Characterization of the Bacteriophage T4 Gene 41 DNA Helicase*

(Received for publication, September 2, 1988)

Ross W. Richardson and Nancy G. Nossal†

From the Section on Nucleic Acid Biochemistry, Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

The T4 gene 41 protein and the gene 61 protein function together as a primase-helicase within the seven protein bacteriophage T4 multienzyme complex that replicates duplex DNA in vitro. We have previously shown that the 41 protein is a 5' to 3' helicase that requires a single-stranded region on the 5' side of the duplex to be unwound and is stimulated by the 61 protein (Venkatesan, M., Silver L. L., and Nossal, N. G. (1982) J. Biol. Chem. 257, 12426-12434). The 41 protein, in turn, is required for pentamer primer synthesis by the 61 protein. We now show that the 41 protein helicase unwinds a partially duplex DNA molecule containing a performed fork more efficiently than a DNA molecule without a fork. Optimal helicase activity requires greater than 29 nucleotides of single-stranded DNA on the 3' side of the duplex (analogous to the leading strand template). This result suggests the 41 protein helicase interacts with the leading strand template as well as the lagging strand template as it unwinds the duplex region at the replication fork. As the single-stranded DNA on the 3' side of a short duplex (51 base pairs) is lengthened, the stimulation of the 41 protein helicase by the 61 protein is diminished. However, both the 61 protein and a preformed fork are essential for efficient unwinding of longer duplex regions (650 base pairs). These findings suggest that the 61 protein promotes both the initial unwinding of the duplex to form a fork and subsequent unwinding of longer duplexes by the 41 protein. A stable protein-DNA complex, detected by a gel mobility shift of 4x174 single-stranded DNA, requires both the 41 and 61 proteins and a rNTP (preferably rATP or rGTP, the nucleotides with the greatest effect on the helicase activity).

In the accompanying paper, we report the altered properties of a proteolytic fragment of the 41 protein helicase and its effect on in vitro DNA synthesis in the T4 multienzyme replication system.

The bacteriophage T4 multienzyme replication system includes seven phage-encoded proteins: T4 DNA polymerase, three polymerase accessory proteins, gene 32 single-stranded DNA (ssDNA) binding protein, and a primase-helicase. The primase-helicase complex, composed of the T4 41 and 61 proteins, catalyzes the synthesis of short RNA primers that initiate new DNA chains on the lagging strand of a replication fork and also acts as a helicase to unwind the duplex ahead of the polymerase on the leading strand (reviewed in Nossal and Alberts (1983)).

The 41 protein is a ssDNA-stimulated nucleotidase that hydrolyzes rATP, rGTP, dATP, and dGTP (Morris et al., 1979; Nossal, 1979; Liu and Alberts, 1981), as well as a DNA helicase which catalyzes the unwinding of restriction fragments annealed to circular ssDNA (Venkatesan et al., 1982). This unwinding requires ATP or GTP and begins at the 3' terminus of the fragment to be unwound. The helicase activity is stimulated by the 61 protein and, to a lesser extent, the 64/65 polymerase accessory proteins, but strongly inhibited by 32 protein, added either before or after the 41 protein. Using a singly nicked circular template, the 41 protein significantly increases the rate at which some chains are elongated by the DNA polymerase, polymerase accessory proteins, and the 32 ssDNA binding protein. The direction of unwinding is consistent with the idea that the 41 protein facilitates DNA synthesis on duplex templates by destabilizing the helix as it moves 5' to 3' along the lagging strand ahead of the leading strand polymerase complex.

The 61 protein alone is capable of synthesizing the dimers, pppApC and pppGpC, in a template-dependent reaction (Hinton and Nossal, 1987; Hinton et al., 1987). However, these dimers are unable to be elongated by the T4 DNA polymerase and polymerase accessory proteins. The synthesis of the biologically relevant pentaribonucleotide primers (mainly pppApC(pN)₅) requires the 41 protein helicase, as well as the 61 protein (Liu and Alberts, 1980, 1981b; Nossal, 1980; Hinton and Nossal, 1987). Therefore, the 41 protein helicase allows short primers to be made by 61 protein and then used by the polymerase to initiate discontinuous lagging strand DNA synthesis.

