DnaB Helicase Stimulates Primer Synthesis Activity on Short Oligonucleotide Templates†

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ABSTRACT: DnaB helicase stimulated the second-order RNA primer synthesis activity of primase by over 5000-fold on DNA templates that were 23 nucleotides long. This template length is the same as the DnaB helicase thermodynamic binding site size [Jezewska, M. J., and Bujalowski, W. (1996) *Biochemistry 35*, 2117−2128]. This phenomenal stimulation was achieved by increasing the template affinity of primase by over 300-fold and increasing the catalytic rate by over 15-fold. It was necessary to determine the optimal amount of DnaB helicase to achieve this stimulation because helicase stimulation was cooperative at low concentration and inhibitory at high helicase concentration. The cooperative stimulation at low concentration indicated the presence of a time-dependent assembly step that preceded the active state. Besides stimulating primase activity, DnaB helicase also prevented primase from synthesizing RNA primers that were longer than the template sequence. In the absence of DnaB helicase, the majority of primers synthesized by primase were longer than the template and were named "overlong primers" [Swart, J. R., and Griep, M. A. (1995) *Biochemistry 34*, 16097−16106]. In contrast, the helicase-stimulated RNA primers were from 10 to 14 nucleotides in length with the 12-mer representing the majority of the total RNA primers produced. It was shown that DnaB helicase stabilized the open or single-stranded conformation of the template, which favored the synthesis of the template-length-dependent primers. In contrast, when primase acted alone, it stabilized the 3′-end hairpin conformation of the template so that the template's 3′-hydroxyl served as a "DNA primer" from which primase elongated to create the overlong primers.

The *Escherichia coli* DNA replication proteins DnaB helicase, primase, and single-stranded binding protein (SSB) are required to initiate DNA synthesis on the lagging strand at replication forks (*1*). DnaB helicase is a ssDNA-translocating enzyme whose 5′ → 3′ polarity causes it to travel on the lagging strand template and to be located at the replication fork. Primase is the special polymerase that synthesizes the nucleic acid oligomers from which the DNA polymerase elongates. Prior to being used as a template by primase, SSB coats the single-stranded lagging DNA strand that was created by the action of the helicase.

DnaB helicase, primase, and SSB have been shown to interact with one another in a variety of replication systems. The primosome was assembled from purified cellular components as being required for the priming of bacteriophage ϕX174 ssDNA (*2*, *3*). The PriA protein of this multienzyme complex has specific affinity for a DNA hairpin called the primosome assembly site (*4*, *5*). Loading of the primosome onto this hairpin does not require primase but does require coating of the ssDNA with SSB. Once the complex is loaded, the DnaB helicase translocates along the viral ssDNA and occasionally stimulates primase to synthesize RNA primers.

The hairpin specificity of the primosome has been used to load DnaB helicase onto a ssDNA tail of an artificial rolling circle synthesis system (*6*, *7*). This system has been used to determine which replication proteins interact during coupled leading and lagging strand synthesis and the order in which they act (*8*−*12*). Neither SSB nor primase were required for leading strand DNA synthesis, but they were required when the synthesis of the two strands was coupled. In addition, it was also shown that primase was involved in the rate-limiting step of lagging strand DNA synthesis and was not continuously associated with the replication fork. In contrast, both the dimeric DNA polymerase III holoenzyme and the DnaB helicase were continuously associated with the DNA. Later studies with modified primase found that its C-terminus was critical for its ability to interact with DnaB helicase in this system such that coupled leading and lagging strand synthesis did not take place (*13*−*15*).

While examining the roles of the various proteins in the primosome, it was found that, in the absence of SSB, the combination of DnaB helicase and primase was able to synthesize RNA primers on all of the natural and synthetic templates that were tested (*16*−*18*). This was termed the general priming system because it lacked ssDNA sequence specificity. Because SSB is certainly present in the bacterium and is essential, this system has been considered to be primarily of biochemical interest. However, biochemical studies of DnaB helicase have also shown that this enzyme is only inhibited if SSB is added to the ssDNA substrate first (*19*). In fact, SSB even enhances the helicase activity when it is added after the helicase has loaded. Thus, the physiological key during DNA replication initiation is to load DnaB helicase before SSB.

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The rolling circle and general priming systems have been used to establish the functions and structural portions of DnaB helicase and primase that are required for their interaction. DnaB helicase is a homohexamer (*20*−*23*) and a variety of studies have shown that the long hinge between helicase's N-terminal head and its homohexameric body must be intact for it to be able to bind and stimulate primase activity (*24*−*26*). DnaB helicase must play a structural role in its stimulation of primase because it does not have to retain either its helicase function (*27*) or its ATPase function (*28*, *29*) to do so. As for primase, two of its C-terminal residues are essential for coupled DNA synthesis in the rolling circle synthesis system (*15*) and for binding to the DnaB helicase (*26*).

Our lab recently developed an assay for initiation site-specific primase activity that only required primase, Mg^{2+}, a short oligonucleotide template, and the appropriate nucleoside triphosphate mixture (*30*, *31*). The critical features of the template were that it contained the trinucleotide initiation sequence d(CTG) and at least 6 nucleotides flanking its 3′ side. By using a template sequence containing only one thymine [the one in the trinucleotide initiation sequence d(CTG)] and γ-[^{32}P]ATP to study RNA primer synthesis, only those primers that initiated from the d(CTG) were observed and they each had 1 radiolabel regardless of their length. Using this template design in which the initiating nucleotide was known, it became a trivial matter to determine the length of RNA primers that were synthesized through the use of controls in which chain-terminating dideoxyribonucleotides were added (*31*). In the study described here, various modifications to this assay were used to characterize the effect that DnaB helicase had on the primer synthesis activity of primase. We found that DnaB helicase greatly stimulated primer synthesis activity on these 23 nucleotide templates which were the same size as the DnaB-binding site size.

