DnaB Helicase Affects the Initiation Specifity of *Escherichia coli* Primase on Single-Stranded DNA Templates†

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ABSTRACT: The effect of DnaB helicase on the initiation specificity of primase was studied biochemically using a series of single-stranded DNA templates in which each nucleotide of the trinucleotide d(CTG) initiation sequence was systematically varied. DnaB helicase accelerated the rate of primer syntheisis, prevented "overlong" primers from forming and decreased the initiation specificity of primase. In the presence of DnaB helicase, all trinucleotides could serve as the primer initiation site although there was a distinct preference for d(CAG). These data may explain the high chromosomal prevalence of octanucleotides containing CTG on the leading strand and its complement CAG on the lagging strand. The specificity of DnaB helicase places it on the lagging strand template where it stimulates the initiation of Okazaki fragment synthesis. In the absence of DnaB helicase, primase preferentially primed the d(CTG) template. In the presence of DnaB helicase, the initiation preference was not only altered but also the preferred initiating nucleotide was found to be GTP rather than ATP, for both the d(CTG) and the d(CAG) templates. This suggested that the specificity of primase for the d(CTG) initiation trinucleotide was predominantly unaffected in the absence of DnaB helicase on short ssDNA templates, whereas in conjunction with DnaB helicase, the specificity was altered and this alteration has significant implications in the replication of *Escherichia coli* chromosome in vivo.

Over 30 proteins participate in the replication of *Escherichia coli* chromosome (*1*). The inability of DNA polymerases to initiate nucleopolymer synthesis creates the need for an RNA polymerase because they are capable of both initiating and elongating nucleopolymers. The special single-stranded-DNA-dependent RNA polymerase that starts DNA synthesis is called primase (*1*−*3*). At the replication origin *oriC* (*4*), DnaB helicase unwinds the duplex DNA in conjunction with DnaA and DnaC proteins to allow primase to initiate DNA synthesis on both strands in both directions from the origin. Primer RNA is synthesized just once to initiate leading strand DNA synthesis and repeatedly on the lagging strand single-stranded DNA to initiate Okazaki fragment synthesis. Each RNA primer is then elongated by DNA polymerase III holoenzyme. The RNA−DNA heteropolymer is called the Okazaki fragment.

Okazaki fragments are an average of 1500 nucleotides with a distribution of lengths from 500 to 2000 nucleotides (*5*−*7*). In vivo studies have shown that, during replication initiation, primer synthesis predominantly initiates from d(CTG) trinucleotide (*8*, *9*) by synthesizing the diribonucleotide 5′-pppApG-3′. The guanosine in the trinucleotide is apparently required for the site-directed initiation but is noncoding in primer synthesis. In vitro studies have shown that three other well-studied primases from bacteriophage T4, T7, and satellite phage P4 also possess initiation specificity. T7 primase has been reported to initiate primer synthesis from the trinucleotide sequence d(GTC) (*10*−*12*), and T4 primase initiates from d(GCT) or d(GTT) (*13*, *14*). In contrast, P4α primase requires the dinucleotide sequence d(CT) to initiate primer synthesis (*15*).

We have developed a simple system that allowed *E. coli* primase to be assayed with or without the influence of auxiliary proteins (*16*). The assay includes a single-stranded DNA template possessing the initiating trinucleotide sequence d(CTG), primase, magnesium and nucleotides. Kinetic analysis has revealed that *E. coli* primase, acting alone, is the slowest RNA polymerase with an in vitro rate of about one primer every thousand seconds (*17*). This in vitro rate is too slow to keep up with the in vivo Okazaki fragment synthesis rate that occurs at about one primer per second. This discrepancy suggested the need to add a stimulatory agent to the assay so that it might achieve in vivo levels. Two likely candidates were SSB[1] and DnaB helicase because both proteins interact with single-stranded DNA and are thought to play important roles on the lagging strand.

We anticipated that DnaB helicase would greatly stimulate primase in our assay, because prior to the development of our oligonucleotide template assay, primase had been shown to be active in vitro in non-G4 origin systems only when DnaB helicase was present (*18*, *19*). This system was termed the general priming system because this two-protein combination could prime virtually every polymeric template. The conundrum with regard to the biological activity was that

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[1] Abbreviations: SSB, single-stranded DNA binding protein; ssDNA, single-stranded DNA.

