2014

Helicase-SSB Interactions In Recombination-Dependent DNA Repair and Replication

Christian Jordan

University of Vermont

Follow this and additional works at: https://scholarworks.uvm.edu/graddis

Part of the Biochemistry Commons, and the Molecular Biology Commons

Recommended Citation

Jordan, Christian, "Helicase-SSB Interactions In Recombination-Dependent DNA Repair and Replication" (2014). Graduate College Dissertations and Theses. 270.
https://scholarworks.uvm.edu/graddis/270

This Dissertation is brought to you for free and open access by the Dissertations and Theses at ScholarWorks @ UVM. It has been accepted for inclusion in Graduate College Dissertations and Theses by an authorized administrator of ScholarWorks @ UVM. For more information, please contact donna.omalley@uvm.edu.
HELICASE-SSB INTERACTIONS IN RECOMBINATION-DEPENDENT DNA REPAIR AND REPLICATION

A Dissertation Presented

by

Christian S. Jordan

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Cellular and Molecular Biology

October, 2014
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Cellular and Molecular Biology.

Dissertation Examination Committee:

______________________________________ Advisor
Scott W. Morrical, Ph.D.

______________________________________
David Pederson, Ph.D.

______________________________________
Robert J. Kelm, Ph.D.

______________________________________ Chairperson
Nicholas H. Heintz, Ph.D.

______________________________________ Dean, Graduate College
Cynthia Forehand, Ph.D.

Date: June 12, 2014
ABSTRACT

Dda, one of three helicases encoded by bacteriophage T4, has been well-characterized biochemically but its biological role remains unclear. It is thought to be involved in origin-dependent replication, recombination-dependent replication, antirecombination, recombination repair, as well as in replication fork progression past template-bound nucleosomes and RNA polymerase. One of the proteins that most strongly interacts with Dda, Gp32, is the only single-stranded DNA binding protein (SSB) encoded by T4, is essential for DNA replication, recombination, and repair.

Previous studies have shown that Gp32 is essential for Dda stimulation of replication fork progression. Our studies show that interactions between Dda and Gp32 play a critical role in regulating replication fork restart during recombination repair. When the leading strand polymerase stalls at a site of ssDNA damage and the lagging strand machinery continues, Gp32 binds the resulting ssDNA gap ahead of the stalled leading strand polymerase. We found that a Gp32 cluster on leading strand ssDNA blocks Dda loading on the lagging strand ssDNA, blocks stimulation of fork progression by Dda, and stimulates Dda to displace the stalled polymerase and the 3’ end of the daughter strand. This unwinding generates conditions necessary for polymerase template switching in order to regress the DNA damage-stalled replication fork. Helicase trafficking by Gp32 could play a role in preventing premature fork progression until the events required for error-free translesion DNA synthesis have taken place. Interestingly, we found that Dda helicase activity is strongly stimulated by the N-terminal deletion mutant Gp32-B, suggesting the N-terminal truncation to generate Gp32-B reveals a cryptic helicase stimulatory activity of Gp32 that may be revealed in the context of a moving polymerase, or through direct interactions of Gp32 with other replisome components.

Additionally, our findings support a role for Dda-Gp32 interactions in double strand break (DSB) repair by homology-directed repair (HDR), which relies on homologous recombination and the formation of a displacement loop (D-loop) that can initiate DNA synthesis. We examined the D-loop unwinding activity of Dda, Gp41, and UvsW, the D-loop strand extension activity of Gp43 polymerase, and the effect of the helicases and their modulators on D-loop extension. Dda and UvsW, but not Gp41, catalyze D-loop invading strand by DNA unwinding. The relationship between Dda and Gp43 was modulated by the presence of Gp32. Dda D-loop unwinding competes with D-loop extension by Gp43 only in the presence of Gp32, resulting in a decreased frequency of invading strand extension when all three proteins are present. These data suggest Dda functions as an antirecombinase and negatively regulates the replicative extension of D-loops. Invading strand extension is observed in the presence of Dda, indicating that invading strand extension and unwinding can occur in a coordinated manner. The result is a translocating D-loop, called “bubble migration synthesis,” a hallmark of break-induced repair (BIR) and synthesis dependent strand annealing (SDSA). Gp41 did not unwind D-loops studied and may serve as a secondary helicase loaded subsequent to D-loop processing by Dda. Dda is proposed to be a mixed function helicase that can work both as an antirecombinase and to promote recombination-dependent DNA synthesis, consistent with the notion that Dda stimulates branch migration. These results have implications on the repair of ssDNA damage, DSB repair, and replication fork regulation, which are highly conserved processes sustained in all organisms.
DEDICATION

I dedicate this work to my family and friends, who have supported me in this journey. I am especially grateful to Curie, Betsy, Eric, Geño, Marie, Tina, Eugenio Alejandro, Andres, Emil, Mariangeli, Maureen, Jaime, James, Kevin, Irmita, Elisa, Mercedes, and Raiza; these are the people I consider my family and whom I hold dearest. I also dedicate this to Brad and my friends Kovi, Sarah, Jess, Dennis, Kiana, Kerby, Tanya, Dave, and Vince; they have made this journey fun and unforgettable.
ACKNOWLEDGEMENTS

I am in debt to the many faculty and colleagues who have been key in my success as a graduate student. First and foremost, I am forever in debt to my mentor, Scott Morrical, for giving me the opportunity to fly freely while always keeping a net underneath me. I have had the pleasure to work alongside Michelle Silva, Robyn Maher, Amy Branagan, Jackie Chen, Jenny Tomczak, Mila Morrical, Xinh Xinh Nguyen, Kristian Finstad, Jenny Klein, and Keri Sullivan, all of whom have been instrumental in my success in the Morrical laboratory. I am grateful to Stephen Lidofsky and Ann Chauncey for building the MD/PhD Program, Mary Tierney, Nick Heintz, Erin Montgomery, and Kirstin van Luling for developing a strong CMB Program, Paula Tracy, Gary Stein, Patty Bosley, Lisa Marshall, Yvonne Green-Putnam, and Taylor Putnam for running a supportive Biochemistry Department, as well as Nick Heintz, David Pederson, and Bob Kelm for their time and guidance as my thesis committee.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Genome stability and DNA replication, repair, and recombination</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Properties of DNA Helicases</td>
<td>15</td>
</tr>
<tr>
<td>1.3. Helicases in human disease</td>
<td>25</td>
</tr>
<tr>
<td>1.4. The Bacteriophage T4 model system for the study of DNA replication, repair, and recombination</td>
<td>30</td>
</tr>
<tr>
<td>1.4.1. Overview of T4 DNA replication, recombination, and repair</td>
<td>31</td>
</tr>
<tr>
<td>1.4.2. T4 origin-dependent replication</td>
<td>39</td>
</tr>
<tr>
<td>1.4.3. T4 recombination-dependent replication</td>
<td>41</td>
</tr>
<tr>
<td>1.5. Dda: a versatile mediator of DNA recombination, replication and repair</td>
<td>46</td>
</tr>
<tr>
<td>1.5.1. Functional characterization</td>
<td>46</td>
</tr>
<tr>
<td>1.5.2. Structure and SF1B homology</td>
<td>52</td>
</tr>
<tr>
<td>1.5.3. Protein-DNA interactions</td>
<td>59</td>
</tr>
<tr>
<td>1.5.4. Protein-protein interactions</td>
<td>63</td>
</tr>
</tbody>
</table>
CHAPTER 2: REGULATION OF THE BACTERIOPHAGE T4 DDA HELICASE BY GP32 SINGLE-STRANDED DNA-BINDING PROTEIN

Abstract

Introduction

Materials and Methods

Results

Dda forms similar tripartite complexes with ssDNA and either Gp32 or Gp32-B. 99
Gp32 and Gp32-B do not bind polyamide nucleic acid (PNA). 103
Effect of Gp32 and Gp32-B on Dda DNA unwinding activity. 105
Gp32-B enhances the DNA unwinding activity of Dda on substrates containing short ssDNA gaps. 112
Gp32-B promotes the DNA unwinding activity of Dda helicase at elevated NaCl concentrations. 114
Gp32-B promotes Dda-catalyzed unwinding of a gapped duplex bound by DNA polymerase. 116
Gp32-B stimulates the ssDNA-dependent ATPase activity of Dda helicase. 119

Discussion

1.5.4.1. Gp32 ................................................................. 63
1.5.4.2. UvsX ............................................................... 71
1.5.5. The role of Dda in regulating strand displacement DNA synthesis .......... 76
1.5.6. Structural and functional comparison with other T4 helicases............... 78
1.5.6.1. Gp41 ............................................................... 78
1.5.6.2. UvsW ............................................................... 79
1.6. Research objectives ........................................................................... 80

Abstract

Introduction

Materials and Methods

Results

Discussion
CHAPTER 3: T4 HELICASES DIFFERENTIALLY REGULATE GP43 POLYMERASE-CATALYZED EXTENSION OF THE INVADING STRAND IN A SYNTHETICALLY CONSTRUCTED RECOMBINATION INTERMEDIATE .... 138

Abstract............................................................................................................................. 139

Introduction .......................................................................................................................... 141

Materials and Methods ...................................................................................................... 144

Results .................................................................................................................................. 150

Construction and validation of D-loop substrates ................................................................. 150
UvsW helicase efficiently unwinds an inverted D-loop substrate.............................................. 153
Dda helicase efficiently unwinds a D-loop substrate .............................................................. 155
D-loops and inverted D-loops are poor substrates for Gp41 helicase. ................................. 157
Gp32 and Gp59, effectors of Gp41 helicase activity, have little effect on Gp41-catalyzed D-loop unwinding........................................................................................................ 159
Gp32-B, but not Gp32, promotes D-loop unwinding by Dda. ................................................. 162
Gp43 and Gp43-D219A catalyze strand displacement DNA synthesis in the D-loop substrate and are stimulated by Gp32................................................................. 165
Dda helicase suppresses D-loop extension by Gp43 polymerase in the presence of Gp32. ......................................................................................................................... 170
Gp41 helicase has minimal effects on Gp43-catalyzed D-loop extension. ...................... 173

Discussion............................................................................................................................ 177
Conflicts of interest statement ................................................................. 182

Acknowledgements .................................................................................. 183

References ............................................................................................... 184

CHAPTER 4: CONCLUSIONS ...................................................................... 192

4.1. Evaluation of major techniques .......................................................... 192
  4.1.1. Quantification of radiolabeled oligonucleotides by phosphor autoradiography and densitometry .................................................. 192
  4.1.2. Spectrophotometric ATPase assays .............................................. 194

4.2. Discussion ......................................................................................... 197
  4.2.1. The role and regulation of Dda in DNA replication repair .............. 197
    4.2.1.1. Uncoupling of leading and lagging strand DNA synthesis .......... 198
    4.2.1.2. Displacement of proteins in a stalled replication fork ............... 200
    4.2.1.3. Interactions with Gp32 ............................................................... 201
  4.2.2. Helicase D-loop processing .......................................................... 202
    4.2.2.1. Branch migration ..................................................................... 203
    4.2.2.2. SDSA and BIR ......................................................................... 204
    4.2.2.3. Crossover control ................................................................. 204

REFERENCES ........................................................................................... 209
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Oligonucleotides used in the construction of DNA substrates.</td>
<td>98</td>
</tr>
<tr>
<td>Table 2</td>
<td>Kinetic parameters of the ssDNA-stimulated ATPase activities of Dda in the absence and presence of Gp32-B.</td>
<td>119</td>
</tr>
<tr>
<td>Table 3</td>
<td>Oligonucleotide compositions of D-loop substrates.</td>
<td>149</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Three major classes of DNA damage repaired by recombination-dependent replication.</td>
<td>6</td>
</tr>
<tr>
<td>Figure 2</td>
<td>One-ended DNA double-strand break and repair by non-homologous end joining.</td>
<td>7</td>
</tr>
<tr>
<td>Figure 3</td>
<td>One-ended DNA double-strand break and repair by homology-directed repair.</td>
<td>9</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Two-ended DNA double-strand break and repair by double Holliday Junction (dJH).</td>
<td>10</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Two-ended DNA double-strand break and repair by synthesis-dependent strand annealing (SDSA).</td>
<td>13</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Schematic of template-switching to bypass a single-strand lesion by homology-directed repair.</td>
<td>14</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Summary of the helicase classification system depicting all six superfamilies and their associated motifs.</td>
<td>23</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Origin-dependent and recombination-dependent modes of initiation of DNA replication in bacteriophage T4.</td>
<td>34</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Circular permutation and terminal redundancy coordinates origin-dependent and recombination-dependent modes of DNA replication initiation in T4.</td>
<td>36</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Packaging of the terminally redundant and circularly permuted T4 genome.</td>
<td>38</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Homologous recombination is coupled to DNA replication in bacteriophage T4 recombination-dependent replication.</td>
<td>44</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Key structural and functional domains and motifs of the Dda-ssDNA complex.</td>
<td>56</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Interaction between the pin and tower structures and ssDNA.</td>
<td>58</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Dda-ssDNA interactions.</td>
<td>62</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Structure of Gp32 SSB core domain.</td>
<td>67</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Model of Gp32 domains and electrostatic interactions mediating DNA binding.</td>
<td>68</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Dda surface electrostatic potential and substrate binding.</td>
<td>69</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Dda protein-protein and protein-DNA interactions during unwinding.</td>
<td>70</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Structure of the UvsX monomer.</td>
<td>74</td>
</tr>
<tr>
<td>Figure 20</td>
<td>UvsX recombination filaments.</td>
<td>75</td>
</tr>
<tr>
<td>Figure 21</td>
<td>Complex formation between Dda and Gp32 and Gp32-B in the presence and absence of ssDNA.</td>
<td>101</td>
</tr>
<tr>
<td>Figure 22</td>
<td>Complex formation between Gp32 and Gp32-B with ssDNA or PNA.</td>
<td>102</td>
</tr>
<tr>
<td>Figure 23</td>
<td>Schematic representation of DNA unwinding assay.</td>
<td>104</td>
</tr>
</tbody>
</table>
Figure 24. Dda unwinding activity of various DNA substrates in the presence and absence of Gp32 or Gp32-B. .................................................................................................................. 110
Figure 25. DNA unwinding activity of Dda helicase as a function of ssDNA gap length. ........................................................................................................................................ 112
Figure 26. Gp32-B stimulates the DNA unwinding activity of Dda helicase at elevated salt concentrations. ................................................................................................. 114
Figure 27. In the presence of Gp32-B, Dda helicase unwinds past a primer/template-bound exonuclease-deficient polymerase. ........................................................................ 115
Figure 28. ATPase activity of Dda helicase as a function of ssDNA concentration, in the presence and absence of Gp32-B. .............................................................................. 117
Figure 29. ATPase activity of Dda helicase as a function of ATP concentration. .......... 118
Figure 30. Proposed model for Gp32 SSB regulation of Dda helicase in replication fork restart during recombination repair. .............................................................................. 127
Figure 31. D-loop substrate construction. ................................................................................................................. 151
Figure 32. UvsW helicase DNA unwinding of D-loop and inverted D-loop substrates. 154
Figure 33. Dda helicase unwinding of D-loop and inverted D-loop substrates. ............... 156
Figure 34. Gp41 helicase unwinding of D-loop and inverted D-loop substrates. .......... 158
Figure 35. Effects of Gp32 SSB and Gp59 helicase loader on Gp41 helicase unwinding of synthetic D-loop DNA substrate. .............................................................................. 161
Figure 36. Effects of Gp32 SSB and the non-cooperative variant Gp32(-)B on D-loop unwinding by Dda helicase. ........................................................................................................ 164
Figure 37. Strand displacement DNA synthesis by Gp43 polymerase and the exonuclease-deficient variant Gp43-D219A in the presence of Gp32 SSB. ................. 167
Figure 38. Schematic of the possible effects of DNA helicase addition on the replicative extension of a D-loop. ................................................................................................. 169
Figure 39. Effects of Gp32 SSB and Dda helicase on strand displacement DNA synthesis by Gp43 polymerase. ........................................................................................................ 173
Figure 40. Effects of Gp41 helicase on strand displacement DNA synthesis by Gp43 polymerase on invading strand of synthetic D-loop in the presence and absence of Gp32 SSB and Gp59 helicase loader. ................................................................. 176
Figure 41. Schematic of the spectrophotometric NADH-coupled ATPase assay. ........ 196
CHAPTER 1: INTRODUCTION

1.1. Genome stability and DNA replication, repair, and recombination

The maintenance of genomic stability is a fundamental challenge that is faced by every biological entity on Earth, from viruses to humans. It is a challenge because our genetic material is continuously damaged by errors in DNA metabolism (e.g., misincorporated bases), byproducts of cellular respiration (e.g., reactive oxygen species generated by oxidative phosphorylation), and the environment (e.g. radiation and mutagenic chemicals). It is estimated that there are 100,000 sites of DNA damage per mammalian cell per day (Brosh and Bohr 2007) and about 50 endogenous double strand breaks per cell cycle (Vilenchik and Knudson 2003). Some lesions, like double strand DNA breaks (DSBs), are so lethal that a single lesion, if left unrepaired, can result in the loss of a very large amount of genetic information (e.g. loss of a chromosome arm) or apoptosis. Therefore, cells have evolved efficient pathways to repair the high volume of diverse DNA damage that is inherent to life (Figure 1). If these pathways become corrupted, the integrity of the genome cannot be maintained and there is a decline in cellular health, which manifests itself as premature aging, developmental abnormalities, and uncontrolled cell growth (i.e., cancer).

The study of the cellular pathways responsible for maintaining the integrity of genomic content and structure is facilitated by their universality. All organisms rely on a similar core set of programs to replicate DNA, repair DNA, and generate genetic diversity. These programs, in turn, are interconnected because they rely on a common
core set of enzymes that can catalyze all the reactions necessary to carry out these different processes (Kreuzer, Saunders et al. 1995, George and Kreuzer 1996, Mueller, Clyman et al. 1996). This versatility imposes a requirement for the coordination and regulation of these enzymes in order to ensure that the correct programs are executed at the correct locations at the correct times. This coordination is well illustrated in the discussion of double strand break repair, which involves all three programs: recombination, replication, and repair.

The simplest way to repair a double strand break is to ligate the broken ends back together, a process termed non-homologous end joining (NHEJ) (Weterings and van Gent 2004) (Figure 2). Even though this mechanism efficiently preserves the structure of the DNA, the content near the break is lost as a result of the repair (van Heemst, Brugmans et al. 2004). In order to restore the content, as well as the structure, that is compromised in a double strand break, a program known as homology-directed repair (HDR) has evolved. In HDR, homologous recombination and DNA replication occur in a synchronized way to fill in the genetic information in and around the break. Once the gap has been filled using a homologous chromosome or sister chromatid as a template, recombination and replication can stop and repair can proceed to ligate the chromosome back together.

The repair of a double strand break by homology-directed repair begins with recombination. The first step in recombination is the generation of a 3’ ssDNA end. In the case of a double strand break, an exonuclease complex resects the broken ends (Paull and Gellert 1998). The 3’ ssDNA is bound by single-strand binding proteins, mediator proteins, and ultimately, a recombinase protein that generates a structure known as a
presynaptic filament. The presynaptic filament is capable of invading a homologous duplex DNA molecule and displacing one of the duplex strands, creating a structure known as a displacement loop (D-loop). Recombination is then finished and replication begins where recombination left off. The D-loop generated by recombination serves as a loading site for the replication enzymes and the 3’ end of the invading strand in the D-loop primes DNA replication by a DNA polymerase. Once enough DNA has been replicated to repair the damaged area, repair takes over and restores the structural integrity of the chromosome (Batty and Wood 2000) (Figure 3). This is the fundamental coordination of DNA recombination, replication and repair; specialized HDR pathways have evolved that are optimized for specific types of damage and circumstances (Kreuzer and Drake 1994, Mosig 1998).

The first HDR pathway to be described is the double Holliday Junction (dHJ) pathway (Szostak, Orr-Weaver et al. 1983), in which both ends of the double strand break generate ssDNA overhangs. Once strand invasion, D-loop formation, and DNA replication take place in one end, the resulting displaced strand anneals to the second ssDNA overhang, a process termed second end capture. This second ssDNA overhang primes DNA replication using the homologous displaced strand as a template and generates a structure known as a double Holliday Junction. This structure can translocate and is resolved by specific endonucleases; depending on how the double Holliday Junction is resolved, the resulting chromosomes may crossover and undergo gene conversion (Helleday, Lo et al. 2007) (Figure 4).
A related but distinct mode of HDR called synthesis-dependent strand annealing (SDSA) begins the same as the dHJ pathway. However, in SDSA, the D-loop translocates because the invading strand is displaced at the trailing edge of the D-loop while it is simultaneously extended at the leading edge; this is known as bubble migration DNA synthesis (Formosa and Alberts 1986). In SDSA, after the invading strand is sufficiently extended to repair the damaged region, it is displaced from the D-loop and anneals to the second ssDNA overhang generated by the DSB, serving as a template for the second round of DNA replication (Figure 5). This mechanism results in conservative DNA synthesis and does not generate chromosomal crossover or gene conversion (Hellday, Lo et al. 2007).

A third HDR pathway, break induced repair (BIR), is highly prone to genetic instability. A single broken end invades a homologous DNA sequence and primes replication that can proceed for hundreds of kilobases (Saini, Ramakrishnan et al. 2013). The ssDNA generated by the extensive replication can serve as a template for lagging strand synthesis, albeit asynchronously. The generation of long stretches of ssDNA makes this repair method mutation-prone, and the conservative mode of replication results in significant loss of heterozygosity (Saini, Ramakrishnan et al. 2013).

HDR is also used to restart stalled replication forks due to single stranded DNA lesions in order to achieve error-free lesion bypass (Helleday, Lo et al. 2007). This mechanism uses the lagging strand Okazaki fragment that is homologous to the damaged template strand in order to bypass the damaged region and restart replication. This requires uncoupling of leading and lagging strand synthesis, so that the stalled daughter
strand can invade the homologous duplex on the lagging strand. The leading strand then uses the Okazaki fragment as a template and replicates the DNA corresponding to the damaged region via bubble migration synthesis. Once sufficient replication has taken place to bypass the damaged region, the daughter strand reanneals to the template strand and replication continues normally (Figure 6) (Helleday, Lo et al. 2007).

The generation of a D-loop is a universal structural feature in all these processes. The conversion of a D-loop into a replication fork is the crucial step in the switch from recombination to replication, and it is tightly regulated (Bleuit, Xu et al. 2001, Maher, Branagan et al. 2011). The loading of DNA helicases onto specific sites and at specific times regulates this conversion; helicase loading completes the assembly of the replisome and permits DNA synthesis to begin (Bleuit, Xu et al. 2001, Maher, Branagan et al. 2011, Branagan, Klein et al. 2014). This fundamental function in DNA replication, recombination, and repair is a major focus of this dissertation, and in the following sections we discuss in detail the properties of DNA helicases and their regulation and function in the coordination of DNA replication, recombination and repair.
Figure 1. Three major classes of DNA damage repaired by recombination-dependent replication. A) DNA double-strand break. B) Replication fork encounters a DNA single-strand break, which results in a one-ended DNA double-strand break. C) A single-strand DNA lesion inhibits lagging or leading strand DNA synthesis, resulting in a daughter strand gap. Adapted from Helleday, Lo et al. 2007.
Figure 2. One-ended DNA double-strand break and repair by non-homologous end joining. A DNA single-strand break results in replication fork breakdown and a one-ended DNA double-strand break. The one-ended DNA double-strand break can be repaired by non-homologous end joining (NHEJ), which can result in misjoining and loss of genetic information. Adapted from Helleday, Lo, et al. 2007.
Figure 3. **One-ended DNA double-strand break and repair by homology-directed repair.** A DNA single-strand break results in replication fork breakdown and a one-ended DNA double-strand break. The 3’ overhang invades the sister chromatid and creates a D-loop that allows replication to proceed beyond the damaged region. The resulting Holliday Junction is resolved and the replication fork is restored. Adapted from Helleday, Lo et al. 2007.
Figure 4. **Two-ended DNA double-strand break and repair by double Holliday Junction (dJH).** Ends of the two-ended break are resected to produce ssDNA overhangs. The 3’ end of a ssDNA overhang invades a homologous dsDNA region creating D-loop. The opposite end of the DNA break anneals to the D-loop displaced strand creating a
double Holliday Junction. Resolution of the Holliday Junctions can preserve the continuity of the flanking sequence or produce a crossover event, depending on the orientation of cleavage. Nonetheless, repair synthesis results in gene conversion at the location of the break. Adapted from Helleday, Lo et al. 2007.
Figure 5. Two-ended DNA double-strand break and repair by synthesis-dependent strand annealing (SDSA). The end of a DNA double-strand break is resected to create single-stranded 3’ overhang that can catalyze strand invasion and prime DNA replication. The resulting Holliday Junction undergoes branch migration and the invading strand is released after DNA synthesis has gone beyond the region of the break. The released extended invading strand reanneals to its complementary strand bridging the region of the break. Adapted from Helleday, Lo et al. 2007.
Figure 6. Schematic of template-switching to bypass a single-strand lesion by homology-directed repair. Parental DNA is depicted as thin lines and daughter strands as thick lines. A single-strand lesion blocks leading strand fork progression. Fork reversal allows the leading strand 3’ end to pair with the complementary portion of the lagging strand and prime DNA synthesis. Junction migration restores the conventional replication fork beyond the single-strand lesion. Adapted from Kadyrov and Drake 2004.
1.2. Properties of DNA Helicases


Helicases, and more generally, translocases, have several fundamental properties that can be used to classify these diverse enzymes. Helicases unwind nucleic acids by translocating on a nucleic acid lattice. The rate of translocation is a fundamental property that is useful in characterizing helicases. Translocation rates vary from several base pairs per second to several thousand base pairs per second. This rate is variable depending on the presence of accessory factors and regulatory proteins; some helicases are not active until they form a complex with a protein partner or are loaded at specific sites. This characteristic is related to another property of helicases, the rate of ATPase activity (Singleton, Dillingham et al. 2007).

A third fundamental characteristic of helicases and translocases in general is the directionality of translocation and unwinding. Single stranded nucleic acids are
asymmetric polar molecules, so motor proteins that translocate on single stranded nucleic acids (type $\alpha$) have a sense of the direction in which they are moving. Translocases that move along a double stranded nucleic acid (type $\beta$) appear to track along just one of the two strands, which overcomes the intrinsic symmetry and lack of polarity of double stranded nucleic acids. The strand on which double stranded nucleic acid helicases load therefore determines the directionality of translocation and unwinding. Hence, helicases are classified as unwinding nucleic acids in a 3’-to-5’ (type A) or 5’-to-3’ (type B) direction (Singleton, Dillingham et al. 2007). Additionally, the type of nucleic acid that helicases unwind is variable; helicases and translocases with specificity for DNA, RNA, or DNA-RNA hybrids have all been identified (Singleton, Dillingham et al. 2007).

A fourth fundamental property of helicases is processivity, which refers to the number of cycles the enzyme catalyzes before it dissociates from the nucleic acid lattice. Highly processive helicases catalyze multiple cycles of unwinding before releasing the product; highly distributive helicases, on the other hand, undergo one or a few rounds of catalysis, dissociate from the nucleic acid, and bind a new substrate for the next reaction. The most processive helicases catalyze separations of several thousand base pair per binding event. Like the rate of translocation, the processivity of helicases can be modulated by interactions with accessory or regulatory proteins (Singleton, Dillingham et al. 2007). Processivity is closely related to the step size of a helicase or translocases. Step size refers to the number of base pairs that are translocated or unwound per catalytic cycle, which may be equivalent to one ATP hydrolysis event (Singleton, Dillingham et al. 2007).
Finally, the mechanism of helicase-catalyzed unwinding can be used to classify helicases as active or passive. Helicases that are highly active unwind duplex nucleic acids by translocating on a single stranded lattice and actively disrupting base pairing interactions to unwind the duplex ahead. On the other hand, passive helicases rely on thermal fraying taking place at the fork and catalyze unwinding by sequestering the strands in the unwound state (Lohman and Bjornson 1996). This mechanistic difference adds to the complexity in the classification, mechanism, and regulation of helicases.