EXPERIMENTAL PROCEDURES

DNA and Enzymes. The oligonucleotides were synthesized either by the University of Nebraska—Lincoln Nucleic Acid Synthesis Facility, Midland Certified Reagent Company (Midland, TX), or Research Genetics (Huntsville, AL). They were then either purified by us as previously described (*31*) or by the company. The concentrations of d(CACACAGACACACACTGCAAAGC) and d(CACACAGACACACACTGCACACA) were determined using their extinction coefficients of 226.0 and 210.1 mM^{-1} cm^{-1} at 260 nm. The purities of all oligonucleotides were checked by 5′-end labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs, Inc.) according to the standard procedure (*33*).

Primase was isolated as previously described (*34*) from a primase-overproducer generously supplied by Dr. Roger McMacken (John Hopkins University). Its concentration was determined using its extinction coefficient of 47 800 M^{-1} cm^{-1} at 280 nm (*30*). DnaB helicase was isolated from overproducing cells according to a procedure similar to the one we used for primase. The DnaB helicase-overproducing plasmid pRLM1038 in an *E. coli* strain was kindly provided by Dr. Roger McMacken. The ATPase activity of helicase was monitored using a continuous spectrophotometric assay

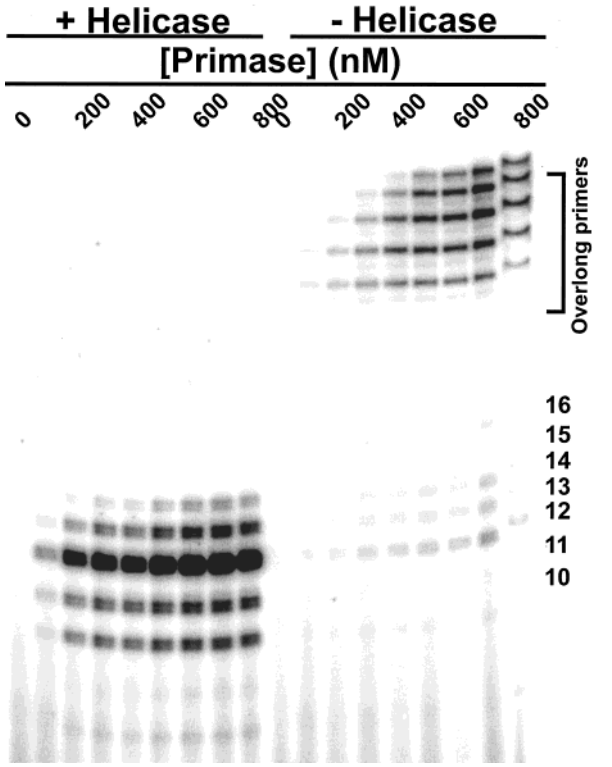

FIGURE 1: Effect of DnaB helicase on RNA primer synthesis by primase. A mixture of all components except primase (200 nM template, 200 μM NTPs including α-[^{32}P]UTP as the label, ±1200 nM DnaB helicase monomer) was preincubated for 5 min at 30 °C. Then, 200 nM primase was added to initiate the reaction. After 10 min at 30 °C, the reaction was terminated and analyzed as indicated in the Experimental Procedures. The approximate number of nucleotides in the RNA polymers are indicated along the right side of the image.

(*35*). The protein concentration was determined using the extinction coefficient for DnaB helicase hexamer of 185 000 M^{-1} cm^{-1} at 280 nm (*22*).

RNA Primer Synthesis Assay. The primer synthesis assays were performed as previously described (*31*). Briefly, a typical 25 μL reaction contained 50 mM Hepes (pH 7.5), 10 mM magnesium acetate, 100 mM potassium glutamate, 10 mM dithiothreitol, 200 μM NTPs, [α-^{32}P]UTP (5.0×10^7 cpm/reaction) or [γ-^{32}P]ATP (7.5×10^7 cpm/reaction), 2 μM ssDNA template, 200 nM DnaB helicase hexamer, and 400 nM primase. All components except for primase were preincubated at 30 °C for 1 min, primase was added, and the reaction run at 30 °C for 1 min. The figure legends indicate the actual primase, helicase, and template concentrations used.

RESULTS

Stimulation of Primase Activity by DnaB Helicase. Primer synthesis activity was measured as a function of primase concentration in the presence or absence of DnaB helicase. In this first set of experiments, the radiolabel was α-[^{32}P]-UTP so that all RNA primers would be visualized regardless of their initiating nucleotide. In the absence of DnaB helicase, primase synthesized two classes of RNA primers (Figure 1, right side) when using the template d(CAGA$(CA)_5$CTG-CAAAGC). One class of RNA primers was between 10 and 16 nucleotides long, and the other class of RNA primers was

about twice that length. Both of these primer classes have been reported previously (*31*) and were, respectively, named the "template-length-dependent" primers and the "overlong" primers. The shorter class was so-named because they had the appropriate sequence and length to have been initiated from the d(CTG) initiation sequence and to have been synthesized complementary to the template. The RNA primer sequence was determined by using dideoxyribonucleotides as chain terminators (*31*), a technique that was also exploited in the present study. Regardless of the primase concentration, the ratio of uracils in the two RNA primer classes remained constant at 29 ± 3 in the overlong primers for every one in the template-length-dependent primers. It appeared that these two classes of primers were not directly related to one another since it was not possible to convert one of them into the other by adding more primase (Figure 1) or by incubating for longer time periods (*31*). It will be shown below that the major difference between these two primer classes was in how they were initiated.

When the optimum amount of DnaB helicase was added to the assay, the classes, lengths, and amounts of RNA primers synthesized by primase were altered when using the 23 nucleotide template with a sequence of d(CAGA-$(CA)_5$CTGCAAAGC) (Figure 1, left side). The change in the primer classes was that helicase prevented the synthesis of the overlong primers even though they had been the most abundant class in the absence of helicase (see previous paragraph). Thus, any mechanism concerning overlong primers will have to be able to explain this feature. The lengths of the template-length-dependent RNA primers were between 10 and 14 nucleotides long in the presence of helicase rather than from 10 to 16 nucleotides as in the absence of helicase. In fact, the majority of them (60%) were 12 nucleotides long (Figure 1) and there was never more than a few percent of RNA primers that was shorter than 10 nucleotides. The third profound effect that DnaB helicase had on primer synthesis activity was that it stimulated it considerably. It was so considerable that, even though the assay was incubated for 1 min with helicase versus 60 min without helicase, the amount of radioactivity in the presence of helicase had to be one-fifth as much to be able to visualize them on the same gel as those synthesized in the absence of helicase.

