg(OAc)$_2$ to final concentrations of 3 mM and 10 mM, respectively.
CHAPTER 4
SYNTHESIS OF 2-AMINOPURINE DEOXYRIBONUCLEOSIDE PHOSPHORAMIDITE

As mentioned before, endeavor in our lab includes the use of modified fluorescent nucleosides to elucidate the mechanism by which RecA protein mediated DNA strand exchange takes place. One nucleoside we are interested in is 2-aminopurine deoxyribose nucleoside 1. In order to incorporate the nucleoside into oligonucleotides, we need to synthesize the synthon, 2-aminopurine deoxyribonucleoside phosphoramidite 3, for the automated synthesis of oligonucleotides.

![Chemical structure of 2-Aminopurine Deoxyribonucleoside Phosphoramidite](image)

2-Aminopurine Deoxyribonucleoside Phosphoramidite

The synthesis of phosphoramidites from nucleosides is relatively straightforward. The approach consists of protecting the 5'-hydroxyl group with 4, 4'-dimethyltrityl group and reacting protected deoxyrobonucleosides 4 with 2-cyanoethyl diisopropylchlorophosphoramidite and N,N-diisopropylethylamine (Scheme 1). The products 5 are isolated by conventional extraction procedures, precipitated, and stored as dry powders.\textsuperscript{58-60}
Scheme 1

Reagents and conditions: (a) DMTCl, DMAP, pyridine; (b) 2-cyanoethyl diisopropyl-chlorophosphoramidite, (iPr)$_2$NEt, DCM.

4.1 Review of Literature Methods for Synthesis of 1

The key issue here involves the synthetic transformation of normal nucleosides into 2-aminopurine deoxyribonucleoside 1. Many methods have been reported for the production of the fluorescent nucleoside. We began by surveying the literature and choosing the most appropriate method. A brief summary of the variously published routes to 1 is presented below.

Fox synthesis (Figure 4-1)$^{61}$ and Nair synthesis (Figure 4-2)$^{62}$ are the synthetic routes of 2-aminopurine ribonucleoside 2. The intermediate product of Fox synthesis was thioguanosine which was treated with Raney Nickel to give 2. Guanosine was converted to 6-chloro-guanosine first in Nair synthesis. Photolysis of 6-chloro-guanosine in dry THF containing 10% Et$_3$N produced 2. The use of Raney Nickel in Fox synthesis does not require anhydrous condition and therefore produces few by-products.

McLaughlin synthesis (Figure 4-3)$^{63}$ and Kobe synthesis (Figure 4-4)$^{64}$ are the syntheses of 1 from 2'-deoxyguanosine. These two methods are similar.
Figure 4-1. Fox Synthesis of 2-aminopurine ribonucleoside. First, treatment of guanosine with benzoyl chloride in pyridine yielded N-benzoyl-2',3',5'-tri-O-benzoylguanosine. This blocked nucleoside was treated with phosphorus pentasulfide to give protected thioguanosine. It was deprotected by sodium methylate to give thioguanosine. Dethiolation of thioguanosine with Raney Nickel gave 2-aminopurine nucleoside.

Reagents and conditions: (a) BzCl, pyridine; (b) P_2S_5, pyridine, H_2O; (c) 2 N NaOMe, MeOH, pH 9; (d) Raney nickel, H_2O.
Reagents and conditions: (a) Ac₂O, Et₃N, DMAP; (b) POCl₃, N,N-diethylaniline; (c) Et₃N, THF, hv; (d) NH₃, EtOH.

Figure 4-2. Nair synthesis of 2-aminopurine ribonucleoside. The starting material for the synthesis was guanosine which was selectively acetylated by using acetic anhydride. Treatment of the triacetylated guanosine with phosphorus oxychloride gave 6-chloro-guanosine. Photolysis of 6-chloro-guanosine in dry THF containing 10% Et₃N produced 2-aminopurine ribonucleoside.
Reagents and conditions: (a) BzCl, pyridine; (b) (iPr)₂NEt, triisopropylbenzene sulfonylchloride, DMAP, DCM; (c) NH₂NH₂, dioxane; (d) Ag₂O, dioxane:H₂O (95:5); (e) 2 N NaOH, MeOH.

Figure 4-3. McLaughlin synthesis of 2-aminopurine deoxyribonucleoside. The synthetic method begins with 2'-deoxyguanosine. A benzoyl group is used to protect the 3', 5' hydroxyl group and 2-amino group, followed by the sulfonation of the guanosine O6 in order to labilize the six position to addition/elimination reactions. The sulfonated 2'-deoxyguanosine derivative is reacted with hydrazine to prepare the purine 6-hydrazino derivative. The hydrazino derivative can be oxidized in the presence of silver (I) oxide to form the protected 2-aminopurine deoxynucleoside. Deprotection of carbohydrate residue and subsequent preparation of the dimethoxytrityl phosphoramidite derivative produced a compound suitable for incorporation into oligonucleotides.
Reagents and conditions: (a) Ac₂O, Et₃N, DMAP; (b) Et₃N, triisopropylbenzene sulfonylchloride, DMAP, DCM; (c) Pd/C/H₂ or Pd/C/triethylammonium formate.

Figure 4-4. Kobe synthesis of 2-aminopurine deoxyribonucleoside. The synthesis is similar to the McLaughlin synthesis in early steps. However, the reduction of the sulfonated 2'-deoxyguanosine derivative was achieved using either Pd/C/H₂ or Pd/C/triethylammonium formate.
in early steps. Kobe and coworkers reported either Pd/C/H₂ or Pd/C/triethylammonium formate system is more effective than silver (I) oxide to reduce of the sulfonated 2'-deoxyguanosine derivative. Both methods suffer from poor yields and high cost of starting materials.

Since the cost of 2'-deoxyguanosine is relatively high as a starting material. Cech and coworkers (Figure 4-5) begins with 6-chloro-guanosine just as Nair synthesis. After the protection of the 3'- and 5'- hydroxyl groups by 1,3-dichloro-1,1,3,3-tetraisopropylsiloxyxane, the 2'-hydroxyl groups of the protected nucleoside was transformed to the phenoxythiocarbonyl ester. Both the 6-position of the heterocyclic base and the 2'-position of the sugar moiety were converted simultaneously giving the 1. The advantages of the synthesis are (1) the procedure does not require any protection of the exocyclic amino function in the starting material; (2) tri-n-butyltin hydride is a mild reducing reagent and doesn’t affect the glycosidic bond.

Sowers synthesis (Figure 4-6) is the combination of the Fox synthesis and the Cech synthesis. However, Sowers group did the protection of the amino group prior to reduction of the sugar moiety because of the instability of deoxyribonucleoside to acylation conditions.

4.2 Synthesis of 2-Aminopurine 2'-Deoxynucleoside Phosphoramidite 3

The method we employed to synthesize our desired 2AP phosphoramidite 3 is Sowers method. The synthetic scheme is shown on Figure 4-6 and the experimental data are described in Appendix IV.
Reagents and conditions: (a) TPDSCl₂, pyridine; (b) PTC-Cl, DMAP, CH₃CN; (c) AIBN, n-Bu₂SnH, benzene; (d) TBAF/THF.

Figure 4-5. Cech synthesis of 2-aminopurine deoxyribonucleoside 1. Similar to Nair synthesis, Cech synthesis begins with 6-chloro-guanosine. After the protection of the 3’- and 5’- hydroxyl groups by 1,3-dichloro-1,1,3,3-tetraisopropylsiloxane, the 2’-hydroxyl groups of the protected nucleoside was transformed to the phenoxythiocarbonyl ester. Both the 6-position of the heterocyclic base and the 2’-position of the sugar moiety were converted simultaneously giving 1.
Figure 4-6. Sowers synthesis of 2-aminopurine deoxyribonucleoside phosphoramidite 1. We employed this synthetic route to synthesize our 2AP phosphoramidite. The method involves protection of the 2-aminopurine ribonucleoside, reduction to the deoxyribonucleoside and standard preparation of phosphoramidite.
The synthesis began with thioguanosine 6. Thioguanosine was treated with Raney nickel in boiling water to give 2-aminopurine ribonucleoside 7. The reaction was completed in 4 hours and gave 70% yield. The yield could be increased if less Raney nickel was used.

Ribonucleoside 7 was treated with trimethylchlorosilane in pyridine for protection of the hydroxyl groups, and then immediately reacted with isobutyryl chloride to effect N-acylation. The trimethylsilyl groups were removed by dilute ammonia to give N-acyl ribonucleoside 8.

Ribonucleoside 8 was treated with 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane/pyridine to afford selective protection as its 3', 5'-O-(1,1,3,3-tetraisopropyldisilox-1,3-diyl) derivative 9. Phenoxythiocarbonylation of the 2'-hydroxyl group of 3', 5'-O-TPDS-nucleoside 9, AIBN-initiated homolytic deoxygenation with tri-n-butyltin hydride, and deprotection with TBAF completed the conversion to 2'-deoxynucleoside 11. The 50% yield is satisfactory.

Deoxyribonucleoside 11 was treated with 4, 4'-dimethoxytrityl chloride and 4-dimethylaminopyridine in dry pyridine to give dimethoxytritylated deoxyribonucleoside 12 with a yield of 50%.58,59

The DMT-deoxyribonucleoside 12 was treated with 2-cyanoethyl diisopropylchlorophosphoramidite and (iPr)₂NET in dry DCM to give our desired final product deoxyribonucleoside-3'-O-(2-cyanoethyl)-N, N-diisopropyl-phosphoramidite 3 with a yield of 50%.60
4.3 Synthesis of 6-Thioguanosine Ribonucleoside 6

Jones and coworkers reported a small-scale one-flask synthesis of thioguanosine (Figure 4-7). We followed his method successfully in a small-scale reaction but had a difficult time to scale it up. The experimental details are also described in Appendix IV.
Figure 4-7. Jones synthesis of 6-thioguanosine ribonucleoside. First, guanosine was treated with (CF$_3$CO)$_2$O in pyridine. This blocked intermediate was treated with sodium hydrosulfide to give thioguanosine.
CHAPTER 5
SYNTHESIS AND PURIFICATION OF OLIGONUCLEOTIDES

5.1 CED-Phosphoramidite Chemistry

The synthesis of DNA molecules is now easily automated using solid
phase chemistry, thanks to the pioneering work of Merrifield\textsuperscript{69} in the field of
solid phase synthesis. There have been several chemical procedures\textsuperscript{70-72}
developed, but the β-cyanoethyl-\textit{N},\textit{N}-diisopropyl phosphoramidite (CED-phos-
phoramidite) method\textsuperscript{73} is generally recognized as the fastest and most efficient
approach.

The CED-phosphoramidite synthesis begins with an initial protected
nucleoside attached via an organic linker to a CPG solid support (controlled
pore glass or CPG) contained in a reactor column. Reagents and solvents can
be pumped through the column to control the addition of successive protected
phosphoramidite nucleotide monomers (Figure 5-1). The addition of each new
nucleoside to elongate the chain goes through the following cycle (Figure 5-2).
Except as noted, all bases include the protecting groups on the exocyclic amine
of the bases. It is important to have an anhydrous environment in the column
since water will react with the activated phosphoramidite.

1. Debloking: Detritylation of the 5'-end of the last support bound
nucleotide by treatment with TCA. Once the DMT is removed, the
exposed 5'-OH moiety is able to react and add the next nucleotide to the
chain.

2. Acetonitrile wash: Removes any free DMT\textsuperscript{+}, Cl\textsubscript{3}CCOO\textsuperscript{-}. 
Figure 5-1. Structures of the standard DNA monomers
Figure 5-2. CED-phosphoramidite synthesis cycle. Detritylation (debloking) of the 5’-end of the last support bound nucleotide by treatment with trichloroacetic acid is followed by reacting with the next nucleotide phosphoramidite (coupling) which is activated by mixing with tetrazole prior to delivery to the column (activate). A small percentage of the 5’-hydroxyl groups will fail to react in each cycle. These failed sequences must be “capped” or blocked from any further chain elongation cycles by mixing acetic anhydride and N-methylimidazole. The addition of an iodine solution is used to oxidize the labile phosphite trivalent phosphorus atom to the more stable phosphate (pentavalent) form found in native DNA (oxidize). Repeat the synthesis cycle until synthesis is complete.
3. Activation: Tetrazole is mixed with the next nucleotide phosphoramidite to be coupled to activate the monomer prior to delivery to the column. This forms a highly reactive species which will readily attach to the now unprotected 5'-OH site on the oligonucleotides.

4. Coupling: The activated phosphoramidite reacts with the exposed 5'-hydroxyl of the growing chain to form a phosphite linkage.

5. Acetonitrile wash: Removes unreacted phosphoramidites.

6. Capping of failure sequences: a small percentage of the 5'-hydroxyl groups will fail to react in each cycle. These failed sequences must be "capped" or blocked from any further chain elongation cycles. This is accomplished by mix acetic anhydride and N-methylimidazole to form an activate acetylating agent, which then reacts with the free 5'-hydroxyl groups.

7. Acetonitrile wash: Removes unreacted capping reagents.

8. Oxidation: The addition of an iodine solution is used to oxidize the labile phosphite trivalent phosphorus atom to the more stable phosphate (pentavalent) form found in native DNA.

9. Acetonitrile wash: Removes unreacted oxidizing reagents.

10. Repeat steps 1-9 until synthesis is complete.

5.2 Oligonucleotide Sequences

The design of our first-generation oligonucleotide substrates was based on a study of chlorambucil-bearing ODNs employed to delineate the effects of
length and nonhomologous base substitutions on RecA-mediated DNA strand exchange. Since primarily alkylates guanine N7 and to a much lesser extent adenine N3 in dsDNA, the efficiency and specificity of alkylation could reflect those of pairing of DNA strands. The researchers concluded that: (1) alkylation required the ODN to be at least 26 nucleotides long and to possess homology to the target in the vicinity of the modification site; and (2) mismatches inhibited alkylation when they perturbed the structure of the strand exchange product near the targeted guanine. Based on their results, we devised the following oligonucleotides sequences:

A: 5’-TAGCTCCTTCCGCTCTCCGATCGTTGTCAG-3’
B: 5’-TAGCTCCTTCCGCTCTCCGATCGTTGTC1G-3’
C: 5’-TAGCTCCTTCCGCTCTCCG1TCGTTGTCAG-3’
D: 5’-TGCTCCTTCCGCTCTCCGATCGTTGTCAG-3’
E: 5’-TGCTCCTTCCGCTCTCCG1TCGTTGTC1G-3’
F: 5’-TAGCTCCTTCCGCTCTCCAG1CCGTTGTCAG-3’
G: 5’-TAGCTCCTTCCGCTCTT1CTATTTGTCAG-3’
H: 5’-CTGACAAAGATCGAGGAGACCCAGAGGACCTA-3’

ODN A-G are almost identical except: B has a 1 substituted for dA near 3’ end; C has a 1 substitute for dA in the middle of the strand; D has a 1 substitute for dA near 5’ end; E has 1 substitute for all three dA; F has a 1 substitute for dA in the middle of the strand and two mismatches around 1; G has a 1 substitute for dA in the middle of the strand and six mismatches around 1. ODN H is complementary to ODN A-G though F and G have some mismatches. Because the homology search is unidirectional and can be
blocked by heterologous sequences,\textsuperscript{75,76} we intentionally placed 1 in different positions in the ODNs and introduced mismatches in some ODNs to test how they will affect DNA strand exchange.

