Roles of the helicase and primase domain of the gene 4 protein of bacteriophage T7 in accessing the primase recognition site

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The 63 kDa gene 4 protein of bacteriophage T7 provides both helicase and primase activities. The C-terminal helicase domain of the gene 4 protein is responsible for DNA-dependent NTP hydrolysis and for hexamer formation, whereas the N-terminal primase domain contains the zinc motif that is, in part, responsible for template-directed oligoribonucleotide synthesis. In the presence of β,γ-methylene dTTP, the protein forms a hexamer that surrounds and binds tightly to single-stranded DNA and consequently is unable to translocate to primase recognition sites, 5′-GTC-3′, or to dissociate from the molecule to which it is bound. Nonetheless, in the presence of β,γ-methylene dTTP, it catalyzes the synthesis of pppAC dimers at primase sites on M13 DNA. When bound to single-stranded DNA in the presence of β,γ-methylene dTTP, the primase can function at recognition sites on the same molecule to which it is bound provided that a sufficient distance exists between the recognition site and the site to which it is bound. Furthermore, the primase bound to one DNA strand can function at a primase site located on a second DNA strand. The results indicate that the primase domain resides on the outside of the hexameric ring, a location that enables it to access sites distal to its site of binding.

Keywords: DNA binding/DNA primase/helicase/β,γ-methylene dTTP

Introduction

Gene 4 of bacteriophage T7 encodes two co-linear proteins, a 63 kDa helicase/primase and a 56 kDa helicase (Dunn and Studier, 1983). The 56 kDa protein, translated from an internal AUG codon, lacks the 63 N-terminal amino acids found in the 63 kDa gene 4 protein. Both of the gene 4 proteins contain the nucleoside 5′-triphosphate-binding site that is required for hexamer formation (Patel and Hingorani, 1993; Notarnicola et al., 1995) and for the DNA-dependent hydrolysis of dTTP (Matson and Richardson, 1983; Notarnicola et al., 1995). Consequently, both proteins bind to single-stranded DNA (ssDNA) and translocate unidirectionally, 5′ to 3′, along the DNA strand (Matson and Richardson, 1985), a reaction that is coupled to the hydrolysis of dTTP (Matson and Richardson, 1983). Upon encountering a duplex region, the proteins can continue their translocation leading to the unwinding of the DNA and hence their designation as DNA helicases.

In addition to its helicase activity, the larger 63 kDa gene 4 protein also catalyzes the template-directed synthesis of di-, tri-, tetra- and pentaribonucleotides on ssDNA (Scherzinger et al., 1977; Mendelman and Richardson, 1991). Both in vivo and in vitro the tetra- and pentaribonucleotides are used as primers by T7 DNA polymerase (Romano and Richardson, 1979; Fujiyama et al., 1981; Tabor and Richardson, 1981). In this communication, however, we refer to the 63 kDa gene 4 protein as T7 primase and to the 56 kDa gene 4 protein as DNA helicase. The trinucleotide sequence 5′-GTC-3′ is the minimum sequence recognized by the T7 primase, and at this site pppAC dimers are synthesized (Mendelman and Richardson, 1991). The 3′-cytidine is required for recognition but is not copied into the primer. The actual RNA primers found at the 5′ termini of Okazaki fragments synthesized in cells infected with phage T7 (Fujiyama et al., 1981) or in reactions containing the T7 primase are tetraribonucleotides, predominately pppACCC/A and pppACAC (Tabor and Richardson, 1981). These tetraribonucleotides arise from the general recognition sites 5′-G/TGGTC-3′ and 5′-GTGTC-3′, respectively, all containing the core recognition sequence, 5′-GTC-3′ (Romano and Richardson, 1979; Fujiyama et al., 1981; Tabor and Richardson, 1981).

The ability of the T7 primase to recognize specific sequences on ssDNA in part derives from the presence of a Cys32 zinc-binding motif in the 63 amino acid N-terminus domain that distinguishes the 63 kDa gene 4 protein from the 56 kDa gene 4 protein (Bernstein and Richardson, 1988a,b). Amino acid alignments of the T7 primase with other prokaryotic primases and helicases, most of which reside in separate polypeptides, assign the primase domain to the N-terminal 245 amino acids and the helicase domain to the C-terminal 294 amino acid residues (Ilyina et al., 1992). Thus, the T7 56 kDa gene 4 protein contains a portion of the primase domain, a conclusion that is substantiated by our previous finding that the zinc motif alone is not sufficient for sequence recognition (Kusakabe and Richardson, 1996) and the observation that the 56 kDa protein contains the active site for phosphodiester bond formation (Bernstein and Richardson, 1988a).

Both helicase and primase functions at the replication fork dictate an interaction with T7 DNA polymerase. T7 DNA polymerase alone is unable to catalyze strand displacement synthesis and thus is incapable of catalyzing DNA synthesis on duplex DNA templates. However, T7 DNA polymerase physically interacts with gene 4 protein, and the helicase activity of the latter protein enables the fork to move at a rate of ~300 nucleotides per second at
30°C (Lechner and Richardson, 1983). Gene 4 protein lacking the 17 C-terminal acidic amino acid residues no longer binds to T7 DNA polymerase and is unable to stimulate the activity of the polymerase on duplex templates although the truncated protein has normal helicase activity (Notarnicola et al., 1997). Initiation of lagging strand synthesis is also dependent on an interaction of the 63 kDa gene 4 protein with T7 DNA polymerase since tetra- and pentaribonucleotides are only extended by the polymerase in the presence of gene 4 protein (Bernstein and Richardson, 1988a; Kusakabe and Richardson, 1997b). However, the altered T7 primase lacking its C-terminus functions normally as a primase with T7 DNA polymerase, suggesting additional polymerase-binding domains on the primase (Notarnicola et al., 1997). Both the polymerase and the gene 4 protein in turn interact with the T7 ssDNA-binding protein, the gene 2.5 protein, an interaction that provides for more efficient transfer of primers to the polymerase (T.Kusakabe and C.C.Richardson, unpublished result).