In this paper, we have further characterized the helicase activity of the 41 protein. Both the T7 gene 4 primase-helicase (Hatano et al., 1983) and the Escherichia coli dNaB helicase (LeBowitz and McMacken, 1986) have been shown to require a preformed forked molecule to observe helicase activity. We now show that the 41 protein helicase unwinds forked DNA hybrids more efficiently than fully complementary DNA hybrids. These forked substrates also lessen the stimulatory effect of the 61 protein on the unwinding of a short duplex region (51 bp). However, the efficient unwinding of longer hybrids requires both a preformed fork and the 61 protein. We also find that both proteins of the primase-helicase complex and rGTP (or rATP) are required to form a stable protein-DNA complex.

In the accompanying paper (Richardson and Nossal, 1989), we examine how the substitution of a proteolytic fragment of the 41 protein helicase for the intact 41 protein affects the...
The properties of the primase-helicase and its function in the T4 multienzyme DNA replication reactions.

**EXPERIMENTAL PROCEDURES**

**Nucleic Acids**—R21M13mp7 (kindly provided by Paul MacDonald and Gisela Mosig, Vanderbilt University) is a derivative of phage M13mp7 which carries the 653-bp bacteriophage T4 EcoRI fragment oriented from map position 15.545 to 16.198 kb into the EcoRI sites of M13mp7 (MacDonald and Mosig, 1984). M13mp2 and M13mp6 phages were isolated from the supernatant of infected E. coli JM105 cells by two sequential (6 and 4% (w/v)) polyethylene glycol 6000, 0.5 M NaCl precipitation as described previously (Yamamoto and Alberts, 1976). The phage were purified by two equilibrium centrifugations in CsCl and dialyzed against TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). The viral DNA was extracted from the phage using 0.1 volume of chloroform at room temperature for 10 min, followed by 0.1 volume of 2% (w/v) sodium dodecyl sulfate and 1 volume of phenol equilibrated with TE buffer at 60 °C for 15 min. The aqueous phase was re-extracted twice with 1 volume of phenol at room temperature. The initial phenol phase was back-extracted with TE buffer and the aqueous phases were combined. The single-stranded viral DNA was dialyzed extensively against TE buffer to remove the phenol and stored at −20 °C. The T4 41 protein, T4 62 protein (Fraction V) (Hinton et al., 1985), and the T4 44/62 protein (Fraction VI) (Hinton and Nossal, 1985), were purified as described (Venkatesan et al., 1982) and 45 proteins (Venkatesan and Nossal, 1982) were purified as described previously (Venkatesan et al., 1982) except Sepharose CL-2B was used to separate the DNA hybrid from free fragment.

**Construction of Helicase Substrates**—The DNA substrates used to determine the length of the noncomplementary 3' tail required for optimal helicase activity were prepared as follows. The 24-base M13 primer (330 ng) was annealed to M13mp19 viral DNA (20 pg), and the primer was extended by the Klenow fragment of E. coli DNA polymerase I which was purchased from Pharmacia. The restriction endonucleases were obtained from New England Biolabs. All DNA concentrations are expressed as molecules.

**Enzymes**—T4 41 protein (Fraction V) (Hinton et al., 1985), T4 61 protein (Fraction V) (Hinton and Nossal, 1985), and the T4 44/62 and 45 proteins were purchased from United States Biochemicals. The restriction endonucleases were purchased from Pharmacia LKB Biotechnology Inc. The M13 oligonucleotide sequencing primer 5'-CCACTGACAAGGGGGGGG-3' (Promega) was purchased from Du Pont-New England Nuclear. Unlabeled deoxynucleoside triphosphates according to the procedure of Speek et al. (1986). The restriction endonucleases were purchased from New England Biolabs. T7 gene 6 exonuclease and T4 polynucleotide kinase were purchased from United States Biochemicals. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim.