Effect of DnaB Helicase and Template Concentration on RNA Primer Synthesis Activity. We examined the relationships between DnaB helicase, template, and primase concentration on RNA primer synthesis activity to establish the nature of the functional interaction between these three components in vitro. At 200 and 400 nM primase, the helicase dependence displayed sigmoidicity at low concentrations and inhibition at high concentrations (Figure 2). At both 200 and 400 nM primase, the apparent optimum helicase concentration was between 1200 and 1800 nM monomer. This helicase concentration at optimum primase activity was equivalent to 200−300 nM helicase hexamer and was much closer in concentration to the primase than to the oligonucleotide template (2000 nM).

The sigmoidal response at low helicase reflected one of two possibilities. Either there was cooperativity with respect to helicase binding when it complexed with primase and the template or there was a time-dependent assembly of primase, helicase, and template. It is unlikely that the cooperativity

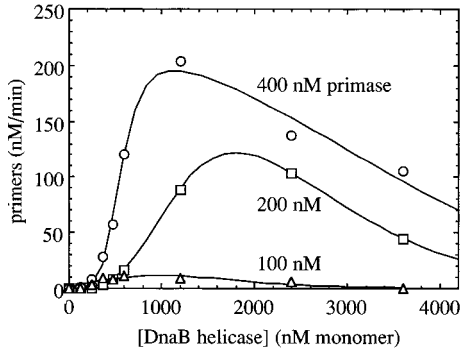

FIGURE 2: RNA primer synthesis activity as a function of DnaB helicase and primase concentration. Mixtures of template (2000 nM), helicase (various concentrations), and the four NTPs (including α-[^{32}P]UTP) were preincubated for 1 min and then incubated for 1 min after the addition of primase [(△) 100 nM; (□) 200 nM; (○) 400 nM].

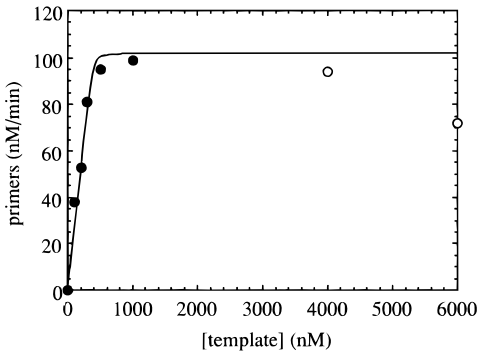

FIGURE 3: DnaB helicase-stimulated RNA primer synthesis activity as a function of template concentration at the optimal primase-to-helicase ratio. The mixture contained 400 nM primase and 200 nM DnaB helicase hexamer, the label was α-[^{32}P]UTP, and the preincubation and incubation times were each 1 min.

involves the binding of multiple DnaB helicase hexamers to the template because our oligonucleotide templates are too small to allow the binding of more than one hexamer to a template (*36*). In addition, the cooperativity is not likely to involve the assembly of DnaB helicase protomers into hexamers because it has been established that it is hexameric even at concentrations as low as 1 μM (*22*). To test whether the apparent cooperativity was due to some time-dependent phenomenon, a series of preincubation experiments was performed. We found that preincubating the primase-NTP−helicase−template complex for between 5 s and 1 min led to the same high level of activity and the same apparent cooperativity at low helicase concentrations. When the preincubations were carried out for longer than 2 min, the levels of primer synthesis at all helicase concentrations were greatly inhibited. As a result of these experiments, all subsequent preincubations and incubations were conducted for 1 min each.

A template-concentration-dependence experiment revealed that RNA primer synthesis activity responded nearly stoichiometrically with respect to primase concentration, which was 400 nM in this experiment (Figure 3). Below 390 nM template, RNA primer synthesis was linearly dependent on template concentration. The stoichiometry of 390 nM correlated with the primase concentration and not the helicase hexamer concentration of 200 nM. When the template concentration was more than 10-fold higher than either primase or DnaB helicase, RNA primer synthesis activity

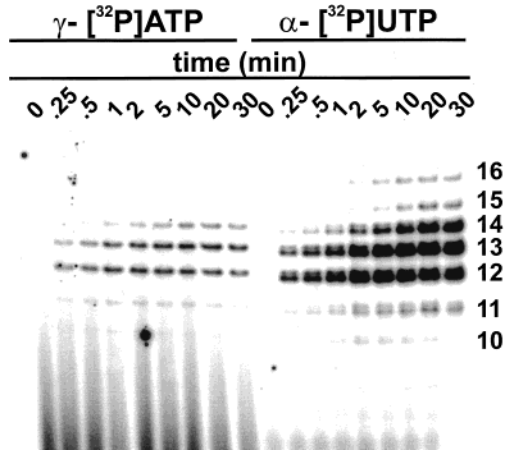

FIGURE 4: Denaturing electrophoretic gel of the DnaB-stimulated RNA primer synthesis time course labeled using either γ-[^{32}P]ATP or α-[^{32}P]UTP. A mixture of 400 nM helicase hexamer and 400 nM template was preincubated for 1 min at 30 °C and then 200 nM primase was added to initiate the reaction.

was inhibited (Figure 3). We hypothesize that, at high template, both primase−template and helicase−template complexes will form and result in less of the high activity primase−helicase−template complex.

The affinity of primase for the template in the presence of DnaB helicase was determined by fitting the data to the equation $v = V_{max}[PD]/P$, where $[PD] = \{P + D + K_d - [(P + D + K_d)^2 - 4PD]^{1/2}\}/2$, P is total primase concentration, D is total template concentration, and K_d is the primase−template dissociation constant. From the fit, the V_{max} was determined to be 102 ± 9 nM/min, the K_d was 2 ± 13 nM, and there was 8.9% error in the fit of the data. The large standard deviation associated with the dissociation constant reflected the necessarily high primase and helicase concentrations that were needed to achieve measurable primer synthesis velocities.