The T7 gene 4 protein (Patel and Hingorani, 1993; Notarnicola et al., 1995), like other prokaryotic DNA helicases (Lohman and Bjornson, 1996), functions as a hexamer, with the ssDNA passing through the center of the ring (Engelman et al., 1995). Hexamer formation is enhanced by the presence of dTDP; dTTP and β,γ-methylene dTTP (Patel and Hingorani, 1993; Notarnicola et al., 1995). The presence of any of these nucleotides supports tight binding of the protein to ssDNA. However, β,γ-methylene dTTP, since it cannot be hydrolyzed to dTDP and Pn, prevents translocation of the helicase and thus essentially locks the protein onto ssDNA (Matson and Richardson, 1985).

Physical association of primases with a helicase is important since the translocation activity of the helicase provides a mechanism by which the primase can reach its recognition site (Liu and Alberts, 1980; Arai and Kornberg, 1981a,b; Hinton and Nossal, 1987; Nossal and Hinton, 1987). The T7 primase differs from the Escherichia coli and phage T4 primases in that the helicase is a part of the same protein molecule. Consequently, it is often difficult to evaluate the relative contributions of each activity to experimental observations. For example, the 5′ to 3′ translocation of the helicase on the template strand must be reconciled with the 5′ to 3′ polymerization of nucleotides during primer synthesis, movements that are opposite to one another. Likewise, the rapid movement of the protein along ssDNA facilitates a search for primase recognition sites but may well interfere with pausing of the protein to initiate primer synthesis. It is not known if the tight binding of the protein that arises from the binding of the helicase domain to ssDNA is coordinated with the weaker binding of the primase domain to its recognition site. Electron microscopy suggests that on binding to ssDNA, the primase domain of the 63 kDa protein is located on the 5′ side of the DNA, with the helicase domain on the 3′ side (Engelman et al., 1995). This result is in agreement with our earlier studies that demonstrated that the T7 primase requires a relatively long stretch of 15–20 nucleotides on the 3′ side of the recognition site, presumably for helicase binding (Kusakabe and Richardson, 1997a).

Early studies of the T7 gene 4 protein demonstrated that β,γ-methylene dTTP does not inhibit the synthesis of oligoribonucleotides on single-stranded φX174 DNA (Scherzinger et al., 1977) even though this non-hydrolyzable analog cannot support 5′ to 3′ translocation of the protein on ssDNA. In the present study, we have used β,γ-methylene dTTP to investigate the relative roles of the multiple activities of the 63 kDa gene 4 protein on oligoribonucleotide synthesis. We show that the binding of the hexameric gene 4 protein to ssDNA stimulates primase activity but that translocation of the protein to reach primase recognition sites need not occur. The evidence suggests that the primase domain resides on the outside of the hexameric ring and is thus able to synthesize oligoribonucleotides at distant primase recognition sites or even on other DNA molecules.

**Results**

**Unidirectional translocation on ssDNA is not required for oligonucleotide synthesis by T7 primase**

T7 DNA primase binds ssDNA and translocates 5′ to 3′ on the DNA strand, a reaction that is coupled to the hydrolysis of dTTP (Matson and Richardson, 1985). Upon encountering a primase recognition site, 5′-GTC-3′, the primase ceases its translocation and synthesizes oligoribonucleotides (Bernstein and Richardson, 1988a). In order to examine the relationship between translocation and oligoribonucleotide synthesis, we have used β,γ-methylene dTTP, a non-hydrolyzable analog of dTTP. β,γ-Methylene dTTP allows for hexamer formation and DNA binding through the helicase domain of the 63 kDa gene 4 protein, but does not support its translocation on ssDNA (Matson and Richardson, 1985).

At the basic primase recognition site, 5′-GTC-3′, T7 primase catalyzes the synthesis of pppAC when provided with ATP and CTP (Mendelman and Richardson, 1991). The dinucleotide is then extended to trimers, tetramers and pentamers at the more general recognition sites. Although dTTP is the required nucleotide for binding and hydrolysis, ATP is also hydrolyzed by T7 primase (Matson and Richardson, 1983). Therefore, in order to eliminate any source of a hydrolyzable nucleotide that could support translocation, we have examined the ability of the primase to extend a ribodinucleotide, 5′-ApC-3′, at primase recognition sites in the presence of [α-32P]CTP. As shown in Figure 1A, T7 primase catalyzes the extension of AC dimers on M13 ssDNA or of a synthetic oligonucleotide containing the recognition sequence 5′-GGGTC-3′ to tetraribonucleotides in the presence but not in the absence of dTTP. When β,γ-methylene dTTP was substituted for dTTP, the dinucleotide was extended on both M13 ssDNA (lane 3) and on the synthetic oligonucleotide (lane 6), albeit at ~20 and 10% the efficiency, respectively. Thus dTTP is required for primer synthesis but it need not be hydrolyzed. The extension of the dinucleotide on the M13 ssDNA template in the presence of CTP is as efficient as the de novo synthesis of oligoribonucleotide in the presence of ATP and [α-32P]CTP (Figure 1B, lanes 1 and 4). The extension reaction is, however, ~4.5-fold more efficient than de novo synthesis on the synthetic oligonucleotide template (lanes 2 and 5). In agreement with our previous study, dinucleotide extension occurred only at primase

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reactions that contained 0.1 mM ATP or AC dimer, 0.1 mM left. (
autoradiography. The oligoribonucleotide species are indicated on the
products were resolved on a 25% urea gel and visualized by
autoradiography. Free DNA and DNA bound by primase
were indicated in Figure 1. The amount of
incubation at 37°C for the identical time, and the reaction products
were incubated on a 25% urea gel as shown in Figure 1. The amount of
were resolved on a 25% urea gel as shown in Figure 1. The amount of
incubation on ssDNA (see Discussion).

of the primase with β,γ-methylene dTTP prior to the addition of ssDNA.