**Construction of Helicase Substrates**—The DNA substrates used to determine the length of the noncomplementary 3' tail required for optimal helicase activity were prepared as follows. The 24-base M13 primer (330 ng) was annealed to M13mp19 viral DNA (20 pg), and the primer was extended by the Klenow fragment of E. coli DNA polymerase I which was purchased from Pharmacia. The restriction endonucleases were obtained from New England Biolabs. T7 gene 6 exonuclease and T4 polynucleotide kinase were purchased from United States Biochemicals. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim.

**Enzymes**—T4 41 protein (Fraction V) (Hinton et al., 1985), T4 61 protein (Fraction V) (Hinton and Nossal, 1985), and the T4 44/62 and 45 proteins (Venkatesan and Nossal, 1982) were purified as described previously (Venkatesan et al., 1982) except Sepharose CL-2B was used to separate the DNA hybrid from free fragment.

**Construction of Helicase Substrates**—The DNA substrates used to determine the length of the noncomplementary 3' tail required for optimal helicase activity were prepared as follows. The 24-base M13 primer (330 ng) was annealed to M13mp19 viral DNA (20 pg), and the primer was extended by the Klenow fragment of E. coli DNA polymerase I which was purchased from Pharmacia. The restriction endonucleases were obtained from New England Biolabs. T7 gene 6 exonuclease and T4 polynucleotide kinase were purchased from United States Biochemicals. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim.

**Enzymes**—T4 41 protein (Fraction V) (Hinton et al., 1985), T4 61 protein (Fraction V) (Hinton and Nossal, 1985), and the T4 44/62 and 45 proteins (Venkatesan and Nossal, 1982) were purified as described previously (Venkatesan et al., 1982) except Sepharose CL-2B was used to separate the DNA hybrid from free fragment.

**Construction of Helicase Substrates**—The DNA substrates used to determine the length of the noncomplementary 3' tail required for optimal helicase activity were prepared as follows. The 24-base M13 primer (330 ng) was annealed to M13mp19 viral DNA (20 pg), and the primer was extended by the Klenow fragment of E. coli DNA polymerase I which was purchased from Pharmacia. The restriction endonucleases were obtained from New England Biolabs. T7 gene 6 exonuclease and T4 polynucleotide kinase were purchased from United States Biochemicals. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim.

**Enzymes**—T4 41 protein (Fraction V) (Hinton et al., 1985), T4 61 protein (Fraction V) (Hinton and Nossal, 1985), and the T4 44/62 and 45 proteins (Venkatesan and Nossal, 1982) were purified as described previously (Venkatesan et al., 1982) except Sepharose CL-2B was used to separate the DNA hybrid from free fragment.

**Construction of Helicase Substrates**—The DNA substrates used to determine the length of the noncomplementary 3' tail required for optimal helicase activity were prepared as follows. The 24-base M13 primer (330 ng) was annealed to M13mp19 viral DNA (20 pg), and the primer was extended by the Klenow fragment of E. coli DNA polymerase I which was purchased from Pharmacia. The restriction endonucleases were obtained from New England Biolabs. T7 gene 6 exonuclease and T4 polynucleotide kinase were purchased from United States Biochemicals. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim.

**Enzymes**—T4 41 protein (Fraction V) (Hinton et al., 1985), T4 61 protein (Fraction V) (Hinton and Nossal, 1985), and the T4 44/62 and 45 proteins (Venkatesan and Nossal, 1982) were purified as described previously (Venkatesan et al., 1982) except Sepharose CL-2B was used to separate the DNA hybrid from free fragment.

