Nucleotide-dependent Binding of the Gene 4 Protein of Bacteriophage T7 to Single-stranded DNA*

(Received for publication, June 20, 1984)

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The gene 4 protein of bacteriophage T7 is a multifunctional enzyme that catalyzes (i) the hydrolysis of nucleoside 5′-triphosphates, (ii) the synthesis of tetraribonucleotides, and (iii) the unwinding of duplex DNA. All three activities depend on binding of gene 4 protein to single-stranded DNA followed by unidirectional 5′ to 3′ translocation of the protein (Tabor, S., and Richardson, C. C. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 205–209). Binding of gene 4 protein to single-stranded DNA, assayed by retention of DNA-protein complexes on nitrocellulose filters, is random with regard to DNA sequence. Although gene 4 protein does not bind to duplex DNA, the presence of a 240-nucleotide-long single-stranded tail on a 7200-base pair duplex DNA molecule is sufficient for gene 4 protein to cause retention of the DNA on a filter. The binding reaction requires, in addition to MgCl₂, the presence of a nucleoside 5′-triphosphate, but binding is not dependent on hydrolysis; nucleoside 5′-diphosphate will substitute for nucleoside 5′-triphosphate. Of the eight common nucleoside triphosphates, dTTP promotes optimal binding. The half-life of the gene 4 protein-DNA complex depends on both the secondary structure of the DNA and on whether or not the nucleoside 5′-triphosphate cofactor can be hydrolyzed. Using the nonhydrolyzable nucleoside 5′-triphosphate analog, 5′,3′-methylene dTTP, the half-life of the gene 4 protein-DNA complex is greater than 80 min. In the presence of the hydrolyzable nucleoside 5′-triphosphate, dTTP, the half-life of the gene 4 protein-DNA complex using circular M13 DNA is at least 4 times longer than that observed using linear M13 DNA.

Biochemical and genetic analysis of bacteriophage T7 has revealed a requirement for the product of gene 4 of the phage for DNA replication (2–6). Initially, purification of gene 4 protein relied on an in vitro complementation assay (7–9). Subsequent studies of the purified protein have shown it to be a multifunctional enzyme with single-stranded DNA-dependent nucleoside 5′-triphosphatase activity (10, 11), site-specific primase activity (9, 12–16), and DNA helicase activity (1, 8, 17, 18). To prime DNA synthesis the gene 4 protein synthesizes tetraribonucleotides, pppACCC and pppACCA, that are complementary to four of the nucleotides found in the specific pentanucleotide (3′-CTGGG/T-5′) recognition sequence (9, 15, 16). The helicase activity of the gene 4 protein, initially inferred from its ability to stimulate DNA synthesis catalyzed by T7 DNA polymerase on duplex DNA templates (8, 12, 17, 19), has been recently demonstrated directly using a novel DNA substrate (1). The gene 4 protein is found in two forms with molecular weights of 58,000 and 66,000 which purify together (4, 8, 9, 19, 20). Two initiation codons located 189 base pairs apart account for the two species of gene 4 protein which have common C termini but different amino ends (21).

Much is already known about the mechanisms by which the gene 4 protein catalyzes primer synthesis and the unwinding of duplex DNA (1, 5, 9–16). Like the nucleoside 5′-triphosphatase activity, both the primase and helicase activities require the presence of single-stranded DNA and a NTP. Furthermore, the addition of a nonhydrolyzable NTP analog, β,γ-methylene dTTP, inhibits both priming and helicase reactions, indicating that NTP hydrolysis is coupled to both activities (1, 10, 11, 19). The relative rates of utilization of priming sites on circular single-stranded DNA suggests that these recognition sites on the single-stranded DNA are encountered by the gene 4 protein in a nonrandom fashion (16). In fact, the utilization of primer sites can be best explained by a mechanism in which the gene 4 protein binds at random sites on the single-stranded DNA and remains bound while translocating 5′ to 3′ along the single strand of DNA (16). Studies on the nucleoside 5′-triphosphatase hydrolysis and the helicase activities of gene 4 protein using defined DNA substrates support such a mechanism (1, 11, 19). Translocation in a single direction must be coupled to some irreversible chemical process, and the obvious candidate is the hydrolysis of NTPs.