5.3 Automatic Synthesis of Oligonucleotides

The ten ODNs were synthesize on DNA synthesizer using our synthesized 3. The protocol for automated synthesis of ODNs is described in Appendix IV.

There is a trityl-off monitor in DNA synthesizer. The raw data of trityl-off could provide some estimate of coupling efficiency. Figure 5-3 is the trityl-off data of our synthesized ODN E which has three 1s. It shows that our synthesized 2AP phosphoramidite 3 performed as well as commercially available dA, dC, dG, and T phosphoramidites. The overall synthesis of all the ODNs is good because all trityl-off peaks are in the same high region.

5.4 Postsynthesis and Purification of Oligonucleotides

When the synthesis has been completed, the oligomer is the same as natural ODNs because it is still attached to CPG column and has protecting groups on its bases and cyanoethyl group in phosphate backbone. The whole post-synthesis and purification steps are summarized as following Figure 5-4. The protocol for purification of ODNs is described in Appendix IV.

We first needed to do cleavage and full deprotection. We treated ODNs with ammonium hydroxide to remove CPG and cyanoethyl group. Then we
Figure 5-3. Trityl-off data of oligonucleotide E which has three Ts. Our synthesized 2AP phosphoramidite 3 performs as well as commercial A, C, T and G phosphoramidites.
Figure 5-4. Procedure of post-synthesis and purification of oligonucleotides. ODNs are treated with ammonium hydroxide to remove CPG, cyanoethyl and amino-protecting groups. RP HPLC is used in order to purify oligonucleotides. Trityl-on oligonucleotides then are easily detritylated by acetic acid. If necessary, another RP HPLC purification is performed in order to remove small impurities.
heated the ODNs with ammonium hydroxide to remove the protecting groups of DNA bases. Figure 5-5 is the typical UV spectrum of ODNs at this stage, just before HPLC. RP HPLC was used in order to purify oligonucleotides. There have been a lot of good reviews about purifying ODNs.77,78

Trityl-on reverse-phase HPLC utilizes the separation of tritylated from non-tritylated oligonucleotides. If all the reactions involved in an oligonucleotide synthesis have worked properly, the only compound bearing the terminal 5'-protecting group, the DMT group, is the full-length product. The failure sequences are acetylated at the 5'-end during capping. Under appropriate solvent conditions, a RP HPLC column will easily separate trityl from non-trityl bearing ODNs. The more hydrophobic tritylated full-length product will be retained longer on the column than shorter failure sequences. Figure 5-6 is the typical HPLC spectrum of trityl-on HPLC purification.

Trityl-on oligonucleotides could be easily detriylated by acid. We treated ODNs with acetic acid and extracted the removed DMT group with ethyl acetate. Great caution must be taken during the step because ODNs will be depurinated under acidic conditions.

As mentioned before, if all the reactions involved in an oligonucleotide synthesis have worked properly, the only compound bearing the terminal 5'-protecting group, the DMT group, is the full-length product. It means the length and sequence of the product after detriylation should be the desired product. However, there might be still some impurities in the sample. If the deprotection step is not complete during postsynthesis, some bases may still have the protecting groups on the exocyclic amines. If depurination happens during detriylation, the product loses the purine bases. Of course, if ethyl acetate
Figure 5-5. Typical UV spectrum of oligonucleotides before RP HPLC purification.
Figure 5-6. Typical spectrum of trityl-on HPLC purification. The main peak is the single trityl-on ODN peak at 15.51 min and it is far away from multiple impurity peaks around 8 min. It is easy to separate our products from impurities using trityl-on HPLC purification.
extraction is not complete during detritylation, the trityl group could be still in the sample. It is necessary for us to do another RP HPLC purification in order to get pure oligonucleotides. Figure 5-7 is the typical HPLC spectrum of trityl-off purification. The main peak is around 12.54 min and the product area accounts for 95% of the whole area. We only collected the fractions around the main peak, so our oligonucleotides are very pure based on HPLC analysis.

Figure 5-8 is the typical UV spectrum of ODNs after trityl-off HPLC purification.

5.5 DNA Base Composition Analysis

We attempted to characterize our synthetic oligonucleotides by digesting the oligodeoxynucleotides to their monodeoxynucleoside constituents with snake venom phosphodiesterase and alkaline phosphatase to yield the free nucleosides.59,79 The nucleosides were then fractionated by reverse phase HPLC. The results are inconclusive so far.
Figure 5-7. Typical spectrum of trityl-off HPLC purification. The main peak is our desired product at 12.54 min. We collected fractions around the peak, so the product is very pure.
Figure 5-8. Typical UV spectrum of oligonucleotides after trityl-off RP HPLC purification.
CHAPTER 6
KINETIC STUDIES OF RecA BINDING TO ssDNA

6.1 Introduction

Polarized light spectroscopy has been used to study the interaction of the RecA protein with DNA. Several different RecA-DNA complexes have been identified and characterized with respect to their relative stoichiometries, chromophore orientations, and nuclease accessibilities.\(^{80}\)

In the absence of ATP or its analogs, RecA binds only one molecule of ssDNA. These RecA•ssDNA complexes found in the absence of cofactor adopt a stiff fiber-like structure with a stoichiometry of one RecA monomer per four DNA bases. The indolic planes of the tryptophan residues in RecA are parallel to the fiber axis. However, the DNA bases are oriented isotropically. This conformation of the RecA•ssDNA complex is known as a "collapsed" filament.

RecA•ssDNA complexes found in the presence of the cofactor ATP, or its slowly hydrolyzed analog ATP\(_\gamma\)S, are characterized as "extended" filaments when compared to those formed without cofactor. The filaments still adopt the stiff fiber structure. The indolic planes of the tryptophan residues in RecA oriented parallel to the fiber axis while DNA bases are oriented perpendicular. The stoichiometry numbers of those filaments indicate that there are at least two type of complexes: one complex with 3 bases per RecA monomer and one with 6 bases per RecA monomer. This finding can be rationalized if a single RecA fiber can bind two molecules of ssDNA in the presence of cofactors. Based on
spectroscopic data, binding of a second ssDNA by RecA·ssDNA does not significantly perturb the structure.

The pre-steady-state kinetics of ATP- and ATPγS-mediated conformational transitions of the [H163W] RecA-ssDNA were examined by the Bryant group using stopped-flow fluorimetry. They found that the kinetics were consistent with a two-step mechanism in which an initial rapid binding of ATP to the RecA-ssDNA complex is followed by a first order isomerization. The rate constant for isomerization is 0.3 s⁻¹, and the isomerization step follows ATP (or ATPγS) binding but precedes ATP (or ATPγS) hydrolysis. The authors suggested that the isomerization occurs between a "closed" and "open" conformation of the filament, but it cannot be concluded that these putative kinetic intermediates are the collapsed and extended filaments, respectively. The steady state rate constant for ATP and ATPγS hydrolysis were measured to be 0.3 s⁻¹ and 0.0003 s⁻¹, respectively. The kinetic scheme proposed by Bryant and coworkers is shown in Figure 6-1(A).

Although the data of Bryant and coworkers has provided insight into the conformational transition of a RecA·ssDNA filament, the details of the closed to open transition is not clear. There are two possible mechanisms. One is that the transition occurs directly by a reorganization of RecA monomers on the ssDNA, with the monomers never leaving the DNA strand. The other is that a rapid dissociation of RecA monomers (in the closed conformation) from ssDNA is followed by ATP binding and rapid reassembly of the monomers on the ssDNA (in the open conformation) before ATP hydrolysis occurs. The Alternative mechanisms are shown in Figure 6-1 (B).
Figure 6-1. (A): The minimal kinetic scheme for ATP turnover consistent with the stopped-flow fluorescence and pre-steady-state kinetic results of Bryant and coworkers.\textsuperscript{11} \textbf{R} represents the RecA protomer and \textbf{O} represents the ssDNA. The subscripts "op" and "cl" represent the quenched fluorescent state (open conformation) and unquenched fluorescent state (closed conformation), respectively. The asterisk (*) represents the complex that has undergone ATP-mediated isomerization and is ready for ATP hydrolysis. The turnover-limiting steps are the two isomerizations indicated by the apparent rate constants 0.3 s\textsuperscript{-1}.

Figure 6-1. (B): Possible kinetic schemes for the closed to open transition of the RecA·ssDNA complex. The pathway on the right-hand side represents the direct reorganization of RecA monomers on the ssDNA. The pathway on the left-hand side (red) represents the alternative involving the dissociation of RecA monomers (in the closed conformation) from ssDNA followed by their rapid reassembly on the ssDNA in the open conformation before ATP hydrolysis begins. The complex in grey is the as yet unobserved putative open intermediate prior to quenching.
In order to better characterize the RecA-ssDNA binding process, we employed ssDNA containing the fluorescent nucleoside 1. Such substrates will provide a DNA-based signal that can complement the protein-based signal observed by Bryant and coworkers. Importantly, because the fluorescence yield of 1 responds to changes in DNA structures and environment, kinetic experiments using ssDNA containing 1 will allow these alternative mechanistic hypotheses for the closed to open isomerization to be tested. Moreover, the relationship between Bryant's closed and open intermediates and the collapsed and extended filaments can be probed using the fluorescence of DNA containing 1.

6.2 Results

6.2.1 DNA Hybridization Control Experiments

In order to test the reliability of our synthesis and purification procedures, we prepared two ODNs which have the same sequence as those for which Kool and coworkers have reported DNA hybridization kinetic data. The two ODN sequences are:

\[ M: \quad 5' - CTTTTCTTTCTT - 3' \]

\[ N: \quad 5' - AAGAAAGAAAAAG - 3' \]

The association of two complementary ssDNAs is a second-order reaction, and the rate (\( V_{assoc} \)) is defined by
\[ V_{\text{assoc}} = k_{\text{assoc}} \cdot [M] \cdot [N] \]  

(1)

where \( k_{\text{assoc}} \) is the rate constant for the association reaction. Rather than measure \( k_{\text{assoc}} \) directly, we measured the pseudo-first-order rate constant \( k_{\text{obs}} \) and derived the second-order rate constant \( k_{\text{assoc}} \).

Under pseudo-first-order conditions such that \([M]\) is 10-fold greater than \([N]\), \([M]\) remains virtually constant throughout the reaction process. Thus, the rate of association can be written by

\[ V_{\text{assoc}} = k_{\text{obs}} \cdot [N] \]  

(2)

where \( k_{\text{obs}} = k_{\text{assoc}} \cdot [M]^0 \) and \([M]^0\) is the initial concentration of M. The reaction is first-order with respect to N. From eq 2, the rate of association is

\[ V_{\text{assoc}} = \frac{d[N]}{dt} = k_{\text{obs}} \cdot [N] \]  

(3)

Eq 3 can be rearranged and evaluated to give an integrated rate equation:

\[ [N]_t = [N]^0 \cdot \exp(-k_{\text{obs}} \cdot t) \]  

(4)

According to Beer's Law, \([N]\) is proportional to the absorbance at 260 nm

\[ [N] = A_{260} / \epsilon \cdot l \]  

(5)
where $\varepsilon$ is the molar extinction coefficient at 260 nm and $l$ is the path length of the cell, thus, the rate of association can be written by

$$A_t = A^o \cdot \exp(-k_{\text{obs}} t)$$

(6)

We can measure the change in absorbance and derive the rate constant $k_{\text{obs}}$ for the pseudo-first-order reaction by fitting a single exponential function to the experimental data. The second-order rate constants were then derived from the apparent first-order rate constants and the known concentration of $M$.

$$k_{\text{assoc}} = k_{\text{obs}}/[M]^o$$

(7)

$M$ was prepared as a 3 $\mu$M solution in the buffer reported in the literature (100 mM NaCl, 10 mM MgCl$_2$, and 10 mM Na-PiPES at pH 7.0).$^{81}$ $N$ was prepared as a 0.3 $\mu$M solution in the same buffer. Both solutions were injected simultaneously into the SX-18 MV flow cell at 24 °C, and the formation of the double-stranded was monitored by the well-known hypochromic change in UV absorption at 260 nm. The time-dependent $A_{260}$ data are in Figure 6-2 and the extracted rate constant is listed in Table 6-1.

As the data in Table 6-1 indicates, we successfully reproduced the published value of $k_{\text{obs}}$. Importantly, this result demonstrated that our oligonucleotides were pure and that our stopped-flow spectrometer was functioning reliably.
Figure 6-2. Stopped-flow time-dependent absorbance data for the hybridization of two 12mer ODNs (M and N). The temperature was 24 °C and the absorbance was monitored at 260 nm. The oligonucleotides M and N were present at concentrations of 1.5 μM and 0.15 μM, respectively, in buffer containing 100 mM NaCl, 10 mM MgCl₂, and 10 mM Na·PIPES at pH 7.0. The data are fit to a single exponential decay as described in the text with a time constant, $k_{\text{obs}} = 6.3 \text{ s}^{-1}$. 
Table 6-1. Kinetic Data of DNA Hybridization (12 mers)

<table>
<thead>
<tr>
<th>ODNs</th>
<th>source</th>
<th>length, n (nts)</th>
<th>method</th>
<th>$k_{\text{obs}}$ (s$^{-1}$)</th>
<th>$k_{\text{assoc}}$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M + N</td>
<td>this work</td>
<td>12</td>
<td>A</td>
<td>6.3$^a$</td>
<td>4.2 x 10$^6$</td>
</tr>
<tr>
<td>M + N</td>
<td>literature</td>
<td>12</td>
<td>A</td>
<td>11.0$^a$</td>
<td>4.4 x 10$^6$</td>
</tr>
</tbody>
</table>

$^a$ The sources of the ODNs for the experiments whose results are tabulated were either described in the Materials and Methods section, or the data are taken from Reference.$^{81}$

$^b$ The time-dependent data were collected using either (A) absorption change at 260 nm.

$^c$ The fitted time constant for the exponential function used to fit the time-dependent data under pseudo-first-order conditions.

$^d$ The calculated associate rate constant derived from $k_{\text{obs}}$ in each case using eq (7).