**Construction of Helicase Substrates**—The DNA substrates used to determine the length of the noncomplementary 3' tail required for optimal helicase activity were prepared as follows. The 24-base M13 primer (330 ng) was annealed to M13mp19 viral DNA (20 pg), and the primer was extended by the Klenow fragment of E. coli DNA polymerase I which was purchased from Pharmacia. The restriction endonucleases were obtained from New England Biolabs. T7 gene 6 exonuclease and T4 polynucleotide kinase were purchased from United States Biochemicals. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim.

**Enzymes**—T4 41 protein (Fraction V) (Hinton et al., 1985), T4 61 protein (Fraction V) (Hinton and Nossal, 1985), and the T4 44/62 and 45 proteins (Venkatesan and Nossal, 1982) were purified as described previously (Venkatesan et al., 1982) except Sepharose CL-2B was used to separate the DNA hybrid from free fragment.

**Construction of Helicase Substrates**—The DNA substrates used to determine the length of the noncomplementary 3' tail required for optimal helicase activity were prepared as follows. The 24-base M13 primer (330 ng) was annealed to M13mp19 viral DNA (20 pg), and the primer was extended by the Klenow fragment of E. coli DNA polymerase I which was purchased from Pharmacia. The restriction endonucleases were obtained from New England Biolabs. T7 gene 6 exonuclease and T4 polynucleotide kinase were purchased from United States Biochemicals. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim.
FIG. 1. **Length of noncomplementary 3' tail required for maximal 41 protein helicase activity.** A, substrates were DNA hybrids consisting of M13mp2 single-stranded viral DNA with an annealed fragment (51 bp of duplex) whose noncomplementary 3' tail length is either 10, 15, 29, 37, or 45 nucleotides. Helicase reactions were carried out as described under "Experimental Procedures" using, where indicated, 85 µg/ml (1.5 nmol/ml) 41 protein and 12 µg/ml (0.3 nmol/ml) 61 protein. B, products of helicase reactions displayed on a 4% agarose gel. The positions of the ssDNA of 66, 80, 88, and 96 bases (b) unwound from hybrids with 3' tails of 15, 29, 37, and 45 bases, respectively, are indicated on the figure. Controls: DNA hybrid incubated without enzyme (lanes 1, 7, 13, and 19); DNA hybrid incubated without enzyme and heat-denatured (lanes 2, 8, 14, and 20). C, time course of DNA unwinding by the 41 protein helicase. Fragment in the no enzyme control (lane 19), due to incomplete hybridization during preparation of the 45-base tail substrate, has been subtracted in calculating the fragment unwound by enzyme.

**FIG. 2.** 41 protein concentration dependence for the helicase reaction. Helicase activity was measured as described under "Experimental Procedures" with the 15-nucleotide-long (solid circles) or 45-nucleotide-long (open circles) 3' tail DNA hybrid (51-bp) for 20 min with the indicated 41 protein levels.
As the 3' extension was increased from 10 to 15 nucleotides, the stimulation by the 61 protein decreased from 17–2.5-fold. With each hybrid, unwinding was measured at a point within the linear time course for that substrate. These results show that the 61 protein has less of an effect on substrates with longer preformed forks and thus suggest that the 61 protein acts, in part, to help the 41 protein helicase begin to open the duplex. 61 protein probably promotes this initial fork formation by stabilizing the binding of the 41 protein helicase to the ssDNA as shown by the gel mobility shift experiments described below.

The effect of the 61 protein is not solely confined to the step of initial duplex opening. Fig. 3 shows that the 61 protein has a strong stimulatory effect on the unwinding of longer (650 bp) hybrids, even if they are attached to preformed forks of greater than optimum size. In this experiment, one helicase substrate consisted of a 650-bp DNA restriction fragment (Section I) or 35 pg/ml 41 protein and, where indicated, 4.8 µg/ml 61 protein (Section II). (See Fig. 1A for diagram of the substrates).