A common feature of the known reactions catalyzed by the gene 4 protein is that each activity requires binding of gene 4 protein to single-stranded DNA. We have now applied a direct filter binding method to demonstrate binding of the protein to DNA. Binding of the gene 4 protein to DNA is found to require nucleotides, and the rate of dissociation of the complex on defined DNA molecules supports unidirectional translocation of the protein on single-stranded DNA.

* The abbreviations used are: NTP, nucleoside 5′-triphosphate; RF1, replicative form I.
EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains and Bacteriophages—Escherichia coli 71.18 has been described (22). Mutants requiring a high concentration (50 µg/ml) of exogenous thymine were isolated as described by Miller (23). Second site mutants requiring a low concentration (2 µg/ml) of exogenous thymine were isolated by plating mutants requiring a high level of exogenous thymine on M-9 minimal media plates containing 2 µg/ml added thymine. Colonies were isolated and determined to have a thymine requirement that was satisfied by the addition of 2 µg/ml thymine. All other bacterial strains and bacteriophages have been described (1, 11).

Enzymes—T7 gene 4 protein, isolated as previously described (11), is greater than 95% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (24) and has a specific activity of 33,000 units/mg. All restriction endonucleases were purchased from New England Biolabs; reaction conditions were those suggested by the supplier. Phage T4 polynucleotide kinase was purchased from New England Nuclear (IICN). Phage T4 DNA polymerase and T4 DNA ligase were purchased from P-L Biochemicals. 3H-labeled nucleotides were from New England Nuclear; [γ-32P]ATP was from ICN.

Methods

Binding Assay—The assay used to detect binding of gene 4 protein to DNA has been described (27). The standard binding reaction mixture (20 µl) contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 50 µg/ml bovine serum albumin that had been warmed at 70°C for 5 min, and 5% glycerol. 3H-labeled single-stranded DNA was produced by growing phage T7 on lawn of E. coli alkaline phosphatase (7000 Ci/mmol) for 45 min at 37°C in the presence of 0.1 µCi of [γ-32P]ATP (7000 Ci/mmol) to label the 5'-ends of the DNA. After phenol extraction, unretracted radioactive rATP was removed from the DNA by gel filtration through a 1.2-ml Sepharose 6B-CL column equilibrated with 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 100 mM NaCl. The void volume of the column, which contained the DNA, was pooled and used in binding reactions.

Preparation of 5' End-labeled Duplex DNA Substrates—The topologically stable replication fork DNA substrate (0.18 µg) was 5' end labeled by incubating the DNA with T4 polynucleotide kinase (18 units) in the presence of 0.1 µCi of [γ-32P]ATP (7000 Ci/mmol) for 60 min at 37°C. After phenol extraction, unretracted radioactive rATP was removed as described above.

Nicked pBR322 dimer DNA (1.25 µg) was 5'-end labeled after incubation with E. coli alkaline phosphatase at 65°C and proteinase K as described above. The dephosphorylated DNA was extracted with phenol and precipitated with ethanol. The DNA was resuspended in a minimal volume of 5 mM Tris-HCl (pH 7.5) and incubated with T4 polynucleotide kinase as described above. The 5'-end-labeled DNA was phenol extracted and the unretracted radioactive rATP removed as described above.

Preparation of Linear Single-stranded M13 3H DNA—M13 [3H]DNA (16 µg) was incubated with 10 ng of pancreatic DNase (Boehringer Mannheim) in 40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 50 µg/ml bovine serum albumin for 1 min at 23°C. The reaction was terminated by the addition of 10 mM EDTA and then heated at 68°C for 2 min. Greater than 90% of the circular DNA molecules were converted to linear molecules as judged by electrophoresis in a 1% agarose gel at 20 V/cm.

Other Methods—Polyacrylamide gel electrophoresis in the presence of 8 M urea was carried out by the method of Maxam and Gilbert (28). DNA concentrations were determined by directly measuring absorbance at 260 nm and are expressed as nucleotide equivalents except as indicated in Fig. 4.