$^e$ The magnitudes of the $k_{\text{obs}}$ parameters are different because the initial concentrations of the 12mers were different.
The previous experiment used absorption to study DNA hybridization. However, we wished to study RecA-DNA interactions using the fluorescence signal of 1. Therefore, we performed a hybridization control experiment using our 30mer ODNs to compare absorption and fluorescence spectroscopy.

Oligonucleotide H was prepared as a 3 μM solution in buffer containing 100 mM NaCl, 10 mM MgCl₂, and 10 mM Na·PIPES at pH 7.0. Oligonucleotide C (1 in the middle of strand) was prepared as a 0.3 μM solution in the same buffer. Both solutions were injected simultaneously at 24 °C into the flow cell of the spectrometer, and the formation of the dsDNA was monitored by the well-known hypochromic changes in UV absorption at 260 nm. To generate a fluorescence signal, an excitation beam of 320 nm was used to probe the flow cell with a cuton (long pass) filter placed between the sample cell and the photomultiplier to remove scattered light below 335 nm. When analyzing the fluorescence signal, F was simply substituted for A in eq 6.

The time-dependent changes in both absorption and fluorescence are shown in Figure 6-3 (A) and 6-3 (B), respectively, and the kinetic parameters are listed in Table 6-2.
Figure 6-3. (A): Stopped-flow time-dependent absorbance data for the hybridization of two 30mer ODNs (H and C). The temperature was 24 °C and the absorbance was monitored at 260 nm. The oligonucleotides H and C were present at concentrations of 1.5 μM and 0.15 μM, respectively, in buffer containing 100 mM NaCl, 10 mM MgCl₂, and 10 mM Na-PIPES at pH 7.0. The data are fit to a single exponential decay as described in the text with a time constant, $k_{\text{obs}} = 10.1$ s⁻¹. (B) Stopped-flow time-dependent fluorescence emission data for the hybridization of two 30mer ODNs (H and C). The excitation wavelength was 315 nm, and the data were recorded under the same conditions reported for panel A. The data are fit to a single exponential decay as described in the text with a time constant, $k_{\text{obs}} = 10.0$ s⁻¹.
Table 6-2. Kinetic Data of 30-mer DNA Hybridization Experiments

<table>
<thead>
<tr>
<th>ODNs</th>
<th>length, $n$ (nts)</th>
<th>Method$^a$</th>
<th>$k_{obs}^b$ (s$^{-1}$)</th>
<th>$k_{assoc}^c$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H + C</td>
<td>30</td>
<td>A</td>
<td>10.1</td>
<td>6.7 x 10$^6$</td>
</tr>
<tr>
<td>H + C</td>
<td>30</td>
<td>F</td>
<td>10.0</td>
<td>6.7 x 10$^6$</td>
</tr>
</tbody>
</table>

$^a$ The time-dependent data were collected using either (A) absorption change at 260 nm or (F) fluorescence emission change upon excitation at 320 nm using a cuton filter to remove scattered light below 335 nm.  
$^b$ The fitted time constant for the exponential function used to fit the time-dependent data under pseudo-first-order conditions.  
$^c$ The calculated associate rate constant derived from $k_{obs}$ in each case using eq 7.
For different DNA sequences, the relative rates of hybridization should be proportional to the square root of the ODN lengths \((n)\):

\[
\frac{k_{\text{assoc}}(n_1)}{k_{\text{assoc}}(n_2)} = \sqrt{\frac{n_1}{n_2}}
\]  \(8\)

Our results are consistent with this expectation, given the previous data obtained for 12 mer ODNs:

\[
\frac{k_{\text{assoc}}(30)}{k_{\text{assoc}}(12)} = \frac{6.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1}}{4.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}} = 1.59
\]

\[
\sqrt{\frac{n(30)}{n(12)}} = \sqrt{\frac{30}{12}} = 1.58
\]

We conclude that the fluorescence of 2-aminopurine is well suited for stopped-flow kinetic studies.

6.2.2 RecA Binding to ssDNA

Because we were concerned that the intrinsic fluorescence of the RecA typtophan residues, W290 and W308, might interfere with the emission from \(1\), Dr. Alberto Roca recorded the steady-state excitation and emission spectra of \(1\) and RecA protein (Figure 6-4 (A)). The excitation spectrum for RecA is at a minimum near 320 nm where the excitation spectrum for ODN C shows a maximum. Hence, nucleoside \(1\) can be selectively probed in RecA-DNA complexes. Next, steady-state fluorescence emission spectra for ODN C, RecA-C, and RecA-ATPyS-C were recorded (Figure 6-4 (B)). The fluorescence
Figure 6-4. Steady-state fluorescence excitation and emission spectra for RecA and ODN C. (A) Fluorescence excitation (dashed lines) and emission (solid lines) for purified RecA protein and ODN C. The spectra for the ODN are indicated by the heavier lines. (B) Emission spectra of C in the absence of RecA and ATPγS (thin solid line), in the presence of RecA only (thick solid line), and in the presence of RecA and ATPγS (dashed heavy line). The spectra were collected using 42 μM-nt C, 27 μM RecA, and 50 μM ATPγS in aqueous buffer (33 mM PIPES, pH 7.2, 52 mM K+, 1 mM Mg(OAc)₂, 5% glycerol, and 1 mM DTT) at 37 °C. The excitation wavelength was 320, and the excitation and emission monochromators were set to a spectral bandwidth of 4 nm.
emission increases dramatically when ODN C binds to RecA in the presence or absence of ATPγS. This may result from DNA strand extension upon RecA-DNA filament formation. Importantly, these spectral data indicate that free C can be delineated from the DNA substrate bound to RecA protein in the presence or absence of cofactor. These fluorescence changes are the basis for the continuous kinetic assay using nucleoside 1.

The necessity of a fluorescence-based assay is demonstrated by the observation that no absorption signal changes accompany the interaction of RecA with ODN C, which is comprised only of natural nucleotides, in the presence or absence of ATPγS (Figure 6-5 (A)). Apparently, the changes in the conformation of the protein and DNA chromophores are minimal so that the more sensitive fluorescence spectrophotometry must be employed. Like the absorption data, the protein tryptophan emission data do not show any signal changes (Figure 6-5 (B)). This data, taken together with that for C described above, validates our approach and demonstrates that a continuous kinetic assay must make use of fluorescently labeled DNA and/or protein.

Using the fluorescence signal originating from nucleoside 1, time-dependent pre-steady-state emission data were recorded for ODN C binding to RecA in the presence of various cofactors. The RecA protein was prepared as a 3 μM solution in aqueous buffer (25 mM Na-PIPES, pH 7.0, 10 mM MgCl₂, 1 mM DTT, and 5% glycerol). The appropriate cofactor, ADP or ATPγS, was also included in the buffer with a final concentration of 50 μM. ODN C was prepared as a 0.3 μM solution in the same buffer and cofactors were included in this solution as well. Both solutions were injected simultaneously into the
Figure 6-5. (A) Stopped-flow absorbance measurements upon mixing 1.5 μM RecA protein with 0.15 μM 30mer ODN C (●), and stopped-flow absorbance measurements upon mixing 1.5 μM RecA protein and 50 μM ATPγS with 0.15 μM 30mer ODN C (○). The protein and ODN were mixed at 25 °C in aqueous buffer (25 mM Na-PIPES, pH 7.0, 10 mM MgCl₂, 1 mM DTT, and 5% glycerol). The monochromator on the incident light was set to 260 ± 4 nm. (B) Stopped-flow fluorescence emission measurements upon mixing 1.5 μM RecA protein with 0.15 μM 30mer ODN A. The data were recorded in the same buffer reported for panel A, with an excitation wavelength of 315 ± 4 nm and a 335-nm cuton filter.
SX18MV flow cell at 24 °C, and the formation of RecA-ssDNA was monitored by the changes in the total fluorescence emission above 335 nm.

The kinetics of RecA binding to C, a 30-nt ssDNA containing 1, in the absence of cofactor was studied (Figure 6-6 (A)). The changes in emission intensity were recorded up to 100 s post-mixing and fitted to a single exponential function with a time constant, \( k_{\text{obs}} = 0.2 \text{ s}^{-1} \). (The fitted parameters for all RecA experiments are listed in Table 6-3.) Time-dependent fluorescence data for RecA binding to C in the presence of 50 μM ADP is shown in Figure 6-6 (B). The signal curve is similar to that observed in the absence of cofactor; however, the data were best described by a double-exponential function with time constants of 1.2 s\(^{-1}\) and 0.2 s\(^{-1}\) for the two phases.

The time-dependent fluorescence data for RecA-ssDNA binding in the presence of ATP analog ATPγS was more complicated than those data reported above. The data for RecA binding to C in the presence of 50 μM ATPγS is shown in Figure 6-6 (C). The signal curve has at least two phases. The first phase is similar in time and amplitude to the changes observed in the absence of cofactor, but the second phase represents a new, slower process. The data were fit to a combination single exponential plus linear function with a fitted time constant of 0.2 s\(^{-1}\) and a slope of 0.01 min\(^{-1}\).

In order to study the isomerization of a RecA-ssDNA filament from the "collapsed" to the "extended" conformation, we first incubated RecA and oligonucleotide C in the same buffer at 24 °C for 2 h to form the RecA-C filament. The binding of this filament to ATPγS was then monitored (Figure 6-6 (D)). The signal curve is similar to RecA-ATPγS binding to C, but also includes a previously unobserved rapid phase. The best-fit double exponential plus
Figure 6-6. Stopped-flow time-dependent fluorescence emission data for mixing 1.5 μM RecA protein with 0.15 μM 30mer ODN C. The data were recorded in the same buffer reported for Figure 7, with an excitation wavelength of 315 ± 4 nm and a 335-nm cuton filter. (A) Time-dependent emission for RecA binding C in the absence of cofactor. The best-fit single-exponential function is shown. (B) Time-dependent emission for RecA binding C in the presence of 50 μM ADP. The best-fit double-exponential function is shown. (C) Time-dependent emission for RecA binding C in the presence of 50 μM ATPγS. The best fit single-exponential + linear function is shown. (D) Time-dependent emission for RecA-C filament (pre-formed) binding ATPγS (50 μM). The best fit double-exponential + linear function is shown. The fitted parameters for all four data panels are listed in Table 6-3.
Table 6-3. Pre-steady-state Kinetic Parameters Fitted to Fluorescence Changes for RecA Binding to ODN C.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Reagent 1\textsuperscript{b}</th>
<th>Reagent 2\textsuperscript{b}</th>
<th>Phase One</th>
<th>Phase Two</th>
<th>Phase Three</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$k_{\text{obs}}$</td>
<td>$\Delta F^c$</td>
<td>$k_{\text{obs}}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(s\textsuperscript{-1})</td>
<td>(mV)</td>
<td>(s\textsuperscript{-1})</td>
</tr>
<tr>
<td>RecA</td>
<td>C</td>
<td>—\textsuperscript{a}</td>
<td>0.22 ± 0.01</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>RecA-ADP</td>
<td>C + ADP</td>
<td>1.2 ± 0.2</td>
<td>22 ± 2</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>RecA-ATP\textsubscript{γ}S</td>
<td>C + ATP\textsubscript{γ}S</td>
<td>—</td>
<td>0.20 ± 0.01</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>RecA-C</td>
<td>ATP\textsubscript{γ}S</td>
<td>11 ± 3</td>
<td>35 ± 8</td>
<td>0.37 ± 0.04</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The parameters for rows one to four were derived from the curve fits in Figures 7A to 7D, respectively. The error limits represent the residual fitting errors. \textsuperscript{b} RecA was present at 1.5 µM and ODN C was present at 0.15 µM (4.5 µM-nts). When present, the concentration was 50 µM and was contained in both reagent syringes so that no dilution occurred upon mixing. \textsuperscript{c} The amplitudes of the fluorescence emission enhancement for each phase ($\Delta F$) are tabulated. \textsuperscript{d} A single dash in a cell indicates that the phase was not observed.
linear function is shown in Figure 6-6 (D). The fitted parameters include exponential time constants of 11 s\(^{-1}\) and 0.4 s\(^{-1}\), and a slope of 0.01 min\(^{-1}\). The rate constants derived from the linear phases of the two experiments conducted in the presence of ATP\(\gamma\)S are consistent with steady-state hydrolysis of the ATP\(\gamma\)S.\(^{11}\)

6.3 Discussion

In order to probe the details of the dynamic processes involved in DNA extension by the RecA protein, one must make use of a signal that responds to changes in the DNA conformation. We have shown that the use of 1 in DNA substrates provides an exceptional way to probe such changes. Specifically, we have performed rapid mixing experiments to investigate the pre-steady-state kinetics of RecA binding to a 30mer ODN in the presence and absence of nucleotide cofactors. These pre-steady-state emission data demonstrate multiphasic behavior for all but the simplest (RecA + C) mixing experiments. In order to connect these data to the mechanistic model, a specific molecular process must be assigned to each observed phase (Figure 6-7).

The common molecular events among the RecA kinetic experiments are the binding of RecA to C and to nucleotide cofactor (when present). Using a fluorescence excitation wavelength of 320 nm, the binding of nucleotide to RecA is accompanied by no changes in the emission above 335 nm. In contrast, the results summarized by Figure 6-4(B) demonstrate that RecA binding to C is accompanied by an emission enhancement in the absence and presence of ATP\(\gamma\)S. Given these facts, the rapid (0.2 s\(^{-1}\) ≤ \(k_{obs}\) ≤ 1.2 s\(^{-1}\)) emission
Figure 6-7. Hypothetical kinetic scheme for the interaction of a RecA protomer (R) with a 30mer ODN (O) and adenosine nucleotide cofactors (ADP, ATP or ATPγS, collectively represented by A). The scheme was constructed using the data in Figs. 8 and 9 and in Table 2. The rate constants for the processes monitored by changes in the fluorescence of ODN C are indicated by the black rate constants, while the other steps are assumed to be rapid. As the primary data indicate, the steps indicated by the green rate constants are accessible only in the presence of ATP or ATPγS. The rate constant of 0.01 min⁻¹ indicates the turnover-limiting step for ATPγS hydrolysis. The "op" and "cl" notations indicate the "open" and "closed" conformations of the RecA protomer filament as proposed by F. R. Bryant and coworkers.¹¹
enhancement observed in each experiment can be attributed to the binding of C to RecA.

Interestingly, all experiments involving ATP\(\gamma\)S show a slow quench as the final phase. This phase can likely be attributed to ATP\(\gamma\)S hydrolysis, given the similarity of the derived steady-state rate constant with the published value of \(k_{\text{cat}}\). Thus, the slow, linear quench likely reflects the steady-state turnover of ATP\(\gamma\)S.