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the addition of SSB prevented both DnaB helicase and primase from binding to the ssDNA template when it was added to the template first (*18*). In the absence of SSB, DnaB helicase-bound primase was able to bind to the ssDNA at relatively low concentration (*19*). We also found that SSB did not stimulate primase in our simple assay (*17*). Likewise, we have found that DnaB helicase was greatly stimulatory in our simple assay (*20*) even though these templates are the same size as the binding site size for DnaB helicase. This was consistent with other observations that DnaB helicase stimulates the activity of primase in other biochemical assays (*21*−*24*). Furthermore, the biochemical strand specificity of DnaB helicase places it on the lagging strand (*22*), where it is well-placed for stimulating primase to initiate Okazaki fragment synthesis.

Given the large stimulation of primase activity by DnaB helicase, it was now of interest to determine whether the primase stimulation by DnaB was at the expense of primase initiation specificity. We studied this by systematically altering the base at each position of the initiation trinucleotide. We also monitored primer synthesis in the presence and the absence of ATP to observe the effect of ATP on primer synthesis from different single-stranded DNA templates in the presence of DnaB helicase.

MATERIALS AND METHODS

Chemicals. Radioactive ribonucleotide triphosphates (4000 Ci/mmol), [γ-^{32}P]ATP, [γ-^{32}P]CTP, [γ-^{32}P]GTP, [γ-^{32}P]UTP, and [α-^{32}P]UTP were purchased from ICN. The ribo- and deoxyribonucleotide triphophates were purchased from Pharmacia, and the dideoxyribonucleotide triphosphates were purchased from Sigma. Glycogen was purchased from Boehringer Mannheim. Ultrapure formamide was purchased from ICN and deionized (*25*) with a mixed-bed resin, AG501-X8 (Bio-Rad), at 100 mL/5 g and stirred for 1 h. The solution was filtered once through Whatman no. 1 paper, snap-frozen in liquid nitrogen, and stored at −70 °C. T4 polynucleotide kinase and 10× phosphorylation buffer were purchased from the New England Biolabs.

DNA. The oligodeoxyribonucleotides d(CA)$_{17}$ and those with the sequence d(CAGA(CA)$_5$**XXX**CACACA), where **XXX** denotes the initiating trinucleotide sequence, NTG, CNG, and CTN with N being the altered base, were synthesized on an ABI DNA synthesizer using phosphoramidate methodology by the University of Nebraska—Lincoln DNA synthesis facility. The oligonucleotides were purified using a 20% denaturing polyacrylamide gel, visualized by UV shadowing (*26*), and electroeluted in a Little Blue Tank from ISCO after cutting them from the gel. The gel-filtration grade oligodeoxynucleotides having the sequence d(CAGA(CA)$_5$**CAN**CACACA), d(CAGA(CA)$_5$**NAG**CACACA), d((CA)$_3$GA(CA)$_3$**CTG**CACACA), and d((CA)$_3$GA(CA)$_3$-**CAG**CACACA) were purchased from the Midland Certified Reagent Company. The concentration of all the oligonucleotides was determined using approximate (±10%) calculated extinction coefficients (*27*) at 260 nm. The purity of all the oligonucleotides was checked by 5′-end labeling using [γ-^{32}P]-ATP and T4 polynucleotide kinase (*28*).

Enzymes. Escherichia coli primase was purified as previously described (*29*) from a primase overproducing strain supplied by Dr. Roger McMacken (Johns Hopkins University). The concentration of primase was determined using its extinction coefficient of 47 800 M^{-1} cm^{-1} at 280 nm (*16*). DnaB helicase was isolated using a procedure very similar for the one for primase, from a helicase-overproducing strain also supplied by Dr.Roger McMacken. The hexamer concentration of DnaB helicase was determined using the extinction coefficient of 185 000 M^{-1} cm^{-1} at 280 nm (*30*).

End-Labeling of Oligonucleotides. The 5′-termini of oligonucleotides were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The typical 25 μL reaction mixture contained 10 pmol of oligonucleotide, 2.5 μL of 10× T4 polynucleotide kinase buffer, 5 μL of [γ-^{32}P]ATP (10 mCi/mL in aqueous solution), and enough water to make up the volume.