T7 primase catalyzes oligoribonucleotide extension in the
presence of β,γ-methylene dTTP. (A) Oligoribonucleotide extension
assays were performed as described in Materials and methods.
Reaction mixtures (10 µl) contained 0.1 mM AC ribonucleotide,
0.1 mM [α-32P]CTP, 10 nM T7 primase (hexamer), either 20 nM M13
ssDNA (lanes 1–3) or 100 nM synthetic oligonucleotide C0:10 (lanes
4–6), and 0.5 mM of dTTP or β,γ-methylene dTTP as indicated.
Oligonucleotide C0:10 contains the primase recognition sequence
5′-GGGTC-3′. After incubation at 37°C for 30 min, the reaction
products were resolved on a 25% urea gel and visualized by
autoradiography. The oligoribonucleotide species are indicated on the
left. (B) Oligoribonucleotide synthesis and extension is carried out in
reactions that contained 0.1 mM ATP or AC dimer, 0.1 mM
[α-32P]CTP, 0.5 mM β,γ-methylene dTTP, 10 nM T7 primase
(hexamer), and either 20 nM M13 ssDNA (lanes 1 and 4), 100 nM
synthetic oligonucleotide C0:15 (lanes 2 and 5) or T0:15 (lanes 3 and
6) as indicated. Oligonucleotides C0:15 and T0:15 contain the primase
recognition sequence 5′-GGGTC-3′ or 5′-GGGT-3′, respectively.

of the primase with β,γ-methylene dTTP or incubated with β,γ-methylene dTTP prior
to the addition of ssDNA. In the first case, 10 nM T7 primase (hexamer)
was mixed with 20 nM M13 ssDNA and then incubated for 5 min
with 0.5 mM β,γ-methylene dTTP. The reaction was then initiated by
the addition of 0.1 mM AC dinitucleotide and 0.1 mM [α-32P]CTP. In
the second case, 10 nM T7 primase (hexamer) was mixed with
0.5 mM β,γ-methylene dTTP and incubated for 5 min. The reaction
was then initiated by the addition of 0.1 mM AC dinitucleotide,
0.1 mM [α-32P]CTP and 20 nM M13 ssDNA. The reactions were
incubated at 37°C for the identical time, and the reaction products
were resolved on a 25% urea gel as shown in Figure 1. The amount of
[α-32P]CMP incorporated into tri- and tetranucleotide was determined
using a BAS100 Fuji Bio-Imaging analyzer. (B) Binding of pre-formed
hexamer to various ssDNAs. T7 DNA primase was added to reaction
mixtures containing four different ssDNA molecule and β,γ-methylene
dTTP or incubated with β,γ-methylene dTTP prior to the addition of
the DNAs. DNA molecules added to each reaction were 5′-32P-labeled
79 nt ssDNA alone (lanes 1 and 5), with a 20 nt oligonucleotide
annealed to its 5′ end (lanes 2 and 6), with a 20 nt oligonucleotide
annealed to its 3′ end (lanes 3 and 7) or with a 20 nt oligonucleotide
annealed to both the 5′ and 3′ ends (lanes 4 and 8). A cartoon of each
DNA is indicated above the lane. In the first case, 100 nM T7 primase
(hexamer) was mixed with each of the DNAs (100 nM) and then
incubated for 5 min with 0.5 mM β,γ-methylene dTTP (lanes 1–4). In
the second case, 100 nM T7 primase (hexamer) was mixed with
0.5 mM β,γ-methylene dTTP and pre-incubated for 5 min, then
incubated each of the DNAs (100 nM) (lanes 5–8). After
incubation at 22°C for 5 min, protein–DNA complex formation was
analyzed by native polyacrylamide gel electrophoresis in the presence of
β,γ-methylene dTTP as described in Materials and methods and
visualized by autoradiography. Free DNA and DNA bound by primase
are indicated on the right.

recognition sites bearing the basic primase recognition
sequence 5′-GTC-3′; the cryptic cytidine is required for
recognition as in the de novo synthesis reaction (Kusakabe
and Richardson, 1997b). Neither CTP nor β,γ-methylene
dTTP, the only nucleotides present in this experiment, are
hydrolyzed by T7 primase (Matson and Richardson, 1983).