The classification system for helicases was developed based on primary structure (Gorbalenya and Koonin 1993) (Figure 7). It has been expanded to include helicases that are members of the group of proteins known as ATPases associated with various cellular activities (AAA$^+$) (Erzberger and Berger 2006), modified to formally include translocases that were originally believed to be putative helicases, and refined to include additional motifs that are characteristic of specific helicase superfamilies or subfamilies (Pause and Sonenberg 1992, Korolev, Yao et al. 1998, Mahdi, Briggs et al. 2003, Tanner, Cordin et al. 2003). Despite the diversity of this large class of enzymes, all helicases and translocases share universal structural and mechanistic properties.

All helicases possess motifs that form signature RecA-like fold core domains either within the same polypeptide chain or between subunits. These RecA-like folds couple the chemical energy released during ATP hydrolysis with the mechanical energy needed to generate conformational changes that drive translocation on a single stranded nucleic acid lattice and/or unwinding of double-stranded nucleic acids (Ye, Osborne et al. 2004). These core domains always include conserved residues involved in the binding
and hydrolysis of ATP, equivalent to the Walker A and B boxes of many ATPases (Walker, Saraste et al. 1982) and an arginine finger that plays a role in coupling of chemical and mechanical energy (Scheffzek, Ahmadian et al. 1997).

The helicase/translocase classification system is divided into six superfamilies, many of which have specific structural motifs unique to them. Superfamily 1 (SF1), one of the best characterized classes, is made up exclusively of type α helicases that unwind in either direction and they all contain two RecA-like folds in a single polypeptide chain, a characteristic they share with superfamily 2 (SF2) enzymes (Singleton, Dillingham et al. 2007). The minimal oligomeric state is monomeric and most are considered to be monomeric or dimeric (Subramanya, Bird et al. 1996, Korolev, Hsieh et al. 1997, Velankar, Soutlanas et al. 1999), even though monomers may act cooperatively to increase unwinding efficiency (Nanduri, Byrd et al. 2002, Brendza, Cheng et al. 2005, Dillingham, Webb et al. 2005, Zhang, Dou et al. 2006). Examples of type A SF1 helicases include Rep and UvrD helicases in gram-negative bacteria and PcrA helicase from gram-positive bacteria. Examples of type B SF1 helicases include T4 Dda helicase, \textit{E. coli} RecD helicase, and the eukaryotic helicases Pif1 and Rrm3 (Ivessa, Zhou et al. 2002). RecD, although monomeric, is part of the RecBCD heterotrimeric complex, and has been studied in both contexts (Taylor and Smith 1995, Nanduri, Byrd et al. 2002). Dda and RecD are the only two SF1B helicases that have been crystallized to date (Singleton, Dillingham et al. 2004, He, Byrd et al. 2012). The ATP binding site of SF1 enzymes is at the interface of the two RecA-like folds contained within the single polypeptide chain that forms the monomeric unit (Singleton, Dillingham et al. 2007).
Superfamily 2 (SF2), the largest group, is composed mostly of type A helicases and translocases that are considered to be monomeric or dimeric. Similar to SF1 helicases, the minimal oligomeric state is monomeric (Subramanya, Bird et al. 1996, Korolev, Hsieh et al. 1997, Velankar, Soultanas et al. 1999) and they all contain two RecA-like folds in a single polypeptide chain (Singleton, Dillingham et al. 2007). Examples of SF2 subfamilies include DEAD-box RNA helicases (Cordin, Banroques et al. 2006), the RecQ-like family (Bennett and Keck 2004), and the Snf2-like enzymes (Flaus and Owen-Hughes 2004, Flaus, Martin et al. 2006). The hepatitis C virus nonstructural protein 3 (NS3) is one of the best-characterized SF2 helicases. It is a monomeric (Dumont, Cheng et al. 2006) type Aα helicase that can unwind RNA and DNA duplexes (Pang, Jankowsky et al. 2002) and requires a single-stranded overhang on which to load (Tai, Chi et al. 1996). NS3 has been crystalized alone and in complex with single-stranded nucleic acid (Kim, Morgenstern et al. 1998). Like SF1 helicases, the interface between the two RecA-like folds in NS3 forms a deep groove that accommodates the helicase motifs, producing the nucleotide-binding pocket and part of the ssDNA-binding site (Singleton, Dillingham et al. 2007). Nonhydrolyzable ATP analogs weaken NS3-ssDNA binding, and NS3 can melt several nucleotides of duplex using binding energy alone (Levin, Gurjar et al. 2003, Levin, Gurjar et al. 2005). These findings are consistent with the proposed “Brownian ratchet” mechanism in which ATP binding reduces the enzyme’s affinity for DNA and results in a brief period of Brownian motion of the helicase relative to the nucleic acid lattice before ATP hydrolysis triggers rebinding biased in the forward direction (Levin, Gurjar et al. 2005). Similarly to SF1
helicases, SF2 helicases demonstrate cooperative interactions between monomers (Levin, Wang et al. 2004, Tackett, Chen et al. 2005).

Enzymes in superfamilies 3-6, unlike SF1 and SF2, are all hexameric helicases. There are structural similarities among these superfamilies, including either a RecA or an AAA$^+$-like ATPase core (a 230-amino acid conserved module in a large family of ring-shaped NTPases) with the nucleotide-binding sites at the interface between monomers. Neighboring units contribute an arginine finger to the nucleotide-binding pocket, which is involved in modulating NTP binding and/or hydrolysis. Functionally, many of these helicases are replicative helicases, are highly processive and require an ATP-dependent loading partner (Davey and O'Donnell 2003).

Superfamily 3 (SF3) enzymes are type A (unwind/translocate 3’-to-5’) hexameric or double hexameric helicases associated with diverse enzymatic activities, such as origin recognition and unwinding (Hickman and Dyda 2005). SF3 enzymes all share four conserved motifs: A and B, which correspond to the canonical Walker A and B boxes, B’, and C, which is SF3 specific and contains the arginine finger. All SF3 helicases have an ori DNA-binding domain and a modified AAA$^+$ core in the helicase domain. The SF3 papilloma virus E1 helicase has been crystalized in complex with a 13-mer ssDNA and ADP and provides insights into the structure and mechanism of this superfamily (Enemark and Joshua-Tor 2006). The E1 structure shows an oligomerization domain that forms a six fold symmetric collar that stabilizes the hexamer. A central channel has the ssDNA bound via interactions between a B’ motif loop and the phosphodiester backbone.
which is similar to the proposed model for other hexameric helicases (Singleton, Dillingham et al. 2007).

Superfamily 4 (SF4) enzymes are replicative helicases that either associate with a primase (e.g., *E. coli* DnaB and DnaG proteins) or contain primase activity. SF4 helicases are all type B helicases, translocating 5’-to-3’ (Singleton, Dillingham et al. 2007). SF4 enzymes are defined by five sequence motifs: H1 and H2, equivalent to Walker A and B motifs, H1a, H3, and H4 (Ilyina, Gorbalenya et al. 1992). Examples of SF4 helicases include the bacteriophage T4 gene 41 protein (Gp41) and bacteriophage T7 gene 4 protein (Gp4). T4 Gp41 helicase associates with the T4 primase gene 61 protein (Gp61) whereas the T7 Gp4 helicase has both primase and helicase domains connected by a linker (Toth, Li et al. 2003).

All superfamily 5 (SF5) proteins characterized to date, like SF4 helicases, are type B helicases (unwind/translocate 5’-to-3’). The prototypical SF5 helicase is the bacterial Rho helicase, which binds specific sequences on nascent RNA during transcription, unwinds the DNA/RNA hybrid, and terminates transcription. Full-length Rho helicase has been crystallized (Skordalakes and Berger 2003) and it is a hexamer similar in structure of the SF4 helicases.

Superfamily 6 (SF6) includes helicases that contain the AAA⁺ core such as the eukaryotic replicative helicase mini chromosome maintenance (MCM) protein complex and the prokaryotic RuvB protein in conjunction with RuvA and RuvC. The MCM protein complex is a six-protein complex (MCM2-7) that is essential for replication initiation and elongation (Labib, Tercero et al. 2000); however, the MCM4,6,7
heterohexamer is the only species that has detectable helicase activity so far (Kaplan and O'Donnell 2004). Archaeal MCM homologs have been crystallized (Chong, Hayashi et al. 2000, Pape, Meka et al. 2003) and show a hexameric ring with a central channel sufficiently large to accommodate dsDNA, indicating it is likely a type β helicase. The prokaryotic RuvB protein, along with RuvA and RuvC, processes Holliday junctions by pumping dsDNA through a central channel in an ATP-dependent manner, and hence, is also a β-type helicase (West 1996).

The fundamental function of nucleic acid unwinding is a universal characteristic of helicases in all six superfamilies. However, the location, rate, and extent of unwinding are highly regulated, given that the majority of cellular DNA is kept double-stranded whenever possible (Singleton, Dillingham et al. 2007). In order for this to be accomplished, helicases are frequently inactivated until they have formed critical protein-protein interactions or are loaded at the specific times and sites at which they are needed. Proteins that regulate the function of helicases are a major function of this thesis and examples of helicase protein-protein interactions are discussed in the following section.
Figure 7. Summary of the helicase classification system depicting all six superfamilies and their associated motifs. A) One example of each superfamily is given in parentheses. Core domains and signature motifs are given for each superfamily; accessory domains denoted are specific to the example family member. Universal motifs present in all helicases are depicted in yellow. B) Structure of the SF1 helicase PcrA monomeric core with N- and C-terminal RecA-like folds. The NTP-binding and
hydrolysis region is shown in yellow and an NTP analogue is shown in black. This core domain is the minimal motor unit of SF1 and SF2 helicases. C) Structure of the SF4 helicase gene 4 protein (Gp4) from bacteriophage T7. SF3 through SF6 enzymes share a core of six individual RecA- or AAA+-like domains (shown in red) arranged in a ring that forms six nucleotide-binding pockets, one at each domain interface. Four NTP analogues are shown in black. D) Helicases are classified by directionality, 3’-to-5’ (type A) or 5’-to-3’ (type B), and by whether the nucleic acid substrate is single-stranded (type α) or double-stranded (type β). Adapted from Singleton, Dillingham et al. 2007.
1.3. Helicases in human disease

Helicases are highly conserved enzymes that are viewed to be playing an increasingly central role in genomic maintenance; they are involved in all processes involving ssDNA, including DNA replication, repair, recombination, and transcription (van Brabant, Stan et al. 2000). Defects in human helicases can result in distinct disorders that are characterized by genomic instability and predisposition to cancer. Mutations in *XPB* and *XPD* can result in Xeroderma Pigmentosum, Cockayne Syndrome, or Trichothiodystrophy. Mutations in the RecQ-like genes *BLM*, *WRN*, and *RECQL4* can result in Bloom Syndrome, Werner Syndrome, and Rothmund-Thomson Syndrome, respectively.

*XPB* and *XPD* both function in nucleotide excision repair and transcription initiation; their deficiency presents with failure to repair mutagenic DNA lesions and defects in the recovery of RNA transcription after UV irradiation. The first disease that can result from deficiency in *XPB* or *XPD* helicases is Xeroderma Pigmentosum, which is characterized by extreme photosensitivity, skin abnormalities in sun-exposed areas, and cataracts. It is an autosomal recessive trait, and is genetically and phenotypically heterogeneous. Patients with XP have a 1,000-fold increased risk of skin cancer and the median age of the first skin neoplasm is 8 years (van Brabant, Stan et al. 2000).

Mutations in *XPB* and *XPD* can also result in Cockayne Syndrome, which is characterized by photosensitivity, cognitive and developmental deficiencies, and skeletal abnormalities, but not increased risk of cancer. The mean lifespan of a person with
Cockayne Syndrome is 12 years (van Brabant, Stan et al. 2000). Finally, mutations in these two genes can lead to a third disorder, Trichothiodystrophy. Patients with Trichothiodystrophy present with sulfur-deficient brittle hair, short stature, cognitive deficiencies, developmental abnormalities and photosensitivity, but no cancer-proneness (van Brabant, Stan et al. 2000).

XPB and XPD are components of the basal transcription complex TFIIH. In nucleotide excision repair they unwind DNA at the site of the lesion to create an open structure. XPB unwinds with 3’-to-5’ directionality, while XPD unwinds 5’-to-3’ (Schaeffer, Roy et al. 1993, Sung, Bailly et al. 1993, Ma, Siemssen et al. 1994, Schaeffer, Moncollin et al. 1994). Patients with mutations in XPB and XPD exhibit either XP, XP with CS, or TTD (Cleaver, Thompson et al. 1999). The syndrome that develops depends on the type of mutation. If the mutation primarily affects nucleotide excision repair, XP develops; however, mutations that result in defects in transcription result in TTD or CS (Hwang, Moncollin et al. 1996).

The founding RecQ family member was discovered in *Escherichia coli* where it functions in the RecF recombination pathway (Nakayama, Nakayama et al. 1984, Umezu, Nakayama et al. 1990) in plasmid recombination (Kolodner, Fishel et al. 1985, Luisi-DeLuca, Lovett et al. 1989), stalled replication fork restart (Courcelle, Carswell-Crumpton et al. 1997, Cox 1997, Galitski and Roth 1997, Courcelle, Crowley et al. 1999, Courcelle and Hanawalt 1999, Cox, Goodman et al. 2000), repair of UV light-induced DNA damage (Kowalczykowski, Dixon et al. 1994), and suppression of illegitimate recombination or recombination between sequences with limited homology (Hanada,
In vitro, *E. coli* RecQ behaves as a 3’-to-5’ helicase and ssDNA-dependent ATPase (Umezu, Nakayama et al. 1990). RecQ can catalyze recombination initiation with RecA and SSB (Harmon and Kowalczykowski 1998) and plasmid DNA catenation with topoisomerase III and SSB (Harmon, DiGate et al. 1999). Some of these functions are conserved in many eukaryotic RecQ proteins, suggesting functional homology among RecQ family members (Wu and Hickson 2001).

So far five human RecQ homologs have been identified: RECQL1, BLM, WRN, RECQL4 and RECQL5. Three of them are associated with autosomal recessive disorders characterized by premature aging, genome instability and cancer predisposition: Werner Syndrome (WS) is associated with defects in WRN protein, Bloom Syndrome (BS) is associated with defects in BLM helicase, and Rothmund Thomson Syndrome (RTS), RAPADILINO Syndrome, and Baller Gerold Syndrome are all associated with defects in RECQL4 helicase (Singh, Ahn et al. 2009). RecQ-like genes function in DNA repair pathways including double-strand break repair, repair of interstrand crosslinks and stalled replication fork restart, and protection from illegitimate recombination during chromosome segregation in mitosis (Chakraverty and Hickson 1999, Shen and Loeb 2000, van Brabant, Stan et al. 2000).

Werner Syndrome (WS) is an autosomal recessive disease characterized by premature aging associated with genome instability and an increased risk of cancer. WS cells show chromosomal aberrations including deletions, translocations and rearrangements as well as increased spontaneous mutations (Hoehn, Bryant et al. 1975, 1978).
WS cells also show selective sensitivity toward many different types of DNA damaging agents, including 5-nitroquinoline-N-oxide, comptothecin and ionizing radiation (Ogburn, Oshima et al. 1997, Okada, Goto et al. 1998, Pichierri, Franchitto et al. 2000, Poot, Gollahon et al. 2002). WRN cells show proliferative defects, limited replicative capacity and premature cellular senescence due to widespread genomic damage that results in the rapid exit from the cell cycle (Faragher, Kill et al. 1993).

Bloom Syndrome (BS) is a rare autosomal recessive disorder that presents with sun-sensitive facial erythema, short stature, hypo- and hyper-pigmented skin lesions, characteristic facies and voice, immunodeficiency, infertility in males and subfertility in females, a predisposition to developing diabetes and a greatly increased predisposition to developing cancer (German and Ellis 1998, van Brabant, Stan et al. 2000). BS is associated with inherent genomic instability (Bachrati and Hickson 2003, Hickson 2003) and elevated levels of chromosomal breaks and translocations. BS cells diagnostically show greatly increased frequency of sister chromatid exchange (SCEs), exchanges between homologous chromosomes and loss of heterozygosity (Chaganti, Schonberg et al. 1974, German 1995). These DNA exchanges mostly arise as part of homologous recombination events that occur during repair of DNA damage in the S and G2 phases of the cell cycle (Singh, Ahn et al. 2009).

Rothmund Thomson Syndrome (RTS) is an autosomal recessive disorder characterized by skin and skeletal deformities, premature hair loss and cataracts (Kitao, Shimamoto et al. 1999, Larizza, Magnani et al. 2006), and cancer predisposition,
particularly to osteosarcomas (Wang, Levy et al. 2001, Wang, Gannavarapu et al. 2003) that results from mutations in the RECQL4 gene. RTS cells show chromosome abnormalities and genomic instability (Der Kaloustian, McGill et al. 1990, Lindor, Devries et al. 1996, Miozzo, Castorina et al. 1998) and are hypersensitive to gamma irradiation and oxidative damage (Vennos and James 1995, Werner, Prahalad et al. 2006). Mutations in the RECQL4 gene can result in two other recessive disorders: RAPADILINO (radial hypoplasia, patella hypoplasia and cleft or arched palate, diarrhea and dislocated joints, little size and limb malformation, nose slender and normal intelligence) (Siitonen, Kopra et al. 2003) and Baller–Gerold Syndrome (BGS), which presents with radial hypoplasia and craniosynostosis (Megarbane, Melki et al. 2000, Van Maldergem, Siitonen et al. 2006). The syndrome that develops is a function of the specific mutation found in RECQL4. Mutations that result in RTS are mostly nonsense or frameshift mutations that result in low levels of truncated protein. The most common mutation in RAPADILINO Syndrome is an in-frame deletion that leaves the helicase region largely intact (Siitonen, Kopra et al. 2003), suggesting the sequences outside of the helicase region mediate important regulatory functions. BGS, on the other hand, is only associated with two specific mutations, a loss-of-function missense mutation and a deletion frameshift mutation (Van Maldergem, Siitonen et al. 2006).
1.4. The Bacteriophage T4 model system for the study of DNA replication, repair, and recombination

It has been well established that helicase function and regulation is essential for the maintenance of genomic sequence and structure and thus for all aspects of human health. They are important players in cancer, genomic stability, and aging. However, helicases work in the context of other proteins that regulate their function by diverse mechanisms that include helicase loading, activation or inhibition, and complex formation. This regulation is paramount; in fact, most helicases are believed to require activation by regulatory proteins and are thought to be inactive in the absence of these protein partners. Thus, the study of helicase function and regulation requires the study of these mediator proteins as well. In humans this is limited by our technical capabilities and the complexity inherent in the human system. However, as for any fundamental cellular process, there are common themes that remain universal across species. Many core mechanisms have been maintained throughout evolution, and therefore the study of helicase function and regulation in a minimal system provides information relevant to the human system. This is the rationale for studying a simpler model system, specifically bacteriophage T4, in order to understand the core processes that have been evolutionarily conserved.
1.4.1. Overview of T4 DNA replication, recombination, and repair

Bacteriophage T4 is an ideal system for evaluating mechanisms of DNA replication, repair and recombination for many reasons, the most important being that all the proteins necessary for DNA replication, repair and recombination, with the exception of RNA polymerase, are encoded by T4 and have been purified and extensively characterized biochemically and genetically (Nossal 1994). There are many similarities in the DNA metabolism mechanisms between T4 and other systems, and even though more proteins are involved in DNA metabolism in eukaryotes, such as those that coordinate replication with the cell cycle, the basic mechanisms are comparable (Kreuzer 1994). In fact, the link between replication and recombination, which is now universally accepted, was first established in the T4 system (Melamede and Wallace 1980, Formosa and Alberts 1986, Morrical and Alberts 1990, Morrical, Hempstead et al. 1994, Mosig 1998, Kowalczykowski 2000, Kreuzer 2000, Bleuit, Xu et al. 2001, Liu and Morrical 2010, Liu, Ehmsen et al. 2011, Maher, Branagan et al. 2011). In essence, T4 provides a simplified system for analyzing the role of specific proteins in DNA replication, repair, and recombination.

T4 uses two major modes of replication initiation (Figures 8 and 9). The dominant mode of DNA replication initiation at the earliest times of infection is the origin-dependent mode of replication, which assembles replication forks at origins of replication. There are five origins of replication in the T4 genome, which are sequences
that serve to generate the RNA primers necessary to initiate DNA synthesis (Kreuzer and Brister 2010). The RNA primer at the origin forms a structure known as an R-loop, which facilitates the assembly of the replication fork machinery and initiates lagging and leading strand DNA synthesis (Mosig, Colowick et al. 1995, Carles-Kinch and Kreuzer 1997, Belanger and Kreuzer 1998). Later in infection, recombination-dependent replication becomes the dominant mode of DNA synthesis initiation, in which recombination intermediates known as D-loops, instead of origin of replication R-loops, are used to initiate DNA replication. Recombination-dependent replication ultimately generates the majority of genomic DNA in T4 (Kreuzer and Brister 2010).

The genomic structure of T4 is optimized to facilitate recombination-dependent replication. Since linear genomes have ends, DNA synthesis occurs in the 5’-to-3’ direction, and polymerases require a pre-existing 5’ primer to start replication, the 3’ ends are not replicated and remain single-stranded in origin-dependent replication. The T4 genome has overcome this problem by having a terminally redundant genome, with the sequences at the ends being copies (Kreuzer 2000). Additionally, T4 particles package a length of DNA that is in excess of that which is needed for a single copy of the genome, and therefore the DNA is also circularly permuted (Figure 10) (Mathews 1994, Kreuzer 2000). The end result is that the end of a chromosome in one phage particle is homologous to the other end, and is also homologous to internal sequences in chromosomes packaged in other phage particles. In this way, T4 is optimized to efficiently initiate DNA replication through either inter- or intra-chromosomal recombination events (Figure 9) (Kreuzer and Brister 2010).
As has been established, recombination intermediates can not only serve to prime DNA replication, but they can serve to facilitate repair of a variety of DNA lesions, from double-strand breaks to single-stranded lesions that result in stalled replication forks, through a process known as homology-directed repair (HDR). Regardless of the downstream resolution of the recombination intermediate, the initial steps are the same. The first step is the generation of a 3’ single stranded DNA overhang, which can be generated by exonucleases acting on a double-strand break or by origin-dependent DNA replication (Kowalczykowski 2000, Maher, Branagan et al. 2011). The next step is the formation of the presynaptic filament on the ssDNA by UvsX, a member of the conserved RecA/Rad51 recombinase family. The presynaptic filament then invades a homologous duplex DNA in a process called strand exchange. The resulting D-loop recombination intermediate serves as a loading site for the components of the replication fork and either RDR or HDR can proceed. (Liu and Morrical 2010). This interconnection between recombination, replication and repair is not unique to bacteriophage T4; it is now clear that recombination processes play critical roles in DNA replication and the maintenance of genome stability across species (Barbour and Xiao 2003, Kreuzer 2005, Lambert, Froget et al. 2007, Aguilera and Gomez-Gonzalez 2008).
Figure 8. **Origin-dependent and recombination-dependent modes of initiation of DNA replication in bacteriophage T4. A and B)** Schematic representation of origin-dependent initiation of DNA replication showing a T4 origin with its complementary RNA strand serving as a primer for initiating DNA replication. The resulting linear chromosome contains a 3’ ssDNA overhang that can participate in homologous recombination and initiate recombination-dependent replication initiation. **C and D)** Recombination-dependent replication initiation begins with strand invasion by a 3’ ssDNA end of a homologous region of duplex DNA, resulting in the formation of a D-
loop. The 3’ end of the invading strand can prime DNA synthesis and the displaced strand becomes the template for lagging strand DNA synthesis. Adapted from (Kreuzer and Morrical 1994).
Figure 9. Circular permutation and terminal redundancy coordinates origin-dependent and recombination-dependent modes of DNA replication initiation in T4. 

**A)** Origin-dependent initiation of DNA replication occurs early in infection and produces **B)** two daughter chromosomes with 3’ ssDNA overhangs. **C)** The 3’ ssDNA overhangs can invade a homologous region of duplex DNA, **D)** which can prime DNA synthesis and initiate leading strand DNA replication; the displaced ssDNA strand consequently
generated can serve as the template strand for lagging strand DNA replication. Adapted from (Trun and Trempy 2003).
Figure 10. **Packaging of the terminally redundant and circularly permuted T4 genome.** A) After enough origin- and recombination-dependent replication has taken place, an initial T4 parental genome can generate B) a concatemeric DNA intermediate containing many circularly permuted genomic copies. C) The concatemeric DNA is cut into lengths that can be packaged in the T4 particles; since each can carry more DNA than a single genome’s length, progeny phage particles contain daughter chromosomes that are terminally redundant and circularly permuted genomes. Adapted from (Elrod and Stansfield 2010).
1.4.2. T4 origin-dependent replication

The existence of T4 origin of replication sequences was brought to light by the fact that DNA produced early during infection originated at specific sites in the T4 genome (Halpern, Mattson et al. 1979, Kozinski, Ling et al. 1980, Macdonald, Seaby et al. 1983). The identity of origins was first confirmed by experiments demonstrating that small DNA fragments with specific sequences were capable of driving autonomous replication of plasmids during a T4 infection (Kreuzer and Alberts 1985, Kreuzer and Alberts 1986). Today we know there are at least five origins in T4 that are active during early infection: oriA, oriC, oriE, oriF, and oriG (Brister and Nossal 2007, Brister 2008). Notably, they do not share any local sequence motifs (Kreuzer and Brister 2010).

All origins are thought to be capable of facilitating formation of RNA primers used to initiate leading strand DNA synthesis (Mosig, Colowick et al. 1995). Furthermore, the resulting R-loop structure potentially holds the origin duplex in an open conformation, giving the T4 Gp41/Gp61 primosome complex access to the non-template strand to facilitate priming necessary for lagging strand DNA synthesis in addition to DNA unwinding (Liu and Alberts 1980, Nossal 1980, Hinton and Nossal 1987). It is the R-loop itself that is the signal for replisome assembly, since a promoter sequence is not necessary for efficient replisome assembly and function (Nossal, Dudas et al. 2001). Once the R-loop is assembled, the proteins required for replication at these R-loops are DNA polymerase (Gp43), Polymerase clamp (Gp45), Clamp loader (Gp44/62) and
single-stranded DNA binding protein (Gp32). The replicative helicase Gp41 is required for processive leading strand synthesis and for lagging strand synthesis to occur, and its loading is greatly enhanced by the presence of the loader Gp59 (Kreuzer and Brister 2010).

Origins start becoming inactivated once the transcriptional program has enough time to shut off middle mode transcriptional promoters and RNA polymerase is converted into the form for late transcription (Stitt and Hinton 1994, Hinton 2010). One of the genes expressed by a late promoter is the helicase UvsW (Derr and Kreuzer 1990). UvsW has a broad specificity for unwinding substrates that includes RNA/DNA duplexes, such as the R-loops that occur at origins of replication (Carles-Kinch, George et al. 1997, Dudas and Kreuzer 2001, Nelson and Benkovic 2007). This way UvsW unwinds R-loops that are currently in place, origin-dependent initiation of DNA replication is inhibited and recombination-dependent DNA replication becomes the dominant mode of DNA synthesis (Kreuzer and Brister 2010).
1.4.3 T4 recombination-dependent replication

When the origin-initiated replication forks reach the ends of the linear T4 chromosome, one of the daughter molecules will contain a 3’ ssDNA end that is competent for strand invasion and D-loop formation, the initial steps in recombination-dependent DNA replication (Doermann and Boehner 1963, Mosig 1963, Womack 1963). In addition, the second daughter molecule is presumably competent for strand invasion following processing to generate a 3’ ssDNA end. The complementary sequence that is invaded can be at the other end of the same chromosome, since the genome is terminally redundant, or in the interior of the chromosome of a co-infecting phage particle, since the chromosome is circularly permuted (Kreuzer and Brister 2010, Liu and Morrical 2010). Regardless, recombination-dependent replication can initiate soon after an origin-initiated fork has reached a genomic end (Kreuzer and Brister 2010).