Primer Synthesis Assay. The assay was conducted as described before (*17*) with [α-^{32}P]UTP (5×10^7 cpm/reaction), 1 μM ssDNA template, 200 nM helicase and 200 nM or 2 μM primase. The reactions were incubated for 45 s or 10 or 30 min as required. To determine the initiating nucleotide in the DNA template, the [γ-^{32}P]phosphate-labeled nucleotide triphosphates were used (7.5×10^7 cpm/reaction). A "control" reaction without primase was also run in each case (*17*). The reactions that lacked primase failed to produce any primers. The length of primers observed in the gel was identical to the ones observed before (*17*). When the 5′-^{32}P-end labeled oligonucleotide was used for analysis, the reaction mixture containing the labeled oligo was heated at 68 °C for 10 min to inactivate the T4 polynucleotide kinase, and then 1 μM of the labeled DNA was used for primer synthesis.

Polyacrylamide Gel Electrophoresis. The procedure employed was identical to the one described before (*17*). After the run, the gels were analyzed either by autoradiography or by using the Molecular Dynamics PhosphorImager SF. For autoradiography, the gels were exposed to Fuji RX-film with a FischerBiotech L-plus intensifying screen at −70 °C. For analysis using the Molecular Dynamics PhosphorImager SF, the gels were exposed to a Molecular Dynamics PhosphorImager screen overnight at −70 °C. The total amount of primers synthesized from the single-stranded DNA templates was quantitated using the ImageQuant software. Standards of known radioactivity, placed on the PhosphorImager screen together with the wrapped gel, generated the standard curve that was used for the quantitative analysis.

RESULTS

All of the single-stranded DNA templates used in this study were 23 nucleotides long except for the 34 nucleotide long d(CA)$_{17}$ template. The templates containing a trinucleotide initiation sequence had a common sequence that was designed using the nucleotide prevalence of the bacteriophage G4 origin (*16*). The role of each position in the initiation trinucleotide was examined by systematically altering the bases at each position (Figure 1). All of the templates possessed the following characteristics: (a) an initiation trinucleotide; (b) 16 nucleotides from their 5′-terminus to the central nucleotide of the initiation; (c) six residues 3′ to the initiating trinucleotide, the minimum required for efficient primer synthesis (*16*); and (d) a single guanine near the 5′-end to enable primer sequence verification (*17*). The primer synthesis assay was used to monitor the amount of RNA

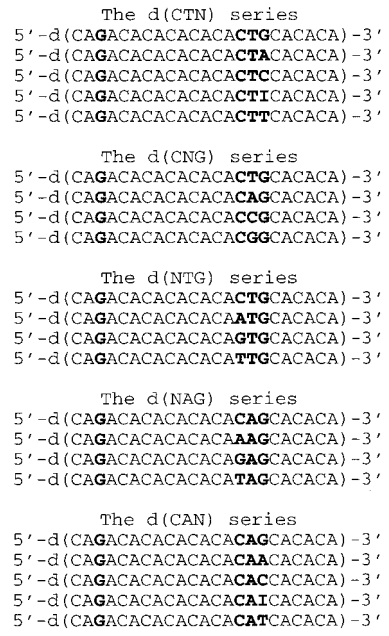

FIGURE 1: The sequences of the different series of oligodeoxynucleotides used for determining primer synthesis initiation specificity. The initiation trinucleotide and the single guanine near the 5′-end are in bold.

primers produced from the template in the presence and the absence of DnaB helicase. When [γ-^{32}P]ATP was used to probe the reaction, only those primers that initiated with ATP were visualized. When [α-^{32}P]UTP was used to probe the reaction, all primers were visualized regardless of their initiating nucleotide because the chosen template was rich in adenine. To visualize the ATP-initiated primers on the denaturing gel, a higher amount of [γ-^{32}P]ATP was used because there was only one thymine on the template. For quantitative analysis, the total yield of primers visualized with [α-^{32}P]UTP was calculated by dividing the radioactivity incorporated per RNA primer by the number of uracils incorporated. In an identical manner, the quantity of primers initiated with an ATP was also calculated.

The low activity of primase when using synthetic oligonucleotide templates (*17*) prompted a search for proteins that would stimulate this reaction. We found that SSB did not stimulate (*17*) but that DnaB helicase did by 5000-fold at its optimum ratio to primase (*20*). As will be presented below, DnaB helicase also altered the types of primers that were synthesized. We hypothesized that DnaB helicase stimulated primase activity in part by reducing its trinucleotide initiation specificity. As a test of this hypothesis, our first study involved the systematic replacement of each position within the initiation trinucleotide d(CTG) in the template and then examining the types and relative amount of primers synthesized in the presence and absence of DnaB helicase. The concentration of DnaB helicase used in the reactions was the optimum amount as previously determined (*20*). The reactions were incubated for 45 s because the helicase-stimulated reaction was complete in less than 2 min.