Effect of DnaB Helicase on the Primer's Initiating Nucleotide. Given that DnaB helicase prevented overlong primers from forming, altered the lengths of primers synthesized, and increased the rate at which primers were synthesized, it was likely that there were also changes in which nucleotides were being used to initiate primer synthesis. This issue was addressed by comparing the helicase-stimulated RNA primers that were visualized using [γ-^{32}P]ATP versus [α-^{32}P]UTP (Figure 4). When [γ-^{32}P]ATP was used as the radiolabel, only those RNA primers that were initiated complementary to the unique template thymine from the trinucleotide d(CTG) were visualized. When [α-^{32}P]UTP was used as the radiolabel, all RNA primers were visualized regardless of their initiating nucleotide because the template had a high adenine composition.

The time course for incorporation of [γ-^{32}P]ATP or [α-^{32}P]-UTP revealed that there were at least two initiation sites on the template and that the majority of primers were not initiated with ATP. When [γ-^{32}P]ATP was present in the assay, each RNA primer length species was a single band (Figure 4, left half). Whereas, when [α-^{32}P]UTP was present in the assay, then each RNA primer length appeared as a doublet (Figure 4, right half). These doublets were observed in all of the experiments that were visualized with [α-^{32}P]-UTP, which indicated that there was more than one sequence of primers being synthesized complementary to the template.

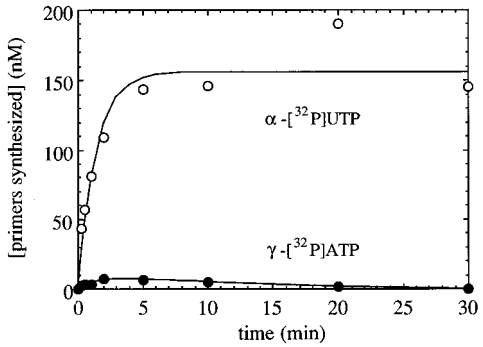

FIGURE 5: Time course for total RNA primers [(○), α-[^{32}P]UTP as the radiolabel] or ATP-initiated RNA primers [(●), γ-[^{32}P]ATP as the radiolabel] synthesized by 200 nM primase in the presence of 200 nM DnaB helicase hexamer and 2000 nM template. The rate of RNA primer synthesis was determined by fitting the [α-^{32}P]-UTP-labeled time course data to the equation [RNA primers] = $f_{total}[PD]_0[1 - \exp(-k_{obsd}t)]$, where f_{total} was the fraction of oligonucleotides that served as a template, $[PD]_0$ was the concentration of primase−ssDNA complex which was taken to be equal to the primase concentration because the ssDNA was saturating, k_{obsd} was the observed catalytic rate, and t was time in minutes.

Table 1: Rate Constants Associated with the Primase−Helicase-Template Complex[a]

radiolabel	f_{total}	k_{obsd} (min^{-1})	k_{inhib} (min^{-1})	% fit error
[α-^{32}P]UTP	0.78 ± 0.04	0.74 ± 0.18		13
[γ-^{32}P]ATP	0.044 ± 0.005	0.93 ± 0.25	0.20 ± 0.03	16

[a] The data were fit to the equation described under Results using a nonlinear least-squares program. The derived parameters for the helicase−primase-template complex were f_{total}, the fraction of templates utilized to make primers containing the indicated radiolabel; k_{obsd}, the rate constant for RNA primer synthesis; k_{inhib}, the rate constant for the inhibition of RNA primer synthesis; and % fit error, the overall error associated with the fit of the data to the derived parameters.

The slightly slower migrating species within each doublet had the same mobility as the [γ-^{32}P]ATP-labeled species. Since the [γ-^{32}P]ATP-labeled primers were necessarily initiating complementary to the unique thymine in the template, we could conclude that the faster-migrating band in the doublet must have a different initiating nucleotide.

Because primase does not transfer very efficiently from a completed primer to a new template, its reaction time course can be described according to single-turnover kinetics (*31*) as follows: [RNA primers] = $f_{total}[PD]_0[1 - \exp(-k_{obsd}t)]$, where f_{total} was the fraction of oligonucleotides that served as a template, $[PD]_0$ was the concentration of primase−ssDNA complex which was taken to be equal to the primase concentration because the ssDNA was saturating, k_{obsd} was the observed catalytic rate, and t was time in minutes. The equation was also multiplied by $\exp(-k_{inhibition}t)$ for the [γ-^{32}P]ATP-visualized primers because the number of RNA primers decreased at long incubation times. To ensure conformity to single turnover conditions, the time course was determined using a primase concentration of 400 nM, the same magnitude as the template concentration (Figure 5). The nonlinear least-squares fit of the single-turnover data indicated that the [γ-^{32}P]ATP- and [α-^{32}P]UTP-visualized primers differed in the fraction of templates used to make them but were similar in their rates of synthesis (Table 1). The fraction of templates used to make [α-^{32}P]UTP-visualized primers was 0.78. This was 18-fold greater than the

fraction used to make the [γ-^{32}P]ATP-visualized primers, which indicated that only a small fraction of RNA primers was being initiated complementary to the unique thymine in the template. Interestingly, the choice of initiating nucleotide did not have much influence on the subsequent rate of synthesis. The rate constants were not significantly different at 0.74 ± 0.18 min^{-1} for [α-^{32}P]UTP-visualized primers versus 0.93 ± 0.25 min^{-1} for [γ-^{32}P]ATP-visualized primers.

Source of the Overlong RNA Primers. In the absence of DnaB helicase, two types of RNA primers were synthesized by primase (*31*). The "template-length-dependent RNA primers" were initiated with [γ-^{32}P]ATP, had a sequence complementary to the template, and were 16 nucleotides in length or shorter. The "overlong RNA primers" were not initiated with [γ-^{32}P]ATP or [γ-^{32}P]GTP and were longer than the template. The overlong RNA primers had been discovered when [α-^{32}P]UTP was used to visualize all of the products of RNA primer synthesis on the 23 nucleotide 5′-d(CAGA(CA)$_5$CTGCAAAGC). This experiment had been performed when quantification revealed that less than 10% of the templates was being used to create the [γ-^{32}P]ATP-initiated RNA primers. When visualized using [α-^{32}P]UTP instead, the majority of RNA primers was revealed to be the overlong RNA primers, and only 8% were the template-length-dependent RNA primers (*31*).