RESULTS

The three activities of the phage T7 gene 4 protein, helicase, primase, and DNA-dependent NTase, all apparently require binding of the protein to single-stranded DNA (see the introduction). However, binding has never been demonstrated directly. The retention of nucleoprotein complexes by nitrocellulose filters has provided a useful method for characterizing interactions between protein and nucleic acids (see Ref. 27). We have applied this method to study the binding of gene 4 protein to various DNAs.

Gene 4 Protein Binds Single-stranded DNA—As shown in Fig. 1, in the presence of the nonhydrolyzable nucleoside 5'-triphosphate analog β,γ-methylene dTTP, the addition of gene 4 protein to single-stranded M13 DNA leads to the retention of more than 70% of the DNA on a nitrocellulose filter. The addition of more gene 4 protein to the binding reaction mixture does not significantly increase the amount of DNA retained. The reason for this is unknown, but it may reflect the efficiency with which the DNA-protein complexes are retained on the nitrocellulose filter under the conditions of the binding assay. When 70% of the single-stranded DNA is retained on the filter there are approximately 100 gene 4 protein molecules/DNA molecule in the reaction mixture. However, binding has never been demonstrated directly. The retention of nucleoprotein complexes by nitrocellulose filters has provided a useful method for characterizing interactions between protein and nucleic acids (see Ref. 27). We have applied this method to study the binding of gene 4 protein to various DNAs.

This estimation of the number of gene 4 protein molecules/DNA substrate molecule is based on the following: (i) all gene 4 protein molecules are active, (ii) a molecular weight for gene 4 protein of 60,000, and (iii) a monomeric active species of gene 4 protein.1

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synthesis catalyzed by form II of T7 DNA polymerase on a preformed replication work fork (19) requires a ratio of gene 4 protein molecules to T7 DNA polymerase molecules of approximately 100:1. The requirement for excess gene 4 protein in both these reactions is unexplained.

The retention of duplex M13 RFI DNA under the same conditions is considerably lower at low concentrations of gene 4 protein (Fig. 1). The binding to duplex DNA is approximately 7-fold lower than binding to single-stranded DNA. The binding interaction observed with M13 RFI DNA may, in fact, reflect the presence of regions of single-stranded DNA in the supercoiled RFI DNA. This interpretation is supported by the finding that nicked circular duplex DNA is far less effective in binding to gene 4 protein. The binding of gene 4 protein to nicked pBR322 DNA is 10-fold less than binding to a circular duplex DNA bearing a single-stranded tail (see Fig. 4).

Requirements for Binding to Single-stranded DNA—The requirements for binding of gene 4 protein to single-stranded DNA are shown in Table I. The MgCl₂ and NaCl requirements for binding to single-stranded DNA are essentially identical to the requirements for helicase activity and for DNA-dependent NTP hydrolysis (1, 11).

Binding to Single-stranded DNA Requires a Nucleotide Co-factor—The binding of gene 4 protein to single-stranded M13 DNA is dependent on the presence of a nucleoside 5'-diphosphate, nucleoside 5'-triphosphate, or a NTP analog (Table I, Fig. 2). In the absence of added nucleotide there is essentially no retention of single-stranded DNA on the filter (less than 3%) in the presence of gene 4 protein. The binding that does occur in the absence of added NTP at high concentrations of gene 4 protein may be due to the presence of trace amounts of NTP in the preparation of gene 4 protein. Although the binding reaction requires the presence of a NTP, it is not dependent on NTP hydrolysis. In fact, the nonhydrolyzable nucleoside triphosphate analog, β,γ-methylene dTTP, is the most effective nucleotide cofactor we have found (Fig. 2). At low concentrations of gene 4 protein more DNA is retained on the filter in the presence of β,γ-methylene dTTP than in the presence of dTTP. The concentration of β,γ-methylene dTTP required to obtain maximal binding is less than 100 μM (data not shown). Increasing concentrations of β,γ-methylene dTTP up to 1 mM have no effect on the amount of DNA bound by gene 4 protein.