Effect of DnaB Helicase on Primase Trinucleotide Initiation Specificity. In the d(CTN) series (Figure 1), the third position in the initiation trinucleotide was varied systematically. Deoxyinosine was among the nucleotides tested in this position because it had been found to have important effects on the activity of the T7 primase−helicase (*31*). When primase was stimulated by DnaB helicase, each member of the d(CTN) series was capable of serving as a template for primer synthesis (Figure 2A). When the total primers were quantified, it was found that the initiation sequence d(CTI) generated 1.5-fold more primers than d(CTG) but that the three other templates generated fewer primers than d(CTG) (Table 1). Despite these differences in the amounts of primers, the pattern of primers synthesized was mostly independent of the template sequence. This indicated that the initiating NTP for these primers was not the complement of this third position within the initiation trinucleotide. Taken together, these data indicated that primase exhibited a structural preference for the third and noncoding position within the templates initiation trinucleotide.

In the d(CNG) series of templates (Figure 1), again all of them were capable of serving as templates for primer synthesis when primase was stimulated by DnaB helicase (Figure 2A). In this series, the template containing the trinucleotide d(CAG) was 6.5-fold more effective than the one containing d(CTG) (Table 1), and the other two were worse than d(CTG). This effect was tested in nine trials using two different preparations of the d(CAG) template, and the ratio was found to vary by only 4%. It was also significant that the pattern of primers synthesized by the d(CTG) template was different from the one synthesized complementary to the others. This suggested that the initiating NTP had changed when the central position of the template trinucleotide was changed. The identity of initiating nucleotide was addressed as described later.

In the d(NTG) series of templates (Figure 2A), the d(TTG) template caused the same pattern of primers to be synthesized as the d(CTG) template but was a slightly more effective template (Table 1). In contrast, the templates containing the initiation trinucleotides d(GTG) and d(ATG) were less effective and caused a subset of the primer types to be synthesized.

Because d(CAG) had proven to be the most effective, two more series of templates were tested. Even though all the members of the d(NAG) and the d(CAN) series were observed to be more effective than nearly every member of the previous three series, the d(CAG)-containing template remained the most effective at stimulating primer synthesis (Table 1). Interestingly, in the sequence context of a central adenine, inosine as the third base was less stimulatory relative to a guanine. This result contrasted with its effect in the sequence context of the d(CTN) family. Thus, the role of the noncoding third nucleotide was sensitive to the identity of the central nucleotide.

As shown above, the ability of DnaB helicase to reduce the primase trinucleotide initiation specificity was so great that virtually all of the templates were capable of directing primer synthesis within 45 s. To establish whether primase acting alone had a high initiation specificity, each of the first three template series was incubated with primase and its substrates for 15 min in the absence of DnaB helicase. The much longer incubation time was needed because primase was so much less active in the absence of DnaB helicase.

In the absence of DnaB helicase, only three templates were capable of serving as templates (Figure 3 and Table 2). The initiation trinucleotide preference order for these templates was d(CTG) > d(CTT) > d(ATG). The number of primers synthesized complementary to the other templates was below