In the binding of C by RecA-ADP, two phases are observed with rates that are consistent with RecA-ODN binding. The simplest explanation is that the RecA-ADP complex is unstable under these conditions and exists as an equilibrium mixture of free and nucleotide-bound RecA. Thus, the two phases would simply represent the two populations of RecA protein binding to C. This conclusion is supported by the observation that the sum of the \(\Delta F\) values for the two phases in the “Rec-ADP” experiment is equal to the \(\Delta F\) observed for the single phase in the no nucleotide experiment. Moreover, when the concentration of ADP was increased from 50 \(\mu\)M to 100 \(\mu\)M, the amplitude of faster phase doubled in magnitude, while the slower phase disappeared. No further changes were observed upon further increases in the concentration of ADP. These results are consistent with the idea that two populations of the RecA protein – nucleotide-bound and unbound-exist at 50 \(\mu\)M RecA protein, and that the independent binding of C by these populations lead to the observation of biphasic fluorescence enhancement.

When preformed RecA-C is mixed with ATP\(\gamma\)S, a unique rapid phase is observed. Given that this phase is more rapid than any other observed during these experiments and that it is accompanied by a fluorescence enhancement
considerably weaker than those accompanying RecA-C binding, this phase most likely represents the isomerization of a "closed" RecA-C filament to the "open" conformation. Because this process \( (k_{\text{obs}} = 11 \text{ s}^{-1}) \) occurs much more rapidly than the rate-limiting isomerization observed by Bryant and coworkers \( (k_{\text{obs}} = 0.3 \text{ s}^{-1}) \), the ATPγS-mediated isomerization likely results in a different intermediate than the pre-ATP-hydrolysis Michaelis complex \([(R\cdot\text{ATP}\cdot\text{O})_{\text{op}} \cdot \text{in} \text{ Figure 6-1 (A)}. Specifically, we propose that this intermediate is the previously unobserved open intermediate prior to quenching of the H163W mutant protein’s emission. Moreover, the fact that similar kinetic intermediates are consistent with both Bryant’s protein-based signal and our DNA-based signal leads us to speculate that Bryant’s closed and open intermediates are indeed the collapsed and extended nucleoprotein filaments.

Somewhat unexpectedly, there appears to be an ODN-binding phase in the experiment involving preformed RecA-C binding to ATPγS. This binding signal implies that the RecA-C filament can rapidly disassemble before or after binding to ATPγS and then reassemble again.

If the disassembly takes place after nucleotide binding, then the rate of disassembly must be similar to that of the closed-to-open isomerization step in order for both pathways to be observed. In such a model, the RecA-C filament would rapidly bind ATPγS to form the closed complex which could then follow either one of two pathways: direct isomerization to the open complex or dissociation of RecA (in the closed conformation) from C followed by their isomerization and rapid reassembly on C in the open conformation. Thus, the "alternative" mechanisms of Figure 2 would be operating in parallel.
If, however, the disassembly occurs before nucleotide binding, a different model likely holds. In this case, the likely origin of the two different pathways would be the inherent instability of the RecA-C filament. The two pathways would arise as a result of the ability of the RecA-C filament to rapidly disassemble. Specifically, if the preformed RecA-C filament loses RecA at a rate that is competitive with that of ATPγS binding, then one would expect to observe the mechanistic pathways originating from both filament-bound and free protein species.

In order to address the question of whether the disassembly of the RecA-C occurs before or after ATPγS binding, the amplitudes and rates of the isomerization and binding phases were measured as a function of [ATPγS] by Hansi Singh. As [ATPγS] was varied from 20 to 400 μM, neither rate constant changed appreciably; however, the amplitude of the isomerization phase increased while that of the binding phase essentially disappeared. The lack of an effect of [ATPγS] on the rates suggests that the nucleotide is influencing a process that is rapid and/or is not accompanied by a change in the DNA conformation. The substantial effect on the relative amplitudes suggests that ATPγS influence the pathway through which the reaction proceeds, presumably by influencing a cofactor-sensitive equilibrium. Based on the minimal kinetic scheme (Figure 6-7), ATPγS likely binds the closed filament prior to filament disassembly, committing the reaction to the isomerization pathway. Hence, the data are consistent with filament disassembly occurring prior to ATPγS binding.

Taken together, our data are consistent with the minimal kinetic scheme shown in Figure 6-7. The scheme is similar to that proposed by Stole and Bryant, but the use of an ODN containing 1 has allowed the definition of more
intermediates. Most notably, evidence for the direct closed to open isomerization of the nucleoprotein filament has been obtained. However, under the reaction conditions used here, the direct isomerization competes with filament disassembly. The coincidence of the rate constant observed here for RecA·ATPγS binding C, and that obtained by Stole and Bryant for steady-state ATP hydrolysis ($k = 0.3 \text{ s}^{-1}$), leads to the conclusion that the process that limits ATP turnover under steady-state conditions is reassociation of ATP-bound RecA monomers to the DNA substrate rather than direct isomerization. A revised minimal kinetic scheme for ATP turnover, consistent with all the data, is presented (Figure 6-8).

6.4 Method and Materials

6.4.1 DNA Hybridization Control Experiments

Oligonucleotides were dissolved in the Hybridization buffer (10 mM Na-PIPES, pH 7.0, 100 mM NaCl, and 10 mM MgCl₂) to a final concentration of either 3 µM (for M and H) or 0.3 µM (for N and C). All the reaction reagent solutions were filtered and degassed before use. Both solutions were injected simultaneously at 24 °C into the flow cell of the SX18MV stopped-flow spectrometer (Applied Photophysics), and the formation of double-stranded DNA was monitored by the changes in UV absorption at 260 nm or fluorescence emission at 370 nm. When measuring fluorescence, a cuton filter was placed between the sample cell and the photomultiplier to remove light below 335 nm.
Figure 6-8. A revised minimal kinetic scheme for ATP turnover consistent with the stopped-flow fluorescence and pre-steady-state kinetic results of both this work and that of Bryant and coworkers.\textsuperscript{11} The symbols are the same as those in Figure 6-1 (A). The direct closed to open isomerization is observable using fluorescent nucleoside 1 (11 s\textsuperscript{-1}), and the turnover-limiting step is the reassembly of the open filament indicated by the apparent rate constant 0.3 s\textsuperscript{-1}. The other steps are presumed to be rapid.
6.4.2 RecA-ssDNA Binding Kinetics

Oligonucleotide C was dissolved in Reaction Buffer (25 mM Na-PIPES, pH 7.0, 10 mM MgCl₂, 1 mM DTT, and 5% glycerol) to a final concentration of 0.3 μM. The RecA protein for the reaction was as a 3 μM solution in the same buffer. All the reaction reagent solutions were filtered and degassed before use. Both solutions were injected simultaneously at 24 °C into the flow cell of the SX18MV, and the reaction progress was monitored by the changes in the fluorescence emission above 335 nm (excitation wavelength 320 nm). The voltage on the PMT was set to 365.5 ± 2.0 V (range: 363.7 – 367.5 V), the offset voltage was set to 3.93 ± 0.05 V (range: 3.87 – 3.98 V), and a 500-μs time filter was employed.

6.4.3 Data Analysis

The time-resolved fluorescence changes were fitted to single- or double-exponential functions with floating end-points (eqs 9 and 10, respectively), single-exponential plus steady-state (eq 12) functions using KaleidaGraph™ (version 3.0.8d; Synergy Software):

\[ F(t) = \Delta F_1 \cdot \exp(-k_1 \cdot t) + F_4 \]  \hspace{1cm} (9)

\[ F(t) = \Delta F_1 \cdot \exp(-k_1 \cdot t) + \Delta F_2 \cdot \exp(-k_2 \cdot t) + F_4 \]  \hspace{1cm} (10)

\[ F(t) = \Delta F_1 \cdot \exp(-k_1 \cdot t) + k_2 \cdot t + F_4 \]  \hspace{1cm} (11)
\[ F(t) = \Delta F_1 \cdot \exp(-k_1 \cdot t) + \Delta F_2 \cdot \exp(-k_2 \cdot t) + k_3 \cdot t + F_4 \] (12)

In eqs 9 - 12, \( k_1 \) and \( k_2 \) represent the observed time constants for the exponential phases of the reactions, \( k_3 \) represents the observed slope of the linear phase of the reactions, \( \Delta F_1 \) and \( \Delta F_2 \) are the amplitudes of the exponential phases of the reactions, and \( F_4 \) is the final fluorescence at the reaction endpoint.
CHAPTER 7
ATTEMPTED SYNTHESIS OF FLUORESCENT NUCLEOSIDE
TRIPHOSPHATE

The importance of phosphorylated nucleosides and analogs in molecular biology and biochemistry has led to the development of improved methods for their synthesis. Our desire to use modified nucleoside triphosphates as a probe for kinetic studies demands an efficient synthetic route of NTP. Many synthetic methods of nucleoside triphosphates have been reported. Most involve the transformation of mononucleotides to reactive intermediates such as morpholidates, imidazolidates, phosphoramidates, salicyl phosphorchlorodite, or 8-quinolates followed by displacement of the leaving group by pyrophosphate.

The most widely used method is to activate the NMPs as nucleoside imidazolates because the reaction of NMPs with 1,1'-carbonyldiimidazole occurs under mild conditions and does not require a purification step. Figure 7-1 shows this general synthetic route developed by Ott and coworkers. Another widely used method is that of Eckstein and coworkers (Figure 7-2). The method involves a rapid one-pot reaction that does not require protection of the nucleobases. Sulfur can also be introduced in the process, so it is very good for the synthesis of thiophosphates. However, the method requires the protection of the ribose 2'- and 3'- hydroxyl groups and, moreover, leads to the formation of by-products, some of which are difficult to remove by conventional DEAE chromatography.
Reagents and conditions: (a) \((\text{CH}_3\text{O})_3\text{PO} + \text{POCl}_3\); (b) 1,1'-carbonyldiimidazole, DMF, pyridine; (c) \(\text{Bu}_3\text{N}/\text{H}_2\text{P}_2\text{O}_7\).

Figure 7-1. Ott synthesis of nucleoside triphosphates. 1,1'-Carbonyldiimidazole reacts with NMP to form an intermediate, which on subsequent reaction with \(\text{Bu}_3\text{N}\) affords the NTP.
Reagents and conditions: (a) salicyl phosphorochlorodite, pyridine, dioxane; (b) tri-n-butylamine, $H_4P_2O_7$; (c) $I_2/H_2O$/pyridine, $NH_3$.

Figure 7-2. Eckstein synthesis of nucleoside triphosphates. 2-Chloro-4\(H\)-1.3.2-benzodioxaphosphorin-4-one phosphitlates the 5'-hydroxyl group of a nucleoside to form an intermediate, which on subsequent reaction with pyrophosphate produces, in a double displacement process, a $P_2$, $P_3$-dioxo-$P_1$-5'-nucleosidylcyclotriphosphite. Oxidation with iodine/water then affords the NTP.
The synthetic method selected for further use in our lab (Figure 7-3) is the Takaku method.\textsuperscript{87} We chose this synthetic method because the intermediate phosphate 13 could be synthesized in large scale and then various nucleosides would be phosphorylated with it. Importantly, this method does not require the protection of the nucleobases. The first two steps have been completed using adenosine as the nucleoside and satisfactory results were obtained.
Reagents and conditions: (a) POC1$_3$, pyridine; (b) diphenyl disulfide, Bu$_3$P, pyridine; (c) Bu$_3$N/ H$_2$P$_2$O$_7$, CuCl.

Figure 7-3. Takaku synthesis of nucleoside triphosphates. First, 8-quinolyl phosphate 13 was synthesized as the general phosphorylating reagent. Next, a nucleoside was treated with 8-quinolyl phosphate in the presence of tributylphosphine and dephenyl disulfide to give 8-quinolyl nucleoside 5'-phosphate 14. The desired NTP was obtained by the reaction of 14 with pyrophosphoric acid in the presence of cupric chloride.
CHAPTER 8
SUMMARY AND PROGNOSIS

8.1 Summary

Homologous genetic recombination is an important biological process. The RecA protein of E. coli is the prototype of recombination proteins. RecA mediates and promotes DNA strand exchange in vitro, providing an experimental window on in vivo homologous recombination. Despite its importance, however, many aspects of the molecular mechanism of the strand exchange reaction are not understood. We want to develop a continuous kinetic assay for monitoring the process to understand the mechanistic details.

RecA protein expression was accomplished using a two-plasmid system in a nuclease-deficient recA<sup>−</sup> cell strain STL327. The expression of recA gene cloned on pAIR79 was transcribed by the T7 RNA polymerase. The T7 RNA polymerase gene was cloned on the pT7POL26 plasmid and was induced upon the addition of lactose or IPTG. The purification of RecA included Polymin P precipitation of DNA and DNA-bound proteins, ammonium sulfate precipitation of resuspended proteins, DEAE column purification, and HAP column purification. We prepared 127 mg RecA protein which is pure and active from 3 L of culture.

2-Aminopurine deoxyribonucleoside phosphoramidite was synthesized from thioguanosine using the method of Sowers and coworkers. The synthetic method involves protection of the 2-aminopurine ribonucleoside, reduction to
the deoxyribonucleoside and standard preparation of the protected phosphoramidite. Our synthesized 2-aminopurine deoxyribonucleoside phosphoramidite 3 was incorporated in oligonucleotides using automated DNA synthesis. Syntheses were performed “trityl on” and ODNs containing the DMT groups were purified by RP-HPLC. After detritylation with acetic acid, ODNs were purified by RP-HPLC again. Our synthesized fluorescent ODNs are very pure and suitable for kinetic studies based on DNA hybridization experiments.

The mechanism of RecA binding to ssDNA was probed by using ODNs containing 1. When RecA binds the ODN, a rapid fluorescence enhancement was observed whose rate and magnitude depended on the presence of nucleotide cofactor. Specifically, the rate varied among the absence of cofactor, the presence of ADP and the presence of ATPγS. In the absence of cofactor or in the presence of ADP, conditions which allow formation of a collapsed nucleoprotein filament, simple binding kinetics were observed. In the presence of ATPγS, a cofactor that favors formation of an extended nucleoprotein filament, both binding and isomerization processes are observed. This latter process represents the first observation of a direct isomerization pathway for RecA monomers associated with DNA. Given a previous observation Stole and Bryant's [conclusion that RecA does not isomerize between closed and open conformations during steady-state turnover of ATP], our data support a model in which association of RecA·ATP to single-stranded DNA is turnover-limiting.