T7 primase forms hexamers in the absence of ssDNA
provided that dTTP, dTDP or γ-methylene dTTP is
present (Notarnicola et al., 1995). Among these effectors
of hexamer formation, β,γ-methylene dTTP is the most
effective. We were therefore curious as to whether a
primase hexamer could assemble on ssDNA as effectively
as monomers. Since the ssDNA passes through the hexa-
mer, one could envisage difficulty in a pre-formed hexamer
threading the end of DNA through its center. In order to
address this question, we examined primase activity using
the dinucleotide extension assay under conditions where
hexamers should exist prior to the addition of the ssDNA
template. These results were then compared with those
obtained in the normal reaction where the DNA, primase
and nucleotide are added simultaneously. Pre-incubation

Four different oligonucleotide constructions were examined, a 5’-32P-labeled 79 nt long oligonucleotide (i) alone, (ii) with a 20 nt complementary oligonucleotide annealed to its 5’ end, (iii) with a 20 nt complementary oligonucleotide annealed to its 3’ end or (iv) with a 20 nt complementary oligonucleotide annealed to both the 5’ and 3’ ends. T7 DNA primase cannot eliminate these duplex regions since helicase activity requires a 6–7 nt 3’ tail on the annealed oligonucleotide. As shown in Figure 2B, pre-incubation of T7 primase with β,γ-methylene dTTP had no effect on binding to any of the ssDNAs as compared with no pre-incubation. However, the presence of a 5’ duplex region did decrease binding in both instances. Since the hole through the center of a T7 DNA primase hexamer cannot accommodate duplex DNA (Stryer, 1995), the hexameric ring must either be able to partially dissociate upon encountering ssDNA or there must be a specific mechanism for entry of the ssDNA.

**T7 primase does not leave the molecule to which it is bound in the presence of β,γ-methylene dTTP**

By what mechanism does T7 primase catalyze the extension of dinucleotides in the presence of β,γ-methylene dTTP? The assumption has been that, in the presence of this analog, the gene 4 hexamer binds randomly to any ssDNA but is locked to the position of its initial binding site since the protein cannot hydrolyze β,γ-methylene dTTP and consequently cannot translocate on ssDNA. In this model, the primase could catalyze the extension of dinucleotides only if it could locate primase recognition sites on another molecule or on the same molecule to which it is bound by forming a loop. Alternatively, however, one could envisage a mechanism by which the hexamer is stably bound to ssDNA but is free to slide in either direction, not unlike the sliding clamps that move freely on duplex DNA molecules and provide processivity to DNA polymerases (Stukenberg et al., 1994; Naktinis et al., 1996). We have therefore examined the ability of the T7 primase to transfer from one linear ssDNA molecule to another in the presence of β,γ-methylene dTTP by different approaches. In the first assay, we have measured directly the physical association of the protein with ssDNA. In the second assay, we have measured the ability of the primase to catalyze the dinucleotide extension reaction on another ssDNA molecule after binding to ssDNA in the presence of β,γ-methylene dTTP.

To follow directly the fate of the primase bound to ssDNA, we first incubated the protein with a radioactively labeled synthetic oligonucleotide (50 nt) lacking a primase recognition site (AE23T) in the presence of β,γ-methylene dTTP and then examined the ability of the same non-radioactively labeled ssDNA to chase the protein off the labeled DNA. T7 primase hexamers bound to radioactively labeled DNA can be detected by non-denaturing gel electrophoresis (Hingorani and Patel, 1993). When a 5’-32P-labeled oligonucleotide was incubated with T7 primase in the presence of β,γ-methylene dTTP and then chased for 5 min with the same unlabeled oligonucleotide, the T7 primase hexamer remained bound to the radioactively labeled oligonucleotide as observed by gel analysis in the presence of β,γ-methylene dTTP (Figure 3, lane 1). However, when T7 primase was first pre-incubated with unlabeled oligonucleotide and then chased with [5’-32P]oligonucleotide, no radioactively labeled hexamer complex was observed (lane 2). In the control shown in lane 3, an equimolar amount of the labeled and unlabeled oligonucleotide was incubated together with the T7 primase in the presence of β,γ-methylene dTTP. Precisely half the amount of radioactivity appeared in the hexamer–oligonucleotide complex. These results indicate that T7 primase bound to ssDNA does not dissociate from the DNA molecule to which it is bound in the presence of β,γ-methylene dTTP. Since the ssDNA molecules are short linear molecules, it seems likely that the gene 4 would slide off the molecule into solution if the hexamer could slide freely in the presence of β,γ-methylene dTTP.

In the second approach to address this question, we pre- incubated T7 primase with various concentrations of a synthetic oligonucleotide (70 nt) lacking a primase recognition site in the presence of β,γ-methylene dTTP. After formation of the T7 primase–ssDNA complex, single-stranded M13 DNA containing multiple primase recognition sites was added and the dinucleotide extension assay was carried out in the presence of [α-32P]CTP. As shown in Figure 4A and C, even at the lowest concentration of oligonucleotide (molar ratio of 1:20 to M13 DNA), there was a >2-fold decrease in dinucleotide extension when M13 DNA was added to the pre-formed complex. When the pre-incubation was carried out with an amount of oligonucleotide identical to the M13 DNA (molar ratio of 1:1), the dinucleotide extension on M13 DNA was reduced by 3.5-fold. The fact that the inhibition eventually reaches a plateau with increasing amounts of oligonucleotide suggests that T7 primase is able to use primase sites on M13 DNA while still bound to the oligonucleotide.

In a control experiment, T7 primase and various concentrations of the oligonucleotide were incubated with M13 DNA in the presence of β,γ-methylene dTTP and then the dinucleotide extension assay was carried out (Figure 4B and C). The presence of the oligonucleotide had no effect on primase activity since on a molar basis the 7249 nt M13 DNA provides the major target of ssDNA to which the gene 4 protein can bind even when the oligonucleotide is present in an equimolar amount.
In this series of experiments, we have also examined the ability of the circular form of the 70 nt oligonucleotide to compete for binding of T7 primase in the presence of β,γ-methylene dTTP. Interestingly, the circular form of the molecule did not inhibit dinucleotide extension on M13 DNA even when it was pre-incubated with T7 primase at a molar ratio of 1:1 (Figure 4A and B). We believe that the primase hexamer cannot bind to a small circle of 70 nt, presumably because the diameter of the circle is too small to accommodate the gene 4 protein (see Discussion).