<table>
<thead>
<tr>
<th>Proteins present</th>
<th>3' Tail length</th>
<th>Time</th>
<th>Fragment unwound</th>
<th>Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nucleotides</td>
<td>min</td>
<td>fmol</td>
<td>-fold</td>
</tr>
<tr>
<td>I. 41</td>
<td>10</td>
<td>10</td>
<td>0.5</td>
<td>17</td>
</tr>
<tr>
<td>41, 61</td>
<td>10</td>
<td>10</td>
<td>8.5</td>
<td>1.7</td>
</tr>
<tr>
<td>41, 61</td>
<td>15</td>
<td>10</td>
<td>5.5</td>
<td>2.5</td>
</tr>
<tr>
<td>41, 61</td>
<td>15</td>
<td>15</td>
<td>14.0</td>
<td>2.5</td>
</tr>
<tr>
<td>II. 41</td>
<td>45</td>
<td>1</td>
<td>3.2</td>
<td>1.6</td>
</tr>
<tr>
<td>41, 61</td>
<td>45</td>
<td>1</td>
<td>5.0</td>
<td>1.6</td>
</tr>
<tr>
<td>41, 61</td>
<td>45</td>
<td>2</td>
<td>6.2</td>
<td>1.5</td>
</tr>
<tr>
<td>41, 61</td>
<td>45</td>
<td>2</td>
<td>9.4</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table I

Effect of the noncomplementary 3' tail on the 61 prime protein stimulation of the 41 protein helicase

Helicase reactions using a 51-bp M13 DNA hybrid with a 3' tail of 10, 15, or 45 nucleotides (see "Experimental Procedures") were incubated with 85 µg/ml 41 protein and, where indicated, 12 µg/ml 61 protein (Section I) or 35 µg/ml 41 protein and, where indicated, 4.8 µg/ml 61 protein (Section II). (See Fig. 1A for diagram of the substrates).

![3' TAIL](Figure 3.png)

**Figure 3.** Forked substrate with 52-base-long 3' tail allows the unwinding of a 702-base fragment by 41 protein helicase alone. Reaction mixtures containing 1 fmol (molecules) of 650-bp or 650-bp with a 52-base 3' tail M13 DNA hybrid (see "Experimental Procedures") were incubated for 30 min with, where indicated, 85 µg/ml 41 protein and/or 12 µg/ml 61 protein. The reaction products were separated on a 1.5% agarose gel. Heat-denatured substrates are shown in lanes 2 and 7.

nucleotide noncomplementary 3' extension. The 41 protein alone gave no detectable unwinding of the fully complementary hybrid (lane 4). However, 12% of this hybrid was unwound by the 41 and 61 proteins together (lane 5). Although the 41 protein alone was able to unwind the same length hybrid with the noncomplementary 3' extension to some extent, unwinding of this hybrid was markedly stimulated by the 61 protein. In lane 9, 18% of the fragment was displaced by the 41 protein alone, whereas 54% was unwound by the 41 and 61 proteins together (lane 10). The 61 priming protein alone showed no helicase activity with either substrate (lanes 3 and 8). We conclude that efficient unwinding of longer duplex regions by the 41 protein helicase requires both a DNA fork ahead of the duplex and the stimulatory activity of the 61 priming protein.

The stimulation of the 41 protein helicase activity by the 61 priming protein is not dependent on concomitant RNA primer synthesis. The 41 and 45 proteins require a minimum of ATP and CTP or GTP and CTP for primer synthesis (Liu and Alberts, 1980; Nossal, 1980). In the experiments shown in Fig. 3 and Table I, all four rNTP were present so that primer synthesis would be expected when the 61 protein was added. The nucleotide concentrations used (0.5 mM ATP and 0.2 mM GTP, CTP, and UTP) were chosen because they are best for DNA synthesis by the seven protein T4 replication system in vitro. However, using a 233-bp hybrid, we have found that unwinding by the 41 and 61 proteins is not decreased when primer synthesis is prevented by omitting CTP and UTP (data not shown). If the GTP concentration is raised to 1 mM (with 0.5 mM ATP and 0.2 mM CTP and UTP), the rate of unwinding of a 84-base fragment without a tail by 41 protein alone, or by 41 and 61 proteins together are each increased about 2-fold (data not shown). Unwinding by 41 protein alone increases as the ATP or GTP concentration is raised to between 5 and 10 mM (Venkatesan et al., 1982). At these very high ATP and GTP concentrations, which inhibit replication in vitro, there is no stimulation of the 41 protein helicase by the 61 protein.