Single stranded DNA ends necessary for strand invasion are generated by origin-dependent replication and by the exonuclease activity of the Gp46/47 complex during double strand break resection (Mickelson and Wiberg 1981). The generated ssDNA regions are coated by the single-strand binding protein Gp32 and recruit the mediator protein UvsY. UvsY loads the UvsX recombinase onto Gp32-covered ssDNA, displacing Gp32 and forming the presynaptic filament (Maher, Branagan et al. 2011). The presynaptic filament invades a homologous duplex and forms the D-loop recombination intermediate. UvsX is thought to spontaneously dissociate once the D-loop is formed.
(Maher, Branagan et al. 2011). The D-loop displaced strand, which is rapidly coated by Gp32 SSB, becomes the lagging strand template in the replication fork (Figure 11).

The Gp59 helicase loader binds the Gp32-covered fork DNA to load the Gp41/Gp61 helicase/primase complex. Gp61 synthesizes the pentaribonucleotides necessary to prime Okazaki fragment DNA synthesis on the lagging strand and Gp41 facilitates leading strand DNA synthesis by processively unwinding the parental DNA ahead of the fork (Maher, Branagan et al. 2011).

Finally, the polymerase clamp Gp45 and the clamp loader Gp44/62 bind to what will become the leading strand of the replication fork and promote the assembly of the Gp43 polymerase, forming the polymerase holoenzyme. Once Gp43 polymerase is loaded, the Gp44/62 clamp loader dissociates from the forming replication fork. The Gp45 sliding clamp greatly increases the processivity of Gp43 polymerase, which catalyzes DNA synthesis in both the leading and the lagging strands in the 5’-to-3’ direction (Kreuzer and Brister 2010).

A second helicase, Dda, is able to load at the lagging strand and unwind the duplex ahead of the replication fork to facilitate leading strand DNA synthesis (Formosa and Alberts 1986, Ma, Wang et al. 2004). However, it does not promote primase loading and is much less processive than the hexameric Gp41, and is therefore not considered to be the primary replicative helicase. It is, however, essential when the Gp59 helicase loader is defective or missing (Doherty, Gauss et al. 1982, Gauss, Doherty et al. 1983, Gauss, Park et al. 1994). Additionally, it interacts with the UvsX recombinase to modulate strand invasion (Kodadek and Alberts 1987, Kodadek 1991) and promote error-
free lesion bypass by replication forks (Kadyrov and Drake 2004). The function and regulation of Dda are discussed in the following section and is one of the major topics of this dissertation.
Figure 11. Homologous recombination is coupled to DNA replication in bacteriophage T4 recombination-dependent replication. UvsX, aided by UvsY, forms a presynaptic filament on a Gp32-covered single-strand DNA overhang generated by
origin-dependent replication. The UvsX-ssDNA nucleoprotein filament invades a homologous region of duplex DNA, forming a D-loop, and the displaced strand is covered by Gp32 SSB. The helicase loader Gp59 loads on the lagging strand parental template at the fork. A) UvsX dissociates from the heteroduplex, allowing the clamp-loader (Gp44/62) to load a sliding clamp (Gp45) onto the 3’ end of the invading strand, initiating the assembly of the polymerase holoenzyme. B) Loading of Gp43 polymerase completes the assembly of the polymerase holoenzyme. C) Gp41 helicase is loaded onto the lagging strand by the Gp59 helicase loader and unwinds the fork ahead of the polymerase, allowing strand displacement DNA synthesis to occur. Gp41 forms a complex with Gp61 primase (not shown) in order to prime Okazaki fragment synthesis and lagging strand DNA replication. Adapted from Maher, Branagan et al. 2011.
1.5. Dda: a versatile mediator of DNA recombination, replication and repair

1.5.1. Functional characterization

The T4 Dda protein was originally identified as a DNA-dependent ATPase (Debreceni, Behme et al. 1970). Infections with phage Dda knockouts were found to have a substantial delay in DNA synthesis at early times of infection, but phage burst size and total DNA amounts were reduced only slightly (Little 1973). Even though Dda knockouts are fully viable (Behme and Ebisuzaki 1975), the helicase is essential for phage replication when the phage also carries a mutation in the Gp59 gene (Doherty, Gauss et al. 1982, Gauss, Doherty et al. 1983, Gauss, Park et al. 1994). These results suggest that Dda has overlapping roles with Gp59 in loading Gp41 at the T4 replication origins (Gauss, Park et al. 1994). Additionally, Dda/Gp61 primase double knockouts are lethal due to poor DNA packaging (Belanger 1997). Excessive ssDNA generated in primase-deficient phage infections promotes excessive recombination and overproduction of packaging-inhibiting DNA branches. In this scenario, Dda might act as an anti-recombinase by reducing the levels of DNA branching and facilitating DNA packaging (Belanger 1997).

Dda has been well characterized as an enzyme. Dda hydrolyzes ATP and dATP, but not other nucleotides, to their respective nucleoside diphosphates only in the presence of DNA. The ATPase activity follows Michaelis-Menten kinetics, with a $K_m$ for ATP of 0.13 mM. Dda preparations have high specific activity, in the range of 170 units/mg,
indicating a high turnover number, on the order of 200 molecules of ATP hydrolyzed per molecule of enzyme per second (Jongeneel, Formosa et al. 1984).

Dda is active between pH 6.0 and 9.0 and is inhibited by salt concentrations greater than 100 mM. Dda activity is maximal when the Mg\(^{2+}\) concentration equals the ATP concentration, suggesting that the Mg-ATP complex is the actual substrate for the helicase (Jongeneel, Formosa et al. 1984). The length of the ssDNA present has little effect on the stimulation of Dda activity; a (dG-dT)\(_5\), which is ten nucleotides in length, stimulates Dda as much as much longer polynucleotides (Krell, Durwald et al. 1979, Jongeneel, Formosa et al. 1984).

Dda is considered a distributive helicase because it exchanges freely between ssDNA molecules. This was demonstrated by incubating duplex DNA and Dda in the absence of ATP; Dda binds the ssDNA regions of the substrate in the absence of ATP (Krell, Durwald et al. 1979). The simultaneous addition of ssDNA (a competitive inhibitor) and ATP results in an inhibition of unwinding of the double-stranded duplex. These results reveal that Dda exchanges with competitor ssDNA during its helicase action, demonstrating that Dda is continuously dissociating and associating with the DNA being unwound (Jongeneel, Formosa et al. 1984).

More recent studies have shown that Dda helicase unwinds DNA as a monomer (Nanduri, Byrd et al. 2002) in a 5’-to-3’ direction (Hacker and Alberts 1992) at a rate of about 250 base pairs per second (Eoff and Raney 2006). Dda efficiently displaces streptavidin from biotin-labeled oligonucleotides (Morris and Raney 1999). Even though Dda molecules are functional monomers (Eoff and Raney 2010), in the presence of
impediments such as streptavidin (Byrd and Raney 2004), proteins bound to DNA (Byrd and Raney 2006) and chemical perturbations in the DNA (Eoff, Spurling et al. 2005), Dda is able to work in tandem via protein-protein interactions to increase displacement efficiency (Byrd and Raney 2004).

Dda is notable for being one of the most “active” (i.e., non-passive) helicases yet studied (Byrd, Matlock et al. 2012). Active helicases actively unwind DNA base pairs; passive helicases sequester ssDNA that forms due to thermal fraying (Manosas, Xi et al. 2010). The contribution of these distinct mechanisms is quantified as the ratio of the rate of unwinding double-stranded DNA to the rate of translocation on single-stranded DNA. Highly active helicases have a ratio approaching 1 and passive helicases have a ratio below 0.25; Dda translocates on ssDNA at nearly the same rate as it unwinds dsDNA (i.e., ratio approaches 1) (Byrd, Matlock et al. 2012). The structural basis for the proposed SF1B helicase 5’-to-3’ mechanism of unwinding (Saikrishnan, Powell et al. 2009) was supported by the crystal structure of the Dda-ssDNA binary complex (He, Byrd et al. 2012).

Even though the in vivo function of Dda remains uncertain, in vitro experiments suggest direct Dda involvement in DNA replication, repair and recombination. In vitro data further support the role of Dda in DNA replication. One important function that Dda possesses is the ability to remove protein blocks that slow down or stall replication fork movement. When the T4 replication fork encounters an E. coli RNA polymerase bound to DNA, movement of the entire fork is blocked. However, if the replication complex includes Dda helicase, the RNA polymerase dissociates from the DNA and does not
block the replication fork. Gp41, however, cannot overcome the replication fork block caused by a DNA-bound RNA polymerase molecule; this seems to be a specialized function unique to Dda (Bedinger, Hochstrasser et al. 1983). Interestingly, Dda is essential for viral DNA replication in an *E. coli optA* mutant strain (Gauss, Doherty et al. 1983). These findings suggest that Dda is in fact essential for T4 DNA replication, but is bypassed *in vivo* by a functionally equivalent host protein. Even though it is unknown whether the *optA* gene product is functionally equivalent to Dda, it has been sequenced (Wurgler and Richardson 1990) and it has at least two fundamental characteristics that suggest it may be a helicase: it has very high affinity for DNA and is an avid GTPase (Beauchamp and Richardson 1988). This is an area that merits further study.

A complementary study investigated the effect of a DNA-bound RNA polymerase on homologous strand exchange catalyzed by T4 UvsX recombinase and T4 Gp32 SSB, both filament-producing enzymes and both Dda binding partners. A DNA-bound RNA polymerase blocks homologous strand exchange catalyzed by UvsX recombinase and Gp32 SSB. The addition of Dda overcomes the RNA polymerase block and allows the T4 recombination machinery to drive branch migration through the RNA polymerase-promoter complex (Salinas and Kodadek 1994). This study further adds credence to the notion that overcoming roadblocks in order to allow DNA replication and repair to proceed is a major physiologic role of Dda.

Similar studies testing the ability of Dda to overcome protein blocks have been performed using the *E. coli trp* repressor DNA binding protein. These studies, performed under single turnover conditions, reveal that one Dda molecule is insufficient to displace
the bound repressor. Two Dda molecules or more, however, efficiently remove the \textit{trp} repressor from DNA (Byrd and Raney 2006). This finding led to the proposal of the cooperative inchworm model, which suggests that multiple Dda molecules can cooperate to unwind DNA and displace proteins more effectively than a single helicase molecule. It is proposed that a single Dda molecule is impeded when it encounters a protein block and slips slightly backward. A second Dda molecule behind it, however, can act as a brake or anchor, preventing the backward slipping of the first Dda molecule ahead of it.

Dda can stimulate the rate of strand displacement DNA synthesis catalyzed by the T4 replisome (Jongeneel, Bedinger et al. 1984). This enhancement is dependent on the presence of Gp32 SSB as well as the polymerase accessory proteins, Gp44/62 and Gp45. Dda, however, does not promote DNA synthesis on a single-stranded DNA template, indicating that it is enhancing the rate of DNA synthesis via its DNA helicase activity, unwinding the duplex ahead of the replication fork. Additionally, Dda does not enhance the rate of DNA synthesis already stimulated by Gp41, indicating they do not act synergistically at the replication fork (Jongeneel, Bedinger et al. 1984).

Dda seems to play a role in DNA recombination and repair. Dda can promote DNA polymerase strand switching to rescue a stalled replication fork \textit{in vitro} (Kadyrov and Drake 2004), suggesting Dda plays a role in survival after DNA damage \textit{in vivo}. When a T4 DNA replication fork faces an abasic site followed by a unique G base in the absence of dCTP, the double lesion abolishes translesion synthesis and the replication fork stalls (Kadyrov and Drake 2003). Dda, in the presence of UvsX, is able to catalyze two sequential template-switching reactions to rescue a DNA replication fork stalled by
the non-coding double lesion. Dda and UvsX each can promote a single template switch reaction individually, but both are required for replication repair to take place (Kadyrov and Drake 2004).

Even though Dda mutants do not show UV sensitivity (Behme and Ebisuzaki 1975) or genetic recombination deficiencies (Gauss, Park et al. 1994), data characterizing Dda interactions with the T4-encoded recombinase UvsX suggests Dda may play a role in homologous recombination. Dda binds the T4 recombinase UvsX (Formosa and Alberts 1984) and is able to stimulate UvsX/Gp32-catalyzed branch migration (Kodadek and Alberts 1987) and inhibit homologous pairing catalyzed by the same two enzymes (Kodadek 1991). The stimulation of branch migration by Dda was observed when Dda was added after synapsis was completely established (Kodadek and Alberts 1987). However, when Dda is added simultaneously with UvsX and Gp32, it strongly inhibits homologous pairing and synapse formation. Gp41, however, does not inhibit UvsX-catalyzed homologous pairing under identical conditions, further establishing specialized roles for the two helicases (Kodadek 1991).
1.5.2. Structure and SF1B homology

An active site point mutant of Dda, K38A, that binds ssDNA and is readily expressed and purified has been crystallized in complex with dT₈ ssDNA (He, Byrd et al. 2012) (Figure 12). The structure of Dda shows a strong similarity to the SF1B helicase RecD2 (Saikrishnan, Powell et al. 2009). The structural core of Dda contains two signature RecA-like domains, 1A in residues 1-173 and 2A in residues 187-257 and 391-439, both spanned by the dT₈ oligonucleotide. Residues 86-102 comprise the pin structure (also known as domain 1B), which is an extended β-ribbon within domain 1A. The pin contains a conserved phenylalanine that serves to mediate transient base-stacking interactions and is essential for DNA unwinding. The pin inserts into and splits the incoming duplex DNA such that one strand passes through the motor while the other stand is displaced (He, Byrd et al. 2012). This pin feature is common of SF1 and SF2 helicases (Korolev, Hsieh et al. 1997, Kim, Morgenstern et al. 1998, Theis, Chen et al. 1999, Velankar, Soultanas et al. 1999, Lee and Yang 2006, Buttner, Nehring et al. 2007, Pike, Shrestha et al. 2009).

Within domain 2A, residues 260 to 389 comprise an SH3-like β-barrel domain 2B in the form of five β strands, which matches a similar domain in RecD2 (Saikrishnan, Powell et al. 2009). What is unique to Dda is the presence of two insertions within the SH3-like domain: a second β-ribbon (residues 275 to 291) termed the “hook” and a β-ribbon/two-helix substructure (residues 305 to 322 and 345 to 383) termed the “tower.” The pin and the tower interact via their distal ends and create an arch through which
ssDNA passes. Trp374, Phe377, and Trp378 together with salt bridges between Glu93, Glu94, and Lys364 comprise the pin-tower interface (He, Byrd et al. 2012).

There is significant similarity between the Dda-ssDNA and the RecD2-ssDNA binary complexes. The two enzymes share the same fundamental architectures; the 1A, 2A, 1B (pin), and 2B (SH3) domains are in equivalent positions with respect to their primary structures. Two key differences between the two helicases are the absence of a tower in RecD2 and the substitution of the hook in Dda with a short β-ribbon in RecD2. The interaction between the helicases and ssDNA, however, is similar, with ssDNA traversing the 1A and 2A domains and the ssDNA 5’ end interacting with the SH3 β-barrel. In fact, most residues that interact with ssDNA in Dda have a direct counterpart in RecD2 (He, Byrd et al. 2012). The most significant difference between the Dda-ssDNA and RecD2-ssDNA interaction is the absence of the triple stack structure in RecD2, which is explained below for Dda. In place of the kink that is created in nucleotide 7 by the triple stack structure formed by Dda, nucleotide 5 is rotated into a small hydrophobic pocket in the RecD2-ssDNA complex (Saikrishnan, Powell et al. 2009). The ssDNA-Dda-ATP ternary complex has not yet been crystallized.

Based on the information obtained from the Dda-ssDNA binary complex structure, functional studies have been carried out disrupting the tower, pin and hook substructures of Dda. One of the notable findings from these structure-based studies is the importance of pin-ssDNA interactions, specifically stacking interactions mediated by Phe98 within the pin with the 3’ side of nucleotide 6 on the dT₈ oligonucleotide. Dda F98A, which has an alanine in place of the phenylalanine to remove the stacking
interaction, binds ssDNA and has ssDNA-dependent ATPase activity. However, the mutation completely abolishes Dda DNA unwinding activity, demonstrating the importance of this residue for Dda DNA unwinding (He, Byrd et al. 2012). Interestingly, Dda F98A translocates on ssDNA at a similar rate as wildtype Dda. Even though translocation on ssDNA is not dependent on this stacking interaction, it is essential for efficient unwinding.

The pin substructure also interacts with the tower substructure (Figure 13). This interaction is critical for the coupling of ATP hydrolysis with DNA unwinding. The residues mediating the electrostatic pin-tower interactions are Glu93 and Glu94 in the pin and Lys364 in the tower. Dda E93A/E94A, which lacks the electrostatic interaction with K364, retains DNA binding and DNA-stimulated ATPase activity. Dda E93A/E94A is able to unwind DNA under multiple cycle conditions. However, under single cycle conditions (where only a single cycle of substrate unwinding can occur per Dda binding event) Dda E93A/E94A unwinds a small quantity of DNA initially and remains constant over the remaining time, an unwinding pattern that is consistent with ATP-independent unwinding and is not observed with wildtype Dda. Dda K364A showed similar results. Furthermore, mutating Trp378, which contributes a hydrophobic pin-tower interaction, to alanine demonstrated similar results. These results establish that disruptions of pin-tower interactions result in the decoupling of ATP binding and hydrolysis from DNA unwinding (He, Byrd et al. 2012). Similarly to Dda F98A, E93A/E94A and K364A translocate on ssDNA at rates similar to wildtype Dda, indicating the tower-pin interactions are critical for efficient DNA unwinding but not translocation on ssDNA.
The crystal structure of the Dda-DNA binary complex offers some insights into the protein-protein interactions sites between Dda and two known binding partners, UvsX recombinase and Gp32 SSB. The surface electrostatic potential of Dda confirms the path of the translocated ssDNA through the arch. Additional positively charged surfaces suggest binding sites for the incoming duplex, the exiting translocating strand, and the displaced strand. The favored model suggests that Dda maintains contact with both the translocating and the displaced strand. Two potential consequences of this sequestration include the prevention of reannealing and/or the handoff of the displaced and/or translocating strand to UvsX and/or Gp32. Supporting the handoff hypothesis, the positively charged surface binding the translocated strand leads directly to a conserved hydrophobic patch on the SH3 domain, which likely represents the docking site for these interacting partners (He, Byrd et al. 2012).
Figure 12. **Key structural and functional domains and motifs of the Dda-ssDNA complex.** A) Stereo view of the structure showing the 1A and 2A RecA-like domains in blue and orange, respectively, the SH3 domain (2B) with tower in cyan, the β-ribbon pin (1B) in yellow, the β-ribbon (hook) extension (2B) in green, and the ssDNA passing through the central arch in magenta. B) Dda primary structure with secondary structures.
labeled and colored as in (A). Helicase motifs and key hydrophobic residues in the tower are underlined in bold. Adapted from He, Byrd et al. 2012.
Figure 13. **Interaction between the pin and tower structures and ssDNA.** A) The pin structure contains highly conserved residues among helicases from T4-like phages. Two completely conserved acidic residues at the end of the pin (asterisks and red box) mediate electrostatic interactions with the tower. The completely conserved proline and phenylalanine (asterisks and green box) form stacking interactions with a base in the translocating ssDNA. B) Interface between the pin, depicted in yellow, and the tower, depicted in cyan, relative to the bound ssDNA, shown in magenta. Completely conserved residues are shown in green and red, as in (A). Adapted from He, Byrd et al. 2012.
1.5.3. Protein-DNA interactions

Dda interactions with ssDNA are modulated in part by the presence of ATP. Dda helicase binds ssDNA tightly in the absence of ATP (Krell, Durwald et al. 1979). In the presence of ATP, Dda is able to dissociate and associate with multiple DNA molecules, which gives it its distributive character (Jongeneel, Formosa et al. 1984). Indeed, ATP binding and/or hydrolysis is thought to be a necessary step in the dissociation of the enzyme-DNA complex; both ADP and ATP induce Dda dissociation from ssDNA (Krell, Durwald et al. 1979).

Fluorescence titration assays have been used to determine the DNA binding-site size of DNA-binding proteins (Kowalczykowski, Paul et al. 1986). Titration of Dda with poly(dT) and 60-mer oligonucleotides have established that individual Dda molecules have a ssDNA binding-site size of 6 nucleotides (Byrd and Raney 2004).

Studies investigating the DNA unwinding activity of Dda in the presence of chemically modified DNA have established the importance of the charge distribution along the backbone of the DNA substrate. Dda is unable to efficiently unwind substrates with a disruption in the charge distribution of the substrate, including a 5’-DNA-PNA-DNA-3’ substrate and an oligonucleotide with a single methylphosphonate replacement on the phosphate backbone. On the other hand, Dda efficiently unwinds a duplex substrate with a single abasic site, which retains the charge distribution of normal DNA and only alters the conformational flexibility of the oligonucleotide (Eoff, Spurling et al. 2005). Dda unwinding is highly dependent on electrostatic interactions between Dda and the DNA substrate.
Dda can efficiently displace a polyamide nucleic acid (PNA) strand that has formed a duplex with DNA according to Watson-Crick base pairing rules. Instead of the deoxyribose phosphodiester backbone found in DNA, PNA contains a \( N-(2\text{-aminoethyl})\text{glycine} \) backbone. The PNA-DNA substrate has a higher melting temperature than the DNA-DNA counterpart. Since Dda can unwind a DNA-PNA substrate at similar rates as DNA-DNA substrates, Dda is insensitive to the chemical nature of the displaced strand and the thermal stability of oligonucleotide substrates (Tackett, Corey et al. 2002).

The crystal structure of the Dda-ssDNA complex has yielded some definitive insights into the nature of the protein-ssDNA interactions. ssDNA traverses the 2A and 1A domains in a 5’-to-3’ orientation. The ssDNA-binding groove of Dda, which is conserved among similar helicases, interacts with the ssDNA sugar-phosphate backbone via hydrogen bonding and ionic interactions. The register of the ssDNA with respect to domains 1A and 2A is likely partly controlled by the positive helix dipoles at the N termini of two topologically equivalent \( \alpha \) helices (Kerr, Sivakolundu et al. 2007), 1A\( \alpha \)3 and 2A\( \alpha \)3 in Dda. The bases, which are mostly stacked, contribute very little to the protein-ssDNA interface (He, Byrd et al. 2012).

There is a bipartite distribution between the ssDNA-binding domains and the ssDNA nucleotides. In this crystal structure, which contains a dT\(_8\) oligonucleotide, domain 2A interacts almost exclusively with nucleotides 1 through 4 while domain 1A interacts almost exclusively with nucleotides 5 through 8. Within domain 2A, Lys243, Asn242, and Thr394 interact with the phosphates of nucleotides 2, 3 and 4, respectively. Within domain 1A, His64, Thr80, and Ser83 interact with the phosphates of nucleotides
6, 7, and 8, respectively. His82 and Asn88 stack against the ribose groups of nucleotides 6 and 7, respectively, while Pro89 and Phe98 stack onto the base of nucleotide 6 from opposite sides of the pin. Together, these interactions create a three-stack structure that creates a kink in the ssDNA (He, Byrd et al. 2012) (Figure 14).
Figure 14. **Dda-ssDNA interactions.** As ssDNA traverses the two RecA-like domains, P89 and F98 form a triple stack interaction with the nucleotide at position X6. RecA-like domains are depicted in blue (1A) and orange (2A), the SH3 domain (2B) with tower in cyan, the β-ribbon pin (1B) in yellow, the β-ribbon (hook) extension (2B) in green, and the ssDNA in magenta. Adapted from He, Byrd et al. 2012.
1.5.4. Protein-protein interactions

1.5.4.1. Gp32

Gp32 is an essential canonical single-strand binding protein (SSB) that plays a role in nearly every aspect of DNA metabolism in T4, including DNA replication, repair, and recombination. Gp32 functions by tightly and cooperatively coating any ssDNA that results from DNA metabolic processes. Gp32 binding to the ssDNA intermediates generated by DNA metabolism might serve at least three important roles: removal of potentially inhibitory secondary structures such as hairpin loops, protection of the ssDNA from host cell nucleases, and the coordination of T4-specific enzymes to bind ssDNA and carry out the catalytic events necessary for efficient DNA replication, recombination, and repair to take place (Kowalczykowski, Lonberg et al. 1981).

As a central mediator of DNA metabolism, Gp32 is able to regulate its own translation in order to maintain adequate concentrations in the host cell. Free Gp32 initially titrates all available ssDNA sites, followed by binding of the SSB to its own messenger RNA, resulting in the specific downregulation of translation of its own mRNA, but not other T4 mRNAs (Krisch, Bolle et al. 1974, Gold, O'Farrell et al. 1976, Russel, Gold et al. 1976). This process is reversible and is a function of the intracellular Gp32 concentration (Lemaire, Gold et al. 1978). It is estimated that there are approximately 10,000 copies of Gp32 per infected cell, a total intracellular concentration around 5 µM.

Even though Gp32 may demonstrate some slight specific affinity for at least some sequences of RNA (as suggested by its specific autoregulation), Gp32 binds ssDNA.
nonspecifically with respect to sequence and in a polar manner. There are three principal
dparameters used to define Gp32 binding to ssDNA: binding size site (n), binding constant
(K), and cooperativity (ω) (McGhee and von Hippel 1974). Gp32 has a binding site n =
7±1, a binding constant K = 10^8 to 10^9 M^-1 in 0.01 M NaCl, and a highly positive
cooperativity constant ω = 1000, reflecting the formation of continuous Gp32 clusters
saturating the ssDNA lattice (Kowalczykowski, Lonberg et al. 1981). These parameters
quantify and help explain the rapid sequestration of ssDNA by Gp32 in vivo.

Gp32 was first identified as a helix destabilizing protein (Alberts and Frey 1970).
It is a 34-kDa, 301-amino acid zinc metalloprotein with three functional domains
identified by proteolysis (Villemain, Ma et al. 2000): the N-terminal basic (B) domain
(residues 1-21), the core domain (residues 22-254), and the C-terminal acidic (A) domain
(255-301) (Williams, Sillerud et al. 1979). The core domain contains the ssDNA-binding
site (Spicer, Williams et al. 1979) and the A domain mediates critical interactions with
heterologous replisome proteins such as Dda and Gp43 (Burke, Alberts et al. 1980,
Krassa, Green et al. 1991). The B domain mediates self-association of Gp32 monomers in
solution and cooperativity of ssDNA binding (Giedroc, Khan et al. 1990). Gp32
associates on ssDNA in continuous clusters, which is mediated by highly basic amino
acids in the B domain. Specifically, the highly basic amino acids 3-7, Lys-Arg-Lys-Ser-
Thr are of particularly importance for Gp32 self-association and cooperativity. (Churchill
and Travers 1991, Casas-Finet, Fischer et al. 1992). Gp32-B is a 30.8-kDa truncated
version of Gp32 that lacks the B domain, is devoid of Gp32-Gp32 interactions, exhibits
no cooperativity (ω= 1) and has severely reduced ssDNA-binding affinity compared with
wildtype (Villemain, Ma et al. 2000) (Giedroc, Khan et al. 1990, Ma, Wang et al. 2004). Gp32-B is therefore a useful tool that separates the DNA-binding component from the protein-protein interaction functionality of Gp32, and thus allows us to directly and specifically test the effects of Gp32 protein-protein interactions.