**Effect of distance of primase recognition site from primase-binding site on dinucleotide extension**

In earlier studies, we showed that oligoribonucleotide synthesis at primase recognition sites on relatively short oligonucleotides occurs via an interaction of the primase with the site by random collision (Mendelman and Richardson, 1991; Kusakabe and Richardson, 1997a). If the length of the flanking sequence on the 3' side of the oligonucleotide template exceeds 15–25 nt, then primer synthesis decreases, presumably due to a dTTP-dependent engagement of 5' to 3' translocation activity on the longer sequence, a reaction that competes for primer synthesis in the opposite direction. We therefore reasoned that in the presence of β,γ-methylene dTTP, which locks the primase to any site on ssDNA at which it initially binds at random, the length of the ssDNA template would be a critical determinant of primer synthesis. On very short DNA templates, there would not be sufficient sequence between a primase recognition site and the site of attachment of the primase to permit looping of the DNA between the two sites so that the primase could not access the site.

In order to examine the effect of the length of DNA between the primase recognition site and the primase/helicase-binding site, we constructed six oligonucleotides, each of which has the primase recognition site 5'-GGGTC-3' with a 5'-flanking sequence of 15 nt but with a variable 3'-flanking sequence of 5–65 nt (Figure 5A). As shown in Figure 5B and C, in the presence of dTTP, maximal extension of the dinucleotide AC occurred on the template having a 3'-flanking sequence of 15 nt; as the length increases, there was a rapid decrease. At a 3' length of 65 nt, dinucleotide extension was only 10% of that observed with a 3' length of 20 nt. Reduced dinucleotide extension on templates shorter than 15 nt is due to a minimal requirement of 3'-flanking sequence for stable binding by the helicase domain (Kusakabe and Richardson, 1997a). In contrast, when β,γ-methylene dTTP replaced dTTP, dinucleotide extension remained relatively independent of the length of the 3'-flanking sequence beyond the optimal 15–25 nt length (Figure 5B and D).

**T7 primase bound to one strand can extend a dinucleotide at a primase recognition site on a second DNA strand**

The fact that T7 primase can interact with recognition sites distal to its attachment site suggests that it can interact with primase recognition sites on another DNA strand to which it is not bound through its helicase domain. In the experiment presented in Figure 6, T7 primase was incubated with a 70 nt ssDNA lacking a primase recognition site in the presence of β,γ-methylene dTTP. Subsequently, a second oligonucleotide containing the primase recognition site, 5'-GGGTC-3', was added and dinucleotide (AC) extension was measured in the standard assay. In one case, the oligonucleotide lacked any homology to the 70 nt ssDNA and in the other case the 3' 20 nt
DNA binding by T7 primase

Fig. 5. Effect of template length on oligoribonucleotide extension in the presence of β,γ-methylene dTTP. (A) Schematic illustration of templates. The six ssDNA templates each have the primase recognition sequence 5’-GCGTC-3’, a 15 nt 5’-flanking sequence and a 3’-flanking sequence varying from 5 to 65 nt. (B) Gel analysis of products of dinucleotide extension. The ssDNA templates depicted in (A) above were analyzed for their ability to support extension of the dinucleotide AC in the presence of [α-32P]CTP, GTP and either dTTP or β,γ-methylene dTTP. The reaction products were resolved on a 25% urea gel and visualized by autoradiography. The length of 3’-flanking sequence from the 3’ cryptic cytidine is shown above each lane and the oligoribonucleotide species are indicated on the left. (C) and (D) Total incorporation of [α-32P]CMP from each reaction shown in (B) is plotted as a function of the length of 3’-flanking sequence.

Fig. 6. Ability of T7 primase bound to one strand to extend dinucleotide at a primase recognition site on a second DNA strand. (A) T7 DNA primase (10 nM hexamer) was first incubated with a 70 nt oligonucleotide lacking a primase recognition site (20 nM CL-1 ssDNA) and 0.5 mM β,γ-methylene dTTP for 5 min at 22°C. The dinucleotide extension assay was initiated by the addition of 0.1 mM AC dinucleotide, 0.1 mM [α-32P]CTP and the indicated concentration of a 40 nt oligonucleotide containing the primase recognition sequence 5’-GGGTC-3’ (CL-nCO) (lanes 1–4) or a 40 nt oligonucleotide containing the primase recognition sequence 5’-GGGTC-3’ a 20 nt 3’ sequence complementary to the CL-1 oligonucleotide (CL-CO) (lanes 6–9). The sequences of the oligonucleotides are given in Materials and methods. Dinucleotide extension on both CL-CO and CL-nCO oligonucleotides was measured directly in the absence of CL-1 ssDNA (lanes 5 and 10). After incubation at 37°C for 30 min, the reaction products were resolved on a 25% urea gel and visualized by autoradiography. The oligoribonucleotide species are indicated on the left. (B) Total incorporation of [α-32P]CMP from each reaction shown in (A) is plotted as a function of the concentration of the ssDNA added. (C) Effect of T7 gene 2.5 protein. The reactions were carried out as described in (A), using 20 nM oligonucleotide CL-CO and in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of T7 gene 2.5 protein (500 nM).

were complementary to the 5’ 20 nt of the 70 nt ssDNA. Dinucleotide extension occurred with both oligonucleotides over a 6-fold range of template concentrations (Figure 6A and B). However, the oligonucleotide capable of partially hybridizing to the first ssDNA showed a concentration dependence with regard to the added nucleotide. In a control experiment, the amount of dinucleotide extension on each template containing a recognition
sequence was shown to be the same (Figure 6A). Thus, the difference observed in Figure 6 was due to partial hybridization of the two oligonucleotides.