Gene 4 protein is known to hydrolyze seven of the eight commonly occurring nucleoside 5'-triphosphates in the presence of single-stranded DNA (rCTP is not hydrolyzed) (11). All eight NTPs were tested separately in binding reactions as well as two nonhydrolyzable nucleoside 5'-triphosphate analogs (Table II). Interestingly, of the hydrolyzable NTPs tested only dTTP can effectively serve as the NTP cofactor required by gene 4 protein for binding to single-stranded DNA. Either nonhydrolyzable NTP analog, β,γ-methylene dTTP or β,γ-methylene rATP, may serve as cofactors for the binding reaction (Table II). Since the nonhydrolyzable analog of ATP, β,γ-methylene rATP, is active we tested the ability of ATP
TABLE II

NTP requirements for binding to M13 DNA

| Nucleotide          | M13 DNA retained (%) |
|---------------------|----------------------|
| dTTP                | 48.0                 |
| dATP                | 6.9                  |
| dGTP                | 4.6                  |
| dCTP                | <3.0                 |
| rATP                | 5.2                  |
| rGTP                | <3.0                 |
| rUTP                | 5.8                  |
| rCTP                | 7.5                  |
| β,γ-Methylene dTTP  | 71.0                 |
| β,γ-Methylene rATP  | 42.0                 |
| dTDP                | 55.0                 |
| dTMP                | 6.9                  |

To function as a cofactor at concentrations up to 5 mM (data not shown). At the highest concentration of ATP tested, this nucleoside 5'-triphosphate failed to promote binding.

Surprisingly, dTDP serves effectively as a cofactor in the gene 4 protein binding reaction (Table II). The binding curve obtained when dTDP is substituted for dTTP in the reaction mixture is essentially the same as the binding curve obtained when dTTP is used as the NTP cofactor (data not shown). In addition, the concentration of nucleoside 5'-diphosphate required to obtain maximal binding is similar to that required of β,γ-methylene dTTP (less than 100 μM). These results indicate that the affinity of gene 4 protein for DNA is unchanged in the presence of dTDP, a product of dTTP hydrolysis. It is unlikely that a contaminant of nucleoside 5'-triphosphate failed to promote binding.

When dTTP is used as the NTP cofactor (data not shown), the concentration of nucleoside 5'-diphosphate required to obtain maximal binding is too low to permit significant levels of nucleoside 5'-triphosphate contamination. Analysis of dTDP by thin layer chromatography indicates contamination by dTTP of less than 2%. 100 μM dTDP is sufficient to promote maximal binding, but 2 μM dTTP is insufficient to observe any retention of DNA on the filter. We conclude that both dTTP and dTDP will serve as cofactors in the gene 4 protein binding reaction; dTMP is not active (Table II).

Binding to Single-stranded DNA Is Random with Respect to DNA Sequence—A comparison of the rates of utilization of primase recognition sites on φX174 DNA (16) suggested that binding of gene 4 protein to single-stranded DNA is random with regard to DNA sequence. In order to directly demonstrate this we have incubated gene 4 protein with single-stranded 5'-end-labeled HaeIII restriction fragments from φX174 DNA and determined which fragments are retained by gene 4 protein on a nitrocellulose filter (Fig. 3). With all concentrations of gene 4 protein used (Fig. 3, lanes 2-4) all the restriction fragments are represented on the polyacrylamide gel. This is a good indication that gene 4 protein binds single-stranded DNA of any sequence. It is important to note that the predominant gene 4 protein primase recognition sequences, 3'-CTGGC/T-5' (16), are not present in all of the restriction fragments, while several of the restriction fragments have more than one primase recognition sequence. Therefore, the recognition sequence is not required for a stable complex between gene 4 protein and single-stranded DNA. In addition, to eliminate the possibility of some oligonucleotide recognition sequence, which does occur in all of the HaeIII restriction fragments, being responsible for the binding we have used poly(dT) as a competitor for the binding of gene 4 protein in this reaction (data not shown). Poly(dT) effectively competes with the labeled φX174 DNA fragments for gene 4 protein in the binding reaction. We conclude that binding of gene 4 protein to single-stranded DNA has little or no sequence dependence.