The C-terminal acidic (A) domain is essential for protein interactions with other T4 replisome components such as Gp43, Gp61 and Dda (Krassa, Green et al. 1991). Studies using Gp32 lacking the A domain (Gp32-A) demonstrate that these protein-protein interactions are critical for T4 replication (Burke, Alberts et al. 1980). The electronegative A domain can adopt two distinct conformations as a function of salt concentration and ssDNA binding (Pant, Karpel et al. 2005, Rouzina, Pant et al. 2005). Under physiologic salt concentrations, the A domain interacts with the electropositive core domain when the protein is not bound to ssDNA (Figure 16). ssDNA binding results in the exclusion of the A domain from the core domain, and the A domain becomes displaced from the core and solvent-exposed upon ssDNA binding. In this state, the A domain is positioned in a way that it can interact with a neighboring N-domain, mediating cooperative binding (Figure 16). Gp32-A, however, binds ssDNA with greater affinity than full-length Gp32, indicating a role in the regulation of Gp32 by heterologous protein-protein interactions (Hosoda and Moise 1978, Waidner, Flynn et al. 2001).

The core domain of Gp32 contains the essential structural motifs for ssDNA binding (Spicer, Williams et al. 1979). The core domain crystal structure reveals a single oligonucleotide/oligosaccharide-binding (OB) fold and a zinc ion coordinated by histidine 64, cysteine 77, cysteine 87 and cysteine 90 (Shamoo, Friedman et al. 1995).
The zinc ion is essential for the structural and functional integrity of Gp32 (Giedroc, Keating et al. 1986, Giedroc, Keating et al. 1987, Pan, Giedroc et al. 1989). Interestingly, the OB fold β-sheets in Gp32 are mostly perpendicular to the DNA α-helix, whereas β-sheets of the zinc finger domains of other DNA-binding proteins typically have a parallel orientation to the DNA α-helix (Shamoo, Friedman et al. 1995).

Dda is among the proteins that interact most strongly with Gp32 (Figure 17). Dda is quantitatively bound to Gp32 during chromatography of T4-infected cells over Gp32 protein affinity columns and elutes at salt concentrations ranging from 0.6 to 2.0 M, the highest of all major bound protein species. (Formosa, Burke et al. 1983) (Jongeneel, Formosa et al. 1984) (Jacobsen, Kazmierczak et al. 2011) (Formosa, Burke et al. 1983) (Ma, Wang et al. 2004) (Jongeneel, Formosa et al. 1984). (Hurley, Chervitz et al. 1993) In vitro ATPase assays have established that Gp32-covered ssDNA does not stimulate Dda ATPase activity and that Gp32 acts as a competitive inhibitor of Dda by making ssDNA binding sites inaccessible to Dda. This suggests that in vivo the site of action of Dda may be on ssDNA not covered by Gp32 (Jongeneel, Formosa et al. 1984).

However, studies have shown a positive interaction between Dda and Gp32 in vitro (Figure 18). Dda stimulates strand displacement DNA synthesis in vitro only in the presence of Gp32 (Cha and Alberts 1989). This stimulation requires direct Gp32-Dda protein-protein interactions since it is relatively unaffected by mutations that destabilize the ssDNA-binding component of Gp32 (Ma, Wang et al. 2004).
Figure 15. **Structure of Gp32 SSB core domain.** A) Ribbon diagram of the tertiary structure of the Gp32 core domain. The structure of subdomain I (dark blue) is determined by the coordination about a Zn$^{2+}$ atom (silver sphere). Subdomain II (red) is composed of five $\beta$-strands that form a twisted $\beta$-sheet. The linking region is shown in light blue. All three regions comprise part of the ssDNA-binding cleft. The positioning of the ssDNA phosphate backbone is shown in pink. B) The secondary structure of Gp32 SSB core domain represented schematically as a topology map. Adapted from Shamoo, Friedman et al. 1995.
Figure 16. **Model of Gp32 domains and electrostatic interactions mediating DNA binding.** The three Gp32 domains are depicted. The electropositive core domain (depicted as a labeled white oval) is binding to electronegative ssDNA. The N-terminal (B) domain (depicted as a solid black line) mediates homologous protein-protein interactions essential for cooperative ssDNA-binding. The C-terminal (A) domain (depicted in gray) mediates heterologous protein-protein interactions and can be bound to the core domain or exposed in solution. **A)** Electrostatic interactions mediate the binding of the largely electropositive Gp32 core domain and the largely electronegative C-terminal (A) domain. **B)** High salt disrupts this interaction; four Cl- ions, depicted as negative signs, are shown bound to the cationic core. **C)** The Gp32 C-terminal domain is exposed to solution when Gp32 is bound to ssDNA; three sodium ions, depicted as positive signs, are shown bound to the anionic C-terminal domain. Adapted from Rouzina, Pant et al. 2005.
Figure 17. **Dda surface electrostatic potential and substrate binding. A and B)**

Positive and negative electrostatic potential are depicted in blue and red, respectively. Neutral electrostatic potential (±5 kT/e) and hydrophobic regions are depicted in white. A model of the full DNA substrate is superimposed in magenta and yellow. Surface region S1 engages the translocating strand; S2 and S3 bind the incoming duplex; S4 and S5 bind the exiting translocated strand and displaced strand, respectively; S6, a conserved hydrophobic patch on the SH3 domain, is optimally positioned to bind UvsX recombinase and/or Gp32 SSB. Adapted from He, Byrd et al. 2012.
Figure 18. **Dda protein-protein and protein-DNA interactions during unwinding.**

Dda, in gray, is schematically depicted bound to a partially unwound DNA substrate. In this schematic, the displaced strand, which corresponds to the leading strand template in a DNA replication fork, interacts with T4 Gp32 SSB, depicted as yellow circles, and/or with replication machinery (not depicted). Adapted from Blair, Tackett et al. 2009.
1.5.4.2. UvsX

UvsX is the second known protein partner of Dda. The UvsX locus was initially defined by mutations that increase T4 sensitivity to UV radiation (Harm 1963) and was later purified and characterized as a DNA-binding, DNA-dependent ATPase important for T4 recombination (Yonesaki, Ryo et al. 1985). Later studies proved that mutations in UvsX also lead to decreased recombination frequencies, a smaller burst size, early arrest of DNA synthesis, aberrant DNA replication, and increased sensitivity to UV radiation and other DNA-damaging agents (Hamlett and Berger 1975, Wakem and Ebisuzaki 1976, Cunningham and Berger 1977, Cunningham and Berger 1978, Melamede and Wallace 1980, Melamede and Wallace 1980, Wakem and Ebisuzaki 1981, Bernstein and Wallace 1983).

UvsX is a ssDNA-dependent ATPase and produces a mixture of ADP + P\textsubscript{i} and AMP + PP\textsubscript{i} (Formosa and Alberts 1986); its ATPase activity is not stimulated by dsDNA (Griffith and Formosa 1985). Like the E. coli recA protein, UvsX forms presynaptic filaments on ssDNA (Ando and Morrical 1998), the obligatory nucleoprotein intermediate in recombination, and catalyzes DNA homologous pairing and strand exchange (Cox and Lehman 1981, Kahn, Cunningham et al. 1981). UvsX-catalyzed synapsis formation is dependent on sequence homology, ATP hydrolysis, and the presence of Gp32 SSB (Griffith and Formosa 1985).

The UvsX filaments have been studied by electron microscopy. These studies have shown that UvsX binds cooperatively to dsDNA and, in the presence of Mg\textsuperscript{2+}, to ssDNA, forming filaments that are 14 nm in diameter with an axial repeat of 12 nm.
Filaments made on dsDNA contain about 42 base pairs per repeat while ssDNA filaments contain 49 base pairs per repeat; in both cases each repeat contains 9-12 UvsX protein monomers, each monomer covering four to five nucleotides. Both ssDNA and dsDNA filaments are nearly identical in appearance and, interestingly, have a tendency to align side-by-side (Griffith and Formosa 1985).

More recently, the crystal structure of UvsX was obtained (Figure 19). The overall architecture of UvsX closely resembles that of RecA, including a highly conserved ATP binding site. The UvsX crystal structure was docked into electron microscopy reconstructions of UvsX-dsDNA filaments and it was found that the ATP binding site sits at the protomer interface, as in the RecA filament crystal structure. The filaments formed in the presence of ATP were found to have a rotation per subunit of 58.5° and an axial rise per subunit of 16.1 Å. In the presence of ADP, UvsX forms compressed ‘inactive’ filaments, which have a rotation per subunit of 55.7° and an axial rise per subunit of 10.8 Å (Gajewski, Webb et al. 2011) (Figure 20).

The ssDNA-binding properties of UvsX have been studied using etheno-derivitized ssDNA, whose fluorescence increases upon binding by UvsX. These studies have enabled the quantification of the binding-site size ($n$) and cooperativity ($\omega$) parameters of UvsX. Consistent with previous studies, UvsX was determined to have a binding-site size of four nucleotides and moderate cooperativity ($\omega = 100$) (Ando and Morrical 1998). In addition to cooperative ssDNA binding, other studies have demonstrated that UvsX ATP hydrolysis is also cooperative; UvsX ATPase activity seems to require that a ssDNA-bound UvsX monomer be surrounded by other ATP-
bound monomers, indicating that cooperativity contributes to reaction mechanism (Formosa and Alberts 1986).

Dda interacts both physically (Hacker and Alberts 1992) and functionally (Kodadek and Alberts 1987) with UvsX. Dda is retained by a UvsX affinity column (Formosa, Burke et al. 1983) and it can stimulate UvsX-mediated strand exchange (Kodadek and Alberts 1987). Furthermore, UvsX can rescue a stalled replication fork by two sequential template-switching reactions only in the presence of Dda (Kadyrov and Drake 2004). This interaction seems to be specific to Dda, and not a generic property of 5’-to-3’ helicases, since Gp41 has no effect on UvsX template switching (Kadyrov and Drake 2004).
Figure 19. **Structure of the UvsX monomer.** The structure shown is of amino acids 30-358 of the UvsX monomer, with α-helices in green, β-strands in purple, and loops in grey. The N-terminal ATP-binding domain is on the right and the helical C-terminal domain is on the left. A phosphate group (orange ball-and-stick) occupies the site that accommodates the ATP β-phosphate. The locations of loops L1 and L2, which are not depicted, are labeled. Adapted from Gajewski, Webb et al. 2011.
Figure 20. **UvsX recombination filaments.** A) An extended UvsX-dsDNA filament (grey) imaged by electron microscopy in the presence of ATP with the UvsX crystal structure superimposed (cyan). The red and yellow residues correspond to those in RecA that are involved in ATP hydrolysis. B) A compressed UvsX-dsDNA filament formed in the presence of ADP with the UvsX structure superimposed (dark blue). Red arrows indicate interactions between monomers. Adapted from Gajewski, Webb et al. 2011.
1.5.5. The role of Dda in regulating strand displacement DNA synthesis

Our discussion of Dda interactions in DNA replication, repair and recombination will conclude with a model for the role of Dda in the regulation of strand displacement DNA synthesis, a process that is directly involved in recombination-dependent DNA replication and in the repair of double-strand breaks and stalled replication forks. This model is based on all the data available on the biochemical characteristics of Dda and the in vivo genetic studies performed to date.

The interactions between Dda and Gp32 and UvsX are interesting and complex; even though they interact physically, their functional interactions seem to be inhibitory rather than stimulatory, yet they work synergistically. Gp32 SSB aids Gp43 holoenzyme to perform strand displacement DNA synthesis via protein-protein interactions and by destabilizing the helix ahead of the polymerase. In the presence of Dda, which is able to load on the lagging strand and unwind ahead of the leading strand polymerase, Gp32 can stimulate strand displacement DNA synthesis further. Importantly, it is the ratio of Dda to Gp32 that determines the degree of stimulation by Dda (Jongeneel, Bedinger et al. 1984). The importance of the ratio of Dda to Gp32 suggests that excess Gp32 is a modulator that keeps the highly active Dda helicase in check.

The unwinding by Dda is a relatively powerful force: it can displace most proteins from ssDNA, and consequently, can act to promote replication, recombination and repair (in the case of an RNA polymerase slowing down or stalling a replication fork, for example) or can act to inhibit these processes (by displacing UvsX and preventing nucleoprotein filament formation and synapsis, for example). Dda can serve versatile
roles in the cell to correct accidents in DNA metabolism that result in the generation of toxic amounts of ssDNA and ssDNA structures such as recombination intermediates and branched structures. The high affinity of Dda for Gp32 is a central component of this hypothesis and underscores the importance placed on the proper regulation of Dda helicase activity.
1.5.6. Structural and functional comparison with other T4 helicases

1.5.6.1. Gp41

Gp41, the primary T4 replicative helicase, was purified in 1979 (Morris, Moran et al. 1979) and is a 5’-to-3’ Superfamily 4 helicase (Perumal, Raney et al. 2010). Despite the current lack of a crystal structure, it has been well established that the Gp41 helicase functional unit is a hexamer (Egelman 1996) and oligomerizes in response to NTP binding (Dong, Gogol et al. 1995).

In vivo, Gp41 is required for initiation of lagging strand synthesis through its interactions with the Gp61 primase (Liu and Alberts 1981) and is believed to be critical for recombination and branch migration (Yonesaki 1994). In vitro, Gp41 enhances DNA synthesis and the rate of leading strand synthesis by Gp43 polymerase (Morris, Sinha et al. 1975) (Dong, Weitzel et al. 1996).

Gp41 is highly processive, translocates at a rate 500 nucleotides per second on ssDNA, and unwinds dsDNA at a rate of 250 basepairs per second. A single Gp41 hexamer can remain associated with the replication fork for the entire 169-kilobase T4 genome (Schrock and Alberts 1996).

Gp41 is loaded onto replication fork structures by Gp59 in association with Gp32, which comprise the helicase loading complex (Branagan, Maher et al. 2012) (Branagan, Klein et al. 2014). In vitro, loading of Gp41 on fork DNA substrates requires an ssDNA region on the lagging strand arm adjacent to the duplex and can be loaded onto Holliday Junctions and three-stranded junction structures that resemble D-loops (Jones, Mueser et al. 2000).
1.5.6.2. UvsW

UvsW is a monomeric 3’-to-5’ SF2 helicase (Hall and Matson 1999) (Carles-Kinch et al. 1997) that is highly conserved among bacteriophages; it has been identified in five T4-like genomes (Sickmier, Kreuzer et al. 2004). It was discovered as a mutant that causes UV hypersensitivity (Derr, Drake 1990) (Derr, Kreuzer, 1990) and is involved in the recombination repair pathway for UV damage (Conkling and Drake 1984) (Drake and Ripley 1994) and the rescue of stalled replication forks (Kerr, Sivakolundu et al. 2007). Biochemically, it is thought to catalyze branch migration or similar reactions during homologous recombination (Sickmier, Kreuzer et al. 2004). Additionally, UvsW mediates transition from early to late modes of T4 replication (Dudas and Kreuzer 2001) by unwinding R loops as they are formed at late times and prevent them from acting as initiation sites of origin-dependent replication (Dudas and Kreuzer 2001).

Its structure reveals two typical helicase RecA-like domains (Kerr, Sivakolundu et al. 2007) and a putative substrate-binding domain not previously observed in helicases (Sickmier, Kreuzer et al. 2004) that resembles the “double-wing” DNA binding domain from the T4 MotA transcription factor that mediates the expression of T4 middle genes (Li et al. 2002). UvsW also has homology to the eukaryotic SF2 helicase, Rad54. (Kerr, Sivakolundu et al. 2007). UvsW contains a bulky arginine/aromatic-rich loop that is highly conserved and optimally positioned to interact with nucleic acid substrates (Sickmier, Kreuzer et al. 2004).
1.6. Research objectives

Dda, the bacteriophage T4 monomeric type B (5’-to-3’) helicase, is a unique helicase in the bacteriophage T4 genome and plays a unique role in its life cycle, despite its similarities with both UvsW, the monomeric type A (3’-to-5’) helicase, and Gp41, the hexameric type B (5’-to-3’) helicase. The genetics of Dda mutants, although complex, offers some insights that complement the data generated by biochemical analysis. Nonetheless, the in vivo role of Dda remains a mystery despite phenotypic analyses and extensive in vitro characterization. Specifically, the assignment of a discrete role or function for Dda in bacteriophage T4 recombination, repair and replication remains a challenge, partially due to its capability to regulate a diverse and extensive number of biochemical processes (strand displacement DNA synthesis, template switching, strand invasion, branch migration and the displacement of DNA bound-proteins including RNA polymerase).

Furthermore, there is evidence supporting both a synergistic and antagonistic interactions between Dda and its two known binding partners, the T4 recombinase UvsX (it can inhibit strand invasion and promote branch migration, both UvsX-catalyzed processes, depending on order of addition) and the T4 SSB Gp32 (it is inhibited by Gp32 due to competition for ssDNA-binding sites and it works as a function of and synergistically with Gp32 to promote strand displacement DNA synthesis by the T4...
polymerase holoenzyme). Based on our understanding of the existing body of knowledge on Dda, we have defined the following research objectives:

1. Determine whether the N-terminal (B) domain of Gp32 SSB is mediating the interaction between Dda and Gp32.

2. Determine the effect of DNA substrate structure on the interactions between Dda and Gp32 and the –B variant.

3. Demonstrate differential interactions between Dda and Gp32 in the context of different recombination intermediates in the presence of T4 polymerase and/or the polymerase holoenzyme complex.

These research objectives were developed based on a model for the dual interactions between Dda and Gp32 that was proposed by studies demonstrating that Gp32(-)B, as well as Gp32, are able to work synergistically with Dda to stimulate T4 polymerase holoenzyme-catalyzed strand displacement DNA synthesis (Ma, Wang et al. 2004). The hypothesis that Dda and Gp32 are interacting via a ssDNA-binding-independent mechanism is both attractive and intuitive, in part because these two proteins bind with an affinity that is almost an order of magnitude greater than any other two T4 proteins involved in DNA metabolism. It is rational to consider that this remarkably strong interaction may have biological significance in the mutual regulation of these two proteins and in their influence on DNA recombination, replication and repair.
These data suggested to us that the physical interaction between Gp32 and Dda has an influence on the ability of Dda to modulate DNA metabolism, specifically DNA replication, and that these interactions are distinct from the indirect interactions modulated by the occupancy of ssDNA binding sites, which have been previously characterized. Our model states that Gp32(-)B is able to enhance the DNA unwinding activity of Dda via physical protein-protein interactions, and that the relationship between Dda and Gp32 is dependent on their relative localization on a D-loop recombination intermediate. The following chapters discuss the experiments carried out to test our hypotheses and explain the significance of our findings. We discuss what we have learned about Dda in T4 DNA replication, recombination, and repair and how our findings contribute broadly to the field of DNA metabolism and genome maintenance.
CHAPTER 2: REGULATION OF THE BACTERIOPHAGE T4 DDA
HELICASE BY GP32 SINGLE-STRANDED DNA-BINDING PROTEIN

The material in this chapter is in preparation for submission to DNA Repair.

Christian S. Jordan¹ and Scott W. Morrical¹*

¹Department of Biochemistry, University of Vermont College of Medicine, Burlington,
VT, United States of America, 05405.

*To whom correspondence should be addressed: Scott W. Morrical, Department of
Biochemistry, University of Vermont College of Medicine, B407 Given, 89 Beaumont
Avenue, Burlington, VT 05405. Tel.: 802-656-8260. Fax: 802-656-8229. Email:
smorrica@uvm.edu.

Abbreviations: NA, nucleic acid; ssDNA, single-stranded deoxyribonucleic acid; SSB,
single-strand deoxyribonucleic acid binding protein; SDS-PAGE, sodium dodecyl sulfate
polyacrylamide gel electrophoresis.
Abstract

Dda, one of three helicases encoded by bacteriophage T4, has been well-characterized biochemically but its biological role remains unclear. It is thought to be involved in origin dependent replication, recombination-dependent replication, anti-recombination, and recombination repair. The Gp32 protein of bacteriophage T4 plays critical roles in DNA replication, recombination, and repair by coordinating protein components of the replication fork and by stabilizing ssDNA. Previous work demonstrated that stimulation of DNA synthesis by Dda helicase appears to require direct Gp32-Dda protein-protein interactions and that Gp32 and Dda form a tight complex in the absence of ssDNA. Here we characterize the effects of this complex through changes in the duplex DNA unwinding and ATPase activities of Dda helicase in the presence of different variants of Gp32 and different DNA repair and replication intermediate structures. Results show that complex formation can be enhancing or inhibitory, depending on the Gp32 domain seen by Dda. Protein-protein interactions with Gp32 stimulate the unwinding activity of Dda, an effect associated with increased turnover of ATP, suggesting a higher rate of ATPase-driven translocation. Dda-Gp32 interactions also promote the unwinding of DNA substrates at higher salt concentrations and in the presence of substrate-bound DNA polymerase. Conversely, the formation of Gp32 clusters on ssDNA can inhibit unwinding, suggesting that Gp32-ssDNA formation sterically regulates which portions of replication and recombination intermediates are accessible for processing by Dda helicase. The data suggest a mechanism of replication
fork restart in which Gp32 promotes Dda activity in template switching while preventing premature fork progression.

**Keywords:** helicase, single-stranded DNA binding protein, recombination, replication, repair
Introduction

The bacteriophage T4 replisome is a well-studied and extremely tractable system for studying the protein-protein and protein-DNA interactions that mediate DNA replication, recombination, and repair. DNA replication can be reconstituted in vitro using purified recombinant T4 proteins, with rate and fidelity comparable to in vivo reactions (Nossal, 1994). T4 DNA replication proteins function to regulate and coordinate leading and lagging strand synthesis (Kreuzer & Brister, 2010; Liu & Morrical, 2010; Maher, Branagan, & Morrical, 2011).

T4 encodes three DNA helicases, two of which (Gp41 and Dda) appear to participate directly in the initiation and/or propagation of DNA replication forks. The processive T4 helicase Gp41, a Superfamily 4 (SF4) helicase, in association with the helicase loader Gp59, is considered the major replicative helicase in T4. However, the Dda helicase is also able to unwind duplex DNA ahead of the leading strand polymerase and appears to be involved in early origin-dependent replication (Jongeneel, Bedinger et al. 1984, Cha and Alberts 1989, Gauss, Park et al. 1994, Ma, Wang et al. 2004). Dda is a Superfamily 1 (SF1) helicase that stimulates strand displacement DNA synthesis by the T4 polymerase in vitro (Alberts, 1987; Formosa & Alberts, 1986; Ma, Wang, Villemain, Giedroc, & Morrical, 2004; Morrical & Alberts, 1990). Dda is a 49.9-kDa monomeric helicase, with 5’ to 3’ polarity and low to moderate processivity (Nanduri, Byrd, Eoff, Tackett, & Raney, 2002; Raney & Benkovic, 1995). It binds tightly and specifically to the T4 ssDNA-binding protein (SSB) Gp32 (Formosa & Alberts, 1984; Hacker & Alberts, 1992; Hurley, Chervitz, Jarvis, Singer, & Gold, 1993). The ability of Dda to
stimulate strand displacement DNA synthesis is hypothesized to be a function of protein-protein interactions between Gp32 and Dda and not Gp32-ssDNA interactions (Ma et al., 2004). This and other findings suggest a model in which Dda-Gp32 protein-protein interactions are critical in organizing, coordinating and regulating the helicase to promote strand displacement DNA synthesis at the T4 replication fork.

Dda also binds tightly and specifically to the T4 UvsX recombinase, an ortholog of E. coli RecA protein (Formosa & Alberts, 1984; Hacker & Alberts, 1992; Hurley et al., 1993). Results of in vitro studies suggest that Dda participates in a variety of DNA repair and recombination processes in concert with UvsX, including DNA branch migration, D-loop dissolution, recombination-dependent replication, and recombination repair by template switching, a form of error-free lesion bypass by replication forks (Alberts, 1987; Bleuit et al., 2001; Formosa & Alberts, 1986; Ma et al., 2004; Morrical & Alberts, 1990).

Gp32 is a highly cooperative ($\omega \geq 1000$) and nonspecific single-stranded DNA-binding protein (SSB) essential for T4 replication (Villemain, Ma, Giedroc, & Morrical, 2000). It is a 34-kDa, 301-amino acid zinc metalloprotein with three functional domains that can be defined by proteolysis (Villemain, Ma et al. 2000); the N-terminal basic (B) domain (residues 1-21), the core domain (residues 22-254), and the C-terminal acidic (A) domain (255-301) (Williams, Sillerud, Schafer, & Konigsberg, 1979). The core domain contains the ssDNA-binding site (Spicer, Williams, & Konigsberg, 1979) and the A domain mediates critical interactions with heterologous replisome proteins such as Dda and Gp43 (Burke, Alberts, & Hosoda, 1980; Krassa, Green, & Gold, 1991). The B domain mediates self-association of Gp32 monomers in solution and cooperativity of
ssDNA binding (Giedroc, Khan, & Barnhart, 1990). Monomers of Gp32 associate on ssDNA in clusters, which is mediated by highly basic amino acids in the B domain. (Casas-Finet, Fischer, & Karpel, 1992; Churchill & Travers, 1991).

Gp32-B is a 30.8-kDa truncated version of Gp32 that lacks the B domain, is devoid of Gp32-Gp32 interactions, exhibits no cooperativity \((\omega = 1)\) and has severely reduced ssDNA-binding affinity compared with wildtype (Giedroc et al., 1990; Ma et al., 2004; Villemain et al., 2000). Gp32-B is therefore a useful reagent that separates the DNA-binding component from the protein-protein interaction functionality of Gp32, and thus allows us to directly and specifically test the effects of Gp32-Dda protein-protein interactions on helicase activity.

Dda is among the proteins that interact most strongly with Gp32. However, the implications of this interaction have been difficult to characterize. Dda is quantitatively bound to Gp32 during chromatography of T4-infected cells over Gp32 protein affinity columns and elutes at salt concentrations ranging from 0.6 to 2.0 M, the highest of all major bound protein species (Formosa, Burke, & Alberts, 1983; Hurley et al., 1993; Jacobsen, Kazmierczak, Lisher, Winkler, & Giedroc, 2011; Jongeneel, Formosa, & Alberts, 1984; Ma et al., 2004). In vitro ATPase assays have established that Gp32-covered ssDNA does not stimulate Dda ATPase activity and that Gp32 acts as a competitive inhibitor of Dda by making ssDNA binding sites inaccessible to Dda. This suggests that in vivo the site of action of Dda may be on ssDNA not covered by Gp32 (Jongeneel, Formosa, et al., 1984).
However, studies have shown a positive interaction between Dda and Gp32 in vitro. Dda stimulates strand displacement DNA synthesis in vitro only in the presence of Gp32 (Cha & Alberts, 1989). This stimulation requires direct Gp32-Dda protein-protein interactions since it is relatively unaffected by mutations that destabilize the ssDNA-binding component of Gp32 (Ma et al., 2004).

The significance of SSB-helicase interactions has been established in other biologic systems, including E. coli and HSV-1 (Boehmer, 1998; Cadman & McGlynn, 2004). In this paper we will discuss our findings on the modulation of Dda DNA binding, strand displacement and ATPase activity by Gp32 protein-protein interactions. We report here the enhancement of Dda helicase DNA unwinding and ATPase activities by the non-cooperative Gp32-B on varied DNA substrates and the rescue of Dda helicase activity under challenging conditions such as high salt and in the presence of a protein block. These and other results suggest that Gp32 may play both positive and negative roles in regulating the helicase activity of Dda during T4 replication, recombination, and repair transactions.
Materials and Methods

2.1. Reagents and resins. Analytic grade chemicals and reagents were purchased from Sigma unless otherwise indicated. ssDNA-cellulose resin was prepared as described (Alberts & Herrick, 1971). DEAE and hydroxyapatite resin was purchased from BioRad. ATP was purchased from USB. [γ-32P]-labeled ATP was purchased from Perkin Elmer. 6x protein-loading dye was purchased from Promega.