Synthesis of fluorescent nucleoside triphosphates is being vigorously pursued. We intend to use the triphosphate of 2-aminopurine ribonucleoside as another probe for our kinetic studies because ATP or its analogs are required for RecA-mediated DNA strand exchange.
A continuous kinetic assay for RecA-mediated DNA strand exchange using a DNA-based fluorescent signal has been successfully developed and the early events of RecA-mediated DNA strand exchange were investigated. The assay we developed provides a view of the discrete steps along the reaction pathway, which is very useful in understanding mechanistic details of fast biochemical reactions such as DNA strand exchange.

8.2 Prognosis

*Chemical Synthesis*

New and more efficient ways to synthesize 3 shall be explored since the one we used is time consuming and results in low overall yield. The proposed synthetic routes of 3 are shown in Figure 8-1. The key issue in 3 synthesis is the formal deoxygenation of an aryl alcohol since we use guanosine and its derivatives as our starting materials. Guanosine and its derivatives have similar structure to 2-aminopurine and its derivatives and they are fairly inexpensive. The most efficient route for conversion of the C-OH bond into the C-H bond could be the ones which begin with \(dG^\text{bu}\) phosphoradimite. The most promising reagent could be acylsulfonyl hydrazide because it provides a very mild reaction condition.\(^{90,91}\) \(Cp_2ZrHCl\) is also a very interesting reagent because it reduces amides and lactams to the corresponding imines, a transformation that is otherwise very difficult to achieve because imines are reduced more rapidly than carboxamides by most metal hydride reagents.\(^{92}\)

The so-called "convertible nucleoside" technology for synthesizing modified DNAs in a combinatorial chemical approach was pioneered by the
Figure 8-1. Proposed synthetic routes to 2AP phosphoramidite 3. The two starting materials are $dG^{bu}$ phosphoramidite and DMT-$dG^{bu}$. All the routes are less than four steps.
groups of Swann, Jones and Verdine. This approach involves the synthesis of a single phosphoramidite containing a suitable leaving group. The monomer can be incorporated into a synthetic DNA strand, and then subsequently transformed into one of a variety of modified nucleosides. We plan to identify the best convertible purine nucleoside from the literature or from our investigations above and incorporate it in oligonucleotides. Following the standard solid-phase synthesis, we will be able to split the oligonucleotide-resin pool and apply special reagents to transform the convertible nucleoside to our desired modified nucleosides, such as 2,4-diaminopurine nucleoside, and thioguanosine, as shown on Figure 8-2. This approach has a number of advantages over the traditional synthetic approach. The convertible nucleoside approach provides a highly convergent set of synthetic routes in that a number of functional group modifications can be effected using the same intermediate phosphoramisite. Moreover, because the ultimate functional group transformations are not performed until after oligonucleotide synthesis, a single oligonucleotide could be synthesized in relatively large scale, and then split into pools, each of which would be treated with the reagent of choice.

*Kinetic Studies of RecA Binding to ssDNA*

More kinetic experiments have to be done in order to elucidate the mechanisms of RecA binding to ssDNA. First, we will investigate binding with ATP as cofactor. This will provide information about the hydrolysis step. Second, we will measure the dependence of fluorescence change on cofactor concentration since concentrations of reagents always affect the kinetic data.
Figure 8-2. Potential uses of convertible purine nucleoside. The scheme shows the conversion of a fully protected support-bound oligonucleotide carrying an easily leaving group into oligonucleotides containing a variety of derivatives.
This will not only find ideal experimental condition but also provide insights about the kinetic rate law and the binding mechanism. In addition, there are many ways to prevent ATP hydrolysis other than the use of ATPγS. One example is to substitute Ca²⁺ for Mg²⁺ in the reaction buffer.⁸⁰ This change also suggests that use of luminescence spectroscopy kinetic studies with Eu³⁺ as the polyvalent metal ion.¹⁰⁰ Other non-hydrolyzable cofactors, such as ADP•AlF₄⁻ and AMPPNP (5'-α-denylyl-β,γ-imidodiphosphate),¹⁰¹ that better mimic ATP or the pentavalent P hydrolysis transition state can also be used. These are some of the alternatives to ATPγS that can be used to investigate the key intermediates in RecA-mediated DNA strand exchange.
APPENDIX I

THE PROTOCOL FOR OVEREXPRESSION OF THE RecA PROTEIN

Reagents

The recipes for the reagent solutions are listed in Table A-1. The following information describes the vendors (and vendor catalog numbers) where common reagents were purchased.

Ampicillin (Amp): Sigma, A 9518
Bacto-tryptone: DIFCO, 90799JA
Yeast extract: DIFCO, 93019JE
Kanamycin (Kan): Sigma, K 4000
α-Lactose: Sigma, L 3625
IPTG: Sigma, I 6758

Centrifuge Bottles: Nalgene
   500 mL Bottle, PPCO, 3120-9500
   250 mL Bottle, PC, 31222-0250
   50 mL Oakridge Bottle, PPCO, 3119-0050

1. Two 150-mL Erlenmeyer flasks containing 30 mL LB each and six 2-L Erlenmeyer flasks containing 500 mL TB each were autoclaved.

2. Amp (100 mg/mL) and Kan (10 mg/mL) were added to the LB in each 150-mL flask to final concentration of 100 μg/mL and 40 μg/mL, respectively.
3. Two colonies of STL327/pAIR79/pT7POL26 were picked with sterile toothpicks and used to inoculate the media in the 150-mL flasks. The 30-mL cultures were incubated at 37 °C for 10 h with shaking.

4. Amp (100 mg/mL) and Kan (10 mg/mL) were added to the TB in each 2-L flask to final concentration of 100 µg/mL and 40 µg/mL, respectively.

5. Each 2-L flask was inoculated with a 5-mL aliquot from the small flasks.

6. The six 500-mL cultures were incubated at 37 °C with shaking for approximately 4.5 h until OD<sub>600</sub> = 0.80. Then lactose (8% w/v) was added to each 500-mL culture to a final concentration of 0.2% (w/v) and the cultures were incubated for another 4 h.

7. OD<sub>600</sub> was checked for a four-fold diluted sample for each flask (250 µL culture diluted to 1000 µL with fresh TB). The mean OD<sub>600</sub> for the size cultures, after accounting for the dilution factor, was 4.1 ± 0.5.

8. Each 500-mL culture was transferred to a 500-mL centrifuge bottle.

9. The bottles were balanced to within ± 0.1 g and the cells were pelleted using a 5000 rpm spin in a GS-3 rotor at 4 °C for 30 min.

10. The supernatant was carefully decanted from each cell pellet, and the total mass of the wet cells was measured to be 10.0 g.
APPENDIX II

THE PROTOCOL FOR PURIFICATION OF THE RecA PROTEIN

Reagents

All reagent solutions, except DTT, were prepared before the purification and stored at 4 °C refrigerator. DTT was stored at -20 °C. The recipes for the solutions are listed in Tables A-2-1 (Cell Lysis), A-2-2 (Polymin P), A-2-3 (DEAE-sepharose column) and A-2-4 (Hydroxyapatite column). DTT was added to the appropriate buffer just prior to use. The following information describes the vendors (and vendor catalog numbers).

AmS: Fisher, A702-500
Brij-35: Sigma, P 1254
DEAE Sepharose: Pharmacia Biotech, 17-0709-01
DTT: Sigma, D 9779
Na₂EDTA: Fisher, 951316
Hydroxyapatite Bio-Gel: Bio-Rad, 130-042
Lysozyme: Sigma, L 7651
50% (w/v) Polymin P: Sigma, P 3143
Sodium Azide: Sigma, S 2002
Sucrose: Sigma, S 9378
Tris base: Fisher, BP 152
Centrifuge Bottles: Nalgene
   250 mL Bottle, PC, 31222-0250;
   50 mL Oakridge Bottle, PPCO, 3119-0050
Cell Lysis

1. Thaw 10 g cells by adding 35 mL 25% sucrose solution and gently but thoroughly resuspend the pellet by aspirating with a serological pipet. Transfer the solution (total volume = 45 mL) into a beaker.

2. Add 9 mL freshly prepared lysozyme solution and stir slowly for 30 min using a stir bar at 4 °C.

3. Add 10 mL EDTA (25 mM) and slowly for 30 min using a stir bar at 4 °C.

4. Add 60 mL Brij-35 and stir for another 30 min using a stir bar at 4 °C.

5. Transfer the cell suspension into a steel beaker and sonicate with output of 75 (Watt) for 3 x 30 sec with 60 second cooling intervals. (Do not overheat sample during sonication. Keep samples in ice water bath during sonication)

6. Bring the total volume to 140 mL using 25% sucrose solution.

7. Distribute the cell lysate to an even number of 250-mL bottles (2 x 70 mL each).

8. Balance the bottles to within ± 0.01 g and ultracentrifuge using a Ti35 rotor at 32,000 rpm for 45 min at 4 °C.

9. Combine the supernatant from all the bottles into beaker.

10. Measure the OD values for a 1:100 dilution of the supernatant in R buffer at 260 nm, 280 nm and 350 nm. (OD$_{260}$ = 0.80; OD$_{280}$ = 0.53; OD$_{350}$ = 0.04)
Polymin P Precipitation

1. Slowly add 15 mL Polymin P (0.111 x volume of supernatant) over 30 min to the combined supernatant. Gently stir for 30 min using a serological pipet at 4 °C.

2. RecA, DNA, and Polymin P will form a white precipitate.

3. Transfer the Polymin P suspension to four 50-ml Oakridge bottles (4 x 37.5 mL each).

4. Balance bottles to within ± 0.01 g and ultracentrifuge using an SA-600 rotor at 9,000 rpm for 15 min at 4 °C.

5. Combine pellets into one beaker by a serological pipet.

6. Resuspend pellets in 100 mL R+150 AmS solution by aspirating with a serological pipet and gently stir for 15 min at 4 °C. (RecA can be lost in R+150 wash. Entire step must be less than 30 min)

7. Distribute the R+150 AmS resuspension into an even number of 50-mL Oakridge bottles (2 x 50 mL each).

8. Balance the bottles to within ± 0.01 g and ultracentrifuge using an SA-600 rotor at 9,000 rpm for 15 min at 4 °C.

9. Combine the pellets into one beaker using a serological pipet.

10. Resuspend the pellets using a serological pipet in 50 mL R+300 AmS solution and gently stir for 30 min.

11. Distribute the R+300 AmS suspension into an even number of 50-mL Oakridge bottles by a serological pipet (2 x 25 mL).

12. Balance the bottles to within ± 0.01 g and ultracentrifuge using an SA-600 rotor at 9,000 rpm for 15 min at 4 °C.

13. Combine the supernatant from all the bottles in one beaker.
14. Re-extract RecA protein from the pellets with 50 mL R+300 AmS solution by aspirating with a serological pipet and gently stir for another 30 min at 4 °C.
15. Distribute the R+300 AmS resuspension into an even number of 50 mL Oakridge bottles (2 x 25 mL).
16. Balance the bottles to within ± 0.01 g and ultracentrifuge using an SA-600 rotor at 9,000 rpm for 15 min at 4 °C.
17. Combine the R+300 supernatant from steps 13 and 16 into one beaker.

AmS Precipitation
1. Measure 0.28 g AmS per mL of the R+300 resuspension from step 17 above. (e.g., 28 g for our 100 mL solution)
2. Grind AmS to a very fine powder with mortar and pestle.
3. Slowly add 28 g AmS to combined supernatant above (AmS final concentration = 0.28 g/mL) over 30 min and gently stir for at least another 30 min using a stir bar. (The AmS suspension may be left stirring overnight)
4. Distribute the AmS solution into an even number of 50-mL Oakridge bottles (2 x 50 mL).
5. Balance the bottles to within ± 0.01 g and ultracentrifuge using an SA-600 rotor at 9,000 rpm for 30 min at 4 °C. RecA is in the fluffy pellet.
6. Carefully pour the supernatant off and resuspend the pellet in 50 mL R + 0.28 g/mL AmS per bottle by aspirating with a serological pipet (2 x 50 mL).
7. Balance the bottles to within ± 0.01 g and ultracentrifuge using an SA-600 rotor at 9,000 rpm for 30 min at 4 °C. RecA is in the pellet.

8. Carefully pour the supernatant off.

9. Repeat R + 0.28 g/mL AmS rinse (steps 6-8) two more times.

10. Resuspend pellets with 25 mL R+50 KCl buffer by aspirating with a serological pipet and combine R+50 KCl resuspensions into one beaker. (total volume = 25 mL)

11. Slowly stir for 30 min using a stir bar at 4 °C.

12. Transfer the solution to a dialysis bag and put the bag into a 1-L R+50 KCl solution at 4 °C for at least 12 h. (change the old buffer to fresh one every 4 h)

13. Carefully pour RecA solution into a sterile storage bottle from the dialysis bag.

14. Add 25 mL R buffer into R+50 KCl solution to bring the total volume to 50 mL.

15. Determine the amount of RecA protein by measuring the absorbance of the protein at 280 nm (ε₂₈₀ = 0.59): OD₂₈₀ = 4.35; OD₂₆₀ = 2.82; OD₃₅₀ = 0.09. Conc = (4.35/0.59) mg/mL = 7 mg/mL. The total amount of RecA is 350 mg.

**DEAE-Sepharose Column**

1. Put 250 mL DEAE into a beaker and slurry it by a glass tubing. Filter the mixture through a glass frit.

2. Slurry the DEAE in 500 mL 0.5 N HCl for 5 min by a glass tubing and filter through the glass frit. Wash the DEAE with 4 x 250 mL mili-Q water.
3. Slurry in 500 ml 0.5 N NaOH for 5 min by a glass tubling and filter through the glass frit. Wash the DEAE with 4 x 250 mL milli-Q water.
4. Slurry the DEAE in 250 mL R+10 mM KCl solution by a glass tubling.
5. Mark the column at the volume of 70 mL by pouring 70 mL water into the column.
6. Slurry the DEAE suspension by a glass tubling and pour it into column until the solution reaches 70-mL mark.
7. Wash the column with 150 mL R+10 mM KCl.
8. Load the solution from step 14 of *AmS Precipitation section* above on the column and collect fractions in one beaker (B1).
9. Elute the column with 200 mL R+10 mM KCl and collect the fraction in another beaker (B2).
10. Develop the column with gradient: R+10 mM KCl to R+300 mM KCl. Collect the fractions (20 mL each) in glass tubes.
11. Monitor protein peaks by SDS-PAGE (Figure 3-2).
12. Combine beaker 1 and beaker 2 for step 16 (total volume = 200 mL).
13. Wash the column with R+300 mM KCl (200 mL) and collect in one beaker (B3). Wash the column with R+2 M KCl (250 mL) and collect in another beaker (B4).
14. Monitor protein peaks by SDS-PAGE (Figure 3-3). It shows Beaker 3 has some RecA protein. Save Beaker 3 for future use.
15. Wash column with R+NaAz (150 mL) and store the DEAE at 4 °C.
16. Slowly add 56 g AmS to the 200 mL combined solution from step 12 (AmS final concentration = 0.28 g/mL) over 45 min and gently stir for at
least another 30 min using a stir bar. (The AmS suspension may be left stirring overnight)

17. Distribute the AmS solution to two 50 mL Oakridge bottles (2 x 50 mL). Balance the bottles to within \( \pm 0.01 \) g. Centrifuge with an SLA-1500 rotor at 13,000 rpm for 30 min at 4 °C and RecA is in the pellet.