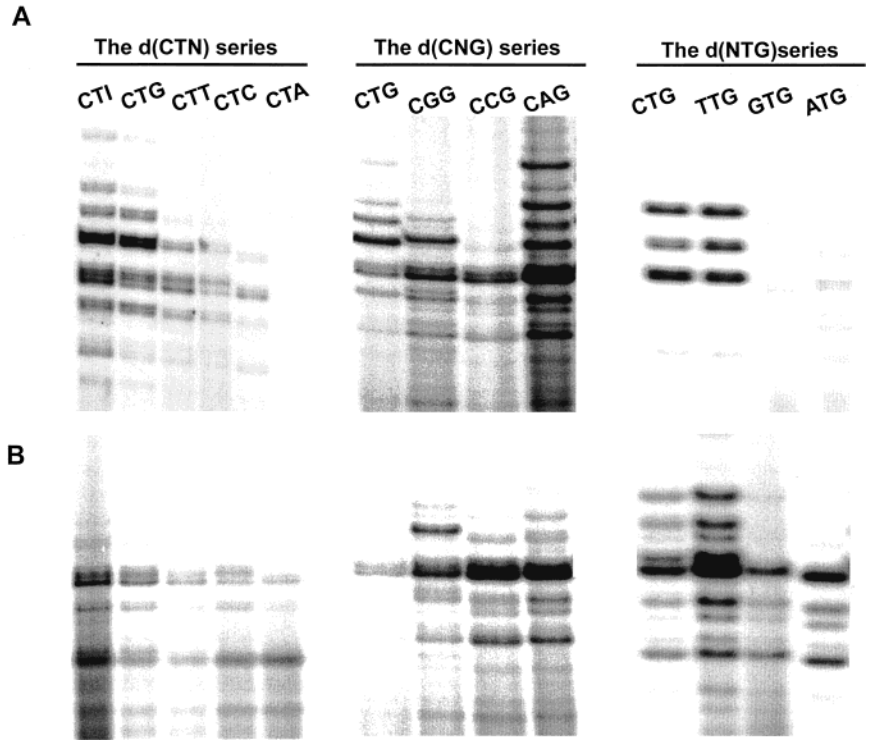

FIGURE 2: Primers synthesized from the three series of DNA templates in the presence (A) and the absence (B) of ATP. The initiation trinucleotides of the template being tested are shown. The radioactive probe used was [α-^{32}P]UTP, and the 45 s reactions were performed using 200 nM primase, 200 nM hexamer DnaB helicase, 200 μM of each NTP, and 1 μM templates. The samples were treated and electrophoresed, and the image of the gel was obtained as described in the Materials and Methods.

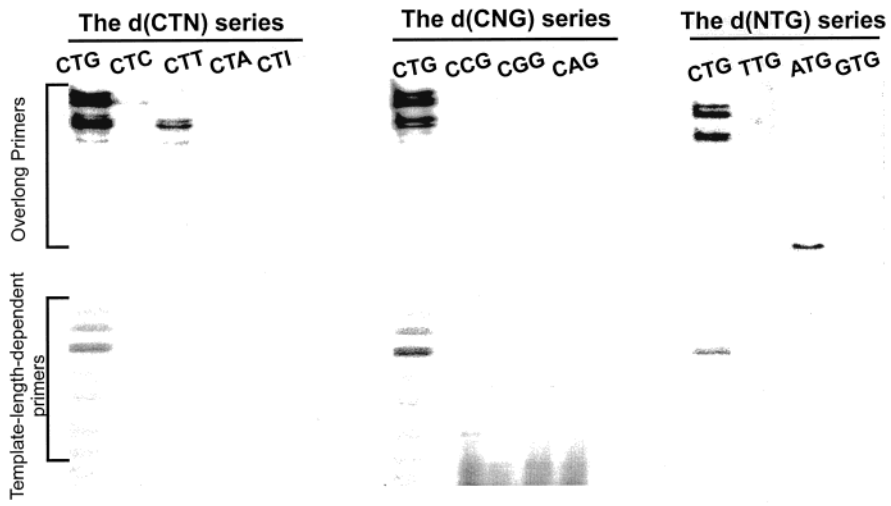

FIGURE 3: Primers synthesized from the three series of DNA templates in the absence of DnaB helicase. The synthesis was monitored with [α-^{32}P]UTP. The primase concentration was 2 μM and the reaction time was 15 min. Otherwise, the conditions were the same as in Figure 2. For the identities of the template-length-dependent and the overlong species please see the following paper in this issue.

the detectable limit. Just as has been previously described (*17*, *20*), the majority of the primers synthesized by primase on oligonucleotide templates such as these were longer than the template. In the previous paper in this issue (*20*), we discovered that these "overlong primers" were created by primase as it elongated from the 3′-terminal nucleotide of the template. Overlong primer synthesis occurred only when the 3′-end of the template could form a hairpin such that the terminal nucleotide had formed a base pair with another portion of the template. Even though these oligonucleotide templates are artificial, the ability of primase to create overlong primers demonstrated its ability to elongate nucleopolymers just as can any other RNA or DNA polymerase. The function that is unique to RNA polymerases is their ability to initiate chain synthesis. With regard to our synthetic oligonucleotide templates, we have termed these types of primers as "template-length-dependent primers" because they retain the 5′-triphosphate of the initiating nucleotide, are initiated complementary to a particular nucleotide within the template, and have a sequence that is directed by the template sequence. Among all of the templates, only the d(CTG)-containing template generated a significant fraction of the template-length-dependent primers within 15 min. By this criterion, primase acting alone has very high trinucleotide