**Stoichiometry of the 41 and 61 Proteins in DNA Unwinding Activity**—To determine the optimal ratio of 41 protein to 61 protein for helicase activity, we titrated three concentrations of 41 protein with 61 protein. The amount of the 61 priming protein required to give maximal stimulation of the 41 protein helicase activity is shown in Fig. 4. With limiting amounts of 41 protein, 300 and 600 pmol/ml of the 41 protein helicase are titrated by about 60 and 120 pmol/ml of 61 protein, respectively, using 2-pmol molecules/ml of the 15-base 3' extended 51-bp DNA hybrid. Assuming all of the protein in each preparation is active, these results give an apparent stoichiometry in the helicase reaction of five monomers of 41 protein per monomer of 61 protein.

**T4 Gene 44/62 and 45 Polymerase Accessory Proteins Increase Unwinding by the 41 Protein Helicase**—The T4 polymerase accessory proteins increase the processivity of T4 DNA polymerase by increasing the binding of the polymerase to the 3'-OH of the primer-template. These proteins are also required for strand displacement synthesis at a duplex replication fork (Liu et al., 1979; Nossal and Peterlin, 1979). Table II shows that these accessory proteins also stimulate unwinding by the 41 protein helicase. In the absence of the 61 protein, the accessory protein stimulation is not altered by the length of the 3' extension. However, in the presence of the 61 protein, increasing the 3' extension from 15 to 45 nucleotides diminishes the stimulatory effect of the accessory proteins. The stimulatory effect of the accessory proteins on the 41 protein helicase is small but reproducible with different hybrid sub-
to ssDNA, we used an agarose gel mobility shift assay to resolve protein-DNA complexes from ssDNA (Fig. 5). The mobility of φX174 ssDNA with rGTP (lane 1) is not changed by addition of the 41 protein (lane 2). Addition of the 61 protein alone induces a small, tight mobility shift (lane 3). In contrast, a large, diffuse mobility shift is observed when both 41 and 61 proteins are added (lanes 4 and 5). In the absence of rGTP, the mobility shift with the 41 and 61 proteins (lane 6) is indistinguishable from that with 61 protein alone, which is the same in the presence (lane 3) or absence (not shown) of GTP. Detergent or phenol extraction destroys the mobility shift (data not shown), indicating that a noncovalent protein-DNA complex is formed. The formation of this protein-DNA complex is temperature-dependent. Very little of the protein-DNA complex is formed at 4 °C as compared to 37 °C. This temperature dependence suggests that the turnover of rGTP by the 41 protein nucleotidase activity might stabilize the formation of the protein-DNA complex. In this regard, the addition of EDTA to inhibit the nucleotidase activity of the 41 protein helicase does not abolish but does reduce the mobility shift (lane 12).

The ribonucleotide requirements for the gel mobility shift showed that the preferred ribonucleoside triphosphates (assayed at 2 mM) were in order: rATP (lane 9) > rGTP (lane 4) > rUTP (lane 10), 3’OMe rGTP (lane 8) > rCTP (lane 11), rGTPyS (lane 7) (Fig. 5). The ribonucleotides rATP and rGTP, which give the greatest mobility shift, also are the nucleotides required for the 41 protein helicase activity. Addition of all four ribonucleotide triphosphates (0.5 mM ATP, 0.2 mM GTP, CTP, and UTP) (lane 14), which permits RNA primer synthesis, does not change the mobility shift beyond that seen when CTP and UTP are omitted (lane 15). These results suggest that the formation of a protein-DNA complex requires the 61 protein, the 41 protein, and the binding of a ribonucleotide. The hydrolysis of rGTP or rATP by the 41 protein helicase forms a more stable protein-DNA complex.
giving a larger mobility shift. The simplest interpretation of these results is that binding of 61 protein to ssDNA facilitates the binding of 41 protein. However, we have not yet demonstrated directly that both 41 and 61 proteins are in the protein-DNA complex detected on the gel.