The ssDNA-binding protein of phage T7, the gene 2.5 protein, stimulates oligonucleotide synthesis catalyzed by T7 primase (Nakai and Richardson, 1988). Furthermore, gene 2.5 protein is known to form a physical complex with both the 56 and 63 kDa gene 4 protein (Nakai and Richardson, 1988; Kim and Richardson, 1994). However, we find no effect of gene 2.5 protein on dinucleotide extension in this assay in which the T7 primase binds the first ssDNA and the extension reaction occurs at the recognition sequence on the second ssDNA fully covered with gene 2.5 protein (Figure 6C, lanes 3 and 4).

In a second approach, we isolated T7 DNA primase bound to ssDNA in the presence of β,γ-methylene dTTP, free of any unbound protein, and then measured the ability of the bound primase to catalyze dinucleotide extension at a primase recognition site on another DNA molecule. T7 primase was bound to a 50 nt oligonucleotide in the presence of β,γ-methylene dTTP. The oligonucleotide did not contain a primase recognition site but it does have a 3′ poly(A) tail of 26 nt. The bound enzyme was then purified by the addition of magnetic beads bearing oligo(dT) which is then annealed to the ssDNA to which the primase is bound via a 3′ poly(A) extension on the latter molecule (Figure 7A). After washing the beads with buffer in the presence or absence of β,γ-methylene dTTP, the ability of the complex to extend the AC dinucleotide on M13 ssDNA in the presence of [α-32P]CTP was measured (Figure 7B). The complex that was maintained in β,γ-methylene dTTP readily extended the dinucleotide (lane 1) whereas the beads that were washed in the absence of β,γ-methylene dTTP were devoid of activity (lane 2).

**T7 primase cannot bind to small circular DNAs**

In an earlier experiment (Figure 4), we found that a linear, but not a circular, 70 nt ssDNA could decrease dinucleotide extension catalyzed by the T7 primase when incubated with enzyme prior to the extension reaction. We have also examined the ability of T7 primase to bind to small circular ssDNA directly. In the presence of β,γ-methylene dTTP, T7 primase binds tightly to both a 70 and 90 nt linear ssDNA as measured by non-denaturing PAGE; no binding was observed with the circular form of either ssDNA (data not shown). As shown earlier, T7 primase readily binds to longer circular ssDNA such as M13 ssDNA. The inability of the protein to bind to these small DNAs suggests that the diameter of the small circular DNA cannot accommodate the protein, thus supporting the model in which the hexamer surrounds the ssDNA (Egelman et al., 1995).

**Discussion**

A unique feature of the DNA primase of bacteriophage T7 is the presence of a helicase domain which allows the primase, a protein composed of a single polypeptide chain, to catalyze both helicase and primase activities (Dunn and Studier, 1983; Bernstein and Richardson, 1988a). The presence of primase and helicase activities within the same polypeptide is not entirely unexpected. In both the *E. coli* and phage T4 systems, the primase and helicase proteins are known to physically interact, and the *E. coli* satellite phage P4 primase, the product of the γ gene, also has a 3′ to 5′ helicase activity (Liu and Alberts, 1980; Arai and Kornberg, 1981a,b; Hinton and Nossal, 1987; Nossal and Hinton, 1987). Furthermore, the primase derives a number of benefits from the associated helicase. In all of these systems, the primase is dependent on the associated helicase activity to translocate it to an appropriate primase recognition site where it can catalyze the synthesis of oligoribonucleotides for use as primers by the DNA polymerase. In addition, the DNA primase, which has a rather weak affinity for DNA and its recognition site, may benefit from the tight binding of the helicase to ssDNA in the presence of an NTP, thus tethering it to the site of oligoribonucleotide synthesis. In the case of the *E. coli* primase, an additional dependency, separate from its translocation activity, on the DNA-bound helicase is known to exist (Arai and Kornberg, 1981a,b).

However, in all of these instances, the question of the
role of the helicase in actual oligoribonucleotide synthesis after the primase recognition site has been accessed can be raised. In the E.coli and T4 systems, there is always the possibility that the primase and helicase dissociate once the primase has reached a primase recognition site, thus obviating this question, but at present such a dissociation at the replication fork is not known. Such an opportunity for the two activities to separate physically clearly does not exist in the T7 helicase/primase system. Consequently, it is reasonable to expect that the 5' to 3' movement of the helicase on the DNA strand may well influence either the initiation or elongation of primers, the latter polymerization of nucleotides progressing along the same strand but in the opposite direction to helicase movement. Likewise, it is difficult to envisage a model in which the synthesis of primers by the T7 primase does not affect the rate of helicase translocation. Other proteins such as the T7 gene 2.5 ssDNA-binding protein may well modulate the two activities, and this protein is known to bind to the gene 4 protein and affect primase activity (Nakai and Richardson 1988; J.Lee and C.C.Richardson, unpublished result). Alternatively, the interactions during primer synthesis may in themselves play an important regulatory role at the replication fork.