Table 1: Relative Efficiency of the Initiation Trinucleotides with Respect to d(CTG) in the Presence of DnaB Helicase[a]

initiation trinucleotide	relative efficiency	initiation trinucleotide	relative efficiency
CAG	6.46	TTG	1.15
CAC	3.58	CTG	1.00
AAG	2.54	CTT	0.52
TAG	2.53	CCG	0.53
CAT	2.49	ATG	0.49
CAA	2.33	GTG	0.36
CAI	2.24	CTC	0.33
CTI	1.49	CTA	0.26
GAG	1.22		

[a] The efficiencies were determined by dividing the yield of uracil incorporated into a given primer length by the number of uracils in that primer and then summing. The values for each template was then normalized to that for the d(CTG)-containing template. The values are averages from three assays and they differ by only 4%.

Table 2: Relative Efficiency of the Initiation Trinucleotides with Respect to d(CTG) in the Absence of DnaB Helicase[a]

initiation trinucleotides	relative efficiency		initiation trinucleotides	relative efficiency	
	overlongs	TLD		overlongs	TLD
CTG	0.85	0.15	TTG	0.04	0.00
ATG	0.23	0.00	CGG	0.03	0.00
CTT	0.22	0.00	CTA	0.03	0.00
CTC	0.14	0.00	CTI	0.03	0.00
CCG	0.05	0.00	CAG	0.01	0.00
GTG	0.05	0.00			

[a] The efficiencies were determined as outlined in Table 1. TLD means template-length-dependent primers.

initiation specificity. However, when primer synthesis was allowed to occur for an hour or more (data not shown), nearly all of the templates directed the synthesis of some primers although more than 10-fold less than the d(CTG)-containing template. DnaB helicase appeared to be stimulating this less efficient type of initiation in vitro.

Effect of ATP on DnaB Helicase-Stimulated Primer Synthesis. The effectiveness of the d(CAG)-containing template for primer synthesis suggested that primer synthesis could initiate without ATP because that template lacked thymines. To test this observation, the DnaB helicase-stimulated primer synthesis assay was carried out using the d(CTN), d(CNG), and the d(NTG) series of templates in the absence of ATP (Figure 2B). Without ATP, there was less difference in the relative amounts of primers synthesized on the various templates and the primer types were very similar. This indicated that the initiation sites on the single-stranded DNA templates had changed and was no longer dependent on the trinucleotide sequence that was being altered.

To investigate further the loss of the requirement for a specific initiation trinucleotide and the relative importance of ATP, primase activity was first tested on a $d(CA)_{17}$ template. The incubation time was prolonged to 10 min because this template was slightly less active than the previous ones tested. A range of primer lengths from 8 to 20 nucleotides was produced at increasing concentration of GTP (Figure 4). The pattern of primers was the same, and the amount of primers formed was nearly the same at each GTP concentration whether or not ATP was present. Thus, neither ATP nor its capacity to be hydrolyzed was crucial for primer synthesis or its stimulation by DnaB helicase. Of course, DnaB helicase can hydrolyze the other NTPs (*32*−*35*) with comparable effectiveness as ATP.