2.2. Nucleic and polyamide nucleic acids. All DNA concentrations are expressed in moles per liter of oligonucleotide molecules. Polyacrylamide gel electrophoresis- and high-performance liquid chromatography-purified mixed sequence oligonucleotides were purchased from IDT. The concentration of each oligonucleotide was calculated using absorbance measurement at 260 nm and extinction coefficients provided by the manufacturer. Polyamide nucleic acid (PNA) was prepared by BioSynthesis, concentrations were calculated using absorbance measurement at 260 nm and extinction coefficients provided by the manufacturer, and was heated to 65 °C for 15 min prior to use. PNA is not a substrate for Dda (Tackett, Corey, & Raney, 2002). The sequences of all oligonucleotides used are listed in Table 1.

2.3. Overexpression and purification of bacteriophage T4 proteins. Recombinant Dda (Morris et al., 2001), Gp32 (Ishmael, Alley, & Benkovic, 2001), and Gp32-B (Giedroc et
proteins were overexpressed and purified from *Escherichia coli* as described. The Gp32 purification protocol had the following modifications: the *E. coli* NCZYM medium was replaced with LB; a Mono Q (GE Healthcare) chromatography step was added after the chitin chromatography step; and the Gp32 storage buffer was modified to be 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM βME, and 10% glycerol. Concentrations of protein stock solutions were calculated from extinction coefficients at 280 nm: 41,306 M$^{-1}$ cm$^{-1}$ for Gp32 and Gp32-B, 59,010 M$^{-1}$ cm$^{-1}$ for Dda, and 136,030 M$^{-1}$ cm$^{-1}$ for Gp43 D219A, all calculated based on the amino acid sequence (Gill & von Hippel, 1989). All stock solutions of Gp32, Gp32-B, and Dda proteins used in this study were nuclease-free according to published criteria (Morrical, Hempstead, & Morrical, 1994). Gp43 D219A, an exonuclease-deficient Gp43 polymerase mutant, was a gift from Dr. Linda Reha-Krantz (University of Alberta). All proteins were determined to be $\geq 95\%$ pure by SDS-PAGE analysis.

2.4. *DNA electrophoretic mobility shift assays.* DNA electrophoretic mobility or gel shift assays were carried out to compare the DNA-binding affinities of Gp32 and Gp32-B to linear ssDNA. Reactions were carried out with 10.5 μM nucleotides (175 nM molecules) of 5'$^{-}$-[$^{32}$P]-radiolabeled mixed-sequence 27-mer (oligo D) or polyT 30-mer ssDNAs at 25 °C in 20 mM Tris-acetate (pH 7.8), 90 mM potassium acetate, 10 mM magnesium acetate in a total reaction volume of 20 μl. Reactions were initiated by the addition of Gp32, Gp32-B, or Dda to a final concentration of 1.5 μM, which represents a protein:nucleotide ratio of 1:7 to ensure saturating protein concentration (each Gp32/Gp32-B protomer
occupies approximately seven nucleotides of ssDNA and stable and cooperative binding of Gp32 on ssDNA requires at least three consecutive Gp32 protomers (Branagan, Maher et al. 2012; Kowalczykowski, Lonberg et al. 1986). Reactions were incubated for ten minutes at 25 °C and prepared for electrophoresis by the addition of non-denaturing gel loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol in 6% glycerol). ssDNA bound by protein was separated from unbound ssDNA by electrophoresis in a 6% non-denaturing polyacrylamide gel in 1X TBE and visualized by exposure on a PhosphorImaging K screen. DNA binding affinity is observed as a decrease in the electrophoretic mobility of the DNA molecule.

2.5. Protein electrophoretic mobility shift assays. Electrophoretic mobility shift assays were carried out to compare the effect of ssDNA (oligo D) and PNA oligonucleotides of complementary sequences and identical lengths (27 bases) on the electrophoretic mobility of Gp32 on a 6% non-denaturing polyacrylamide gel. 10.5 μM nucleotides (175 nM molecules) PNA or ssDNA and 1.5 μM Gp32, Gp32-B, or Dda were incubated at 25 °C in 20 mM Tris-acetate (pH 7.8), 90 mM potassium acetate, 10 mM magnesium acetate. After ten minutes, non-denaturing gel loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol in 6% glycerol) was added, samples were loaded on a non-denaturing 6% polyacrylamide gel, electrophoresed at low voltage (45 V), and proteins were visualized by staining with Coomassie blue.
2.6. Strand labeling DNA for unwinding substrates. Oligonucleotides were 5'-\textsuperscript{32}P-labeled by incubating [\gamma-\textsuperscript{32}P]-labeled ATP and T4 polynucleotide kinase at 37 °C for 1 h in T4 polynucleotide kinase reaction buffer (NEB). The kinase was heat-inactivated at 95 °C for 5 min.

2.7. Tailed, nicked, and gapped duplexes. Tailed duplex substrate was prepared by addition of 1.2 equivalents of unlabeled oligonucleotide A to the 5'-\textsuperscript{32}P-labeled oligonucleotide B in 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl, heating to 95 °C for 5 min, and slowly cooling to room temperature over a period of three hours. Gapped substrates were prepared under the same conditions, mixing one equivalent of oligonucleotide D with 1.2 equivalents of oligonucleotide C (for the six-nucleotide gap duplex), oligonucleotide K (for the four-nucleotide gap duplex), oligonucleotide L (for the two-nucleotide gap duplex), oligonucleotide M (for the one-nucleotide gap duplex), or oligonucleotide N (for the nicked duplex).

2.8. Pseudofork DNA substrate. Pseudofork substrate was made by annealing 5'-\textsuperscript{32}P-labeled oligonucleotide E with 1.5 equivalents of unlabeled oligonucleotide F in 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl and heating to 95 °C for 5 minutes, followed by 60 min at 68 °C. The heated DNA was allowed to slowly cool to room temperature over a period of three hours and then purified by centrifugation through a Probe Quant G-50 spin column (Amersham Biosciences).
2.9. *Fork and inverted fork mimics.* Fork and inverted fork mimics were made by annealing 5'-32P-labeled oligonucleotide E with unlabeled oligonucleotide F and either oligonucleotide G (for the inverted fork mimic) or H (for the fork mimic) at a ratio of 1:1.5:10 E:F:(G or H) under the same conditions used to anneal the pseudofork DNA substrate. Fork and inverted fork substrates were purified by electroelution from 6% polyacrylamide gels in 1× TBE (100 mM Tris, 90 mM boric acid, 1 mM EDTA) buffer using an electroelution device (Owl Scientific). Following electroelution, substrates were concentrated using Microcon YM10 centrifuge filter devices (Millipore).

2.10. *Occluded and inverted occluded fork mimics.* Occluded and inverted occluded fork mimics were made by annealing 5'-32P-labeled oligonucleotide E with unlabeled oligonucleotides F, H, and I (for the occluded fork mimic) or oligonucleotides F, G, and J (for the inverted occluded fork mimic) at a ratio of 1:1.5:10:10 (E:F:H:I or E:F:G:J) under the same conditions used to anneal the pseudofork DNA substrate. Occluded and inverted occluded fork mimics were purified the same as fork and inverted fork mimics.

2.11. *DNA unwinding assays.* DNA unwinding assays were performed as described (Nanduri, Eoff, Tackett, & Raney, 2001). 1 nM Dda was first incubated with 125 nM DNA substrates for two minutes, then with 100 nM Gp43 D219A, if present, for two minutes, and finally with indicated concentrations of Gp32 or Gp32-B for two minutes at 25 °C in reaction buffer (25 mM HEPES (pH 7.5), 10 mM potassium acetate, 0.1 mM EDTA, 2 mM β-mercaptoethanol, 100 μg/ml BSA, 10 U/ml phosphoenolpyruvate
kinase, 15.4 U/ml lactate dehydrogenase, 4 mM phosphoenolpyruvate (pH 7.5)) and the reaction was initiated by the simultaneous addition of 4.6 mM ATP, 5 mM magnesium acetate, and 300 nM PNA. Aliquots were withdrawn at various times and added to an equal volume of 200 mM EDTA to quench the reaction. Non-denaturing gel loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol in 6% glycerol) was added to each sample and each sample was analyzed on native polyacrylamide gels ranging from 4% to 20%, depending on product size, visualized by exposure on a PhosphorImaging K screen, quantified in a BioRad Molecular Imaging FX, and analyzed by densitometry using BioRad Quantity One software. Fraction of unwound product was determined by calculating the ratio of single-stranded product to the total density per lane, as described (Amaratunga & Lohman, 1993).

2.12. ATPase assay. ATPase assay was based on published spectrophotometric-coupled assays (Morrical et al., 1994). For all ATPase assays a Varian Cary Bio 50 UV-visible spectrophotometer was used. The buffer used for ATPase reactions comprised 25 mM Tris-acetate, pH 7.5, 150 mM potassium acetate, and 10 mM magnesium acetate. Components were incubated in a cuvette in a total volume of 150 μl at 25 °C: 25 μM oligonucleotides, 44 nM Gp32-B, and 11 nM Dda. Each reaction was initiated by the addition of 4.5 mM ATP. Absorbance was monitored at 340 nm over a time course of 10 minutes following ATP addition. The rate of ATP hydrolysis was calculated by converting NADH absorbance to NADH concentration using an extinction coefficient of NADH at 340 nm of 6,200 M⁻¹ cm⁻¹ and plotting NADH concentration versus time. Rates
of ATP hydrolysis were determined by calculating the slope at the linear portion of each absorbance scan. The decrease in concentration of NADH (µmoles L⁻¹ s⁻¹) is equivalent to the concentration of ADP released (µmoles L⁻¹ s⁻¹) by Dda, and thus the rate of ATP hydrolysis.

2.13. Statistical analysis. Error bars represent the standard error of the mean. Statistical analysis was performed using GraphPad Prism software. Significance was assessed at the p ≤0.05 level.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tailed duplex</td>
<td>A + B</td>
</tr>
<tr>
<td>Gapped duplex (6-nt gap)</td>
<td>C + D</td>
</tr>
<tr>
<td>Gapped duplex (4-nt gap)</td>
<td>D + K</td>
</tr>
<tr>
<td>Gapped duplex (2-nt gap)</td>
<td>D + L</td>
</tr>
<tr>
<td>Gapped duplex (1-nt gap)</td>
<td>D + M</td>
</tr>
<tr>
<td>Nicked duplex</td>
<td>D + N</td>
</tr>
<tr>
<td>Pseudofork</td>
<td>E + F</td>
</tr>
<tr>
<td>Inverted fork</td>
<td>E + F + G</td>
</tr>
<tr>
<td>Fork</td>
<td>E + F + H</td>
</tr>
<tr>
<td>Occluded fork</td>
<td>E + F + H + I</td>
</tr>
<tr>
<td>Inverted occluded fork</td>
<td>E + F + G + J</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5' to 3' for DNA; N to C for PNA)</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GCCAGACATCAGCG</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>TTTTTTTTTTTTCGCTGATGTCG</td>
<td>24</td>
</tr>
<tr>
<td>C</td>
<td>TGAGCGCCGCACTCTGAAAGCATCAGCTTCTG</td>
<td>84</td>
</tr>
<tr>
<td>D</td>
<td>TGCCCATAGAAATCTCGACTATCGCAAT</td>
<td>27</td>
</tr>
<tr>
<td>E</td>
<td>TAAGCTATTCAGATACCTCTCGATCTCTGACT</td>
<td>56</td>
</tr>
<tr>
<td>F</td>
<td>GATCATGCAGGAGACAGTCGGATCGCAACC</td>
<td>60</td>
</tr>
<tr>
<td>G</td>
<td>GTACGAGGTATCTTGAATAACGT</td>
<td>24</td>
</tr>
<tr>
<td>H</td>
<td>CTGAATCAGCTACTATATGACACAC</td>
<td>24</td>
</tr>
<tr>
<td>I</td>
<td>TACAGAGTACGGATCTTGAATAACGT</td>
<td>30</td>
</tr>
<tr>
<td>J</td>
<td>CTGAATCAGCTACTATATGAACGACAG</td>
<td>30</td>
</tr>
<tr>
<td>K</td>
<td>AGTGAAGCGCGCATCTGAAGCATCAGGTT</td>
<td>86</td>
</tr>
<tr>
<td>L</td>
<td>GCACTGACAGCGCATCTGAGAAGCAGCATCAGGTTCTTGAGCATTAGCTGCAAGATTCTATGGCA</td>
<td>88</td>
</tr>
<tr>
<td>M</td>
<td>CGCAGTGAAGCGCGCATCTGAAGCAGCATCAGGCTTGGAGCATGCACTGGCACTTGCAGAGATTCTATGGCA</td>
<td>89</td>
</tr>
<tr>
<td>N</td>
<td>GCCGAGTGAAGCGCGCATCTGAAGCAGCATCAGGCTTGGAGCATGCACTGGCACTTGCAGAGATTCTATGGCA</td>
<td>90</td>
</tr>
<tr>
<td>PNA</td>
<td>ATTTGCGATAGTCGAGATTCTATGGCA</td>
<td>27</td>
</tr>
</tbody>
</table>
Table 1. **Oligonucleotides used in the construction of DNA substrates.** *Upper Panel:* Oligonucleotide compositions of the various DNA substrates used in unwinding reactions. The structures of relevant substrates are shown schematically in each figure. *Lower Panel:* Lengths and sequences of oligonucleotides used to assemble DNA substrates for unwinding reactions. The PNA oligo was used as a trap in unwinding reactions.
Results

Dda forms similar tripartite complexes with ssDNA and either Gp32 or Gp32-B.

Native gel electrophoretic mobility shift assays (Figure 21) demonstrate that Dda forms complexes with either Gp32 or Gp32-B, both in the presence and absence of 5'-'32P-labeled ssDNA. Experiments were performed in the absence of ATP to prevent Dda translocation on and release of ssDNA. As shown in Figure 21A, Gp32 alone (Figure 21A, lane 1) migrates as a diffuse band with relatively low apparent molecular weight. The smearing of this band is likely due to the formation of small mixed oligomers of Gp32 under native conditions (Carroll, Neet, & Goldthwait, 1975). The mobility of the Gp32 band appears to be shifted upon addition of Dda (Figure 21A, lane 2), while Dda alone migrates as a diffuse band at relatively high apparent molecular weight (Figure 21A, lane 9). This result is consistent with Gp32-Dda interactions occurring in the absence of ssDNA. On the native gel, ssDNA and Gp32 co-migrate as a uniform band with a relatively high apparent molecular weight (Figure 21A, lane 3) that is quantitatively supershifted upon addition of Dda (Figure 21A, lane 4). The ssDNA that is present in the Gp32-ssDNA complex is also quantitatively supershifted upon addition of Dda (Figure 21B, lanes 3-4). In the absence of Gp32, the addition of ssDNA causes Dda to migrate as a more uniform band with slightly higher apparent molecular weight than is seen with Dda alone (Figure 21A, lanes 9-10). The band contains ssDNA as shown in Figure 21B, lane 10. The electrophoretic mobility of the Dda-ssDNA complex (Figure
21A-B, lane 10) is distinct from that of the Dda-Gp32-ssDNA complex (Figure 21A-B, lane 4). Together, the native gel data in Figure 21 indicate that Dda, Gp32, and ssDNA form a stable tripartite complex in the absence of ATP.

On the native gel, Gp32-B alone (Figure 21A, lane 5) migrates at a uniform apparent molecular weight due to its monomeric (non-cooperative) nature and appears to undergo a mobility shift in the presence of Dda (Figure 21A, lane 6). A small fraction of Gp32-B migrates at a slightly higher apparent molecular weight in the presence of ssDNA (Figure 21A, lane 7), consistent with the very weak and non-cooperative interactions of Gp32-B with ssDNA (Giedroc et al., 1990). Both Gp32-B (Figure 21A, lane 8) and ssDNA (Figure 21B, lane 8) bands are supershifted by the addition of Dda. The electrophoretic mobility of ssDNA in the Dda/Gp32-B/ssDNA mixture is distinct from that seen in the Dda-ssDNA complex (Figure 21B, lanes 8 & 10), and similar to that seen in the Dda-Gp32-ssDNA complex (Figure 21B, lane 4). These observations indicate that Dda, Gp32-B, and ssDNA form a tripartite complex similar to the Dda-Gp32-ssDNA complex in the absence of ATP.
Figure 21. **Complex formation between Dda and Gp32 and Gp32-B in the presence and absence of ssDNA.** Electrophoretic shift assays (EMSAs) were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 20 mM Tris-acetate (pH 7.8), 90 mM potassium acetate, and 10 mM magnesium acetate in a total reaction volume of 20 µl. Reactions components were added in the following order: 10.5 µM nucleotides (175 nM molecules) ssDNA, 1.5 µM Gp32 or Gp32-B, and 1.5 µM Dda. Reactions were incubated for ten minutes at 25 °C. **A)** Result of EMSA by 6% non-denaturing polyacrylamide gel electrophoresis and Coomassie blue protein staining. **B)** Result of same EMSA gel visualizing 5’-[³²P]-labeled ssDNA by autoradiography. DNA binding affinity is observed as an increase in the apparent molecular weight of the DNA molecule.
Electrophoretic shift assays (EMSAs) were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 20 mM Tris-acetate (pH 7.8), 90 mM potassium acetate, and 10 mM magnesium acetate in a total reaction volume of 20 µl. Reactions components were added in the following order: 10.5 µM nucleotides (175 nM molecules) ssDNA or PNA (denoted by “+” above) or 21 µM nucleotides (350 nM molecules) PNA (denoted by “++” above) and 1.5 µM Gp32 or Gp32-B. Reactions were incubated for ten minutes at 25 °C. Result of EMSA by 6% non-denaturing polyacrylamide gel electrophoresis and Coomassie blue protein staining showing the formation of complexes between Gp32 and Gp32(-)B with either 10.5 µM
nucleotides ssDNA (lanes 4 and 8), 10.5 µM nucleotides PNA (lanes 2 and 6), or 21 µM nucleotides PNA (lanes 3 and 7). ssDNA or PNA binding affinity is observed as an increase in the apparent molecular weight of the protein.

**Gp32 and Gp32-B do not bind polyamide nucleic acid (PNA).**

Gp32 and Gp32-B could interfere with helicase assays by binding to the ssDNA trap that is used to prevent re-annealing of the unwound products. To guard against this possibility we employed a polyamide nucleic acid (PNA) trap that can anneal via base pairing to the ssDNA product but lacks a negatively charged sugar-phosphate backbone and therefore does not bind to Gp32 or Gp32-B. To confirm the lack of binding, we performed the EMSA experiment shown in Figure 22. We compared the effects of a PNA molecule 27 bases in length, and of a ssDNA oligonucleotide of the same length and complementary sequence, on the electrophoretic mobility of Gp32 and Gp32-B in a 6% non-denaturing polyacrylamide gel. The addition of ssDNA caused a significant decrease in the electrophoretic mobility of Gp32 (Figure 22, lanes 1 & 4), whereas PNA had no effect on the mobility of Gp32 (Figure 22, lanes 1-3). The electrophoretic mobility of Gp32-B was not affected by either PNA or ssDNA (Figure 22, lanes 5-8). These data indicate that Gp32 and Gp32-B do not bind PNA, a conclusion that is supported by fluorescence data (unpublished results). The data in Figure 22, in addition to published data showing Dda does not bind PNA (Nanduri et al., 2001), make PNA an ideal trap for helicase DNA unwinding assays.
**Figure 23. Schematic representation of DNA unwinding assay.** A) Helicase unwinding of DNA substrate with a single strand, depicted in red, 5’ radiolabeled with $^{32}\text{P}$, depicted by an asterisk. DNA unwinding assays were based on previously published protocol (Nanduri et al., 2001) and performed as described in Experimental Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 10 mM potassium acetate, 0.1 mM EDTA, 2 mM β-mercaptoethanol, 100 μg/ml BSA, 10 U/ml phosphoenolpyruvate kinase, 15.4 U/ml lactate dehydrogenase, and 4 mM phosphoenolpyruvate (pH 7.5). Reaction components were added in the following order: 125 nM molecules DNA, 1 nM Dda, followed by a two-minute incubation at room temperature, 100 nM Gp43 D219A, if present, followed by another two-minute incubation at room temperature, and indicated concentrations of Gp32 or Gp32-B, if present, followed by a final two-minute incubation at room temperature. Reactions were initiated by the simultaneous addition of 4.6 mM
ATP, 5 mM magnesium acetate, and 300 nM molecules PNA, and allowed to proceed for the specified times. Aliquots were withdrawn at each time point and quenched by the addition of an equal volume of 200 mM EDTA. B) Intact and unwound DNA substrates were resolved by non-denaturing PAGE, visualized by autoradiography, and quantified by densitometry. Fraction of unwound product was determined by calculating the ratio of single-stranded product to the total density per lane, as described (Amaratunga & Lohman, 1993).

**Effect of Gp32 and Gp32-B on Dda DNA unwinding activity.**

DNA unwinding assays, an example of which is depicted schematically in Figure 23A, were conducted using heteroduplex DNA substrates of different structures and varying ssDNA lengths. 5’-[\(^{32}\)P]-labeled substrates were designed so that unwinding catalyzed by Dda helicase and trapping by PNA would generate a labeled product that can be separated from the substrate by non-denaturing PAGE, allowing the reaction to be quantified by phosphorimaging. Figure 23A illustrates this principle for a substrate that mimics a replication fork. The data in Figure 23B shows a time course for the unwinding of the fork mimic by Dda helicase. Quantitative analysis of this data is presented in Figure 24G, below.

Experiments in Figure 24A-G were designed to investigate the possibility that Gp32 and Gp32-B have differential effects on Dda DNA unwinding activity in the context of different DNA structures, which are shown schematically in each panel of Figure 24. The structures model a number of different intermediates that Dda helicase
might encounter during DNA replication/recombination/repair. The substrates tested included a tailed duplex (Figure 24A), gapped duplex (Figure 24B), pseudo-fork (Figure 24C), inverted fork mimic (Figure 24D), fork mimic (Figure 24E), occluded fork mimic (Figure 24F), and inverted occluded fork mimic (Figure 24G). ssDNA gaps, when present, were 6 nucleotides in length in all experiments in Figure 24. A constant amount of Dda was incubated with variable amounts of Gp32 or Gp32-B. Under all conditions and in all substrates, the observed rate constants of DNA unwinding decreased in the presence of Gp32, and, in all substrates except the simple 12:24 tailed duplex (Figure 24A), the rate of unwinding increased in the presence of Gp32-B (Figure 24B-G). Dda helicase unwinds the 12:24 tailed duplex substrate very efficiently and the rate of unwinding is not increased by Gp32-B at any concentration tested (data not shown).

DNA unwinding assays were performed with substrates designed to mimic features present with replication/recombination/repair processes to identify the precise structures unwound by Dda alone, in the absence of Gp32 or Gp32-B (Figure 24A-G). The data show that Dda alone unwinds a wide variety of partially single-stranded substrates including pseudoforks and fork mimics. As predicted by the 5’-to-3’ polarity of the helicase, no unwinding products were observed with the occluded fork mimic (Figure 24F). The highest rates of unwinding by Dda alone were observed with substrates containing 5’ ssDNA extensions of 12-30 nucleotides. This includes, in descending order, tailed duplex DNA (Figure 24A), fork mimic (Figure 24E), and pseudo-fork substrates (Figure 24C). Substrates containing 6-nucleotide ssDNA gaps, including gapped duplex (Figure 24B), inverted fork mimic (Figure 24D), and inverted
occluded fork mimic (Figure 24G), were also unwound by Dda alone, but at reduced rates compared to substrates with longer ssDNA extensions. Dda was not able to unwind the occluded fork mimic, which lacks lagging strand ssDNA (Figure 24F). These observations suggest that to unwind a fork-shaped substrate, Dda requires lagging strand ssDNA or its equivalent. Dda tolerates a fully duplex leading strand arm of a fork substrate (Figure 24G), but does not tolerate a fully duplex lagging strand arm (Figure 24F). The implications of Dda’s substrate preferences for its DNA repair functions are described in the Discussion.

The enhancement of DNA unwinding in the presence of Gp32-B was specific to the specific DNA substrate structure. The effect was most pronounced in the inverted occluded fork (Figure 24G), the inverted fork mimic (Figure 24D), the gapped duplex (Figure 24B), the pseudo-fork (Figure 24C), and the fork mimic (Figure 24E) structures, in descending order. The effect was least pronounced in the tailed duplex (Figure 24A), while the occluded fork mimic (Figure 24F) was not unwound significantly in the presence or absence of Gp32-B. These results are consistent with the notion that Gp32-B can help Dda load onto lagging strand ssDNA to promote replication fork progression (Ma et al., 2004).

Full-length Gp32 severely inhibits Dda-catalyzed unwinding of the inverted fork mimic (Figure 24D). This is in marked contrast with the inverted occluded fork mimic (Figure 24G), which is similar in structure and was only slightly inhibited by Gp32. This can be explained by the structural difference between the substrates and suggests that Gp32 bound to an extended patch of leading strand ssDNA can prevent Dda from binding
to the ssDNA gap on the lagging strand. The implications of this finding are explored in the Discussion. The unwinding of other DNA substrates by Dda was moderately inhibited by full-length Gp32 (Figure 24A-C,E), except for the occluded fork mimic, which was not unwound significantly under any conditions (Figure 24F).
Figure 24. **Dda unwinding activity of various DNA substrates in the presence and absence of Gp32 or Gp32-B.** Helicase unwinding of DNA substrate with a single strand, depicted in red, with a 5’-[\textsuperscript{32}P] radiolabel, depicted by an asterisk. DNA unwinding assays were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 10 mM potassium acetate, 0.1 mM EDTA, 2 mM β-mercaptoethanol, 100 µg/ml BSA, 10 U/ml phosphoenolpyruvate kinase, 15.4 U/ml lactate dehydrogenase, and 4 mM phosphoenolpyruvate (pH 7.5). Reaction components were added in the following order: 125 nM molecules DNA, 1 nM Dda, and 100 nM Gp32 or Gp32-B, if present. Controls were performed in which Gp32 and Gp32-B were individually replaced with their respective storage buffers. Reactions were initiated by the simultaneous addition of 4.6 mM ATP, 5 mM magnesium acetate, and 300 nM molecules PNA, allowed to proceed for the specified time points, and quenched by the addition of an equal volume of 200 mM EDTA. Results of DNA unwinding reactions using **A** tailed duplex, **B** gapped duplex, **C** pseudofork, **D** inverted fork mimic, **E** fork mimic, **F** occluded fork mimic, and **G** inverted occluded fork mimic. Intact and unwound DNA substrates were resolved by non-denaturing PAGE, visualized by autoradiography, and quantified by densitometry. Fraction of unwound product was determined by calculating the ratio of single-stranded product to the total density per lane.
Figure 25. **DNA unwinding activity of Dda helicase as a function of ssDNA gap length.**  
A) Unwinding reaction schematic. ssDNA gap length was varied in the partial duplex substrate. Unwinding reactions were carried out in the absence or presence of Gp32-B or Gp32 using otherwise identical substrates containing ssDNA gap lengths of B) 6 nucleotides, C) 4 nucleotides, D) 2 nucleotides, E) 1 nucleotide, and F) 0 nucleotides (nick).