We used a template sequence with a different 3′-terminal sequence, 5′-d((CA)$_3$GA(CA)$_3$CTGCACACA), so that we could examine the relationship between its template-length-dependent RNA primer synthesis requirements and those for its overlong RNA primers. An important observation was made when we measured RNA primer synthesis in the presence and absence of ATP. Total RNA primer synthesis was visualized using [α-^{32}P]UTP incorporation, and the concentration of GTP was varied to examine its effect. In the presence of ATP (left side of Figure 6), the template-length-dependent as well as the overlong species was synthesized. This was as previously reported for the original template (*31*). Also, as before, the optimum GTP concentration was 200 μM for both template-length-dependent and overlong RNA primers. By 500 μM GTP, there was considerable inhibition of both types of RNA primers which correlated with the synthesis of shorter lengths of both types of RNA primers.

When ATP was omitted from the RNA primer synthesis reaction, only the overlong species were observed (right half of Figure 6) and in the same amounts as in the presence of ATP. This was surprising because ATP had been shown to be essential for overlong primer synthesis when using the template that terminated with d(CAAAGC) (*31*). Even though the absence of ATP slightly altered the pattern of overlong RNA primer lengths at the different GTP concentrations (compare left and right sides of Figure 6), the ATP played a nonobligatory role. Since the major difference between these two templates involved their 3′-terminal sequences, we hypothesized that the two terminal nucleotides were forming a very short hairpin with bases near the initiation trinucleotide (Figure 7). This mechanism predicted that the 3′-hydroxyl of the template would serve as a primer for RNA synthesis, that rGMP would be the first base added, and that the longest overlong polymer would be a 38-mer composed of the 23 deoxyribonucleotide template extended from its 3′-end with 15 ribonucleotides with a sequence

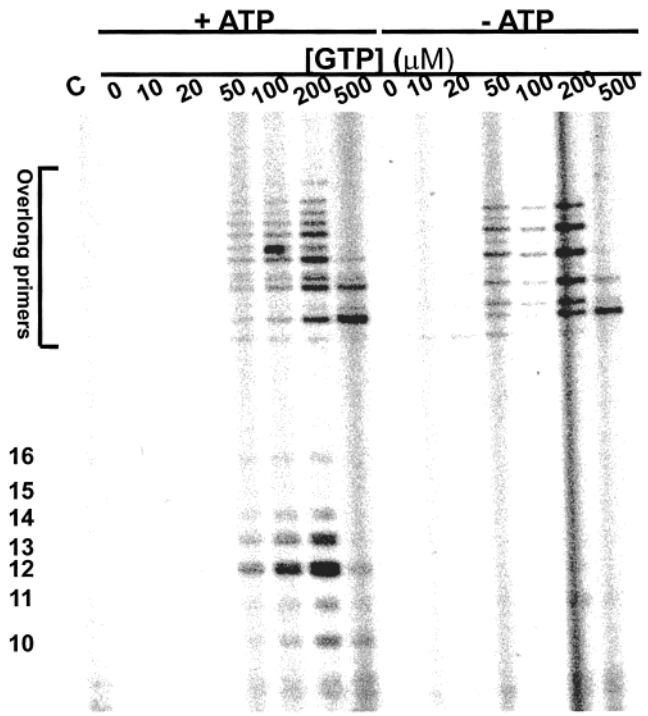

FIGURE 6: The effect of ATP on the GTP concentration dependence of primer synthesis by primase alone. Primase (200 nM), with or without ATP (200 μM), CTP (200 μM), UTP (200 μM), and various concentrations of GTP were incubated for 30 min with 23 deoxynucleotide template (200 nM). The reactions were quenched, the species resolved, the image developed in the usual manner described in the Materials and Methods. The control lane C represents a sample from which the primase was omitted.

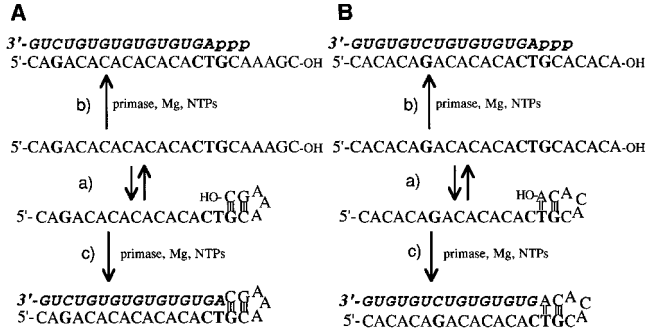

FIGURE 7: Proposed overlong primer synthesis mechanism. Reactions a are the equilibrium between the single strand and the 3′-hairpin conformations of the two templates used in this study. Reactions b show the primer synthesis product when primase initiates de novo from the single strand template conformations. In this case, the primers retain their 5′-triphosphate. Reactions c show the primer synthesis products when primase elongates from the 3′-hydroxyl of the template in their hairpin conformations.

complementary to the 5′-end of the template. Each of these predictions was tested.