In the present study, we have attempted to address the role of ssDNA binding through the helicase domain on oligoribonucleotide synthesis at the primase domain. To do so, it was necessary first to find conditions under which the gene 4 protein could be stably bound to ssDNA without active translocation or diffusion from that site. Our results show that in the presence of a non-hydrolyzable analog of dTTP, the T7 primase remains firmly attached to the molecule to which it is initially bound, thus enabling us to examine primase activity in the absence of translocation.

Earlier studies had firmly established that β,γ-methylene dTTP is a non-competitive inhibitor of ssDNA-dependent hydrolysis of dTTP and helicase activity (Kolodner and Richardson, 1977; Matson and Richardson, 1985). However, it strongly promoted binding to ssDNA, even more so than dTTP (Matson and Richardson, 1985). The molecular mechanism by which NTPs and their hydrolysis mediate binding of DNA helicases to ssDNA and fuel their unidirectional movement along that DNA is not yet fully understood. Of the six dTTP-binding sites located on the subunits of the hexamer, three are postulated to be catalytic sites for the hydrolysis of dTTP while the other three are postulated to bind the nucleotide without hydrolyzing it (Hingorani et al., 1997). Nucleotide occupancy of these latter three sites may be important in hexamer formation; in the absence of ssDNA, the binding of nucleotide by the non-catalytic site is tight (Kd ≈ 0.008 ± 0.002/s) (Hingorani et al., 1997). In the experiment presented in Figure 2C, it is clear that the hexameric primase can bind to ssDNA even when the hexamer is pre-assembled in the presence of β,γ-methylene dTTP. Assuming that at least one of the subunits would have to release its nucleotide for opening of the ring, this experiment implies that ssDNA can mediate the dissociation of the tightly bound NTP from its non-catalytic site.

As pointed out in the Introduction, the ability of β,γ- methylene dTTP to stimulate the synthesis of oligoribonucleotides by the T7 DNA primase on large ssDNAs was puzzling since it could not do so by translocating the primase to recognition sites. One scenario, a random diffusion of the hexamer on ssDNA in the presence of β,γ-methylene dTTP, was eliminated in this study. T7 primase in the presence of β,γ-methylene dTTP remains tightly bound to the DNA strand to which it is initially bound and cannot translocate. Thus the stimulatory effect of β,γ-methylene dTTP on primer synthesis must be related directly to the ability of this analog to tether the protein to ssDNA, a conclusion supported by our results.

Our data show that the strong stimulatory effect of β,γ-methylene dTTP on primer synthesis on large molecules derives from the fact that the concentration of primer sites available to the primase is increased greatly by the ability of the analog to lock the primase onto ssDNA, thus allowing the primase domain to explore for primase recognition sites on the DNA strand to which it is attached. First, the efficiency of oligoribonucleotide synthesis by the primase in the presence of β,γ-methylene dTTP is dependent on the length of DNA sequence flanking the primase recognition site. A minimum flanking sequence of at least 15 nucleotides is required for binding of the helicase domain, but increasing the length of the flanking sequence beyond this critical length has little effect whereas longer flanking sequences are far less effective if dTTP replaces β,γ-methylene dTTP. The latter result has been attributed to the ability of the protein to translocate away from the primase recognition sequence on such long sequences (Kusakabe and Richardson, 1997a). Based on these results, we present a model in which the primase domain catalyzes the synthesis of primers at a recognition site distal from the site to which the helicase domain is bound (Figure 8). Again, the ability of the protein to synthesize primers on a second DNA strand, distinct from the one to which it is bound, supports this model. When the second strand has a region of homology to the strand to which the primase is bound then there is an enhancement of the utilization of the primase recognition site, probably due to the close proximity of the binding site.

The ability of the gene 4 protein to catalyze primer synthesis distal to its binding site could be important in vivo. Prior to the formation of coupled leading and lagging strand synthesis at a replication fork, there is a delay in the initiation of lagging strand synthesis (J.Lee and C.C.Richardson, unpublished results). The ability of the primase domain, in the case of T7, covalently linked to the helicase at the replication fork, to initiate primer synthesis at distal sites of the lagging strand could provide for more efficient lagging strand DNA synthesis. In any case, our results show that the indirect binding of the primase to ssDNA through its association with the helicase bound directly to the ssDNA has important consequences on primer synthesis and that the sole function of helicase need not be to provide translocation activity. In this regard, we believe that our data can be extended to other primase/helicase systems where the association of the two activities need not be covalent but instead involve protein–protein interactions. It is likely that the phenomenon of E.coli DnaB helicase stimulation of the DnaG primase reaction in the presence of ATPγS arises through a similar mechanism (Arai and Kornberg, 1981b; Wahl et al., 1989).

Electron microscopy suggests that the hexameric T7
The primase has a hole through its center estimated to be 25–30 Å in diameter (Egelman et al., 1995). Such a hole could accommodate only a single strand of DNA since two strands of ssDNA have an effectively greater diameter than does the 23.7 Å B-DNA (Stryer, 1995). Consequently, we conclude that the primase domain must reside on the external surface of the hexamer since two strands cannot pass through the center. Such a location is supported by the requirement for an interaction of the primase domain with the DNA polymerase in order for primer transfer from the primase to the polymerase to occur (Scherzinger et al., 1996). Based on the polarity of the translocation reaction and the orientation of the helicase and primase domains, it seemed likely that the site for the protein–protein interaction was located on the helicase protein if it encircled the ssDNA. We believe that these results support a model in which the ssDNA passes through the center of the hexameric gene 4 protein.