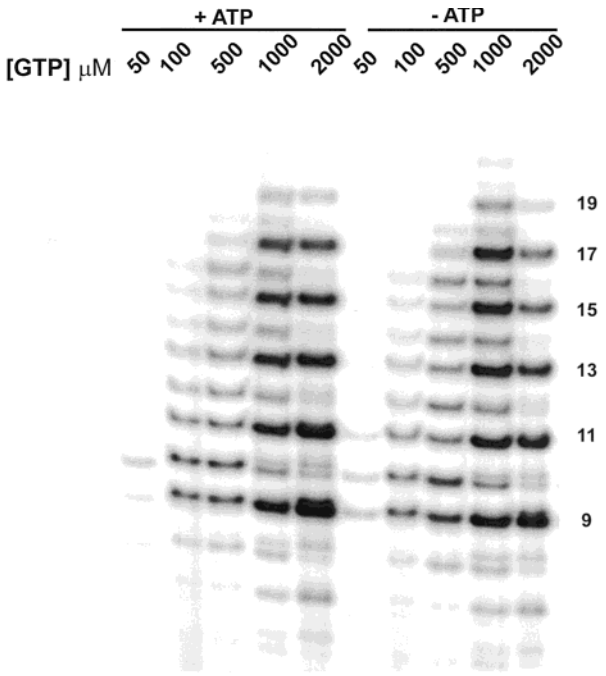

FIGURE 4: GTP titration of primer synthesis from the $d(CA)_{17}$ template in the presence of DnaB helicase. The reaction was monitored using [α-^{32}P]UTP. Primer synthesis was observed in the presence and the absence of 200 μM ATP. The concentration of GTP is indicated at the top of the gel. The concentration of the other NTPs were kept at 200 μM. At the right-hand side of the figure, the lengths of the primers are indicated in comparison with those obtained from the d(CTG) template.

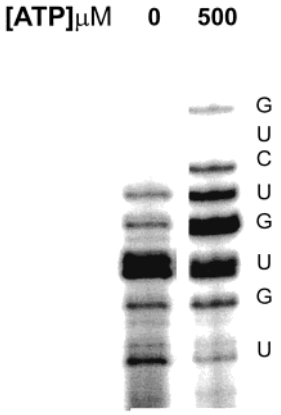

FIGURE 5: The effect of ATP on DnaB helicase-stimulated primer synthesis on the ssDNA template, 5′-d(CA**G**ACACACACACA**CT-G**CACACA)-3′. [α-^{32}P]UTP was the radioactive probe. The 3′-terminal sequence of the longest primers (*16*) are indicated on the right side of the figure.

The ATP dependence on primer synthesis from the d(CTG)-containing template was also examined (Figure 5). It was clearly evident that primer synthesis can initiate without ATP as observed before (Figure 2B), but it was stimulated at higher concentrations of ATP. When ATP concentration was increased from 0 to 500 μM, there was 1.7-fold increase in the amount of primers. The ATP caused a slightly different pattern of primers to be synthesized. The amount of the non-ATP-dependent primers decreased only slightly as ATP concentration was increased. This result affirmed that the primers were initiating from different

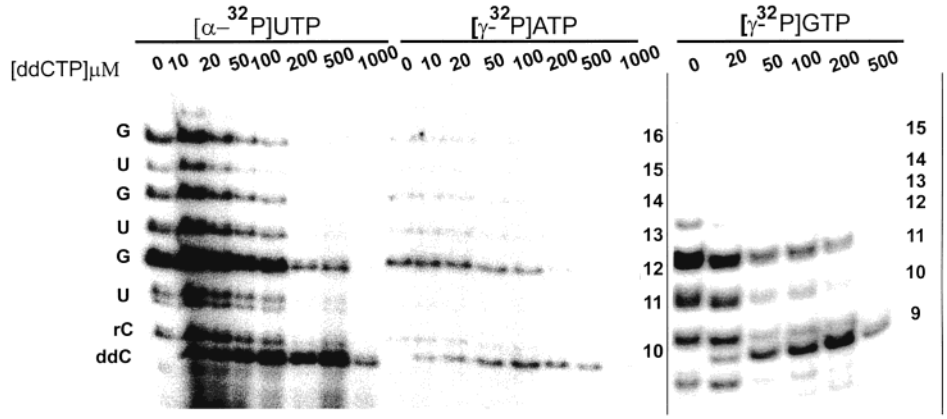

FIGURE 6: ddCTP titration of primer synthesis using [α-^{32}P]UTP, [γ-^{32}P]ATP, and [γ-^{32}P]GTP. Primase (200 nM) and DnaB helicase (200 nM) were incubated with 1 μM template, 5′-d(CACACA**G**ACACACA**CTG**CCACA)-3′, for 2 min. The concentration of ddCTP is indicated at the top of the figure. Along the left side of the image is the 3′-terminal sequence of the longest primers and along the right side are the RNA primer lengths for both the ATP- and the GTP-initiated primers. The lengths of the ATP-initiated primer are known because these primers initiate complementary to the thymine of the initiation trinucleotide 5′-d(CTG)-3′.

sequences on the template and that it would be a complex task to identify the actual initiating nucleotide on the basis of primer sequence alone.