DISCUSSION

The T4 gene 41 DNA helicase is believed to play an important role in the coordinated synthesis of the leading and lagging strands during the replication of duplex DNA. Previous studies have shown that the 5’ to 3’ helicase activity of the 41 protein is responsible for increasing the rate of strand displacement DNA synthesis by the T4 DNA polymerase, the three polymerase accessory proteins, and the 32 ssDNA binding protein (Alberts et al., 1980; Venkatesan et al., 1982). The 41 protein is also an essential component of the T4 primase.

While the 61 protein alone can synthesize small amounts of oligomers (primarily dimers), the 41 protein must be added to make large amounts of the pentamers (pppApC(pN)3) used to prime synthesis on T4 DNA in vivo and in vitro (Hinton and Nossal, 1987, Nossal and Hinton, 1987). Together these results support a model in which the T4 gene 41 DNA helicase destabilizes the helix ahead of the growing leading strand as it moves 5’ to 3’ on the lagging strand toward the next primer site (Nossal and Alberts, 1983).

In this report we have extended earlier biochemical studies of the 41 protein helicase activity to show that forked DNA hybrids are the preferred substrates. Our results are consistent with reports that other replicative helicases, such as the T7 gene 4 primase-helicase (Matson et al., 1983) and E. coli dnaB helicase (LeBowitz and McMacken, 1986), require a forked DNA molecule to observe unwinding activity. The T7 gene 4 primase-helicase required at least a 7-base 3’ extension, while the E. coli dnaB helicase needed more than a 40-base 3’ extension to detect helicase activity. On the other hand, the 41 protein helicase activity can be detected in the absence of a 3’ extension (Venkatesan et al., 1982). However, we have determined that a 3’ extension longer than 29 nucleotides on a 51-bp DNA hybrid is required for maximal 41 protein helicase activity. This result suggests that the 41 protein helicase interacts with both the leading strand and lagging strand templates at the replication fork as it unwinds the duplex region.

The T4 gene 61 priming protein stimulates the unwinding by the 41 protein, especially with DNA hybrids without a 3’ extension. The 61 protein stimulated the unwinding of the DNA hybrid with a 16-base 3’ extension by 17-fold; however, only a 2.5-fold stimulation was observed for the DNA hybrid with a 15-base 3’ extension (Table I). These results suggest that the 61 protein increases the 41 protein helicase activity, in part, by facilitating the initial opening of the duplex to form a fork, the preferred substrate for the 41 protein helicase. As discussed below, the 61 protein binds tightly to ssDNA (Silver and Nossal, 1982; Burke et al., 1985) and is required to show interaction of the 41 protein with ssDNA in a mobility shift assay. Thus, the 61 protein may promote fork formation by stabilizing the binding of 41 protein to the ssDNA ahead of the duplex. The role of the 61 protein is not limited to facilitating the initial opening of the duplex to form a fork. With longer hybrids such as the 650-bp duplex used in Fig. 3, the 61 protein markedly stimulates unwinding even if the molecule has a preformed fork.