18. Resuspend the pellet in 10 mL P buffer solution by aspirating with a serological pipet.

19. Transfer the solution to a dialysis bag and put the bag into a 1-L P solution at 4 °C for at least 8 h. (change the old buffer to fresh one every 4 h)

20. Carefully pour RecA solution into a sterile storage bottle from the dialysis bag.

21. Determine the amount of RecA protein by measuring the absorbance of the protein at 280 nm \((\varepsilon_{280} = 0.59)\): \(\text{OD}_{260} = 10.0\); \(\text{OD}_{350} = 0.4\). \(\text{Conc} = (10.0/0.59) \text{mg/mL} = 17 \text{mg/mL}\). The total amount of RecA is 170 mg.

**HAP Column**

1. Weigh 17 g Bio-Gel HAP (Expected to be 35 mL) and add 210 mL P buffer with gentle swirling. (do not use stir bar)

2. Allow the slurry to settle for at least 10 min and decant the cloudy upper level.

3. Repeat the wash process again.

4. Put a funnel on the top of the column and pour the HAP slurry into the column and allow the slurry to settle to 70 mL.

5. Add 150 mL P buffer to wash the column.
6. Load the sample solution from step 20 of *DEAE-Sepharose Column section* above into column and collect the fraction in Beaker 1 (B1).

7. Add 150 mL P buffer and collect the fractions in 3 x 50 mL tubes (sample 1'~3').

8. Develop the column with 700 mL gradient: P to P+350. Collect the fractions (4 mL each) in glass tubes.

9. Monitor protein peaks by SDS-PAGE (Figure 3-4).

10. Pool fractions 50-80. Slowly add 52 g AmS to combined fractions (AmS final concentration = 0.52 g/mL) over 45 min and gently stir for at least another 30 min using a stir bar. (The AmS suspension may be left stirring overnight)

11. Distribute AmS solution to an even number of 50 mL Oakridge bottles (2 x 50 mL).

12. Balance bottles to within ± 0.01 g and centrifuge with an SLA-600 rotor at 13,000 rpm for 30 min at 4 °C. RecA is in the pellet.

13. Resuspend pellet in 10 mL R Buffer solution by aspirating with a serological pipet and measure OD value. (OD$_{600}$ = 5.0, conc. = 9 mg/mL)

14. Add R buffer to the solution to a final volume of 30 mL (Conc. = 3 mg/mL) using a serological pipet

15. Transfer the solution to a dialysis bag and put the bag into a 1-L R solution at 4 °C for at least 12 h. (change the old buffer to fresh one every 4 h)

16. Carefully pour RecA solution into a sterile storage bottle from the dialysis bag.
17. Since it is cloudy, add another 30 mL R buffer and stir slowly overnight using a stir bar.

18. Distribute the solution to an even number of 50 mL Oakridge bottles (2 x 30 mL).

19. Balance bottles to within ± 0.01 g and centrifuge with an SLA-600 rotor at 13,000 for 30 min at 4 °C.

20. Pour the supernatant from the bottles in a beaker. (total volume = 60 mL)

21. Determine the amount of RecA protein by measuring the absorbance of the protein at 280 nm ($c_{280} = 0.59$): $OD_{260} = 0.80$; $OD_{260} = 1.256$; $OD_{350} = 0.20$. Conc = (1.256/0.59) mg/mL = 2.12 mg/mL. The total amount of RecA is 127 mg.

22. Store the RecA protein in -80°C freezer.

23. Wash the column with 150 mL P+1M buffer and with 150 mL NaAz buffer

Put HAP back to storage flask in 4 °C refrigerator.

Notes

- Perform all purification steps either in cold room, 4 °C refrigerator, or using ice-water bath.
- Check stock solutions and buffer supplies before purification.
- Do not let DEAE and HAP columns run dry.
- Save all waste rinses and pellets in labeled bottles until purification is complete.
- Absorbance readings must be between 0.2 and 1.0 for accuracy. If solution concentration is too high, it should be diluted before OD reading.
- RecA extinction coefficient is 0.59 mL/mg at 280 nm.
Table A-2-1. Solutions for Cell Lysis of RecA Purification

<table>
<thead>
<tr>
<th>solution</th>
<th>stock solution</th>
<th>amount (g/mL)</th>
<th>FW (g/mL)</th>
<th>notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris•HCl</td>
<td>Tris base</td>
<td>121.1 g</td>
<td>FW 121.1</td>
<td>pH to 7.5 w/</td>
</tr>
<tr>
<td>1 M HCl</td>
<td></td>
<td>792 mL</td>
<td>Conc.</td>
<td>10 N NaOH</td>
</tr>
<tr>
<td>pH 7.5 ddH₂O</td>
<td></td>
<td>→1 L</td>
<td></td>
<td>autoclave</td>
</tr>
<tr>
<td>EDTA</td>
<td>Na₂EDTA</td>
<td>18.61 g</td>
<td>FW 372.24</td>
<td>pH to 7.5 w/</td>
</tr>
<tr>
<td>0.5 M ddH₂O</td>
<td></td>
<td>80 mL</td>
<td></td>
<td>10 N NaOH</td>
</tr>
<tr>
<td>pH 7.5 ddH₂O</td>
<td></td>
<td>→100 mL</td>
<td></td>
<td>autoclave</td>
</tr>
<tr>
<td>20 x R 1 M Tris•HCl</td>
<td>Tris•HCl</td>
<td>800 mL</td>
<td></td>
<td>pH 7.5</td>
</tr>
<tr>
<td>pH 7.5 0.5 M EDTA</td>
<td>Na₂EDTA</td>
<td>8 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>→2 L</td>
<td></td>
<td>autoclave</td>
</tr>
<tr>
<td>DTT</td>
<td>DTT</td>
<td>7.71 g</td>
<td>FW 154.2</td>
<td>add just before use</td>
</tr>
<tr>
<td>1 M ddH₂O</td>
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<td>40 mL</td>
<td></td>
<td></td>
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<tr>
<td>ddH₂O</td>
<td></td>
<td>→50 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose 25 % (w/v)</td>
<td>sucrose</td>
<td>125 g</td>
<td>FW 342.3</td>
<td>pH 7.5</td>
</tr>
<tr>
<td>pH 7.5 ddH₂O</td>
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<td>200 mL</td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>→500 mL</td>
<td></td>
<td></td>
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<tr>
<td>Lysozyme</td>
<td>lysozyme</td>
<td>100 mg</td>
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<tr>
<td>1 M Tris•HCl</td>
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<td></td>
</tr>
<tr>
<td>5 mg/mL ddH₂O</td>
<td></td>
<td>15 mL</td>
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<td></td>
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<td></td>
<td>→20 mL</td>
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</tr>
<tr>
<td>EDTA</td>
<td>0.5 M EDTA</td>
<td>1.5 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mM ddH₂O</td>
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</tr>
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<td>ddH₂O</td>
<td></td>
<td>→30 mL</td>
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<td>Brij-35 1% (w/v)</td>
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<td>2 g</td>
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<td>0.4 mL</td>
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<td></td>
</tr>
<tr>
<td>ddH₂O</td>
<td></td>
<td>→200 mL</td>
<td></td>
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Table A-2-2. Solutions for Polynin P Precipitation of RecA Purification

<table>
<thead>
<tr>
<th>solution</th>
<th>stock solution</th>
<th>amount</th>
<th>FW</th>
<th>notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 X P</td>
<td>KH₂PO₄</td>
<td>27.22 g</td>
<td>FW 136.09</td>
<td>for total 0.4M</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>K₂HPO₄</td>
<td>34.84 g</td>
<td>FW 174.18</td>
<td>pH 6.8</td>
</tr>
<tr>
<td></td>
<td>0.5 M EDTA</td>
<td>4 mL</td>
<td></td>
<td>autoclave</td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>900 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>→1 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymin P</td>
<td>50 % (w/v)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% w/v</td>
<td>Polymin P</td>
<td>5.4 g</td>
<td></td>
<td>use falcon tube</td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>30 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td>conc HCl</td>
<td>2.5-3 mL</td>
<td></td>
<td>use pH paper</td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>→50 mL</td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td>R 150 AmS</td>
<td>20 X R</td>
<td>10 mL</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>glycerol</td>
<td>20 g</td>
<td>neat</td>
<td>15.9 mL</td>
</tr>
<tr>
<td>0.15 M</td>
<td>AmS</td>
<td>3.96 g</td>
<td>FW 132.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 M DTT</td>
<td>200 µL</td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>→200 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 300 AmS</td>
<td>20 X R</td>
<td>25 mL</td>
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<td></td>
<td>glycerol</td>
<td>50 g</td>
<td>neat</td>
<td>39.7 mL</td>
</tr>
<tr>
<td>0.30 M</td>
<td>AmS</td>
<td>19.82 g</td>
<td>FW 132.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 M DTT</td>
<td>500 µL</td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>→500 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 0.28 AmS</td>
<td>20 X R</td>
<td>50 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>glycerol</td>
<td>100 g</td>
<td>neat</td>
<td>79.4 mL</td>
</tr>
<tr>
<td>0.28 g/mL</td>
<td>AmS</td>
<td>280 g</td>
<td>FW 132.14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 M DTT</td>
<td>1 mL</td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>→1 L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R + 50 KCl</td>
<td>20 X R</td>
<td>500 mL</td>
<td></td>
<td>20X</td>
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<td></td>
<td>glycerol</td>
<td>1000 g</td>
<td>neat</td>
<td>794 mL</td>
</tr>
<tr>
<td>0.05 M</td>
<td>KCl</td>
<td>37.28 g</td>
<td>FW 74.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 M DTT</td>
<td>10 mL</td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>→10 L</td>
<td></td>
<td></td>
</tr>
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### Table A-2-3. Solutions for DEAE Column of RecA Purification

<table>
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<tr>
<th>solution</th>
<th>stock solution</th>
<th>amount</th>
<th>FW</th>
<th>notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>R + 10</td>
<td>20 X R</td>
<td>50 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>glycerol</td>
<td>100 g</td>
<td>neat</td>
<td>79.4 mL</td>
</tr>
<tr>
<td>0.01 M</td>
<td>KCl</td>
<td>746 mg</td>
<td>FW 74.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 M DTT</td>
<td>1 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>900 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>→ 1 L</td>
<td></td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td>R + 2 M</td>
<td>20 X R</td>
<td>50 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>glycerol</td>
<td>100 g</td>
<td>neat</td>
<td>79.4 mL</td>
</tr>
<tr>
<td>2 M</td>
<td>KCl</td>
<td>149.12 g</td>
<td>FW 74.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 M DTT</td>
<td>1 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>→ 1 L</td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td>R NaN₃</td>
<td>20 X R</td>
<td>50 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 g/L</td>
<td>glycerol</td>
<td>100 g</td>
<td>neat</td>
<td>79.4 mL</td>
</tr>
<tr>
<td></td>
<td>NaN₃</td>
<td>0.1 g</td>
<td>FW 65.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 M DTT</td>
<td>1 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>→ 1 L</td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td>R</td>
<td>20 X R</td>
<td>350 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>glycerol</td>
<td>700 g</td>
<td>neat</td>
<td>555.5 mL</td>
</tr>
<tr>
<td></td>
<td>1 M DTT</td>
<td>7 mL</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>ddH₂O</td>
<td>→ 7 L</td>
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Table A-2-4. Solutions for HAP Column of RecA Purification

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<th>amount</th>
<th>FW</th>
<th>notes</th>
</tr>
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<td>P</td>
<td>20 X P</td>
<td>200 mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glycerol</td>
<td>400 g</td>
<td>neat</td>
<td>317.5 mL</td>
<td></td>
</tr>
<tr>
<td>1 M DTT</td>
<td>4 mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ddH₂O</td>
<td>→4 L</td>
<td></td>
<td></td>
<td>filter</td>
</tr>
<tr>
<td>P 350</td>
<td>KH₂PO₄</td>
<td>11.91 g</td>
<td>FW 136.09</td>
<td>for total 0.35 M</td>
</tr>
<tr>
<td>HAP</td>
<td>K₂HPO₄</td>
<td>15.24 g</td>
<td>FW 174.18</td>
<td>317.5 mL</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>100 μL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glycerol</td>
<td>50 g</td>
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<tr>
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<td>filter</td>
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<tr>
<td>P 1M</td>
<td>KH₂PO₄</td>
<td>68.04 g</td>
<td>FW 136.09</td>
<td>for total 1 M</td>
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<tr>
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<td>FW 174.18</td>
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<tr>
<td>ddH₂O</td>
<td>→1 L</td>
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<tr>
<td>P NaN₃</td>
<td>20 X P</td>
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APPENDIX III

THE EXPERIMENTAL SECTION OF SYNTHESIS OF 2-AMINOPURINE
DEOXYRIBONUCLEOSIDE PHOSPHORAMIDITE

Technical Notes

Nuclear magnetic resonance (NMR) spectra were determined on a Bruker AC250 NMR spectrometer. Chemical shifts of $^1$H NMR are reported in parts per million downfield from tetramethylsilane ($\delta = 0$) as internal standard. Chemical shifts of $^{31}$P NMR are reported in parts per million downfield from 85% phosphoric acid ($\delta = 0$) as internal standard. The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and b = broad.

Analytical TLC was performed on Whatman K5F silica plates (0.25 mm layer thickness). Spots were visualized with UV light and/or staining with anisaldehyde visualization solution (6 g anisaldehyde in 250 mL ethanol plus 2.5 mL conc. $\text{H}_2\text{SO}_4$). All the reactions were monitored by TLC, unless otherwise indicated.

Column chromatography was carried out on 230-400 mesh Spectrum silica gel with the flash technique.\textsuperscript{102}

All reactions sensitive to oxygen or moisture were conducted under an argon or nitrogen atmosphere.
Reagents

All commercial chemicals and solvents were used as supplied with the following exceptions: pyridine was distilled from KOH; DCM, TMSCl, benzene and toluene were distilled from CaH₂; NaSH was dried by evaporation of anhydrous benzene and pyridine; isobutyryl chloride was distilled from PCl₅ and redistilled from quinoline;¹⁰³ POCl₃ was distilled from sodium metal; all nucleosides were dried by evaporation of dry reaction solvents.