**Gp32-B enhances the DNA unwinding activity of Dda on substrates containing short ssDNA gaps.**

A series of DNA unwinding assays was conducted using a set of partial duplex DNA substrates that varied only in the size of the ssDNA gap present, ranging from a gap size of zero (single-strand nick) to a six-base gap, to test whether Gp32 or Gp32-B can promote unwinding of substrates that Dda helicase alone does not unwind effectively (Figure 25A). Consistent with published data (Byrd & Raney, 2005), it was determined that Dda alone unwinds a 6-base gapped substrate efficiently (Figure 25B), but its unwinding activity decreases dramatically as the gap length is shortened to 4, 2, or 1 bases (Figs. 25C-E). No unwinding activity is detectable on the nicked substrate (gap length 0) (Figure 25F). Even though Dda does not efficiently unwind substrates with gaps of fewer than six bases, the rates are enhanced by the presence of Gp32-B in all cases except the nicked duplex, which did not undergo any detectable unwinding (Figure 25B-F). In contrast, Gp32 slightly inhibits unwinding at all gap lengths tested (Figure 25B-E), and did not facilitate unwinding of the nicked substrate (Figure 25F).
Figure 26. **Gp32-B stimulates the DNA unwinding activity of Dda helicase at elevated salt concentrations.** A) Schematic representation of the DNA unwinding reaction employing a partial duplex substrate with a 6-nucleotide ssDNA gap. The DNA unwinding activity of Dda was measured in the absence and presence of Gp32-B or Gp32 under B) low salt conditions (10 mM potassium acetate), C) intermediate salt conditions (75 mM sodium chloride plus 10 mM potassium acetate), and D) high salt conditions (150 mM sodium chloride plus 10 mM potassium acetate).

**Gp32-B promotes the DNA unwinding activity of Dda helicase at elevated NaCl concentrations.**

Consistent with published data (Jongeneel, Formosa, et al., 1984), the DNA unwinding activity of Dda helicase is inhibited by the addition of 75-150 mM NaCl (Figure 26B-D). Addition of Gp32-B, but not Gp32, raises Dda DNA unwinding activity under all salt conditions tested (Figure 26B-D), using the six-base gapped substrate (Figure 26A). The initial rate of DNA unwinding by Dda is dramatically higher in the presence of Gp32-B than in its absence, even as the initial rate decreases with increasing salt concentration. The final extent of unwinding, however, appears to be independent of salt concentration in the presence of Gp32-B (Figure 26B-D).
Figure 27. In the presence of Gp32-B, Dda helicase unwinds past a primer/template-bound exonuclease-deficient polymerase. A) Schematic of the reaction. Gp43 D219A exonuclease-deficient T4 polymerase is incubated with the 6-base gapped DNA substrate in the absence of dNTPs before the unwinding reaction is initiated by the addition of Dda helicase and Mg-ATP. B) Time courses of unwinding reactions catalyzed by Dda alone (blue), in the presence of Gp43-D219A (orange), in the presence of Gp43-D219A and Gp32-B (green), and in the presence of Gp43-D219A and Gp32 (red).
**Gp32-B promotes Dda-catalyzed unwinding of a gapped duplex bound by DNA polymerase.**

Gp43-D219A polymerase, the exonuclease-deficient T4 polymerase mutant, was incubated with the six-base gapped DNA substrate in the absence of dNTPs to create conditions simulating a stalled DNA polymerase (Figure 27A). Under these conditions, addition of Gp43-D219A inhibits Dda unwinding activity in the absence of Gp32-B (Figure 27B). Addition of Gp32-B, but not of Gp32, stimulates Dda strand displacement activity to levels similar to those seen with Dda + Gp32-B in the absence of polymerase (Figure 27B). Gp32 is unable to promote unwinding of the polymerase-bound DNA substrate by Dda, since the time courses of reactions containing Dda and Gp43-D219A are indistinguishable in the presence/absence of Gp32 (Figure 27B).
Figure 28. ATPase activity of Dda helicase as a function of ssDNA concentration, in the presence and absence of Gp32-B. Steady-state reaction velocities were determined by measuring ADP release in a continuous coupled assay at saturating ATP concentration (4.5 mM).
Figure 29. ATPase activity of Dda helicase as a function of ATP concentration. Steady-state reaction velocities were determined by measuring ADP release in a continuous coupled assay at saturating ssDNA concentration. A) Kinetics of Dda-catalyzed, ssDNA-dependent ATP hydrolysis in the presence and absence of a four-fold excess of Gp32-B over Dda concentration. ssDNA concentration was 25 µM and ATP
concentration was varied. B) Effect of Gp32-B concentration on the velocity of Dda-catalyzed, ssDNA-dependent ATP hydrolysis at saturating concentrations of both ssDNA (50 µM) and ATP (4.5 mM).

<table>
<thead>
<tr>
<th></th>
<th>Dda</th>
<th>Dda + Gp32-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (µM s$^{-1}$) (11 nM)</td>
<td>0.9 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (µM s$^{-1}$) (22 nM)</td>
<td>2.5 ± 0.1</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ (s$^{-1}$) (ATP titration)</td>
<td>86 ± 5</td>
<td>141 ± 4</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ (s$^{-1}$) (ssDNA)</td>
<td>107 ± 3</td>
<td>157 ± 9</td>
</tr>
<tr>
<td>$K_{m}$ (ATP) (mM)</td>
<td>0.20 ± 0.08</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>$K_{m}$ (ssDNA) (µM)</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>$k_{\text{cat}} / K_{m}$ (ATP) (M$^{-1}$ s$^{-1}$)</td>
<td>0.4 ± 0.2</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>$k_{\text{cat}} / K_{m}$ (ssDNA) (M$^{-1}$ s$^{-1}$)</td>
<td>3.6 ± 1.3</td>
<td>5.2 ± 0.3</td>
</tr>
</tbody>
</table>

Table 2. Kinetic parameters of the ssDNA-stimulated ATPase activities of Dda in the absence and presence of Gp32-B. [ssDNA] is in molecular molarity.

Gp32-B stimulates the ssDNA-dependent ATPase activity of Dda helicase.

Previous studies established that Gp32 inhibits the ssDNA-dependent ATPase activity of Dda helicase (Jongeneel, Formosa, et al., 1984) (Krell, Durwald, & Hoffmann-Berling, 1979). Here, the effects of Gp32-B on the kinetics of Dda ATPase activity were explored. With ssDNA as the variable ligand, and at saturating ATP, the addition of a four-fold excess of Gp32-B with respect to Dda concentration caused an increase in ATPase activity at all ssDNA concentrations examined (a 1000-fold range) (Figure 28). Similarly, with ATP as the variable substrate, and at saturating ssDNA, a four-fold excess of Gp32-B increased Dda ATPase activity at all ATP concentrations examined (Figure
As reported in Table 2, the stimulation of Dda ATPase activity by Gp32-B was due to an 80-90% increase in the $V_{\text{max}}$ of the reaction in the presence of a four-fold excess of Gp32-B. Gp32-B also lowered the $K_m$ for ATP by approximately 2-fold, but it appeared to have little effect on the apparent $K_m$ for ssDNA (Table 2). Therefore Gp32-B increases the catalytic efficiency of Dda-catalyzed ATP hydrolysis, as reflected by the increase in $k_{\text{cat}} / K_m$ values, primarily by increasing enzyme turnover (Table 2). The data were identical when the ssDNA used was a mixed sequence 20mer (data shown in Figures 28 and 29), or one of the homopolymers (dT)$_{20}$ or (dT)$_{60}$ (data not shown).

The ATPase activity of 11 nM Dda increases linearly as the Gp32-B concentration increases from zero to 110 nM (a ten-fold excess), suggesting that the concentrations of Gp32-B used were below the $K_d$ for its interactions with Dda-ssDNA. Alternatively, the stimulation of Dda by Gp32-B has a more complex dependence on protein concentration, as we explore in the Discussion.
Discussion

Dda helicase unwinds a variety of DNA substrates containing accessible amounts of single-stranded DNA. The data show that Dda most efficiently unwinds substrates containing relatively long 5’ ssDNA extensions of 12-30 nucleotide residues, such as the tailed duplex, pseudo-fork, and fork mimic substrates visited in Figure 24A,C and E. Dda also unwinds substrates containing ssDNA gaps. A 6-nucleotide gap in an otherwise duplex substrate is sufficient to allow unwinding by Dda, however the rate of unwinding decreases dramatically as the gap length decreases, and Dda fails to unwind a nicked substrate (Figure 25). The requirement of a 6-nucleotide or greater ssDNA gap for efficient unwinding activity is consistent with the binding site size of monomeric Dda on ssDNA, which was previously determined to be 6 nucleotides (Byrd & Raney, 2004; Kowalczykowski et al., 1986). The substrate specificity of Dda, combined with its 5’ → 3’ polarity, is consistent with a helicase that can translocate on lagging strand ssDNA to stimulate strand displacement DNA synthesis by the leading strand polymerase. The resistance of the occluded fork mimic substrate, which has a fully duplex lagging strand arm, to unwinding by Dda (Figure 24F) further emphasizes the requirement for lagging strand ssDNA as an assembly site for the enzyme at the replication fork. Meanwhile, the ability of Dda to initiate unwinding from a single-stranded gap in otherwise duplex DNA suggests a mechanism for promoting the restart of stalled replication forks (see below).

Previous studies demonstrated that full-length Gp32 inhibits the ssDNA-dependent ATPase activity of Dda by competing for binding sites on the ssDNA
Nevertheless Dda requires moderate concentrations of either Gp32 or Gp32-B in order to stimulate strand displacement DNA synthesis by the T4 DNA polymerase holoenzyme in vitro (Cha & Alberts, 1989; Formosa & Alberts, 1986; Jongeneel, Bedinger, & Alberts, 1984; Ma et al., 2004; Villemain et al., 2000). Results of this study show that, with one key exception, the unwinding of a variety of DNA substrates by Dda helicase is weakly inhibited by Gp32 but strongly stimulated by Gp32-B. The exception is the inverted fork mimic shown in Figure 24D. Unwinding of this substrate by Dda is severely inhibited by full-length Gp32, but strongly stimulated by Gp32-B. The structure of this substrate resembles a replication fork in which the leading strand polymerase has stalled at DNA damage, leaving an extended region of ssDNA on the leading strand, while Okazaki fragment synthesis has continued on the lagging strand. Thus the inverted fork structure resembles a DNA intermediate that is proposed to occur during the process of recombination repair (Kadyrov & Drake, 2004).

Figure 30 presents a possible model for how Gp32 might direct the activities of Dda helicase in order to help bring about replication fork restart during recombination repair. When the leading strand polymerase stalls at DNA damage, leading and lagging strand synthesis are uncoupled. The uncoupling allows synthesis of an overlapping Okazaki fragment by the lagging strand synthesis machinery, while allowing an ssDNA gap to open up in front of the stalled leading strand polymerase. This gap would attract a Gp32 cluster, which data suggest would regulate Dda activity in the following ways: First, the Gp32 cluster blocks the stimulation of fork progression by Dda, as suggested by the strong inhibition of unwinding of the inverted fork mimic (Figure 24D). Second,
interactions with Gp32 may stimulate Dda to remove the stalled polymerase and unwind the 3’ end of the daughter strand from its template, as suggested by results in Figure 27. The unwinding of this strand is a prerequisite for template switching promoted by UvsX recombinase in the presence of Dda (Bleuit et al., 2001; Kadyrov & Drake, 2004). As shown by Drake and colleagues, successive UvsX- and Dda-dependent template switching events are necessary to regress a lesion-stalled replication fork and subsequently to promote error-free translesion DNA synthesis (Kadyrov & Drake, 2004). The results of this study suggest that helicase trafficking by Gp32 could play a role in preventing premature fork progression until the required template switching events have been completed.

Full-length Gp32 does not stimulate the DNA unwinding activity of Dda towards any of the DNA substrates that we have tested. In general, Gp32 weakly inhibits unwinding by Dda, with the exception of the reaction involving the inverted fork mimic, which is strongly inhibited by Gp32. These results seem to be at odds with previous studies showing that Dda requires Gp32 to stimulate strand displacement DNA synthesis (Cha & Alberts, 1989; Formosa & Alberts, 1986; Jongeneel, Bedinger, et al., 1984; Ma et al., 2004; Villemain et al., 2000). The N-terminal truncation to generate Gp32-B reveals a cryptic helicase stimulatory activity of Gp32, however. It seems probable that at the wild-type T4 replication fork in vivo or in vitro, this cryptic activity may be revealed in the context of a moving polymerase, or through direct interactions of Gp32 with other replisome components including polymerase.
The increased rates of unwinding of various DNA substrates observed for Dda in the presence of Gp32-B could be the result of an increase in translocation velocity, an increase in processivity, or both. Gp32-B increases the catalytic efficiency of Dda for ATP hydrolysis (Table 2). This suggests an increase in the efficiency of Dda as a helicase, which means a higher probability of a productive step coupled to each ATP hydrolytic event. This effect in itself could result in a higher observed rate of unwinding. An SSB functioning as a processivity factor for a DNA helicase has been described in at least two different systems (Boehmer, 1998; Shereda, Bernstein, & Keck, 2007). Processivity in Dda is enhanced when multiple copies of Dda interact with the same region of ssDNA, giving rise to the notion of a “cooperative inchworm” mechanism for Dda helicase activity (Byrd & Raney, 2005; Eoff & Raney, 2010; Spurling, Eoff, & Raney, 2006). We propose the working hypothesis that Gp32-B (or cryptically, Gp32) promotes the assembly of oligomeric Dda species on ssDNA as it is exposed through the unwinding activity of the initial monomeric helicase, leading to the observed rate acceleration via an enhanced cooperative inchworm mechanism. Detailed pre-steady-state and steady-state kinetics measurements will be needed to address this hypothesis, in future studies.

Understanding the mechanism of DNA helicase activity and its regulation by other DNA binding proteins is essential for progress in the fields of genome stability, oncology, and drug development. Since DNA helicases are molecular motors that translocate on ssDNA, it stands to reason that ssDNA-binding proteins play important roles in regulating helicase activity. Results of this study indicate that in the T4 system,
the Gp32 SSB can have a dual regulatory function with respect to Dda helicase activity: it can interfere with Dda loading onto certain DNA structures, and thereby regulate the manner in which different substrates may be recognized and processed by Dda. This effect may be important for the mechanism of recombination repair (Figure 30). In addition, within the context of the replication fork, Gp32 may stimulate the translocation rate and/or processivity of Dda to enhance unwinding. These findings indicate that SSBs, far from being passive players in the disruption of duplex DNA by helicases, are more likely to be active players in coordinating helicase activities with other events at the replication fork or in repair and recombination complexes.
Figure 30. Proposed model for Gp32 SSB regulation of Dda helicase in replication fork restart during recombination repair. A) When leading strand polymerase (blue figure) stalls at DNA damage (depicted by a bold X), leading and lagging strand syntheses are uncoupled. The lagging strand synthesis machinery can synthesize an Okazaki fragment (green line) overlapping the damaged site, while allowing an ssDNA gap to open up in front of the stalled leading strand polymerase, which is covered by Gp32 (orange figures). B) The Gp32 cluster blocks Dda (red figure) stimulation of fork progression by preventing its loading onto the lagging strand and promotes Dda unwinding of the 3’ end of the template daughter strand. C) UvsX recombinase and Dda catalyze template switching (Bleuit et al., 2001; Kadyrov & Drake, 2004) to regress the lesion-stalled replication fork and D) allow subsequent error-free translesion DNA synthesis (Kadyrov & Drake, 2004). This model describes a mechanism by which Gp32 regulation of Dda helicase can prevent premature fork progression and allow replication fork restart and error-free translesion DNA synthesis during recombination repair.
Conflicts of interest statement

The authors declare that there are no conflicts of interest.
Acknowledgements

The authors thank Dr. Stephen J. Benkovic for providing the overexpression vector for Gp32 and Jennifer Tomczak for technical support. This work was supported by National Institutes of Health research grant no. R01GM48847 to SWM. CSJ was supported by NIH training grant no. T32ES07122. The sponsors had no involvement in study design, the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the article for publication.
References


CHAPTER 3: T4 HELICASES DIFFERENTIALLY REGULATE GP43
POLYMERASE-CATALYZED EXTENSION OF THE INVADING STRAND IN
A SYNTHETICALLY CONSTRUCTED RECOMBINATION INTERMEDIATE

Christian S. Jordan, Keri Sullivan, and Scott W. Morrical*

DNA REPAIR

Department of Biochemistry, University of Vermont College of Medicine, Burlington VT 05405

*To whom correspondence should be addressed: Department of Biochemistry, University of Vermont College of Medicine, B407 Given, 89 Beaumont Avenue, Burlington, VT 05405. Email: smorrica@uvm.edu

Pages: 57, Figures: 13, Tables: 1

†Abbreviations: NA, nucleic acid; ssDNA, single-stranded deoxyribonucleic acid; SSB, single-stranded deoxyribonucleic acid binding protein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.
Abstract

DNA repair by homologous recombination is essential for genome stability. One method to repair DNA damage such as double-strand breaks, called homology-directed repair, relies on recombination between the damaged DNA molecule and a sister chromatid or homologous chromosome. Homology-directed repair relies on the formation of a displacement loop (D-loop), the structure created when single-stranded DNA (ssDNA) from the end of a processed double-strand break invades a sister chromatid or homologous chromosome and forms a heteroduplex with its complementary sequence. The invading strand can be used as a DNA synthesis primer in a process known as D-loop extension by strand displacement DNA synthesis. In this study we used the bacteriophage T4 DNA replication and repair model system to examine the D-loop unwinding activity of all three T4-encoded helicases (Dda, Gp41, and UvsW), the strand extension activity of Gp43 polymerase in the context of a D-loop, and the effect of the helicases and their modulators on D-loop extension. Dda and UvsW, but not Gp41, are able to catalyze DNA unwinding of the D-loop invading strand. The activity of Dda D-loop unwinding competes with D-loop extension by Gp43 polymerase in the presence of Gp32, resulting in a decreased frequency of invading strand extension when all three proteins are present. Dda could therefore act as a form of anti-recombinase to negatively regulate the replicative extension of D-loops. A lower level of invading strand extension is still observed when Dda is added to reactions with Gp43 polymerase in the absence or presence of Gp32, demonstrating that invading strand extension and unwinding can occur
simultaneously. The resulting translocating D-loop or “bubble migration synthesis” is a hallmark of break-induced repair (BIR) and synthesis-dependent strand annealing (SDSA), where the extended invading strand is actively displaced from the template by branch migration. Gp41 did not unwind D-loops, even in the presence of the Helicase Loading Complex components, Gp32 and Gp59, due to its hexameric ring structure and/or helicase loading onto the displaced strand of the D-loop and not the invading strand (Gp59 helicase loader has greatest affinity for replication fork DNA structures). These data implicate Dda as a mixed function helicase that can work both as an anti-recombinase and to promote recombination-dependent DNA synthesis, consistent with the notion that Dda works to promote branch migration. These results have implications on the regulation of DNA replication, chromosomal crossover, and the repair of double-strand breaks in all organisms.
Introduction

Genomic recombination, repair, and replication are intertwined processes essential to all life. The DNA displacement loop (D-loop) is a structural intermediate that is common to all three processes, and thus lies at the interface of the three. D-loops are ubiquitous structures that play a central role in recombination-dependent initiation of DNA replication, the restart of stalled DNA replication forks in response to ssDNA damage, and the repair of double-strand breaks by homologous recombination.

T4 recombination-dependent DNA replication and homology-directed repair of double-strand breaks begin with UvsX recombinase catalyzing the homologous DNA strand invasion event, in which a ssDNA strand invades the double-stranded DNA of a homologous chromosome (Liu and Morrical 2010). The invading strand can be used by Gp43 polymerase holoenzyme to initiate DNA synthesis (Formosa and Alberts 1986). The conversion of the resulting D-loop into a replication fork represents the transition from DNA recombination to DNA replication (Kreuzer and Morrical 1994). T4 recombination-dependent DNA replication, which represents its major mode of chromosomal replication, is a well-characterized in vitro model with which to study the mechanisms controlling homology-directed repair and recombination-dependent replication (Mosig 1998, Kowalczykowski 2000, Kreuzer 2000). DNA helicases are known to regulate the transition from DNA recombination to DNA replication and are involved in nearly all aspects of T4 DNA metabolism (Alberts 1987, Nossal 1994).

Bacteriophage T4 encodes three DNA helicases (Perumal, Raney et al. 2010).
Gp41, a Superfamily 4 (SF4) helicase, in association with the helicase loader Gp59, is the major replicative helicase in T4. It is an essential replicative helicase member of the ring hexameric helicase family that stimulates DNA replication by translocating processively 5’ to 3’ on the lagging strand (Alberts 1987, Nossal 1994, Dong, Gogol et al. 1995). Gp41 serves as an essential component of the primosome (helicase-primase complex) and thus lagging strand DNA synthesis (Cha and Alberts 1986, Nossal and Hinton 1987).

The Dda helicase is also able to unwind duplex DNA ahead of the leading strand polymerase and appears to be involved in early origin-dependent replication. Dda is a Superfamily 1 (SF1) helicase that stimulates T4 strand displacement DNA synthesis in vitro (Formosa and Alberts 1986, Alberts 1987, Morrical and Alberts 1990, Ma, Wang et al. 2004). Dda is a distributive-to-moderately processive 5’ to 3’ monomeric helicase (Raney and Benkovic 1995, Nanduri, Byrd et al. 2002). Dda is believed to participate in anti-recombination (Liu, Ehmsen et al. 2011), in recombination repair, a form of error-free lesion bypass by replication forks, (Kadyrov and Drake 2004), in recombination-dependent replication reactions in vitro, (Formosa and Alberts 1986, Alberts 1987, Morrical and Alberts 1990, Ma, Wang et al. 2004) and in DNA branch migration reactions, all initiated by or involving the T4 UvsX recombinase (Kodadek and Alberts 1987).

UvsW is a SF2 helicase implicated in a variety of DNA repair and recombination pathways in T4. UvsW is involved in the transition from an origin-dependent mode of DNA replication to the recombination-dependent mode of replication in T4 (Dudas and

Given the central role helicases play in DNA recombination, replication, and repair, we tested the ability of all three T4-encoded helicases to unwind D-loop substrates and modulate strand displacement DNA synthesis taking place on D-loops. The data presented in this study provide insights into the mechanisms regulating genome stability that are common to all organisms to repair DNA damage.
Materials and Methods

2.1 Reagents and resins. Concentrations of reagents and buffer components given in the text are final concentrations. Analytical grade chemicals and reagents were purchased from Sigma unless otherwise indicated. ssDNA-cellulose resin was prepared as described (Alberts and Herrick 1971). DEAE and hydroxyapatite resin was purchased from BioRad. ATP and deoxyribonucleotides were purchased from USB. \( \gamma^{[32P]} \)-labeled ATP was purchased from PerkinElmer. T4 polynucleotide kinase and bovine serum albumin were purchased from New England Biolabs. 6x protein loading dye was purchased from Promega. Buffers and solutions were all prepared using filter sterilized water deionized by reverse osmosis.

2.2 Nucleic acids. All DNA concentrations given are final concentrations in units of molecules, unless otherwise specified. Oligonucleotides were purchased from IDT and PAGE-purified by the manufacturer. M13mp18 ssDNA was purchased from New England Biolabs. Oligonucleotide sequences are provided in Table 1. The sequences of Oligos 12 and 13 were obtained from previously published work (Bachrati, Borts et al. 2006) in which a similar D-loop substrate was used for functional assays with BLM helicase. Oligonucleotide concentrations were calculated by measuring absorbance at 260 nm using the extinction coefficients provided by the manufacturer.

2.3 Overexpression and purification of bacteriophage T4 proteins. All protein concentrations given in the text are final concentrations. Recombinant Dda (Morris,
Tackett et al. 2001), Gp41 (Ishmael, Alley et al. 2001), Gp59 (Ishmael, Alley et al. 2001), Gp32 (Ishmael, Alley et al. 2001), Gp32-B (Giedroc, Khan et al. 1990), Gp44/62 (Morris, Hama-Inaba et al. 1979), and Gp45 (Morris, Hama-Inaba et al. 1979) proteins were purified as described previously. The Gp32 purification protocol had the following modifications: the *E. coli* NCZYM medium was replaced with LB; a Mono Q (GE Healthcare) chromatography step was added after the chitin chromatography step; and the Gp32 storage buffer was modified to be 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM β-ME, and 10% glycerol. The Gp59 purification method was modified by the addition of a final Biogel-HTP hydroxyapatite column chromatography step (BioRad) to remove contaminating nuclease activity. Gp59 was eluted using a 50 mM to 500 mM potassium phosphate gradient (pH 6.8) in buffer containing 100 mM NaCl, 2 mM EDTA, 1 mM β-ME, and 10% glycerol. Purified fractions were dialyzed into storage buffer containing 10 mM Tris-acetate (pH 7.8), 25 mM potassium acetate, 5 mM magnesium acetate, 2 mM dithiothreitol, and 20% glycerol, and stored at -80 °C.

Concentrations of protein stock solutions were calculated from extinction coefficients at 280 nm: 41,306 M\(^{-1}\) cm\(^{-1}\) for Gp32 and Gp32-B, 59,010 M\(^{-1}\) cm\(^{-1}\) for Dda, 136,030 M\(^{-1}\) cm\(^{-1}\) for Gp43 and Gp43-D219A, 76,000 M\(^{-1}\) cm\(^{-1}\) for Gp41, 37,800 M\(^{-1}\) cm\(^{-1}\) for Gp59, 73,920 M\(^{-1}\) cm\(^{-1}\) for UvsW, 123,000 M\(^{-1}\) cm\(^{-1}\) for Gp44/62, and 57,200 M\(^{-1}\) cm\(^{-1}\) for Gp45, all calculated based on the amino acid sequence (Gill and von Hippel 1989). UvsW was a gift from Dr. Kenneth Kreuzer (Duke University). Gp43-D219A, an exonuclease-deficient form of the Gp43 T4 polymerase, was a gift from Dr. Linda Reha-Krantz (University of Alberta). Wild-type Gp43 was purchased from New England
Biolabs. All proteins used in this study were nuclease-free based on a published nuclease contamination assay (Morrical, Hempstead et al. 1994). All proteins were confirmed to be ≥95% pure by SDS-PAGE analysis.

2.4 Labeling of DNA substrates. Labeled oligonucleotides were 5’ end-labeled with [³²P] by incubating [γ-³²P]-labeled ATP and T4 polynucleotide kinase at 37 °C for 1 hour in T4 polynucleotide kinase reaction buffer (NEB). The kinase was heat-inactivated at 95 °C for 5 minutes.

2.5 D-loop substrates. D-loop design was based on a published protocol (McGlynn, Al-Deib et al. 1997). DNA annealing reactions were based on previously published protocol (Nurse, Liu et al. 1999). Unless otherwise noted, the invading strand (oligo 12 or 13, Table 1) was [γ-³²P]-5’ end-labeled in all D-loop DNA substrates used. Oligonucleotides annealed to give the various substrates are given in Table 1. Annealing reaction buffer contained (final concentrations): 10 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, and 200 mM NaCl. Annealing reactions (400 µl) contained the following components (in final concentrations): 500 nM [γ-³²P]-labeled invading strand (oligo 12 for the 3’ invading D-loop or 13 for the 5’ invading (inverted) D-loop), 500 nM unlabeled invading strand (oligo 12 or 13), 3 µM unlabeled template strand (oligo 7), and 5 µM unlabeled displaced strand (oligo 10). Reactions were heated and gradually cooled using a PCR thermal cycler (PerkinElmer) from 95 °C to 25 °C in 10 °C steps, with a 10 minute transition time between steps and holding at each step temperature for 10 minutes (i.e., 95 °C for ten minutes, ten-minute cool down to 85 °C, 85 °C for ten minutes, etc.). Annealed substrates
were then purified by 6% native PAGE and eluted by soaking the gel slices overnight in 300 mM sodium acetate, 10 mM magnesium acetate, and 1 mM EDTA. The DNA was concentrated by ethanol precipitation and resuspending in 40 μl.