We were able to show that GMP was the first base incorporated at the end of the template to begin the overlong RNA primers when we compared the primers visualized by [α-^{32}P]GTP and [α-^{32}P]UTP (left and right panels of Figure 8). Both of these labels were expected to be incorporated into the RNA primers and result in similar proportions of each kind of primer because the total number of cytosines and adenines in the template were similar. For the most part, the pattern of RNA primers and their relative intensities were almost identical for the two different radioactive labels. The

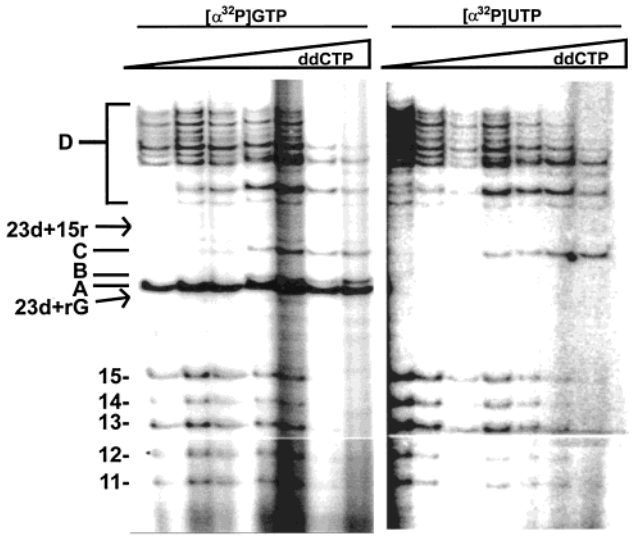

FIGURE 8: Identification of the first nucleotide added by primase during the synthesis of overlong primers. Primase (200 nM) and ribonucleoside triphosphates (200 μM each and including either α-[^{32}P]GTP or α-[^{32}P]UTP) were incubated for 10 min with 23 deoxynucleotide template (200 nM) with various concentrations of dideoxyribocytidine triphosphate. The reactions were quenched, the species resolved, the image developed in the usual manner described in the Materials and Methods. The arrow labeled "23d+rG" along the left side of the image indicates the migration position of the 5′-[^{32}P]phosphorylated standard 5′-d(CACACAGACACACACTGCACACA)-r(G) and the arrow labeled "23d+15r" indicates the migration of the 5′-[^{32}P]phosphorylated standard 5′-d(CACACAGACACACACTGCACACA)-r(GUGUGUGUCUGUGUG).

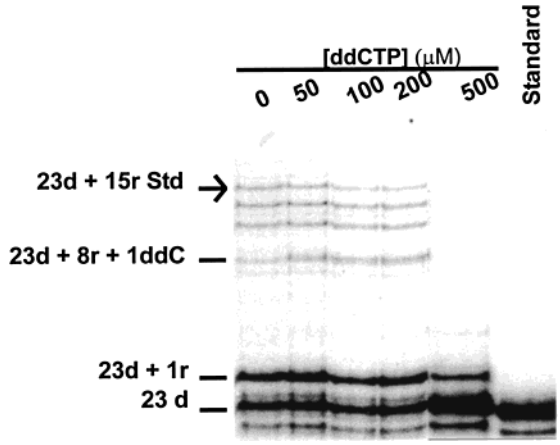

FIGURE 9: Extension of the template by primase to create the overlong primers. Primase (200 nM) and nonradioactive ribonucleoside triphosphates (200 μM each) were incubated for 10 min with the 5′-[^{32}P]phosphorylated 23 deoxynucleotide template (about 1 nM) with various concentrations of dideoxyribocytidine triphosphate. The reactions were quenched, the species resolved, the image developed in the usual manner described in the Materials and Methods. The lane marked standard indicates a sample containing only radiolabeled template. Along the left side of the image, the arrow points to the migration position of the 5′-[^{32}P]phosphorylated standard 5′-d(CACACAGACACACACTGCACACA)-r(GUGUGUGUCUGUGUG). The three other species that are identified along the side of the image are the 5′-[^{32}P]phosphorylated 23 nucleotide template d(CACACAGACACACACTGCACACA), the first ribonucleotide addition product labeled "23d + 1r", and the dideoxycytidine-terminated species labeled "23d + 8r + 1ddC".

major difference was that two extra bands (labeled A and B in Figure 8) were visualized only in the presence of [α-^{32}P]-GTP. From the distance that these species migrated in the denaturing gel, we estimated that they were about 22 ribonucleotides long. We reasoned that a 23 mer deoxyribonucleotide with a terminal riboguanosine would also migrate with this velocity because deoxyribopolymers migrate slightly faster than ribopolymers of similar length. Because the intensity of the special band A did not diminish at increasing ddCTP concentrations, we inferred that the additional nucleotide was a riboguanosine. Because the special band B appeared only as the concentration of ddCTP was increased, we inferred that it was the product of adding a dideoxycytidine after adding the radiolabeled riboguanosine. The identity of band A was confirmed when we found that the migration of a 23 deoxyribonucleotide oligomer of the same sequence as our template and with a 3′-terminal riboguanosine but 5′-labeled with ^{32}phosphate migrated slightly ahead of band A (the arrow labeled "23d + rG" in Figure 8 shows its approximate location). This was reasonable because the shortest overlong primer would not be phosphorylated (at the 5′-end of the template) and its electrophoretic mobility would be slightly less than our standard, which differed only in that it was 5′-phosphorylated.

There were several bands of interest that appeared when the primers were labeled with either [α-^{32}P]GTP or [α-^{32}P]-UTP (Figure 8). The band labeled C accumulated as the ddCTP concentration was raised. It migrated faster than a 5′-^{32}P-labeled oligonucleotide with a sequence of the 23 deoxyribonucleotide template and covalently linked to 15 ribonucleotides with a sequence complementary to the template (its migration is shown by the arrow labeled "23d + 15r"). Because it accumulated at high ddCTP and because of its mobility, it was likely to be the overlong primer created by elongating eight ribonucleotides from the 23 nucleotide template and then terminating with a dideoxycytidine. Such a species indicated that the overlong primers were being synthesized complementary to the template sequence even though they were being initiated in a unusual manner. There were numerous lengths of overlong primers that were labeled D. Their migration relative to the "23d+15r" indicated that they were longer than just one template plus its complementary ribopolymer. These primer species accumlated and increased in length when the concentration of template approached and exceeded that of primase (for instance, compare to Figure 9). These overlong primers were synthesized in a template-directed manner as indicated by the appearance of only two primer bands as the concentration of ddCTP was increased. The overlong primers labeled D probably resulted from some combination of template slipping and template jumping as has been observed for other polymerases (*37*−*40*).

When these same types of experiments were performed using a template with a different initiation trinucleotide, namely d(ATG) in place of the d(CTG), a special overlong RNA primer band similar to band A in Figure 8 was observed only when the overlong RNA primers were visualized with [α-^{32}P]UTP (see third panel of Figure 3 in the following manuscript). This provided additional evidence that the first position of the initiation trinucleotide was coding for the first inserted nucleotide of the overlong RNA primers synthesized on this template.