Gene 4 Protein Can Bind to a Single-stranded Tail on Duplex DNA—Gene 4 protein stimulates DNA synthesis catalyzed by Form II of T7 DNA polymerase on a duplex DNA molecule containing a preformed replication fork (19). The DNA molecule containing a preformed fork is a circular duplex of 7.2 × 10^6 base pairs (M13mp6 DNA) from which arises, at a unique BamHI recognition site, a noncomplementary 5'-phos-

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**FIG. 3.** Binding to single-stranded DNA is not sequence specific. Binding assays were as described under "Experimental Procedures" using 5'-end-labeled single-stranded HaeIII restriction fragments from φX174 DNA except that each reaction mixture contained 15 mM NaCl. The nitrocellulose filters with bound protein-DNA complexes were incubated with 0.4 ml of 20 mM Tris-HCl (pH 7.5), 1% sodium dodecyl sulfate at room temperature with constant shaking for 60 min to release the bound DNA. The radioactivity in an aliquot (0.1 ml) was measured to verify removal of the DNA-protein complexes from the filter. The samples were then mixed with 0.1 ml of deionized formamide, 0.1% bromphenol blue, 0.1% xylene cyanol and heated at 95 °C for 1 min prior to loading onto an 8% polyacrylamide gel containing 8 M urea. Lane 1, no gene 4 protein; lane 2, 24 ng of gene 4 protein; lane 3, 61 ng of gene 4 protein; lane 4, 122 ng of gene 4 protein; lane 5, unreacted DNA fragments applied directly to gel as a control.
phoryl-terminated single strand of 237 nucleotides (SV40 DNA). The single-stranded 5'-tail on the DNA molecule is absolutely required for DNA synthesis catalyzed by a combination of T7 DNA polymerase and gene 4 protein. We have proposed that gene 4 protein requires the single-stranded region for binding to the fork (19). To test whether gene 4 protein can bind to this structure we have measured the amount of DNA retained on a nitrocellulose filter by gene 4 protein using both a nicked, duplex circular DNA molecule and the preformed replication fork DNA substrate (Fig. 4). Gene 4 protein causes the retention of the DNA molecule containing the replication fork on the nitrocellulose filter. Retention of the nicked duplex DNA molecule on the filter by gene 4 protein is nearly an order of magnitude lower. These data suggest that the presence of a single-stranded segment of DNA is sufficient to allow binding of gene 4 protein to a DNA molecule that is otherwise completely duplex. We conclude that gene 4 protein does not stimulate DNA synthesis catalyzed by Form II of T7 DNA polymerase on a nicked duplex DNA template because it is unable to bind the DNA substrate and unwind the duplex for the DNA polymerase.

**Dissociation Rate of Gene 4 Protein-DNA Complex**—In order to assess the stability of gene 4 protein-DNA complexes, the half-time for dissociation of gene 4 protein from single-stranded DNA when challenged with a high concentration of an unlabeled competing DNA was determined using both linear and circular single-stranded labeled DNA (Fig. 5). In the presence of β,γ-methylene dTTP, the half-time for dissociation of gene 4 protein from circular single-stranded DNA is greater than 20 min. Even after 80 min no significant reduction in the amount of labeled DNA retained on the filter was observed (data not shown). When dTTP is used, allowing NTP hydrolysis and translocation of gene 4 protein along the DNA molecule, the half-life of the DNA-gene 4 protein complex is approximately 3 min at 37 °C (Fig. 5). Thus, the gene 4 protein forms a relatively stable complex with single-stranded DNA even under conditions of NTP hydrolysis. If, on the other hand, the same measurements are made using linear single-stranded M13 DNA we obtain a different set of results. The half-time for dissociation of gene 4 protein from an equal-sized linear DNA molecule is still greater than 20 min in the presence of β,γ-methylene dTTP (data not shown). In addition, there is virtually no difference between the binding curves obtained using either linear or circular single-stranded DNA in the presence of β,γ-methylene dTTP. However, using dTTP the half-time for dissociation from linear molecules is about 45 s at 37 °C. This result indicates that, under conditions that permit NTP hydrolysis, the gene 4 protein dissociates rapidly from linear single-stranded DNA. Since NTP hydrolysis is coupled to unidirectional translocation of gene 4 protein along single-stranded DNA, this may be interpreted to mean that translocation to the end of the DNA results in dissociation of the gene 4 protein-DNA complex. On a circular DNA molecule gene 4 protein remains bound to the DNA, even under conditions of NTP hydrolysis, for a relatively long period of time as it processively translocates along the single-stranded DNA since ends are not encountered.