2.6 Helicase unwinding assays. Helicase unwinding assays were based on previously published protocol and carried out at room temperature. Reaction buffer contained 25 mM HEPES (pH 7.5), 2 mM βME, 100 μg/ml BSA, and 10 mM potassium acetate. Reaction components were added in the following order in a total reaction volume of 100 μl for time courses and 10 μl for each individual time point: 50 nM D-loop (or inverted D-loop) substrates and 75 nM UvsW, 300 nM Gp41, 1 nM Dda, and/or 100 nM Gp32 or Gp32-B (if present). Unwinding reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5 mM magnesium acetate and quenched by the addition of an equal volume of 200 mM EDTA. Controls were performed in which individual enzymes were individually replaced with their respective storage buffers. Reaction products were resolved by 6% non-denaturing PAGE and visualized by autoradiography on a phosphorimaging K-Screen using a Molecular Imager FX (Bio-Rad). Intact and unwound D-loops were quantified by densitometry using Quantity One software (BioRad). Fraction of unwound D-loop was calculated as the ratio of unwound product measured to the total density per lane measured.

2.7 D-loop strand displacement DNA synthesis reactions. DNA synthesis reactions were based on a previously published protocol. Buffer contained the following components: 25 mM HEPES (pH 7.5), 2 mM βME, 100 μg/ml BSA, and 40 mM sodium acetate. dATP,
TTP, dCTP, and dGTP were added to the buffer of all DNA synthesis reactions to final concentration of 150 μM each. Reactions components were added in the following order: 50 nM D-loop substrate, 1 μM Gp32 SSB, 1 μM Gp59 helicase loader, 10 nM Dda helicase or 1 μM Gp41 helicase, and 250 nM Gp43 or Gp43-D219A polymerase (Gp43-D219A is deficient in exonuclease activity and was used to test for substrate degradation). Controls were performed in which Gp32, Gp59, Dda, and Gp41 were replaced with the respective storage buffers. Reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5 mM magnesium acetate, allowed to proceed for one minute, and quenched by the addition of an equal volume of 200 mM EDTA. Products were separated by 12% denaturing (6 M urea) PAGE, visualized by autoradiography using a phosphorimaging K-Screen, a Molecular Imager FX (Bio-Rad) and quantified by densitometry Quantity One software (BioRad). Fraction invading strand extended was calculated as the ratio of extended invading strand to the total density per lane.

2.8 Statistical analysis. Error bars represent the standard error of the mean. Statistical analysis was performed using GraphPad Prism software. Significance was assessed at the p ≤ .05 level.
<table>
<thead>
<tr>
<th>Oligo</th>
<th>Strand</th>
<th>Length</th>
<th>Sequence (5’ → 3’)</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>“12”</td>
<td>Invading strand (3’ invasion)</td>
<td>41</td>
<td>TAA GAG CAA GAT GTT CTA TAA AAG ATG TCC TAG CAA GGC AC</td>
<td>D-loop</td>
</tr>
<tr>
<td>“13”</td>
<td>Invading strand (5’ invasion)</td>
<td>41</td>
<td>AAA GAT GTC CTA GCA AGG CAC GAT CGA CCCG GAT ATC TAT GA</td>
<td>Inverted D-loop</td>
</tr>
<tr>
<td>“7”</td>
<td>Template strand</td>
<td>61</td>
<td>TGC CAT AGA ATC TCG ACT ATC GCA AAT CAC GCT GCC GAA TTC TAC CAG TGC CTT GCT AGG AGA ACA TCT TTG CCC ACC TGC AGG TTC ACC C</td>
<td>D-loop Inverted D-loop</td>
</tr>
<tr>
<td>“10”</td>
<td>Displaced strand</td>
<td>61</td>
<td>GGG TGA ACC TGC AGG TGG GCG GCT GCT CAT CGT AGG TTA GGT GGT AGA ATT CGG CAG CGT CAT TTG CGA TAG TCG AGA TTC TAT GCA</td>
<td>D-loop Inverted D-loop</td>
</tr>
<tr>
<td>Trap</td>
<td>Trap</td>
<td>27</td>
<td>ATT TGC GAT AGT CGA GAT TCT ATG GCA</td>
<td>D-loop Inverted D-loop</td>
</tr>
</tbody>
</table>

Table 3. **Oligonucleotide compositions of D-loop substrates.** Lengths and sequences of oligonucleotides used to assemble DNA D-loop substrates for unwinding and strand extension reactions. The trap oligo was used as a trap in unwinding reactions.
Results

Construction and validation of D-loop substrates.

To test the ability of T4 helicases to unwind D-loop substrates and modulate Gp43 polymerase-catalyzed D-loop extension, oligonucleotide-based D-loop substrates were constructed by modifying published protocols, as described in Experimental Methods. The construction of the modified D-loops was validated by non-denaturing PAGE (Figure 31).

The full D-loop was annealed as described in Experimental Methods using three oligonucleotides: [$^{32}$P]-labeled invading strand, unlabeled complementary strand, and unlabeled displaced strand. A control substrate was prepared identically except that the displaced strand was excluded from the reaction. A third control, the invading strand alone, was used as a marker. Non-denaturing PAGE shows that that the full D-loop (Figure 31, lane 1) migrates at a higher molecular weight than the control missing the displaced strand (Figure 31, lane 2), which itself migrates at a higher molecular weight than the invading strand alone (Figure 31, lane 3). The full D-loop thus contains all three oligonucleotides and remains intact under the conditions used in non-denaturing PAGE.

DNA unwinding reactions were conducted using either D-loop or inverted D-loop substrates. In the D-loop substrate, the invading strand was configured to form a 3’ heteroduplex and 5’ ssDNA tail (Figure 32A). In the inverted D-loop substrate, the invading strand was configured to form a 5’ heteroduplex and 3’ ssDNA tail (Figure 32B). In either case, the invading strand was 5’-[${}^{32}$P]-labeled so that unwinding catalyzed
by a helicase would generate a labeled product that can be separated from the substrate by non-denaturing PAGE without the need for a trap, allowing the reaction to be quantified by phosphorimaging.

Figure 31. **D-loop substrate construction.** A) Construction of D-loop substrate was confirmed by electrophoresing the presumed D-loop and two control structures in a 6%
non-denaturing polyacrylamide gel and autoradiography. “+” and “-” signs above each lane denote the presence of absence, respectively, of each of the three oligonucleotide strands. In all reactions, only the invading strand is \(^{32}\text{P}\)-labeled. The full D-loop (lane 1) migrates at a higher molecular weight than the invading strand annealed to the complementary strand (lane 2), which migrates at a higher molecular weight than the invading strand alone (lane 3). **B)** Lengths of individual oligonucleotides, dsDNA regions, and ssDNA regions in the D-loop substrate. Note the invading strand is complementary with the entire ssDNA region in the D-loop and therefore there is no gap. The 5’ invading strand in the inverted D-loop has the same lengths of dsDNA (21 nucleotides) and ssDNA (20 nucleotides).
UvsW helicase efficiently unwinds an inverted D-loop substrate.

Experiments in Figure 32A-E were designed to investigate the DNA unwinding activity of UvsW in the context of the two different D-loop DNA structures, which are shown schematically in each panel of Figure 32. The D-loop (Figure 32A) and inverted D-loop (Figure 32B) structures model two different intermediates that UvsW helicase might encounter during DNA replication/recombination/repair. UvsW exhibits strong unwinding activity towards the inverted D-loop substrate (Figure 32D-E). However, UvsW is much less efficient at unwinding the D-loop substrate (Figure 32C and E). The preference of UvsW for the inverted D-loop is logical given the 3’-to-5’ translocation polarity of this helicase; the 20-nucleotide 3’ ssDNA tail of the inverted D-loop appears to offer an optimal loading site for UvsW to unwind the invading strand, which is not available in the 20-nucleotide 5’ ssDNA tail of the D-loop substrate. It is possible that the low level of UvsW unwinding activity towards the D-loop substrate seen in Figure 32C and E may represent an ability of this helicase to load on ssDNA exposed via thermal fraying at the ssDNA nick on the complementary strand formed between the displaced strand and the invading strand, from which the enzyme conceivably could unwind the heteroduplex from its 5’ end.
Helicase unwinding assays were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 2 mM βME, 100 µg/ml BSA, and 10 mM potassium acetate. Reaction components were added in the following order: 50 nM D-loop (or inverted D-loop) substrates and 75 nM UvsW. Unwinding reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5 mM magnesium acetate and quenched by the addition of an equal volume of 200 mM EDTA. Reaction schematic for helicase unwinding assay using the A) D-loop and B) inverted D-loop substrates. Results of DNA unwinding assays by non-denaturing PAGE autoradiography for reactions with C) D-loop and D) inverted D-loop substrates. Intact and unwound D-loops were quantified by densitometry. Fraction of unwound D-loop was calculated as the ratio of unwound product to the total density per lane. E)
Reaction time course showing fraction unwound as a function of time for reactions with D-loop (open squares) and inverted D-loop (open circles) substrates.

**Dda helicase efficiently unwinds a D-loop substrate.**

Similar DNA unwinding assays were carried out to test the ability of Dda helicase to unwind D-loop and inverted D-loop substrates (Figure 33). The substrates used were identical to those used with UvsW helicase in Figure 32, which are shown schematically in Figure 33A-B. Dda was found to unwind the D-loop substrate efficiently, (Figure 33C and E), while the inverted D-loop substrate was unwound very poorly by Dda (Figure 33 D-E). These results are consistent with the 5’ to 3’ unwinding polarity of Dda helicase, which is opposite that of UvsW. The 20-nucleotide 5’ ssDNA tail of the D-loop provides an optimal assembly site for Dda to initiate the unwinding of the invading strand from its 5’ end. Dda appears to access the heteroduplex end of the invading strand even less efficiently than UvsW, perhaps because Dda requires an ssDNA gap size of 6 nucleotides or larger to initiate an unwinding reaction (see Chapter 2, Figure 25). The result is a very strong substrate bias on the part of Dda. These findings are consistent with the known ability of Dda to stimulate 5’ \( \rightarrow \) 3’ branch migration and replication fork progression reactions *in vitro.*
Helicase unwinding assays were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 2 mM βME, 100 µg/ml BSA, and 10 mM potassium acetate. Reaction components were added in the following order: 50 nM D-loop substrate and 1 nM Dda. Unwinding reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5 mM
magnesium acetate and quenched by the addition of an equal volume of 200 mM EDTA. Results of D-loop unwinding assay using A) D-loop and B) inverted D-loop substrates by non-denaturing PAGE autoradiography. Intact and unwound D-loops were quantified by densitometry. Fraction of unwound D-loop was calculated as the ratio of unwound product to the total density per lane. C) Reaction time courses showing fractions unwound as a function of time.

**D-loops and inverted D-loops are poor substrates for Gp41 helicase.**

Similar DNA unwinding assays were carried out to test the ability of Gp41 helicase to unwind D-loop and inverted D-loop substrates (Figure 34). The substrates used were identical to those used with UvsW and Dda helicases in Figures 32-33, which are shown schematically in Figure 34A-B. Gp41 failed to catalyze significant unwinding of either the D-loop or the inverted D-loop substrate (Figure 34C-E). As a 5’-to-3’ helicase, Gp41 might be expected to unwind the D-loop substrate preferentially with respect to the inverted D-loop substrate. However it is known that Gp41 alone requires a relatively long 5’ ssDNA extension on which to initiate translocation and unwinding (Young, Schultz et al. 1994, Jones, Mueser et al. 2000). Therefore the results might be explained by the hexameric nature of Gp41 helicase and the steric limitations imposed by the oligonucleotide-based D-loop substrate.
Helicase unwinding assays were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 2 mM βME, 100 µg/ml BSA, and 10 mM potassium acetate. Reaction components were added in the following order: 50 nM D-loop substrate and 300 nM Gp41. Unwinding reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5
mM magnesium acetate and quenched by the addition of an equal volume of 200 mM EDTA. Results of D-loop unwinding assay using A) D-loop and B) inverted D-loop substrates by non-denaturing PAGE autoradiography. Intact and unwound D-loops were quantified by densitometry. Fraction of D-loops unwound was calculated as the ratios of unwound product to the total density per lane. C) Reaction time courses showing fractions unwound as a function of time.

Gp32 and Gp59, effectors of Gp41 helicase activity, have little effect on Gp41-catalyzed D-loop unwinding.

We were interested in exploring the effect of two known effectors of Gp41 helicase, Gp59 and Gp32 proteins, on the D-loop unwinding activity of Gp41. Gp59 is a mediator protein that loads Gp41 onto ssDNA and nascent replication forks (Ishmael, Alley et al. 2001, Jones, Green et al. 2004, Delagoutte and von Hippel 2005, Branagan, Klein et al. 2014); Gp32 is the T4 ssDNA-binding protein. Gp59 and Gp32 together form Helicase Loading Complexes on ssDNA or on fork DNA that promote Gp41-DNA assembly (Branagan et al. 2012; Branagan et al. 2014). The D-loop unwinding assay was staged as described previously and represented schematically in Figure 35A. The D-loop remained a very poor substrate for Gp41 under all conditions tested (Figure 35B-C). The extent of unwinding never exceeded 3% after one-minute reactions with Gp41 alone or in the presence of Gp59, Gp32, or both Gp59 and Gp32 (Figure 35C). The addition of Gp59 to Gp41 had no effect on D-loop unwinding activity (Figure 35C). The addition of Gp32 to Gp41 caused a small but statistically significant increase in D-loop unwinding that was
largely suppressed by the further addition of Gp59 (Figure 35C). This result strongly suggests that Gp59 and Gp32 are unable to form a Helicase Loading Complex (HLC) on the 5’ ssDNA tail of the D-loop substrate. A previous study demonstrated that HLC formation requires at least 20 nucleotides of ssDNA to accommodate a minimal Gp32 cluster of 3 monomers (Branagan et al. 2012). It seems likely that the D-loop as constructed is sterically incompatible with HLC formation and therefore remains largely inaccessible to Gp41 assembly on the invading strand. Implications of these finding are explored in the Discussion section of this chapter.
Figure 35. Effects of Gp32 SSB and Gp59 helicase loader on Gp41 helicase unwinding of synthetic D-loop DNA substrate. Helicase unwinding assays were based on previously published protocol and performed as described in Experimental
Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 2 mM βME, 100 µg/ml BSA, and 10 mM potassium acetate. Reaction components were added in the following order: 50 nM D-loop substrate, 300 nM each Gp32, Gp59, and Gp41. Reactions were performed in the presence and absence of both Gp32 and Gp59. Controls were performed in which Gp32 and Gp59 were individually replaced with their respective storage buffers. Unwinding reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5 mM magnesium acetate, allowed to proceed for one minute, and quenched by the addition of an equal volume of 200 mM EDTA. 

A) Reaction schematic for helicase D-loop unwinding assay. B) Results of D-loop DNA unwinding assay by non-denaturing PAGE and autoradiography. Intact and unwound D-loops were quantified by densitometry. Fraction of D-loops unwound was calculated as the ratios of unwound product to the total density per lane. C) Quantified fraction invading strand unwound by Gp41 (black), Gp41 + Gp59 (red), Gp41 + Gp32 (green), and Gp41 + Gp32 + Gp59 (blue). p-values calculated using a two-tailed paired Student’s t-test.

**Gp32-B, but not Gp32, promotes D-loop unwinding by Dda.**

Based on findings using DNA substrates that resemble DNA replication forks (Chapter 2), we investigated the possibility that the Gp32-B-mediated enhancement of Dda helicase activity is differentially affected under different DNA structural contexts, which could have implications for the regulation of DNA replication, recombination, and repair. We performed D-loop unwinding assays, depicted in Figure 36A, with Dda in the presence of either Gp32 or Gp32-B. As observed with replication fork intermediates
(Chapter 2), Dda unwinding of the D-loop substrate is significantly enhanced by Gp32-B, but it is not significantly affected by full-length Gp32 (Figure 36B-C).
Figure 36. **Effects of Gp32 SSB and the non-cooperative variant Gp32(-)B on D-loop unwinding by Dda helicase.** Helicase unwinding assays were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 2 mM βME, 100 μg/ml BSA, and 10 mM
potassium acetate. Reaction components were added in the following order: 50 nM D-loop substrate, 100 nM Gp32 or Gp32-B, and 10 nM Dda. Reactions were performed in the presence and absence of either Gp32 or Gp32-B. Controls were performed in which Gp32 and Gp32-B were individually replaced with their respective storage buffers. Unwinding reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5 mM magnesium acetate, allowed to proceed for two minutes, and quenched by the addition of an equal volume of 200 mM EDTA. A) Reaction schematic for helicase D-loop unwinding assay. B) Results of D-loop DNA unwinding assay by non-denaturing PAGE and autoradiography. Intact and unwound D-loops were quantified by densitometry. Fraction of D-loops unwound was calculated as the ratio of unwound product to the total density per lane. C) Quantified fraction invading strand unwound by Dda (black), Dda + Gp32-B (red), and Dda + Gp32 (green). p-values calculated using a two-tailed paired Student’s t-test.

Gp43 and Gp43-D219A catalyze strand displacement DNA synthesis in the D-loop substrate and are stimulated by Gp32.

We tested the ability of Gp43 and the exonuclease deficient variant Gp43-D219A to catalyze strand displacement DNA synthesis using the D-loop substrate. D-loop strand displacement assays, depicted schematically in Figure 37A-B, were conducted using the D-loop DNA substrate with a [\(^{32}\)P]-5’ end-labeled invading strand. Substrates were designed so that strand extension catalyzed by the polymerases would generate a [\(^{32}\)P]-
labeled product that can be separated from the substrate by denaturing PAGE, allowing the reaction to be quantified by phosphorimaging.

Gp43-D219A, the exonuclease deficient mutant, as well as the WT Gp43 polymerase, were able to perform strand displacement DNA synthesis using the invading strand of the D-loop as a primer (Figure 37C-F). Gp32 significantly enhanced the rate of polymerization in a concentration-dependent manner, enhancing optimally at 1 µM and less effectively at 2 µM. Under the conditions tested, Gp43-D219A catalyzed invading strand extension more efficiently than WT Gp43 (Figure 37C-F). The latter is proficient in 3’→5’ exonuclease activity, therefore primer degradation is in competition with strand displacement synthesis, which likely accounts for the lower efficiency of DNA synthesis seen with WT Gp43 alone vs. Gp43-D219A alone (Tanguy Le Gac, Delagoutte et al. 2004). Gp32 enhancement of DNA synthesis was more pronounced with WT Gp43 than with Gp43-D219A (Figure 37 C-F). Gp32 is known to stimulate strand displacement synthesis by Gp43 (Alberts and Frey 1970, Jongeneel, Bedinger et al. 1984, Chase and Williams 1986, Kodadek 1990), which in this assay would help to overcome the competition from primer degradation by the polymerase’s 3’→5’ exonuclease activity. The addition of polymerase holoenzyme components Gp45 (sliding clamp), Gp44/62 (clamp loader), and ATP had no effect on the rate of DNA synthesis in the D-loop substrate (data not shown), indicating that the polymerase subunit is able to carry out strand displacement synthesis through a 20-basepair duplex, especially when Gp32 is present to sequester the displaced strand.
Figure 37. **Strand displacement DNA synthesis by Gp43 polymerase and the exonuclease-deficient variant Gp43-D219A in the presence of Gp32 SSB.** Invading strand extension assays were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 2 mM βME, 100 µg/ml BSA, and 40 mM sodium acetate. 150 µM each of dATP, TTP, dCTP, and dGTP, was added to the buffer of all strand displacement DNA synthesis reactions. Reactions components were added in the following order: 50 nM D-loop substrate, 1 µM or 2 µM Gp32 as specified, and 250 nM Gp43 or Gp43-D219A polymerase. Reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5
mM magnesium acetate, allowed to proceed for one minute, and quenched by the addition of an equal volume of 200 mM EDTA. **A)** Reaction schematic of strand displacement DNA synthesis reactions using Gp43 WT or Gp43-D219A. Results of the strand displacement DNA synthesis assays using **B)** Gp43-D219A and **C)** Gp43 WT in the presence and absence of Gp32 SSB by denaturing PAGE and autoradiography. Extended and unextended invading strands were quantified by densitometry. Fraction invading strand extended was calculated as the ratio of extended product to the total density per lane. **D)** Quantified fraction invading strand extended by Gp43-D219A (**red**), Gp43-D219A $+ 1 \, \mu$M Gp32 (**black**), and Gp43-D219A $+ 2 \, \mu$M Gp32 (**blue**). **E)** Quantified fraction invading strand extended by Gp43 (**green**), Gp43 $+ 1 \, \mu$M Gp32 (**purple**), and Gp43-D219A $+ 2 \, \mu$M Gp32 (**orange**). p-values calculated using a two-tailed paired Student’s t-test.
Figure 38. Schematic of the possible effects of DNA helicase addition on the replicative extension of a D-loop. A) D-loop substrate with invading strand (*illustrated in red*) with a 5’ [*32P*] radiolabel (*depicted by an asterisk*). B) Helicases that preferentially catalyze D-loop unwinding will inhibit strand displacement DNA synthesis and produce an unwound invading strand without extension. C) Partial invading strand extension would result from a mixed function helicase that participates both in strand displacement
synthesis and D-loop unwinding. **D)** Helicases that preferentially promote strand displacement synthesis will increase the fraction of invading strand that is extended by polymerase. **E)** Idealized appearance of bands on a denaturing polyacrylamide gel under the three scenarios described in panels B-D.

**Dda helicase suppresses D-loop extension by Gp43 polymerase in the presence of Gp32.**

The structure of a D-loop potentially presents multiple sites of action for a helicase, and hence different opportunities to regulate DNA synthesis within the D-loop. Figure 38 illustrates this principle. A helicase that preferentially unwinds D-loops might fully inhibit strand displacement DNA synthesis primed by the invading strand, which would suggest an anti-recombination function for that helicase (Figure 38B, E). A replicative helicase would preferentially enhance the rate of strand displacement synthesis, producing more extended products (Figure 38D-E), and a mixed-function helicase that promotes both strand displacement synthesis and D-loop unwinding might produce a ladder of intermediately sized products (Figure 38C, E).

We tested the effects of Dda helicase on Gp43-catalyzed strand displacement DNA synthesis using the D-loop substrate as depicted schematically in Figure 39A. We observed no change in the rate of strand displacement DNA synthesis in the presence of Dda alone (Figure 39B-C). This finding is consistent with previous reports that the stimulation of strand displacement DNA synthesis by Dda requires Gp32 (Jongeneel, Bedinger et al. 1984, Ma, Wang et al. 2004). Gp32 alone enhances strand displacement
DNA synthesis (Figure 39B-C), consistent with other experiments (Figure 37C and E). Surprisingly, the addition of Dda to reactions containing Gp43 and Gp32 largely suppressed the stimulation of DNA synthesis that was seen with Gp32 alone, with most of the labeled DNA appearing as unextended invading strand (Figure 39B-C). The data suggest that, at least for the D-loop substrate used in these experiments, the D-loop unwinding activity of Dda dominates over the stimulation of strand displacement DNA synthesis. This suggests that Dda negatively regulates D-loop extension by Gp43 polymerase, which is consistent with an anti-recombination function for this helicase.
Figure 39. **Effects of Gp32 SSB and Dda helicase on strand displacement DNA synthesis by Gp43 polymerase.** Invading strand extension assays were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 2 mM βME, 100 μg/ml BSA, and 40 mM sodium acetate. dATP, TTP, dCTP, and dGTP to a final concentration of 150 μM each was added to the buffer of all reactions. Reaction components were added in the following order: 50 nM D-loop substrate, 1 μM Gp32, 10 nM Dda, and 250 nM Gp43. Reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5 mM magnesium acetate, allowed to proceed for one minute, and quenched by the addition of an equal volume of 200 mM EDTA. A) Schematic of the strand displacement DNA synthesis assay. B) Results of strand displacement DNA synthesis assays in the presence and absence of Gp32 SSB and Dda helicase by denaturing PAGE and autoradiography. Extended and unextended invading strands were quantified by densitometry. Fraction invading strand extended was calculated as the ratio of extended product to the total density per lane. C) Quantified fractions of extended invading strand in the presence of Gp43 polymerase (*red*), Gp43 polymerase + Dda helicase (*gray*), Gp43 polymerase + Gp32 SSB (*black*), and Gp43 polymerase + Gp32 SSB + Dda helicase (*blue*). p-values calculated using a two-tailed paired Student’s t-test.

**Gp41 helicase has minimal effects on Gp43-catalyzed D-loop extension.**

Despite the absence of Gp41-catalyzed D-loop unwinding, we tested the possibility that Gp41 has an effect on strand displacement DNA synthesis in the D-loop substrate;
reactions were carried out in the absence or presence of Gp32 SSB and Gp59 helicase loader (Figure 40A). As shown in Figure 40B-C, the addition of Gp41 alone does not have a significant effect on DNA synthesis catalyzed by Gp43 polymerase. The addition of Gp32 alone stimulates DNA synthesis (Figure 40B-C) as we observed previously (Figure 39). The reactions run in the presence of both Gp41 and Gp32 are indistinguishable from those run with Gp32 alone, however, indicating that Gp41 neither stimulates strand displacement synthesis nor unwinds the D-loop under these conditions (Figure 40B-C). In the absence of Gp32, the simultaneous addition of Gp59 and Gp41 yields a slight increase in the amount of primer extension catalyzed by DNA polymerase; however the reaction containing Gp32, Gp59, and Gp41 is indistinguishable from the reaction with Gp32 alone (Figure 40B-C). These results indicate that ssDNA regions of both the invading and displaced strands of the D-loop remain inaccessible to Gp41 helicase even as DNA synthesis catalyzed by Gp43 and stimulated by Gp32 ensues. The fact that Gp59 does not improve the situation indicates that formation of the Gp59-Gp32-ssDNA Helicase Loading Complex is not accommodated by the D-loop structure used in these experiments.
Figure 40. **Effects of Gp41 helicase on strand displacement DNA synthesis by Gp43 polymerase on invading strand of synthetic D-loop in the presence and absence of Gp32 SSB and Gp59 helicase loader.** Strand displacement DNA synthesis assays were based on previously published protocol and performed as described in Experimental Procedures. Reaction buffer contained 25 mM HEPES (pH 7.5), 2 mM βME, 100 µg/ml BSA, and 40 mM sodium acetate. 150 µM each of dATP, TTP, dCTP, and dGTP, was added to the buffer of all DNA synthesis reactions. Reaction components were added in the following order: 50 nM D-loop substrate, 1 µM Gp32, 1 µM Gp59, 1 µM Gp41, and 250 nM Gp43 polymerase. Reactions were initiated by the simultaneous addition of 4.6 mM ATP and 5 mM magnesium acetate, allowed to proceed for one minute, and quenched by the addition of an equal volume of 200 mM EDTA. **A)** Schematic of the strand displacement DNA synthesis assay. **B)** Results of strand displacement DNA synthesis assays by denaturing PAGE and autoradiography. Extended and unextended invading strands were quantified by densitometry. Fraction invading strand extended was calculated as the ratio of extended product to the total density per lane. **C)** Quantified fractions of extended invading strand in the presence of Gp43 (*black*), Gp43 + Gp32 (*red*), Gp43 + Gp41 (*blue*), Gp43 + Gp59 (*green*), Gp43 + Gp32 + Gp41 (*magenta*), Gp43 + Gp41 + Gp59 (*orange*), or Gp43 + Gp32 + Gp41 + Gp59 (*purple*). p-values calculated using a two-tailed paired Student’s t-test.


Discussion

The data presented here show the interactions between T4 DNA replication, recombination and repair components in the context of a D-loop, the initial DNA intermediate in homologous recombination produced by strand invasion. The data provide evidence supporting the involvement of helicase activity in the regulation of recombination-dependent DNA synthesis.