To demonstrate that the template was providing the 3′-hydroxyl from which the overlong RNA primers were elongated, we tested whether a 5′-radiolabeled template could

be elongated by primase using an unlabeled ribonucleoside triphosphate mixture (Figure 9). The pattern of RNA primers that was synthesized in this experiment resembled the pattern of template-length-dependent primers. According to the migration distance of the control oligonucleotide labeled 23d+15r, the longest primers in this experiment were equal to the length of the template plus a covalently attached complementary primer. As the concentration of ddCTP was raised from 0 to 250 μM, one of the bands accumulated at the expense of the others. Because the migration of this species was consistent with the location of the special guanosine in the template relative to the initiating nucleotide, it was labeled 23d+8r+1ddC in Figure 9. When ddCTP concentration was raised further, all RNA primer synthesis was inhibited as usual. All of this evidence indicated that the overlong RNA primers had a sequence that was complementary to the template.

As noted above, the migration distance of the control oligonucleotide labeled "23d+15r" indicated that the longest overlong primers in the Figure 8 experiment were longer than the overlong primers in the Figure 9 experiment. It is probably significant that the template concentration in the Figure 9 experiment was several orders of magnitude lower than in the Figure 8 experiment. This fact supports the mechanism that the overlong primers in the Figure 8 experiment, but not the Figure 9 experiment, were caused by template jumping. Template jumping does not occur when the template concentration is much lower than the primase concentration (Figure 9) and the resulting polymer is no longer than the template plus its complementary primer.

Finally, we hypothesized that it should be possible to inhibit the synthesis of overlong primers by blocking the template's 3′-hydroxyl. This was tested with a 23 nucleotide template of the same sequence used above but that was terminated with a 3′-ether linkage to 1,3-propanediol. When this template was used in the primer synthesis reaction and α-[^{32}P]UTP was used as the radiolabel to visualize all of the primers synthesized, 93 ± 2% of all RNA primers were shorter than the template (Figure 10) regardless of the ddCTP concentration. The resulting small percentage of overlong primers proved that blocking the template's 3′-hydroxyl was effective in inhibiting their production.

At the highest ddCTP, only those primers that have terminated at the special template guanosine should be synthesized. Interestingly, there were about a half dozen bands observed at the highest ddCTP concentration (Figure 10). Because all 3′-dideoxycytidine-terminated primers should have the same 3′-terminal sequence, this meant that these RNA primers differed in their 5′-initiating sequence. Thus, it appeared that initiation sequences other than the trinucleotide d(CTG) were being used when primase was inhibited from creating overlong primers.

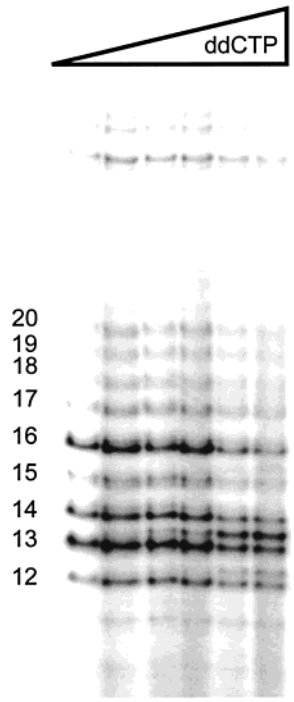

FIGURE 10: Inhibition of overlong primer synthesis by blocking the 3′-terminus of the template. The template in this experiment had the same sequence as that used in Figures 8 and 9 but it differed in that its 3′-hydroxyl was blocked by a dihydroxypropane group as described in the Experimental Procedures. Primase (2 μM), template (200 nM), and a mixture of ribonucleoside triphosphates (200 μM each) including [α^{32}P]UTP were incubated for 10 min with various concentrations of dideoxyribocytidine triphosphate. The reactions were quenched, the species resolved, the image developed in the usual manner described under Experimental Procedures. The lengths of the template-length-dependent primers are shown along the left side of the image.

DISCUSSION

Nature of Overlong Primers. We find that *E. coli* primase will only synthesize overlong primers when it acts in the absence of DnaB protein. Because primase is able to elongate efficiently from a deoxyribonucleotide (*31*, *41*), we hypothesized that when primase acts alone it stabilizes a 3′-end hairpin template conformation (Figure 7) and then it extends from the 3′-hydroxyl of the template to create the overlong primers. This overlong primer mechanism was tested in several ways and found to hold true. It should be noted that these overlong primers are different from the modal length primers synthesized by many eukaryotic primases (*42*, *43*). The modal length primer mechanism is that primase dissociates after about 8−10 nucleotides of RNA synthesis and then reassociates and elongates from the end of the RNA primer (*44*−*46*).

The first evidence relevant to the overlong primer synthesis mechanism was that AMP was not incorporated as part of the overlong primers (Figure 6). The template used in the present study has a sequence such that the overlong primers should not incorporate any AMP if the mechanism presented in Figure 7B is true. Likewise, the mechanism predicts that the template-length-dependent primer should initiate with ATP complementary to the thymine in the initiation trinucleotide sequence d(CTG). When [α-^{32}P]GTP is used to visualize all of the types of primers that are synthesized, both of these predictions are found to be true: (1) only the overlong primers are synthesized when ATP is omitted from the primer synthesis reaction and (2) both the overlong primers and the template-length-dependent primers are synthesized when ATP is present. The template-length-dependent RNA primers in this and the earlier study using a different template (*31*) are known to initiate with ATP because they are only visualized when using [γ-^{32}P]ATP. Because it is possible to find conditions in which only the overlong primers are synthesized, it proves that the two types of primers are not interconvertable and arise according to

different mechanisms. There must be an equilibrium between the unfolded and the hairpin-folded template, each form of which leads to one of the two types of primers (Figure 7). Primase must influence the equilibrium to favor the hairpin-folded conformation because there are more overlong primers synthesized than template-length-dependent ones. When DnaB helicase is present, there are no overlong primers synthesized and the equilibrium must be shifted in the opposite direction.