Materials and methods

DNA, nucleotides, enzymes and biochemicals

M13mp18 ssDNA was purified as described (Sambrook et al., 1989). All nucleotides were purchased from Amersham Corp. The synthetic dinucleotide, AC, was purchased from Sigma Corp. Oligonucleotide templates for assay of oligoribonucleotide synthesis were chemically synthesized by C.Dahl (Harvard Medical School) or Integrated DNA Technologies, Inc. The nucleotide sequences of the oligonucleotide templates are as follows: AE23T, 5’–TCCACCTTCC CCAATTATTTGACCATCAACC TTCACCTCAC CCCGCCCTCC CCAATATTGGAC-3’; CL-1, 5’–CACCATAATC TCCACACCC TCCAAATTT GCATCAACCC TCATCAATTC-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’. If a 15, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; C0:15, 5’–GGGTCACCGA GATCCTTCAG-3’. If a 10, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’. If a 5, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’. If a 25, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’; HR3, 5’–CCCCCCCC CCCCCGCGTC TTTTTTTTTT TTTTTTTTTT-3’; AD-1, 5’–GGGGTTCAAGA GATCCTTCAG-3’; 26AA, 5’–TTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTT-3’.
The 6% polyacrylamide gel was prepared in buffer containing 375 mM (0.25% bromophenol blue and 15% Ficoll) was added to the samples. Non-denaturing polyacrylamide gel electrophoresis was performed as described (Hingorani and Patel, 1993). T7 primase (100 or 500 nM) was added to the oligonucleotides by incubation overnight at 16°C using T4 ligase. The 70 nt closed circular DNA was isolated on a 10% urea gel. Formation of 32P-radiolabeled circular DNA was carried out as follows. Five pmol of oligonucleotide CL-1 (70 nt) were phosphorylated by γ-[32P]-methylene dTTP at 22°C for 5 min. The T7 primase bound to the oligo(dT) particle complex. After incubation at 37°C for 30 min, the reactions were stopped by the addition of 10 μl of sequencing dye [98% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol FF, 0.1% bromophenol blue]. The reaction mixtures were then heated at 95°C for 5 min and the labeled products were separated by electrophoresis through 25% polyacrylamide gels containing 2 M urea.

Formulation of closed circular DNA

Formation of 3P-radiolabeled circular DNA was carried out as follows. Five pmol of oligonucleotide CL-1 (70 nt) were phosphorylated by [γ-32P]ATP using T4 polynucleotide kinase and mixed with 5 pmol of oligonucleotide AD-1 in 100 mM Tris–HCl (pH 7.5). The mixture was heated at 95°C for 5 min and slowly cooled to anneal, following by ligation of the oligonucleotides by incubation overnight at 16°C using T4 ligase. The 70 nt closed circular DNA was isolated on a 10% urea gel.

Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was performed as described (Hingorani and Patel, 1993). T7 primase (100 or 500 nM) was incubated with 100 mM NaCl in standard reactions (10 μl containing 40 mM Tris–HCl (pH 7.5), 10 mM MgCl2, 10 mM dithiothreitol (DTT), 50 μg/ml bovine serum albumin (BSA), 50 mM potassium glutamate, 0.5 mM γ-[32P]-methylene dTTP. After incubation at 22°C for 5 min, 2 μl of the native gel loading dye (0.25% bromophenol blue and 15% Ficoll) was added to the samples. The 6% polyacrylamide gel was prepared in buffer containing 375 mM Tris–HCl (pH 8.8), 100 μM β-γ-methylene dTTP and 10 mM MgCl2. The 3% polyacrylamide stacking gel was prepared in buffer containing 125 mM Tris–HCl (pH 6.8), 100 μM β-γ-methylene dTTP and 10 mM MgCl2. Electrophoresis was performed in Tris-glycine buffer (pH 8.3) at constant current (20 mA). Protein-[32P]DNA complexes were visualized by autoradiography.

Oligoribonucleotide synthesis catalyzed by T7 primase bound to Magnetight® oligo(DT) particles

Magnetight® oligo(DT) particles were purchased from Novagen, Inc. Preincubation reactions (10 μl containing 0.5 pmol of nucleoside (hexamer) and 1 pmol of oligonucleotide 26AA (50 nt) in primase buffer [40 mM Tris–HCl (pH 7.5), 10 mM MgCl2, 10 mM DTT, 50 μg/ml BSA and 50 mM potassium glutamate] were mixed with 0.5 mM β-γ-methylene dTTP. The mixture was incubated with 0.2 μg of Magnetight® oligo(DT) particles equilibrated with primase buffer containing 0.5 mM β-γ-methylene dTTP at 22°C for 5 min. The T7 primase bound to the oligo(DT) particles was washed with primase buffer containing 0.5 mM β-γ-methylene dTTP and 20 mM oligonucleotide 26AA. As a negative control, the same reaction was carried out in the absence of β-γ-methylene dTTP. Oligoribonucleotide synthesis was initiated by the addition of 10 μl of 40 mM Tris–HCl (pH 7.5), 10 mM MgCl2, 10 mM DTT, 50 μg/ml BSA, 50 mM potassium glutamate, 0.5 mM β-γ-methylene dTTP, 20 mM γ-32P-CTP, 0.1 mM AC dimer and 0.1 mM [γ-32P]-methylene dTTP to the protein–oligo(DT) particle complex. After incubation at 37°C for 30 min, the reactions were stopped by the addition of 10 μl of sequencing dye [98% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol FF, 0.1% bromophenol blue]. The reaction mixtures were then heated at 95°C for 5 min and the labeled products were separated by electrophoresis through 25% polyacrylamide gels containing 2 M urea.

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