Common solvents and reagents are purchased from Rice University Chemistry Stockroom which carries ACS grade Fisher products. The information that describes the vendors (and vendor catalog numbers) where common reagents were purchased is listed in Table A-3-1.
Thioguanosine (6)

To a stirred suspended solution of guanosine (1 g, 3.5 mmol) in dry pyridine (70 mL) in an ice bath under a nitrogen atmosphere was added dropwise trifluoroacetic anhydride (5.93 mL, 42 mmol) by syringe over 5 min. After 90 min, a suspension of anhydrous sodium hydrogen sulfide (9.42 g, 168 mmol) in 90 mL DMF was added in portions. The reaction mixture was allowed to warm to room temperature. After a further 24 h, the reaction mixture was poured into a 175 mL portion of 0.24 M ammonium bicarbonate, with vigorous stirring. The mixture was then concentrated to dryness and the residue tiritrated with 300 mL methanol and filtered. The filtrate was again concentrated to dryness and the residue triturated with 200 mL 0.1 M triethylammonium acetate, filtered and the filtrate was brought to dryness again. Ammonium hydroxide (50 mL, 0.1 M) was added to pH 10 and the mixture was shaken and stirred thoroughly. The mixture was filtered and the filtrate was acidified with glacial acetic acid to pH 5 and cooled in freezer overnight. The tan solid was collected and weighed: (510 mg, 50% yield).
Characterization of 6:

$^1$H NMR (DMSO-$d_6$):  
8.15 (s, 1H), 6.83 (br, 2 H), 5.69 (d, 1H), 5.46 (d, 1H),  
5.17 (d, 1H), 5.06 (b, 1H), 4.39 (t, 1H), 4.09 (b, 1H),  
3.87 (b, 1H), 3.56 (m, 2H)

Mol Formula: $C_{10}H_{13}N_5O_4$ (mw= 299.3)

TLC:  
Rf 0.25 (UV active; DCM/MeOH, 3:1)
\[
\text{NiAl}_2 + 6 \text{NaOH} \rightarrow \text{Ni} + 2\text{Na}_3\text{AlO}_3 + 3\text{H}_2
\]

**Raney Nickel**

A solution of NaOH (12.7 g) in distilled water (50 mL), contained in 150 mL beaker, was cooled in an ice bath to 10 °C. Nickel Aluminum (10 g) was added to the solution in small portions, with stirring, at such a rate that the temperature did not rise above 25 °C. The beaker was allowed in the ice bath. When all the alloy was added, the beaker was removed from the ice bath and allowed to come to RT. After the evolution of hydrogen became slow, the mixture was heated in oil bath until the evolution of hydrogen again became slow \((T = 110 \, ^\circ\text{C})\). During the time the volume of the solution was maintained constant by adding distilled water.

After heating for 6 h, the nickel was allowed to set and most of the liquid was decanted. Distilled water was then added to bring the solution to original volume. The nickel was suspended by stirring, again allowed to settle, and the solution was decanted.

The nickel was then transferred to 100 mL beaker with the aid of distilled water and the water was decanted. A Solution of NaOH (1.7 g) in distilled water (17 mL) was added. The nickel was suspended and allowed to settle and the water was decanted. The nickel was washed repeatedly by suspension with water until the washings were neutral \((\text{pH} = 7)\). The washing process is repeated with 95% alcohol \((3 \times 10 \, \text{mL})\) and absolute alcohol \((3 \times 10 \, \text{mL})\). The catalyst was then stored under absolute alcohol.
2-Amino-9-(β-D-ribofuranosyl)purine (7)

Thioguanosine (1.8 g, 6 mmol) was dissolved in boiling water (100 mL) in 250 mL 3-neck RBF and treated with the slurry of Raney Nickel in water (approximately 4 g). The reaction was refluxed at 100 °C for 4 h. TLC showed a single fluorescent spot. The reaction mixture was filtered and the Raney nickel was leached with boiling water (3 x 20 mL). The washings and filtrate were combined, treated with charcoal, and concentrated in vacuo to a syrup which was dissolved in toluene and reconcentrated. The residue was dissolved in DCM/MeOH (1:2), and silica gel (70-230 mesh, 2.0 g) was added. The suspension was dried again and under vacuum overnight. The crude product was purified by silica gel chromatography (25 g of 230-400 mesh silica), eluted with a gradient of MeOH in EtOAc (15% to 35%), to give the product (1.06 g, 70% yield) as white solid.
Characterization of 7:

$^1$H NMR (DMSO-$d_6$): 8.60 (s, 1H), 8.31 (s, 1H), 6.58 (s, 2 H), 5.83 (d, 1H), 5.46 (d, 1H), 5.17 (d, 1H), 5.06 (t, 1H), 4.50 (m, 1H), 4.11 (m, 1H), 3.66-3.11 (m, 2H)

Mol Formula: $C_{10}H_{13}N_5O_4$ (mw = 267.3)

TLC: Rf 0.45 (Fluorescent and UV active; DCM/MeOH, 3:1)
To a stirred solution of 7 (0.75 g, 2.8 mmol) in dry pyridine (35 mL) in an ice bath under a nitrogen atmosphere was added dropwise trimethylchlorosilane (2.73 mL, 21 mmol) by syringe over 10 min. After 1 h, isobutryl chloride (1.50 mL, 14 mmol) was added and the reaction mixture was allowed to warm to room temperature. After a further 6 h, the reaction mixture was cooled in an ice bath and cold water (12 mL) was added. After 15 min, concentrated aqueous ammonia (15 M, 7 mL) was added to give a solution approximately 2 M ammonia. After another 45 min, the solution was then evaporated to near dryness and the residue was dissolved in water (50 mL). The solution was washed once with EtOAC/ether (2:1, 50 mL) and the organic layer was extracted with water (2 x 15 mL). The aqueous layers were combined and concentrated to dryness. The residue was dried under vacuum overnight. The crude product was purified by silica gel chromatography (25 g 230-400 mesh silica), eluted with a gradient of methanol in EtOAc (0% to 20%), to give the product (0.48 g, 50% yield) as white solid.
Characterization of 8:

$^1$H NMR (DMSO-$d_6$): 10.63 (s, 1H), 9.01 (s, 1H), 8.68 (s, 1H), 5.95 (d, 1H), 5.55 (br, 1H), 5.22 (br, 1H), 4.96 (br, 1H), 4.65 (m, 1H), 4.19 (m, 1H), 3.95 (m, 1H), 3.7-3.5 (m, 2H), 2.83 (m, 1H), 1.10 (d, 6H)

Mol Formula: $\text{C}_{14}\text{H}_{19}\text{N}_2\text{O}_5$ (mw= 337.3)

TLC: Rf 0.38 (UV-active; no Fluorescence; DCM/MeOH, 8:1)
\[ N\text{-Isobutryryl-2-amino-3',5'-O(1,1,3,3-tetraisopropyl-1,3-cisiloxanediyl)9-(\beta-D-\text{ribofuranosyl})purine (9)} \]

To a stirred solution of ribonucleoside 8 (385 mg, 1.14 mmol) in dry pyridine (12 mL) under a nitrogen atmosphere in ice bath was added dropwise TPDSCl₂ (365 µL, 1.142 mmol) by syringe. The mixture was stirred at 0 °C for 15 min and RT for 6 h. After the reaction was complete, pyridine was evaporated and the residue was partitioned between EtOAc (70 mL) and H₂O (15 mL). The organic phase was washed with HCl (1 N, 2 X 15 mL), saturated NaHCO₃/H₂O (2 X 15 mL) and brine (20 mL), dried (Na₂SO₄), filtered, and evaporated to give the white residue.

Characterization of 9:

Mol Formula: \[ C_{26}H_{45}N_{5}O_{6}Si_2 \ (mw= 579.7) \]

TLC: Rf 0.70 (UV-active; no Fluorescence; DCM/MeOH, 9:1)
Phenoxythiocarbonylated Ribonucleoside (10)

To a stirred solution of protected ribonucleoside residue 9 in dry MeCN (20 mL) under a nitrogen atmosphere was added anhydrous DMAP (286 mg, 2.34 mmol), and PTC-Cl (230 µL, 1.656 mmol, dropwise). Then solution was stirred at RT overnight. After the reaction was complete, solvent was evaporated and the residue was partitioned between ether (70 mL) and H₂O (15 mL). The organic phase was washed with HCl (1 N, 2 X 15 mL), saturated NaHCO₃/H₂O (2 X 15 mL) and brine (20 mL), dried (Na₂SO₄), filtered, and evaporated to give the yellow residue.

Characterization of 10:

Mol Formula: \( C_{33}H_{49}N_{5}O_{7}SSi_{2} \) (mw= 715.8)

TLC: Rf 0.80 (UV-active; no Fluorescence; DCM/MeOH, 19:1)
**N-Isobutyryl-2-aminopurine-2'-deoxyribonucleoside (11)**

To a stirred solution of phenoxythiocarbonylated Ribonucleoside 10 in dry toluene (28 mL) under a nitrogen atmosphere was added AIBN (48 mg, 0.28 mmol) and $n$-Bu$_3$SnH (460 μl, 1.713 mmol). The solution was degassed with nitrogen (PP) for 35 min and then heated at 75 °C for 4 h and 50 °C overnight. TBAF/THF (2.3 mL, 2.284 mmol) was added and the reaction was at 75 °C for 2.5 h. After the reaction was complete, solvent was evaporated and the residue was dissolved in H$_2$O (75 mL). The aqueous solution was washed with Et$_2$O (2 x 20 mL) and then concentrated to dryness. The crude product was purified by silica gel chromatography (25 g 230-400 mesh silica), eluted with a gradient of methanol in EtOAc (15% to 30%), to give the product (200 mg, 55% yield) as white solid.
Characterization of 11:

$^1$H NMR (DMSO-$d_6$): 10.59 (s, 1H), 8.99 (s, 1H), 8.66 (s, 1H), 6.39 (t, 1H), 5.36 (d, 1H), 4.91 (d, 1H), 4.45 (m, 1H), 3.86 (m, 1H), 3.63-3.49 (m, 1H), 2.85-2.74 (m, 2H), 2.31 (m, 1H), 1.09 (d, 6H)

Mol Formula: $C_{14}H_{19}N_9O_4$ (mw= 321.3)

TLC: Rf 0.74 (UV-active; DCM/MeOH, 3:1)

Rf 0.40 (UV-active; DCM/MeOH, 5:1)
**N-Isobutyryl-2-aminopurine-5'-O-(4, 4'-dimethyltrityl)-2'-deoxyribonucleoside**

To a stirred solution of deoxyribonucleoside 11 (210 mg, 0.65 mmol) in dry pyridine (5 mL) under a nitrogen atmosphere in ice bath was added DMAP (5 mg, 0.04 mmol) and DMTCI (266 mg, 0.78 mmol). The mixture was stirred at RT for 3 h and more DMTCI (85 mg, 0.25 mmol) was added because of reaction incompletion. The reaction was stirred overnight and cold methanol (5 mL) was added to quench the reaction. The mixture was stirred for 15 min and the solvent was evaporated. The residue was dissolved in chloroform (75 mL) and washed with saturated NaHCO₃·H₂O (2 x 20 mL) and brine (20 mL). The organic layer was concentrated to dryness. The crude product was purified by silica gel chromatography (25 g 230-400 mesh silica), eluted with a gradient of hexane in EtOAc (15% to 0%), to give the product (201 mg, 49% yield) as slightly colored solid.
Characterization of 12:

$^1$H NMR (DMSO-$d_6$): 10.54 (s, 1H), 8.98 (s, 1H), 8.55 (s, 1H), 7.3-6.7 (m, 13 H), 6.42 (t, 1H), 5.33 (d, 1H), 4.56 (m, 1H), 3.96 (m, 1H), 3.70 (m, 2 H), 3.69 (s, 6H), 2.85-2.74 (m, 2H), 2.31 (m, 1H), 1.06 (d, 6H)

Mol Formula: $C_{35}H_{37}N_9O_6$ (mw= 623.6)

TLC: Rf 0.19 (UV-active; EtOAc)
N-Isobutyryl-2-aminopurine-5'-O-(4,4'-dimethyltrityl)-2'-deoxyribonucleoside-3'-O-(2-cyanoethyl)-N, N-diisopropylphosphoramidite (3)

To a stirred solution of DMT-deoxyribonucleoside 12 (200 mg, 0.32 mmol) in dry DCM (4.5 mL) under a nitrogen atmosphere was added (iPr)₂NEt (280 μL, 1.6 mmol) and 2-cyanoethyl diisopropylchloro-phosphoramidite (114 μL, 0.48 mmol). The mixture was stirred at RT for 4 h. Cold methanol (2 mL) was added to quench the reaction. The mixture was stirred for 15 min and the solvent was evaporated. The residue was dissolved in cold EtOAC (50 mL) and washed with saturated NaHCO₃/H₂O (2 x 10 mL) and brine (2 x 10 mL). The organic layer was dried by anhydrous sodium sulfate for 2 h and concentrated to dryness. The product was isolated by silica gel chromatography (20 g 230-400 mesh silica), eluted with a gradient of Hexane in ethyl acetates (30% to 0%), to give lightly colored oil. The oil was dissolved in EtOAc (3 mL) and was added slowly to stirred hexane (175 mL) in ice bath. The mixture was filtered to give phosphoramidite as white crystals (136 mg, 52% yield)
Characterization of 3:

$^1$H NMR (CDCl$_3$): 8.96 (s, 1H), 8.24-7.98 (m, 2H), 7.40-6.76 (m, 13 H), 6.44 (t, 1H), 4.75 (b, 1H), 4.13 (m, 1H), 3.9-3.4 (m, 10H), 2.80-2.60 (m, 6H), 2.45 (m, 1H), 1.29-1.10 (m, 18H)

$^{31}$P NMR (CDCl$_3$, H-decoupled):
149.385 (s), 149.279 (s)

Mol Formula: C$_{44}$H$_{54}$N$_{10}$O$_7$P (mw = 823.6)

TLC: Rf 0.72 (UV-active; EtOAc:DCM: Et$_3$N 45:45:10)
$^{31}$P NMR (H-decoupled) spectrum of 2 AP phosphoramidite 3

Our final product consists of phosphoramidite diastereoisomers due to the asymmetric phosphorus atom.
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<td>Bu$_3$SnH</td>
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<td>CIL</td>
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APPENDIX IV
THE PROTOCOL FOR SYNTHESIS, CLEAVAGE, FULL DEPROTECTION AND PURIFICATION OF OLIGONUCLEOTIDES

Reagents

All the reagents and solutions for DNA synthesizer were purchased from Perspective. The following are their catalog number.