_UvsW and Dda, but not Gp41, unwind D-loop substrates._ We tested the ability of the three T4-encoded helicases to unwind a D-loop invading strand. We found that Dda and UvsW, but not Gp41, are able to catalyze DNA unwinding of the D-loop invading strand. UvsW, which is the only T4 helicase to unwind with a 3’ to 5’ polarity, could only efficiently unwind the inverted D-loop substrate in which the invading strand contained a 3’ ssDNA tail and a 5’ heteroduplex end (Figure 32). Even though most models of recombination initiated from strand invasion assume that the invading strand of the D-loop contains a 3’ heteroduplex end, so as to allow for the priming of leading strand DNA synthesis (Kreuzer and Brister 2010, Liu and Morrical 2010, Lo Piano, Martinez-Jimenez et al. 2011, Maher, Branagan et al. 2011), studies have shown that recombination can also initiate with an invading 5’ end (Rosenberg and Hastings 1991).

Dda efficiently unwinds a D-loop substrate containing a 5’ ssDNA tail and a 3’ heteroduplex end (Figure 33), consistent with the 5’ → 3’ polarity of this helicase. D-loop unwinding by Dda is stimulated by Gp32-B (Figure 36), consistent with the effects
of this variant in previous studies using replication fork substrates (Chapter 2). Full-length Gp32 has a slight inhibitory effect on Dda unwinding of the D-loop substrate (Figure 36), whereas it exhibits weak to strong inhibition of Dda unwinding of various replication intermediates (Chapter 2). The D-loop unwinding activity of Dda competes with D-loop extension catalyzed by Gp43 polymerase in the presence of Gp32, resulting in a decreased frequency of invading strand extension when all three proteins are present (Figure 39). This D-loop unwinding activity of Dda could therefore act as a form of anti-recombination, to negatively regulate the replicative extension of D-loops (Bachrati, Borts et al. 2006). The inhibition of D-loop extension by Dda is not absolute, however. A lower level of invading strand extension is still observed when Dda is added to reactions with Gp43 polymerase in the absence or presence of Gp32 (Figure 39). Therefore under the right conditions it is possible to have invading strand extension and unwinding going on at the same time. The result would be a translocating D-loop or “bubble migration synthesis”, a hallmark of SDSA, in which the extended invading strand is actively displaced from the template via branch migration (Figure 5). This is exactly what is observed in a more complete in vitro system for bubble migration synthesis containing DNA polymerase holoenzyme, Dda, Gp32, UvsX recombinase, and long, M13-derived ssDNA and dsDNA molecules (Formosa and Alberts, 1986).

Studies have shown that Dda works to displace a polymerase that is stalled due to an abasic site followed by a guanosine nucleotide on the template strand in the absence of dCTP. In these studies, Dda promoted bypass of the DNA damage by catalyzing strand switching of the polymerase, which could play a key role in survival after DNA damage
(Kadyrov and Drake 2004). In those experiments, Dda loading was restricted to the template strand ahead of the polymerase, displacing the polymerase and unwinding the nascent strand while translocating 5’ to 3’ on the template strand. We demonstrated a similar effect in Figures 24 and 27, Chapter 2 of this thesis, but also noted that the activity of Dda in unwinding the primer terminus is regulated by Gp32. In our D-loop substrate, however, no ssDNA gap is present on the template strand ahead of the polymerase, therefore Dda loading is restricted either to the ssDNA tail of the invading strand or to the displaced strand. In such a configuration Dda would not have the opportunity to displace a bound polymerase, which may account for the residual level of D-loop extension activity observed when Dda is added to reactions with Gp43 +/- Gp32 (Figure 39). These results imply that whether Dda promotes or inhibits strand displacement DNA synthesis primed by a D-loop invading strand is likely to be a function of the site of Dda loading, which in turn may be controlled by interactions with other factors including Gp32 and UvsX recombinase.

*Gp41 helicase does not catalyze D-loop invading strand unwinding.* Even though UvsW and Dda were able to unwind their preferred D-loop substrates efficiently, Gp41 did not unwind D-loops, even in the presence of the Helicase Loading Complex components, Gp32 and Gp59. One key difference between Gp41 and Dda and UvsW that could explain this discrepancy is that UvsW and Dda function as monomeric helicases, whereas Gp41 forms a hexameric ring that encircles the DNA (Liu and Alberts 1981, Venkatesan, Silver et al. 1982, Richardson and Nossal 1989, Young, Schultz et al. 1994, Dong, Gogol
et al. 1995). It is possible the twenty-base ssDNA tail on the invading strand of the D-loop substrate is insufficient for efficient helicase loading and/or assembly. We note from previously published work that formation of Gp59-Gp32-ssDNA Helicase Loading Complexes is efficient on ssDNA lengths of 40 nucleotides or greater, weak on 20 nucleotides of ssDNA, and non-existent on 12 nucleotides of ssDNA (Branagan et al. 2012). Furthermore, given the affinity preference of Gp59 helicase loader for replication fork DNA structures, it is expected that Gp59 would facilitate loading onto the displaced strand of the D-loop, and not necessarily on the invading strand (Branagan et al. 2014).

The absence of a stimulatory effect of Gp41 +/- Gp59 on D-loop extension in our assays indicates that small D-loop structures are inaccessible to processing by Gp41 helicase. This may indicate a physiological niche for Dda and possibly UvsW in processing smaller recombination intermediates, perhaps to set the stage for later assembly of Gp41 helicase once a D-loop has been extended beyond a certain size threshold.

Our findings on the modulation of D-loop invading strand extension by helicases in the T4 system have implications on the role of helicases in recombination. In T4, Dda promotes invading strand branch migration without displacing Gp43 polymerase, supporting the notion that Dda promotes bubble migration and strand annealing DNA synthesis during recombination-dependent DNA replication. Gp41 cannot perform the same task on our D-loop substrates, perhaps a reflection of their differences between Dda and Gp41 as monomeric and hexameric helicases, respectively. The size and availability of ssDNA regions generated during DNA metabolism and their accessibility due to the
relative abundance of Gp32 SSB may all play important roles in regulating their functional roles.

Understanding the regulation of D-loops by DNA helicases is of interest in the fields of DNA repair and in the study of diseases in which genomic instability plays a pathological role, such as cancer. Our findings indicate that helicases play important regulatory functions beyond their unwinding activity and are likely to be involved in coordinating SSB protein-protein interactions with other enzymes at the replication fork and in DNA repair and recombination intermediates.
Conflicts of interest statement

The authors declare that there are no conflicts of interest.
Acknowledgements

The authors thank Dr. Stephen J. Benkovic for providing the overexpression vector for Gp32 and Jennifer Tomczak for technical support. This work was supported by National Institutes of Health (NIH) research grant R01GM48847 to SWM. CSJ was supported by NIH training grant T32ES07122. The sponsors had no involvement in study design, the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the article for publication.
References


CHAPTER 4: CONCLUSIONS

4.1. Evaluation of major techniques

In this dissertation we have investigated the roles and regulation of helicases in the bacteriophage T4 DNA replication, recombination, and repair systems. Our experimental approaches include both novel and established analytic techniques, which require their own analysis and consideration. The techniques described in chapters two and three are critical in the study of the functional regulation of Dda helicase and its interactions with other T4 proteins. The techniques are evaluated in this section and the advantages and limitations of each are discussed.

4.1.1. Quantification of radiolabeled oligonucleotides by phosphor autoradiography and densitometry

Radioactivity is an exceptionally useful tracer for biological molecules. $^{32}$P, the isotope most commonly used to label DNA, is the isotope we use in our laboratory and in the studies described in this dissertation. $^{32}$P is covalently bound to the 5’ hydroxyl of a DNA strand by the enzymatic activity of T4 polynucleotide kinase. DNA samples that are separated by agarose or polyacrylamide gel electrophoresis can be visualized by exposing the gel to a phosphor screen or an x-ray film. Phosphor screen autoradiography has replaced x-ray film autoradiography due to its shorter exposure time, increased sensitivity, greater linear dynamic range, room temperature exposure, reusability, and simplicity (no chemicals, darkroom, or special treatments).
Phosphors are compounds that absorb energy at one wavelength, store it, and re-emit it at another wavelength when stimulated by light of a particular energy. A phosphor screen (we use a Kodak K-screen) is a flexible polyester film with a grains that are 5 micrometers in size that contain fine crystals of barium fluorobromide with a trace amount of bivalent europium that functions as the luminescence center (BaFBr:Eu$^{+2}$).

When the radioactive sample is placed on the phosphor screen, the high-energy radiation from the $^{32}$P-labeled oligonucleotides excites Eu$^{+2}$ electrons and Eu$^{+2}$ is oxidized to Eu$^{+3}$ (while BaFBr is reduced to BaFBr$^-$). The compounds remain oxidized and reduced after the phosphor screen is removed from the sample; the screen retains more than 80% of the signal after three hours.

The screen is scanned with a helium-neon laser that emits red light at 633 nm. The charged BaFBr$^-$ complex absorbs light in that range and the absorbed energy frees electrons and reduces Eu$^{+3}$ to Eu$^{+2*}$. As Eu$^{+2*}$ returns to the ground state, it releases energy as blue light; the level of the signal is proportional to the amount of radioactivity. A fiber-optics bundle collects the blue light and passes it to a photomultiplier tube (PMT), which converts the light into an electrical current and a digital image.

This is the optimal technique for detecting DNA of different molecular weights in our functional assays, which depend on the physical separation of starting reagents and reaction products. The extent of product formation can be quantified as a ratio of the quantified reagents and products. A principal advantage of this approach is that a single DNA strand can be selectively monitored; all other unlabeled strands do not add noise or confound the analysis. Another major advantage is the sensitivity of this method
compared to other DNA-visualizing methods such as staining with UV-fluorescent intercalating agents such as ethidium bromide or SYBR Gold. This has two benefits. The first is that it allows us to use less reagents such as costly and difficult-to-purify enzymes. The second benefit is that UV-fluorescent intercalating agents stain the gel matrix and increase the background of the gel image. This is important because the results using radiolabeled DNA is more quantitative than stained DNA due to the much higher signal-to-noise ratio. Finally, the $^{32}$P-labeling of DNA is inexpensive and simple.

In this dissertation we have modified the application of phosphorimaging that were developed previously to compare the DNA unwinding activity of Dda under different conditions, different DNA substrates, and in the presence of other T4 proteins.

4.1.2. Spectrophotometric ATPase assays

Another important technique employed in the studies presented in this dissertation is the spectrophotometric assay for the quantification of helicase ATPase activity. This approach was designed after a previously published ATPase assay in which the consumption of NADH is coupled to ATP hydrolysis (Morrical, Lee et al. 1986; Morrical, Hempstead et al. 1994). This assay was used to test the ATPase rate of Dda using substrates of different sequences and lengths in the presence of Gp32 and Gp32(-)B.

In this assay, hydrolyzed ATP is continuously regenerated, a process that is coupled to the oxidation of NADH. After each cycle of ATP hydrolysis, phosphoenolpyruvate kinase catalyzes the phosphorylation of ADP to ATP, consuming
one molecule of phosphoenolpyruvate and generating pyruvate. Pyruvate is immediately converted to lactate by lactate dehydrogenase, resulting in the oxidation of NADH to NAD$^+$. The amount of NADH in the reaction is monitored spectrophotometrically by measuring absorbance at 340 nm. The rate of decrease in absorbance is proportional to the rate NADH oxidation and the rate of steady-state ATP hydrolysis. The constant regeneration of ATP allows monitoring the ATP hydrolysis rate in real time continuously over the course of the assay.

One of the main advantages of using this assay is that the absorbance of NADH is measured in real time, and therefore we are able to calculate enzymatic rates from that measurement; in other words, it is a functional quantitative assay. This has been very useful for determining the effects of Gp32 on Dda ATPase function. Since ATP is continuously regenerated, initial velocity conditions are maintained over the course of the entire assay. This is due to the fact that there is no product inhibition by ADP and the concentration of ATP remains constant. A disadvantage of this technique is the large amount of reagents required, making it costly with respect to materials needed. An alternative method we use in our lab to measure ATPase activity is using thin layer chromatography (TLC), which is a direct method of measuring relative levels of ATP vs. ADP during a reaction. However, given there are sufficient starting materials for the coupled spectrophotometric ATPase assay, it is an advantageous quantitative technique and has been used successfully in these studies.
Figure 41. **Schematic of the spectrophotometric NADH-coupled ATPase assay.** ATP is hydrolyzed to ADP by Dda helicase and regenerated by phosphoenolpyruvate (PEP) kinase, generating pyruvate from PEP. Lactate dehydrogenase converts pyruvate to lactate by consuming NADH and generating NAD\(^+\). The concentration of NADH, which absorbs light at 340 nm, is continuously measured spectrophotometrically. The rate of consumption of NADH directly correlates with the rate of ATP hydrolysis.
4.2. Discussion

Our studies have added to our understanding of the function of helicases in the regulation of DNA replication, recombination and repair in bacteriophage T4. The major overall conclusions can be summarized as follows: 1) Gp32 and Dda form a high affinity complex, both with each other and with ssDNA, and these two interactions mediate two types of regulation between Dda and Gp32. Gp32 can inhibit Dda from loading onto ssDNA binding sites and can interact to stimulate strand displacement DNA synthesis by T4 polymerase holoenzyme. Gp32 and Dda regulate each other by two distinct mechanisms, directly and indirectly. 2) Gp32(-)B, which does not bind ssDNA cooperatively, can form a complex with Dda and stimulate Dda DNA unwinding and ATPase activities. This variant likely exposes a Dda-binding site that is kept cryptic in Gp32 in the absence of the polymerase holoenzyme complex. In this section we will discuss our major conclusions with respect to DNA replication, repair, and recombination and the broader field of genome maintenance. We will also discuss potential future directions that address remaining unanswered questions.

4.2.1. The role and regulation of Dda in DNA replication repair

Dda is a versatile highly active monomeric helicase that can bind ssDNA gaps as small as six nucleotides in length and unwind DNA and displace DNA-bound proteins in the 5’-to-3’ direction. Despite the monomeric nature of the enzyme, multiple Dda molecules bound to the same ssDNA can act cooperatively via an inchworm cooperative
mechanism (Byrd and Raney 2005, Spurling, Eoff et al. 2006, Eoff and Raney 2010). These characteristics make Dda a potentially highly versatile regulator of DNA metabolic processes including replication, recombination and repair. Even though it has been characterized biochemically, its \textit{in vivo} functions remain an active area of research. Our studies on Dda, its activity on different DNA substrates, and its regulation by the SSB Gp32 presented in Chapter 2 have provided novel insights on its biological roles and the implications of these findings are discussed here.

4.2.1.1. Uncoupling of leading and lagging strand DNA synthesis

The coupling of leading and lagging strand DNA synthesis is important to maintain the amount of ssDNA in the cell at a minimum. The generation of excess ssDNA is undesirable because of increased susceptibility to enzymatic cleavage, thermal (spontaneous) damage and mutagenesis, damage and mutagenesis due to chemicals, radiation, and free radicals (\textit{i.e.}, reactive oxygen and nitrogen species), and the generation of secondary structures that can interfere with DNA metabolism, transcription, segregation and packaging.

Leading and lagging strand DNA synthesis can become uncoupled when the leading strand polymerase is stalled by the presence of a ssDNA lesion on the template strand; stalling can be due to diverse types of ssDNA damage including but not limited to abasic sites, oxidized bases, and ssDNA breaks. Uncontrolled unwinding ahead of the DNA replication fork in the presence of a stalled leading strand polymerase results in the uncoupling of lagging and leading strand synthesis; the lagging strand replication
machinery continues to generate Okazaki fragments and the DNA ahead of the damaged site (the stalled polymerase) remains single-stranded and susceptible to additional damage.

Even though Dda is not the replicative helicase in the bacteriophage T4 system, it can stimulate strand displacement DNA synthesis in the presence of Gp32 (Jongeneel, Bedinger et al. 1984), allow the replicative machinery to displace DNA-bound protein blocks (Bedinger, Hochstrasser et al. 1983, Byrd and Raney 2006), and there is evidence that suggests that Dda can substitute for the replicative helicase in T4, Gp41, when the Gp41-loader Gp59 is absent (Doherty, Gauss et al. 1982, Gauss, Doherty et al. 1983, Jongeneel, Bedinger et al. 1984, Gauss, Park et al. 1994). Finally, Gp32-B, a non-cooperative variant of Gp32, is able to substitute Gp32 in strand displacement DNA synthesis reactions, indicating that their physical association is playing a role in the stimulation of leading strand DNA replication (Ma, Wang et al. 2004).

Under normal conditions, when leading and lagging strand synthesis are coupled, Dda loading is restricted to the parental lagging strand, positioning it so it can unwind the DNA replication fork ahead of the leading strand polymerase, as does Gp41. However, in the case of leading strand polymerase stalling due to DNA damage, ssDNA is generated on the parental leading strand where Dda can load. Under these conditions, Dda can translocate on the parental leading strand, displace the stalled polymerase, and unwind the 3' end of the daughter leading strand. This generates a 3' ssDNA overhang that can form a nucleoprotein filament with UvsX recombinase that can catalyze DNA recombination with a homologous duplex (i.e. the daughter lagging strand).
4.2.1.2. Displacement of proteins in a stalled replication fork

Our findings suggest that Gp32 plays an active role in restricting Dda loading to the correct ssDNA loci in a stalled replication fork in order to stimulate DNA repair and prevent further uncoupling of leading and lagging strand DNA synthesis. An ssDNA gap in front of the stalled leading strand polymerase is immediately covered by Gp32. Our findings demonstrate that this cluster acts to block Dda loading at the lagging strand but does not interfere with Dda loading at the leading strand. Our data using an inverted fork mimic DNA substrate shows that a Gp32 cluster on leading strand ssDNA prevents Dda from unwinding the replication fork, potentially by inhibiting loading of Dda to the lagging strand (Fig. 24D). However, Dda is able to unwind Gp32-covered ssDNA (Figure 24E) and is able to displace a stalled polymerase and unwind the 3’ end of the daughter strand from its template (Figure 27).

The unwinding of a stalled daughter leading strand is necessary for template switching promoted by UvsX recombinase in the presence of Dda. Successive UvsX- and Dda-dependent template switching regresses a lesion-stalled replication fork and allows for error-free translesion DNA synthesis by Gp43 polymerase (Bleuit et al., 2001; Kadyrov & Drake, 2004). The results presented in Chapter 2 suggest that helicase trafficking by Gp32 could play a role in preventing premature fork progression, stimulating template switching events, and coordinating the steps necessary for error-free translesion DNA synthesis in the process of replication fork restart. Gp32 may have similar functions in other forms of recombination-dependent DNA repair.
4.2.1.3. Interactions with Gp32

Our studies show that the interaction between Gp32 and Dda is DNA substrate-specific. In all but one substrate tested, Gp32 weakly inhibit Dda unwinding activity; in the context of an inverted fork mimic, which models a structure that can result from a stalled replication fork, Gp32 strongly inhibits Dda unwinding. Their interaction is also dependent on the presence of other replication components. Studies performed in our and others’ laboratories show that either Gp32 or Gp32-B, a non-cooperative variant, are required for Dda to stimulate strand displacement DNA synthesis by DNA polymerase holoenzyme (Cha & Alberts, 1989; Formosa & Alberts, 1986; Jongeneel, Bedinger, et al., 1984; Ma et al., 2004; Villemain et al., 2000).

Differing from the results obtained with Gp32, we found that Gp32-B stimulates Dda ATPase and unwinding activities, enhances its protein-displacing activity, and stimulates unwinding in the presence of increasing salt concentrations. This can be explained by the possibility that the N-terminal truncation that generates Gp32-B exposes a cryptic helicase stimulatory site that is revealed in the wild-type T4 replication fork in vivo or in vitro as a consequence of a moving polymerase or through direct interactions of Gp32 with other replisome components.

The increased rate of Dda unwinding activity and ATP hydrolysis catalytic efficiency (Chapter 2, Table 2) in the presence of Gp32-B could represent an increase in translocation velocity and/or processivity as well as an increase in the helicase efficiency/turnover of Dda (i.e. a greater probability of a productive step coupled to each
ATP hydrolysis event). The processivity of Dda is also increased by the presence of multiple Dda molecules translocating on the same region of ssDNA via a “cooperative inchworm” mechanism (Byrd & Raney, 2005; Eoff & Raney, 2010; Spurling, Eoff, & Raney, 2006). Our working hypothesis is that Gp32-B (or cryptically, Gp32) promotes the assembly of multiple Dda molecules on ssDNA generated by unwinding by an initial monomeric helicase. The observed rate acceleration may represent an enhanced cooperative inchworm mechanism that may be studied using pre-steady-state and steady-state kinetics assays. SSBs functioning as helicase processivity factors have been described in at least two other systems (Boehmer, 1998; Shereda, Bernstein, & Keck, 2007).

4.2.2. Helicase D-loop processing

The repair of chromosomal double strand breaks (DSBs) is crucial for the maintenance of genomic integrity. DSBs can be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). The D-loop DNA structure generated during homologous recombination is a universal feature of HDR and its processing is one of the determining factors in the ultimate mechanism of repair. Our findings on the specificity of the three T4-encoded helicase in the processing of D-loops support and add insights to the increasingly important notion that helicases play critical roles in DNA recombination repair and replication, which has implications for the study of mutagenesis, loss of heterozygosity, translocations and copy number variations, all hallmarks of

4.2.2.1. Branch migration

Helicases can process D-loop to inhibit recombination or to promote either conservative or semi-conservative recombination-dependent DNA replication. Helicases can inhibit recombination-dependent replication and repair by disassembling D-loops, unwinding the invading strand and removing the 3’ hydroxyl required to prime DNA replication. Helicases in the RecQ family, for example, can function to inhibit illegitimate recombination in vitro and in vivo (Hanada, Ukita et al. 1997, Harmon and Kowalczykowski 1998, Hanada, Iwasaki et al. 2000, Wu and Hickson 2001, Bachrati, Borts et al. 2006). Alternatively, helicases can unwind the invading strand behind an active polymerase without inhibiting DNA synthesis in a process known as branch migration. Branch migration coupled to DNA replication can result in synthesis dependent strand annealing (SDSA) or break-induced repair (BIR), two related but distinct forms of HDR used to repair single and double stranded DNA breaks.

In the T4 system, Dda and UvsW, but not Gp41, are able to catalyze DNA unwinding of a D-loop invading strand, suggesting a role in branch migration during recombination-dependent replication initiation and in SDSA and/or BIR. Furthermore, invading strand DNA unwinding by Dda does not inhibit invading strand extension by Gp43 polymerase, indicating Dda does not displace Gp43 polymerase when translocating
on the nascent strand, further supporting the role of Dda in branch migration. Branch migration coupled with invading strand extension results in a translocating D-loop or “bubble migration synthesis”, a hallmark of synthesis break-induced repair (BIR) and synthesis-dependent strand annealing (SDSA) (Figure 5), two major pathways of HDR.

4.2.2.2. SDSA and BIR

The finding that Dda can catalyze branch migration supports a role for Dda in recombination-dependent replication and repair. However, the notion that Dda does not displace the invading strand once strand extension has begun suggests Dda may promote DNA synthesis by break-induced repair (BIR), in which branch migration is coupled with extensive invading strand extension. BIR can produce a bona fide replication fork with two semiconservatively replicated molecules when the displaced strand is used as a template for lagging strand DNA synthesis (Malkova, Naylor et al. 2005, Lydeard, Lipkin-Moore et al. 2010) or conservative DNA synthesis when there is asynchronous leading and lagging strand replication (Smith, Llorente et al. 2007, Llorente, Smith et al. 2008, Malkova and Ira 2013).

Our findings support a role for Dda in either SDSA or BIR. Dda unwinding of the invading strand supports a role for Dda in both processes. The ability for Dda to displace the invading strand without inhibiting invading strand extension also supports a role for Dda in both BIR and SDSA.

4.2.2.3. Crossover control
The choice of the HDR pathway determines whether there will be chromosomal crossover or loss of heterozygosity as a consequence of the repair. A D-loop in which the invading strand is not processed by helicases (i.e., no branch migration) will undergo repair by the double Holliday Junction (dHJ) pathway. The D-loop generated by the displaced strand is expanded by invading strand extension, the second ssDNA overhang generated by the DSB anneals to it during second end capture. This second ssDNA overhang primes DNA replication using the homologous displaced strand as a template and generates a double Holliday Junction. This structure translocates and depending on how the double Holliday Junction is resolved, the resulting chromosomes may crossover and undergo gene conversion (Helleday, Lo et al. 2007) (Figure 4).

Unlike UvsW and Dda, which unwound their preferred D-loop substrates efficiently, Gp41 did not unwind D-loops, alone or in the presence of Gp32 and Gp59, which comprise the Helicase Loading Complex. Gp41 is a hexameric helicase (Liu and Alberts 1981, Venkatesan, Silver et al. 1982, Richardson and Nossal 1989, Young, Schultz et al. 1994, Dong, Gogol et al. 1995), and relative to the monomeric helicases UvsW and Dda, may require longer ssDNA for efficient loading. Furthermore, the affinity preference of Gp59 helicase loader for replication fork DNA structures suggests that Gp59 would facilitate loading onto the displaced strand of the D-loop (Branagan et al. 2014). Our findings suggest Gp41 does not promote SDSA or BIR and instead favors the dHJ mechanism of HDR in DBR. The trafficking of D-loops down different repair pathways controls the generation of genomic crossovers and regions of loss of heterozygosity.
The helicase substrate specificity demonstrated in the T4 system has implications in the fields of genome instability, oncology, and therapeutics. Helicases are receiving increased attention with respect to their central role in processing DNA replication, recombination, and repair intermediates such as D-loops. The specialization of helicases in the promotion or inhibition of crossover-generating modes of DSB repair amplifies the importance of helicases in genetic recombination and genomic integrity.
III. Future directions

Our findings on the nature and function of Gp32-Dda interactions have provided new hypotheses to lead future research directions. It needs to be definitively established that Gp32 is displaced from ssDNA by Dda, and this displacement needs to be characterized as a function of the length of the Gp32 cluster, number of Dda molecules loaded on a DNA strand, etc. These studies will clarify the mechanism of the inhibition of Dda helicase by high concentrations of Gp32. One possible approach is the use of fluorescence spectroscopy and fluorescein- or rhodamine-labeled Gp32. The fluorescence of these species is expected to change in the ssDNA-bound and unbound state, and the effect of Dda on this parameter can be tested directly. The most significant potential limitation of this approach is the interruption of Gp32-Dda protein-protein interactions by the extrinsic fluorophore. Other approaches include DNA unwinding assays with carefully-designed DNA substrates to directly test the hypothesis that Dda displaces Gp32 from ssDNA. These DNA substrates must be designed to have few regions of ssDNA in order to restrict loading of proteins to the desired area.

Similar DNA unwinding assay substrates can be designed to probe further into the effect of Gp32 clusters on leading strand ssDNA on Dda loading onto lagging strand ssDNA. The most important unanswered question in this scenario is whether Dda is preferentially loaded onto the leading strand template under these conditions. An assay incorporating two radiolabeled oligonucleotides of significantly different lengths can be easily designed to answer this question directly and unequivocally.
Dda is unique among the T4 helicases in its ability to displace DNA-bound proteins such as *lac* transcriptional repressor, RNA polymerase, and DNA polymerase. The effect of the topological relationship between Dda and the DNA-bound protein is a variable that has not been fully appreciated or studied. Our studies suggest that Dda is able to displace Gp43 DNA polymerase only if it is translocating on the strand the polymerase is using as a template; Dda loaded on the nascent strand does not seem to displace Gp43 or inhibit its function. This hypothesis also needs to be investigated comprehensively using radiolabeled oligonucleotides and non-denaturing PAGE.
REFERENCES