**DISCUSSION**

The gene 4 protein of bacteriophage T7 catalyzes three distinct reactions: (i) DNA-dependent hydrolysis of NTPs,
(ii) site-specific template-directed synthesis of tetraribonucleotides, and (iii) unwinding of duplex DNA. All three of these reactions require binding of gene 4 protein to DNA (1, 10, 11, 16). The primase (9, 14, 16) and single-stranded DNA-dependent NTPase (10, 11) activities of gene 4 protein are manifest only on single-stranded DNA, and gene 4 protein requires single-stranded DNA adjacent to duplex DNA for unwinding of the duplex to occur (1, 19).

The gene 4 protein requires the presence of single-stranded DNA for nucleoside 5'-'triphosphatase activity (10) suggesting that the gene 4 protein must bind DNA during the catalytic cycle of nucleotide hydrolysis. In this paper we have shown that the gene 4 protein can form a stable complex with single-stranded DNA in the presence of a nucleotide cofactor. Taken together, these observations support a model (Fig. 6) in which the gene 4 protein interacts with a nucleoside 5'-triphosphate, perhaps in the absence of DNA (Fig. 6, I), but is unable to catalyze the hydrolysis of the NTP unless bound to single-stranded DNA. The gene 4 protein-NTP complex binds to single-stranded DNA (Fig. 6, II), perhaps leading to a conformational change in the gene 4 protein, such that it will now catalyze the hydrolysis of the bound NTP. It is interesting that not all NTPs are capable of promoting the formation of a stable complex between gene 4 protein and single-stranded DNA. In fact, of the ones tested, the only hydrolyzable nucleoside 5'-triphosphate capable of serving as a cofactor for binding is dTTP. Previous results indicate that although seven of the eight NTPs tested are hydrolyzed by gene 4 protein and, therefore, presumably interact with gene 4 protein, only four of these are hydrolyzed to an appreciable extent (11). It should be noted that of these four NTPs, dTTP also has the lowest $K_m$ value for both the NTP hydrolysis reaction and for unwinding DNA. It is likely that dTTP promotes the strongest binding interaction between gene 4 protein and single-stranded DNA. The assay used to detect this interaction is relatively insensitive when compared to the DNA-dependent NTP hydrolysis assay. Interactions between gene 4 protein and NTPs detected in the NTP hydrolysis assay may not be observed with the less sensitive DNA-binding assay (1, 11). The nonhydrolyzable analog of rATP, $\beta\gamma$-methylene rATP, does serve as a cofactor for the binding reaction, albeit not as well as $\beta\gamma$-methylene dTTP. However, even at high concentrations, rATP does not promote binding of gene 4 protein to single-stranded DNA. Apparently the gene 4 protein-rATP-DNA complex is weak. The reason for this result is unknown; however, it is clear that the interactions between gene 4 protein and rATP must be different from those of the other nucleotides since rATP is also the initiating nucleotide of the tetraribonucleotide primer synthesized by gene 4 protein (13, 15).

It is equally interesting that dTDP promotes stable binding, although dTMP does not. Even in the presence of one of the products of hydrolysis, the gene 4 protein remains bound to single-stranded DNA. This is consistent with the ability of the gene 4 protein to translocate along single-stranded DNA. The enzyme would be expected to remain bound to the DNA throughout the hydrolytic cycle (11, 16).

The binding between gene 4 protein and single-stranded DNA is not sequence specific (Fig. 6, III). The gene 4 protein binds all the single-stranded HaeIII restriction fragments of φX174, indicating no sequence specificity in the binding interaction. In addition, poly(dT) effectively competes with the fragments for binding of gene 4 protein. The original studies which demonstrated the gene 4 protein to prime DNA synthesis at specific sites also suggested that the protein binds randomly to single-stranded DNA and translocates in the 5' to 3' direction (16). This behavior is in contrast to that of some of the prepriming and priming proteins of *E. coli*. Both the dnaG protein and the n' protein recognize and interact with specific sequences on the DNA template (30, 31). Binding to single-stranded DNA without regard for sequence is, however, expected of an enzyme that is responsible for opening the helix at a replication fork. Nonspecific binding to single-stranded DNA is consistent with results presented earlier (11) suggesting that gene 4 protein dose not remain bound for determinable periods of time at primase recognition sequences under the conditions of primer synthesis.