The second piece of evidence relevant to the overlong primer synthesis mechanism is that the first ribonucleotide added to the template is GMP. The first-added ribonucleotide should be GMP because the 3′-terminal sequence d(CA) should anneal to the unique d(TG) sequence located within the initiation trinucleotide. Also consistent with the mechanism is that this shortest of the overlong primers is not visualized by [α-^{32}P]UTP, although the longest overlong primers are (Figure 8). The pattern of overlong primers also indicates that the addition of the first ribonucleotide to the 3′-end of the template is slow, that subsequently added ribonucleotides are added much faster until a certain length of primer has been reached, and then that synthesis becomes slow again. Such a pattern of phosphodiester bond formation rates is reminiscent of that described for the template-length-dependent primer synthesis (*31*). However, the first synthetic step in overlong primer synthesis is fundamentally different because it involves the addition of a ribonucleotide to a deoxyribonucleotide. It may be that the shortest overlong primer is visualized because primase is slower to elongate from a deoxyribonucleotide than from a ribonucleotide (*31*, *41*).

The third piece of evidence is that the overlong primer length pattern is sensitive to ddCTP concentration but that the total numbers of overlong primers synthesized is not. The template includes a special guanine near its 5′-end which allows primers to be terminated at a specific site regardless of where or how they are initiated. Since the special site is not the same as the initiation site, termination at this site should influence their lengths but not the total numbers of primers synthesized. It does not. In addition, the lengths of the overlong primers decreases to create certain discrete lengths of primers (Figure 8) when the ddCTP concentration is increased in the presence of constant CTP.

The last two pieces of evidence relevant to the overlong primer synthesis mechanism involve the template itself. Because a 5′-phosphorylated template can be elongated by a mixture of primase and nonradiolabeled NTPs (Figure 9), it indicates that the template itself acts as the "DNA primer" from which the primase elongates. The products of this elongation share important similarities with the previously described overlong primers. The most abundant product involves the addition of a single ribonucleotide to the end of the template. Thus, primase appears to stall after adding the first ribonucleotide just as was seen in the Figure 8 experiment. The longer products have lengths which are sensitive to ddCTP concentration, which indicates that they are being synthesized complementary to the 5′-end of the template sequence. The last evidence relevant to the overlong primer synthesis mechanism is that they are not created when the 3′-hydroxyl of the template is blocked with propanediol (Figure 10). This demonstrates the essential role that the 3′-end of the template plays in this process.

Table 2: Summary of Primer Synthesis Constants[a]

	$k_{observed}$ (s^{-1})	K_M (M)	$k_{observed}/K_M$ ($M^{-1}s^{-1}$)	ref
without DnaB	8.9×10^{-4}	7.2×10^{-7}	1.2×10^{3}	*25*
with DnaB	1.2×10^{-2}	2×10^{-9}	6.2×10^{6}	this study

[a] The constants were obtained using 23-nucleotide templates incubated at 30 °C in 50 mM Hepes, 100 mM potassium glutamate, and 5 mM magnesium acetate at pH 7.5.

Now that we understand the relationship between the template's sequence and the nature of overlong primers, we will be able to design templates that are more useful in our template-sequence-dependent primer synthesis system. In turn, this will lead to a better understanding of primase function. The ability of primase to elongate from a 3′-hairpin demonstrates that primase is able to elongate primed DNA just as does nearly every other nucleic acid polymerase. Because primase makes more "overlong primers" than "template-length-dependent" primers on our short oligonucleotide templates, this enzyme is better at elongating than at initiating.

DnaB Protein Stimulates Primase Activity on Oligonucleotide Templates. The oligonucleotide templates that were used in this and the accompanying study (see the following paper in this issue) were 23 nucleotides. Even though this length is nearly equal to the DnaB helicase binding site size of 20 ± 3 nucleotides independent of the template nucleotide composition (*36*, *47*), DnaB helicase was capable of greatly stimulating primer synthesis activity on these short templates with defined sequence. Because we also measure primer synthesis directly rather than via a coupled RNA−DNA synthesis system as has been done in the past (*17*, *18*), our much simpler system has allowed us to answer some questions that it has not been possible to address.

A profound change is seen in the length and rate of synthesis of RNA primers produced by primase when DnaB helicase is present. The length of RNA primers is shortened to 10−14-nucleotides, correlating well with the 11 ± 1 nucleotide RNA primers seen in vivo (*48*). These results show that the change in the lengths of RNA primers when DnaB helicase is added to primase occurs because the hairpin conformation of the template is prevented from forming.

The high affinity of the primase−helicase complex for the template reported here ($K_M \approx 2$ nM) was in striking contrast to the much weaker affinity of primase for the template when it acted alone ($K_M \approx 720$ nM) (*31*). The rate of template-length-dependent synthesis was also stimulated 14-fold from 0.053 min^{-1} (*31*) by primase alone to 0.74 min^{-1} when DnaB helicase is present in its optimum ratio with primase (Table 2). These values indicate that DnaB helicase stimulates the second-order rate of template-length-dependent primer synthesis by over 5000-fold. The major effect, though, is to increase the affinity between primase and the template. Helicase may place the ssDNA into an unfolded conformation that is favorable to primase binding or it may bind primase and the template simultaneously to achieve this effect.

The sigmoidal dependence of primer synthesis activity with respect to low DnaB helicase concentration indicates that there is a kinetic barrier to the formation of the highly active primase−helicase−template complex. The active form of the complex appeared within 1 min, but longer preincu-

bation times resulted in less active complexes. Also of interest is that high DnaB helicase inhibits primer synthesis stimulation. In fact, experiments are underway to identify which step in the assembly of the complex is the slowest at low helicase concentration and to try to establish why high helicase is inhibitory. The other possibility for the low helicase sigmoidicity was that it reflected hexamer assembly on the oligonucleotide templates. However, this is much less likely because current evidence suggests that the hexamer is quite stable even at concentrations below 1 μM (*22*). It is not likely that primase destabilizes the helicase hexamer because it is known to enhance DnaB protein activities (*24*, *49*) rather than to inhibit them.

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