The ratio of the 41 and 61 proteins required to give optimal helicase activity was determined by titrating the 41 protein helicase with limiting amounts of the 61 protein. At initial times, such that the kinetics of the reactions were linear, the optimal ratio of 41 protein to 61 protein was found to be about 5:1. Given the large single-stranded character of the helicase substrates and the possibility of inactive protein, this ratio can only be considered an approximation. A high concentration of 41 protein relative to 61 protein was also found to be essential for pentamer primer synthesis (Silver and Nossal, 1982; Hinton and Nossal, 1987). There is a nonlinear dependence on 41 protein concentration in the DNA helicase assay (Fig. 2 and Venkatesan et al. (1982)) and in assays for 41 complementation (Morris et al., 1979; Nossal, 1979). RNA primer synthesis (Liu and Alberts, 1981b; Silver and Nossal, 1982), and ssDNA-stimulated GTPase (Liu and Alberts, 1981a). In addition, preincubation of 41 protein with GTP or GTPyS has been shown to increase its sedimentation coefficient time 4.9 to 6.1 S and to change the 41 protein concentration dependence for primer synthesis and primer-dependent DNA synthesis from strongly sigmoidal to linear (Liu and Alberts, 1981a; Silver and Nossal, 1982). 61 protein is active as a monomer (Silver and Nossal, 1982). These results are all consistent with the proposal that 41 protein is active as a multimeric complex which interacts with a monomer of the 61 protein to form a functional primase-helicase. In all of our experiments, the concentrations of 41 and 61 proteins are much higher than the concentration of DNA molecules. For example, in Fig. 4 unwinding increased as 41 protein was increased from 300 to 1500 pmol/ml with the helicase substrate at 2 pmol/ml (14,400 pmol of ssDNA nucleotide/ml). The high concentrations of 41 and 61 proteins required for unwinding are probably due mainly to the binding of these proteins to the long ssDNA regions in the helicase substrates used. Clearly, further studies with duplex substrates with limited single-stranded regions will be needed to determine whether the 41/61 protein helicase unwinds catalytically.

It must be emphasized that although the requirement for both the 61 and 41 proteins for RNA primer synthesis and optimal helicase activity suggests that a 41/61 protein complex is formed, to date a direct physical interaction between the two proteins has not been demonstrated. The 61 protein is a basic protein which behaves as a monomer in solution and tightly binds to ssDNA (Silver and Nossal, 1982; Burke et al., 1985). The 41 protein has a nucleotidase activity which is stimulated by ssDNA, but does not bind to ssDNA in a stable manner (Morris et al., 1979; Nossal, 1979; Liu and Alberts, 1981a). We have shown (Fig. 5) that a stable protein-DNA complex formed in the presence of the 61 protein, 41 protein, and rNTP is much more retarded in a gel mobility shift assay than a complex of only ssDNA and 61 protein under the same conditions. The most effective ribonucleotides that will induce this gel mobility shift are rATP and rGTP, the nucleotides required for the 41 protein helicase activity. Since preincubation with rGTP increases the sedimentation coefficient of 41 protein (see above), the stable oligomerization of 41 protein with the 61 protein on the ssDNA is an attractive interpretation of the gel shift experiments. However, we have not shown directly that both proteins remain in the complex.

In an analogous interaction, the E. coli dnaG primase has been shown to enhance the stability of a ternary complex of the dnaB helicase, ssDNA, and ATP (Arai and Kornberg, 1981). The T4 gene 44/62 and 45 polymerase accessory proteins have also been shown to stimulate the 41 protein helicase alone and in the presence of the 61 protein (Table II and Venkatesan et al., 1982). The polymerase accessory proteins do not by themselves unwind these fragments. In the accompanying paper (Richardson and Nossal, 1989), we show that the three polymerase accessory proteins (and even the 44/62
protein complex alone to some extent) relieve the inhibition of 41/61 protein-catalyzed primer synthesis by the gene 32 ssDNA binding protein, and show that tryptic cleavage in the COOH-terminal region of 41 protein interferes with this function of the polymerase accessory proteins. We defer to the accompanying paper our discussion of how specific protein-protein interactions between the polymerase accessory proteins and the 41 and 61 proteins might promote primase and helicase activity at the replication fork.

Acknowledgments—We thank Drs. Deborah M. Hinton, Barbara Funnell, and Edith W. Miles (National Institutes of Health) for helpful discussions, and Helen Jenerick for typing this manuscript.

REFERENCES