0.5 g DNA monomer kit GEN066040
1.0 g DNA monomer Kit GEN066041
Standard solutions kit GEN089600
0.5 g dA\textsuperscript{bz} GEN066000 1.0 g dA\textsuperscript{bz} GEN066001
0.5 g dC\textsuperscript{bz} GEN066000 1.0 g dC\textsuperscript{bz} GEN066001
0.5 g dG\textsuperscript{bu} GEN066000 1.0 g dG\textsuperscript{bu} GEN066001
0.5 g T GEN066000 1.0 g T GEN066001
dA\textsuperscript{bz}-CPG (1 μmol) GEN061550
dC\textsuperscript{bz}-CPG (1 μmol) GEN061560
dG\textsuperscript{bu}-CPG (1 μmol) GEN061570
T-CPG (1 μmol) GEN061570

All the solvents for HPLC purification should be HPLC grade. The following information describes the vendors (and vendor catalog numbers) where common reagents were purchased.

MECN: B&J, 015
MeOH: Fisher, A452
DCM: Aldrich, 27,056-3
**Automated Synthesis of Oligonucleotides**

1. Prepare reagents and chemicals before DNA synthesis
   a) The scale of synthesis is 1.0 µmol
   b) Since purification will be by reverse-phase HPLC, we will leave the final DMT group attached to the 5'-OH group
   c) Calculation of the number of bases and CPG column

   CPG column: 3-end base column
   
   \[
   \begin{array}{cccc}
   dA: & 1 & dC: & 0 \\
   dG: & 8 & T: & 1
   \end{array}
   \]

   Monomers
   
   \[
   \begin{array}{cccc}
   dA: & 34 & dC: & 79 \\
   dG: & 51 & T: & 82
   \end{array}
   \]
   d) 0.5 g amidite is enough only for 60 times of DNA cycles.
   e) The time for every cycle is 3.3 min

2. 0.5 g dA, dG, dC, and T phosphoramidite are dissolved in 10 mL dry acetonitrile respectively and put in the corresponding position in DNA synthesizer.

3. 130 mg 2AP phosphoramidite is dissolved in 1.65 mL dry acetonitrile in a 10 x 75 mm tube. The tube is put in a normal amidite bottle and hooked to the 5-position of DNA synthesizer

4. Program the synthesizer and synthesize our oligonucleotides

**Cleavage and Deprotection of ODNs**

1. Remove the column from the instrument or get the column from freezer and label them clearly.
2. With the plunge fully depressed into barrel, attach a slip-tip luer syringe to one end of the column (1 mL syringe for 0.05, 0.2 or 1.0 μmol scales; 5 mL for 15 μmol).

3. Draw 1 mL of fresh NH₄OH (5 mL for 15 μmol) into a second 1 mL or 5 ml luer top syringe. (NH₂OH is put in a beaker in an ice bath. After use, NH₂OH is stored in -20°C freezer)

4. Attach the syringe to the other end of the column.

5. Depress the syringe plunger to force the solution back and forth over the support in the column. Repeat four times to ensure that the support in the column is fully saturated. (Attention: Do not leave air bubble in the column)

6. Let the assembly rest at R. T. for 45 min.

7. Push the solution back and forth four times. (Do not leave air bubble in the column)

8. Let the assembly rest for another 45 min.

9. Draw all the solution into one of the syringe and carefully remove the syringe from column. (Ammonium hydroxide is very volatile)

10. Deposit the solution into a screw-cap tube and securely cap the tube and seal with parafilm.

11. Heat the tube at 55°C overnight in a hot-block. (55°C is for standard phosphoramidite protecting group only; use less vigorous condition for other reagents)

12. Remove the sealed tube from hot-block and allow to cool the tube to R. T.

13. Carefully remove the cap and leave the tube in the back of hood for 6-18 hour to remove NH₃.
14. Make holes in the cap with one 16-gauge syringe needle and freeze the tube in liquid nitrogen.
15. Remove the water from tube by SVC (speed vac concentrator) about 4 h.
16. Label it and keep it in freezer.

Reverse-phase HPLC Purification of Tritylated Synthetic Oligonucleotides

Trityl-on HPLC is performed on System Gold from Beckman, with a 3 micron, 10 mm X 70 C18 Ultrasphere column.

1. Prepare buffers:

   *Buffer A:* 0.1 M TEAA, pH 6.8 with 2% MeCN.
   
   900 mL milli-Q water and 20 mL HPLC grade MeCN. Then add 13.94 mL TEA (0.1 mol). Adjust to pH 6.8 by HOAc.
   
   Add more ddH₂O to 1000 mL. Filter the solution by 0.2 µm Nylon membrane filter (VWR, 28159-723).

   *Buffer B:* 0.1 M TEAA, pH 6.8 with 50% MeCN
   
   400 mL milli-Q water and 500 mL HPLC grade MeCN. Then add 13.94 mL TEA (0.1 mol). Adjust to pH 6.8 by HOAc.
   
   Add more ddH₂O to 1000 mL. Filter the solution by 0.2 µm Nylon membrane filter (VWR, 28159-723).

2. All the buffers must be filtered prior to use and degassed once each day prior to use. (Use 0.2 µm Nylon membrane filters)

3. Dissolve the oligomer in 2000 µL buffer A by vortex (for 1.0 µmol scale). Microcentrifuge it to remove particulate materials, and transfer supernatant to a fresh tube.
4. Prime the pump before using the HPLC and after changing buffer each time.

5. Run 20 μL sample solution first for analysis. Table A-4-1 is the time program for trityl-on analytical HPLC.

6. Run 100 μL for each sample. Table A-4-2 is the time program for trityl-on preparation HPLC.

7. Run blank HPLC between each sample in order to remove all the remaining ODNs on the column.

8. Collect the product in Falcon tubes and cover tube with kim wipe.

9. Freeze each solution thoroughly and dry in lyophilizer overnight to remove all the solvent and keep the samples in -20 °C Freezer.

*Detritylation of Tritylated Synthetic Oligonucleotides*

Great caution should be taken during detritylation steps. (Especially do not expose ODNs to acid for long time)

1. Suspend each ODN sample in 100 μL 80% acetic acid in ddH₂O.

2. After a 20 min incubation at R. T., the sample should be again brought to dryness by SVC.

3. Resuspend pellet in 0.5 mL of 1% TEA in ddH₂O, then concentrate to dryness by SVC.

4. The dried sample is resuspended in 300 μL of ddH₂O.

5. 300 μL ethyl acetate is added and the sample is vortexed. The upper layer which contains free DMT group is then discarded. Repeated this procedure for two additional times.
6. Concentrate samples to dryness by SVC and store them at -20 °C freezer.

**RP HPLC Purification of Trityl-off Synthetic Oligonucleotides**

Trityl-off HPLC is performed on System Gold from Beckman, with a 3 micron, 10mm X 70 mm C18 Ultrasphere column.

1. Prepare buffers:

   *Buffer A*: 0.1 M AA, pH 6.8 with 2% MeCN

   900 mL milli-Q water and 20 mL HPLC grade MeCN. The add 7.71 g AA (0.1 mol). Adjust to pH 6.8 by HOAc. Add ddH₂O to 1000 mL. Filter the solution by 0.2 μm Nylon membrane filter (VWR, 28159-723).

   *Buffer B*: 0.1 M AA, pH 6.8 with 50% MeCN

   400mL milli-Q water and 500 mL HPLC grade MeCN. Then add 7.71 g AA (0.1 mol). Adjust to pH 6.8 by HOAc. Add more ddH₂O to 1000 mL. Filter the solution by 0.2 μm Nylon membrane filter (VWR, 28159-723).

2. All the buffers must be filtered prior to use and degassed once each day prior to use. (Use 0.2 μm Nylon membrane filters)

3. Dissolve the oligomer in 1000 μL buffer A by vortex (for 1.0 μmol scale). Microcentrifuge it to remove particulate materials, and transfer supernatant to a fresh tube.

4. Prime the pump before using the HPLC and after changing buffer each time.
5. Run 20 µL sample solution first for analysis. Table A-4-3 is the time program for trityl-off analytical HPLC.

6. If there are some impurity peaks in analytical run, do the preparation RP HPLC for all the sample in same tube. If no impurity peak, it is not necessary to do it.

7. For reparation HPLC, Run 100 µL for each sample. Table A-4-4 is the time program for trityl-off preparation HPLC.

8. Run blank HPLC spectrum between each sample in order to remove all the remaining ODNs on the column.

9. Collect the product in Falcon tubes and cover tube with kim wipe.

10. Freeze each solution thoroughly and dry in lyophilizer overnight to remove all the solvent and keep the samples in -80 °C Freezer.
Table A-4-1. Time program for Analytical Trityl-on HPLC
(70 mm C18 HPLC column)

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<th>Value</th>
<th>Duration</th>
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Note: the flow rate is 3 mL/min
Table A-4-2. Time Program for Preparation Trityl-on HPLC (70 mm C_{18} HPLC column)

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Note: the flow rate is 3 mL/min
Table A-4-3. Time Program for Analytical Trityl-off HPLC
(70 mm C18 HPLC column)

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Note: the flow rate is 3 mL/min
Table A-4-4. Time Program for Preparation Trityl-off HPLC
(70 mm C₁₈ HPLC column)

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Note: the flow rate is 3 mL/min
APPENDIX V

THE EXPERIMENTAL SECTION OF SYNTHESIS OF FLUORESCENT NUCLEOSIDE TRIPHOSPHATE

Technical Notes

Nuclear magnetic resonance (NMR) spectra were determined on a Bruker AC250 NMR spectrometer. Chemical shifts of $^1$H NMR are reported in parts per million downfield from tetramethylsilane ($\delta = 0$) as internal standard. Chemical shifts of $^{31}$P NMR are reported in parts per million downfield from 85% phosphoric acid ($\delta = 0$) as internal standard. The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and b = broad.

Analytical TLC was performed on Whatman K5F silica plates (0.25 mm layer thickness). Spots were visualized with UV light and/or staining with anisaldehyde visualization solution (6 g anisaldehyde in 250 mL ethanol plus 2.5 mL conc. $H_2SO_4$). All the reactions were monitored by TLC, unless otherwise indicated.

Column chromatography was carried out on 230-400 mesh Spectrum silica gel with the flash technique.$^{102}$

All reactions sensitive to oxygen or moisture were conducted under an argon or nitrogen atmosphere.
Reagents

All commercial chemicals and solvents were used as supplied with the following exceptions: pyridine was distilled from KOH; DCM, TMSCl, benzene and toluene were distilled from CaH₂; NaSH was dried by evaporation of anhydrous benzene and pyridine; isobutyril chloride was distilled from PCl₅ and redistilled from quinoline;¹⁰³ POCl₃ was distilled from sodium metal; all nucleosides were dried by evaporation of dry reaction solvents.

Common solvents and reagents are purchased from Rice University Chemistry Stockroom which carries ACS grade Fisher products. The following information describes the vendors (and vendor catalog numbers) where common reagents were purchased.

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<th>Chemical</th>
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<td>(0.05% v/v TMS)</td>
<td>2206-27-1</td>
<td>CIL</td>
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</table>
8-Quinolyl phosphate (13)

To a stirred dry pyridine (35 mL) under a nitrogen atmosphere in ice bath was added POCl₃ (3.85 mL, 41.4 mmol). The mixture was stirred at 0 °C for 15 min. 8-hydroxyquinoline (2.00 g, 13.8 mmol) was added to dry pyridine (15 mL) to form a yellow-green type solution. The solution was added slowly to the reaction mixture by syringe over 10 min. The reaction mixture was stirred in ice bath for 5 h and stored in -20 °C freezer overnight. Then it was filtered to give yellow filtrate and the filtrate was evaporated in vacuo to give yellow solid. The residue was co-evaporated with dry pyridine (2 x 30 mL) to remove POCl₃. The residue then was dissolved in pyridine (25 mL) and dropped into a mixture of water (40 mL) and pyridine (20 mL). The mixture was stirred at 0 °C for 1 h and then evaporated in vacuo. The residue was co-evaporated with water (3 X 20 mL) to give yellow powder. The residue was dissolved in water (35 mL) and added CH₂CN (35 mL). White solid was formed and filtered to give product as white crystals (2.07 g, 67% yield).
Characterization of 13:

$^1$H NMR (DMSO-$d_6$): 8.95 (d, 1H), 8.44 (d, 1H), 7.93-7.75 (m, 4H)

$^{31}$P NMR (DMSO-$d_6$, H-decoupled):

-4.65 (s)

Mol Formula: $C_9H_8NO_4P$ (mw= 225.2)

TLC: Rf 0.29 (UV-active; DCM/MeOH, 9:1)

The product does not dissolve very well in DMSO-$d_6$, so the peaks are relatively small.
$^{31}$P NMR (H-decoupled) spectrum of 8-quinolyl phosphate 13
Pyridinium salt of 8-quinolyl adenosine 5'-phosphate (14)

Adenosine (180 mg, 0.67 mmol) was dried by evaporation of dry pyridine (3 X 10 mL). The dry adenosine was dissolved in dry pyridine (10 mL) and stirred. To the stirred solution was added 8-quinolyl phosphate 13 (228 mg, 1.01 mmol) and diphenyl disulfide (370 mg, 1.69 mmol). Tri-t-butylphosphine (0.42 mL, 1.69 mmol) was then added by syringe. The reaction mixture was stirred at R.T. for 6 h and quenched by the addition of water (2 mL). The mixture was further stirred for another 2 h. It was evaporated in vacuo to give yellow solid. The residue was dissolved in water (40 mL) and the aqueous solution was washed with chloroform (3 X 20 mL). The aqueous layer was concentrated in vacuo. The residue was dissolved in water (1 mL) and MeOH (20 mL) was added to form slightly yellow solid. The mixture was filtered to give product as slightly yellow crystals (0.34 g, 90% yield)
Characterization of 14:

$^1$H NMR (DMSO-$d_6$): 8.94 (d, 1H), 8.76-8.41 (m, 3 H), 8.19 (s, 1H), 7.90-7.47 (m, 9H), 5.90 (t, 1H), 4.57 (d, 1H), 4.22-3.96 (m, 2H), 3.7-3.52 (m, 2H)

$^{31}$P NMR (DMSO-$d_6$, H-decoupled):

-4.94 (s)

Mol Formula: $C_{25}H_{24}N_7O_4P$ (mw = 555.4)
$^{31}$P NMR (H-decoupled) spectrum of Pyridinium salt of 8-quinoly1 adenosine 5'-phosphate 14. We did not use standard to correct the chemical shift of the spectrum. So, we added some 13 into NMR tube and got the following NMR spectrum.
$^{31}$P NMR (H-decoupled) spectrum of 13 and 14

The chemical shift of 14 is -4.94 based on the chemical shift of 13 we measured before.
REFERENCES


57. Weinstock, G.M. Biochimie 64, 611-616 (1982).