The complex formed between gene 4 protein and single-stranded DNA is extremely stable in the presence of a non-hydrolyzable nucleotide cofactor. Under the conditions we have used, the half-life of the complex is greater than 80 min at 37 °C. When dTTP is substituted for $\beta\gamma$-methylene dTTP the half-life of the complex is reduced to approximately 3 min at 37 °C. This, however, is still a remarkably stable complex considering the fact that the protein is actively hydrolyzing NTP and translocating along the single strand of DNA (Fig. 6, IV). The stability of the complex formed in the presence of dTTP depends on the secondary structure of the DNA. The complex with circular single-stranded DNA has a half-life of more than 150 s; the half-life of the complex with linear single-stranded DNA is about 45 s. These data suggest that the gene 4 protein is translocating along the single-stranded DNA effector as it hydrolyzes NTP. The circular DNA is infinitely long and, therefore, the gene 4 protein remains bound for a considerable period of time. When linear DNA is substituted for circular DNA, the gene 4 protein reaches a terminus and is forced to dissociate and rebind a new DNA molecule.

The binding interaction between gene 4 protein and single-stranded DNA is more complex than originally envisioned in that it requires the presence of a nucleotide cofactor. The model presented in Fig. 6 is consistent with data currently available. The gene 4 protein (I) binds NTP (II) and then the gene 4 protein-NTP complex binds DNA (III) or, alterna-

![Fig. 6. Model for gene 4 protein binding to single-stranded DNA. Gene 4 protein forms a complex with nucleoside 5'-triphosphate which causes a conformational change in the gene 4 protein allowing an interaction with single-stranded DNA to occur. Upon binding to single-stranded DNA the NTP is hydrolyzed. The gene 4 protein remains complexed with the DNA and migrates processively in the 5' to 3' direction.](image-url)
tively, free gene 4 protein may bind weakly to DNA but this complex is stabilized by subsequent binding of NTP. Once bound to the DNA, the gene 4 protein translocates unidirectionally 5' to 3' (16) along the single-stranded DNA template (IV). The unidirectional translocation is coupled to NTP hydrolysis and is essential for both the primase and helicase activities of gene 4 protein (1, 11, 16, 18, 19). Since both dTDP and dTTP promote binding of gene 4 protein to single-stranded DNA, both complexes III and IV (Fig. 6) are relatively stable. Displacement of dTDP by dTTP must occur in such a way that the gene 4 protein remains bound to the DNA since additional cycles of nucleotide hydrolysis and translocation occur without release of the DNA.

Recently, two enzymatically distinct forms of T7 DNA polymerase have been described (20, 26, 29). Form I of T7 DNA polymerase catalyzes strand displacement synthesis on a nicked DNA primer-template; its rate of DNA synthesis is stimulated by gene 4 protein severalfold. Form II of T7 DNA polymerase does not catalyze a strand displacement synthesis reaction and, in addition, cannot be stimulated by T7 gene 4 protein on a nicked duplex primer-template. If, however, a stable replication fork containing a single-stranded 5'-tail is used, the rate of synthesis catalyzed by Form II of T7 DNA polymerase can be stimulated by gene 4 protein (19). In view of the results presented here, we conclude that gene 4 protein is unable to bind and initiate unwinding of a nicked duplex DNA molecule. When a single-stranded region is present, either created by strand displacement synthesis (by Form I of T7 DNA polymerase) or as a pre-existing part of the DNA substrate, the gene 4 protein binds to it and translocates 5' to 3' to the fork to unwind the DNA in concert with the advancing DNA polymerase. The result is an increased rate of DNA synthesis catalyzed by the combined action of gene 4 protein and T7 DNA polymerase. The enzymatic activities of gene 4 protein coupled with those of T7 DNA polymerase are sufficient to accomplish synthesis on both strands of template DNA.

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