Primer Initiation Sites. When [α-^{32}P]UTP was used to label primers, all the primers synthesized were observed, but the identity of the initiating nucleotide was obscured. Each band on the gel may have resulted from initiation at a different site as long as the final primer length was the same and its composition was such that it comigrated during electrophoresis. To identify the initiation site on the ssDNA template, it was necessary to use [γ-^{32}P]-labeled nucleoside triphosphates in conjunction with a dideoxynucleotide that would cause termination of primer synthesis at a site downstream from the initiation site. We only tested the d(CTG)- and d(CAG)-containing templates because they represented the best-studied and the most effective initiation trinucleotides. The templates used in this test, 5′-d(CACACA**G**ACACACA**CTG**CACACA)-3′ and 5′-d(CACACA**G**ACACACA**CAG**CACACA)-3′, differed from those studied above in that they contained the unique guanine at position 10 from the central nucleotide of the trinucleotide rather than at position 14. This special guanine was placed within 11 ± 1 of the central nucleotide because DnaB helicase caused the majority of primers to be that length.

Primer synthesis was first carried out in the presence of [α-^{32}P]UTP, [γ-^{32}P]ATP, and [γ-^{32}P]GTP (Figure 6). With the α-label, all the primers, irrespective of their initiation sites, were visualized on the gel. The assay was performed at various concentration of 2′,3′-dideoxycytidine 5′-triphosphate (ddCTP). At increasing concentration of ddCTP, there was an accumulation of the dideoxycytidine-terminated primers. Even though the total primer synthesis diminished considerably at higher concentrations of ddCTP, it still provided a useful primer termination signal. When primer synthesis was probed with [α-^{32}P]UTP, each length of primer was visualized as a doublet. This indicated that distinctly different species of primers were generated from the same template and that they most likely possessed different initiating nucleotides. The [γ-^{32}P]ATP initiated primers on the d(CTG)-containing template also diminished in amount with increasing concentration of ddCTP. A direct comparison of the singlet bands visualized with [γ-^{32}P]ATP and the doublets visualized with [α-^{32}P]UTP revealed that the ATP-initiated primers were the slower migrating species within each [α-^{32}P]UTP-visualized doublet. Quantitative analysis of the doublets showed that 6% of the total primers synthesized was initiating with ATP complementary to the thymine of the trinucleotide CTG and the rest were initiating elsewhere.

Similar analysis was also carried out with the three other [γ-^{32}P]phosphate-labeled nucleoside triphosphates. Very few of the total primers synthesized were initiated with either [γ-^{32}P]CTP or [γ-^{32}P]UTP (data not shown). Most of the primers were initiating with [γ-^{32}P]GTP (Figure 6) and the pattern of those visualized primers was very similar to those observed in the presence of [α-^{32}P]UTP. This suggested that most primers initiated using GTP and not ATP when DnaB helicase was present. We found that high concentration of ddCTP resulted in the generation of multiple dideoxycytosine terminated species instead of a single species observed in the presence of [γ-^{32} P]ATP. This indicated that there was more than one initiating site on the template d(CACACA**G**ACACACA**CTG**CACACA). Any one of the several cytosines may serve as an initiation site. Similarly, we found that most of the primers were initiating with GTP from the d(CAG)-containing template, 5′-d(CACA**CAG**ACACACA**CAG**CACACA)-3′ (data not shown). We also observed that the total primers synthesized on this template was now less than for the equivalent d(CTG)-containing template. One important factor to consider in the modified template was the sequence. The change of the position of the unique guanine from 14 to 10 with respect to adenine led to the generation of a second initiation site on the template [the two 5′d(CAG)-3′ initiation trinucleotides are shown in bold in the sequence]. We also found that that the amount and the pattern of primers generated from the above and the original 5′-d(CA**G**ACACACACACA**CAG**CACACA)-3′ templates were different (data not shown). On the basis of these observations, we hypothesized that apparently the presence of two highly active primer initiation sites in the same ssDNA template resulted in the inhibition of primer synthesis, when they were both located away from the ends of the template. However, this is purely an in vitro observation. But it does signify the importance of the sequence of the template DNA in performing these kind of in vitro studies.

DISCUSSION

E. coli primase is a single-stranded-DNA-dependent RNA polymerase. An in vitro assay was developed (*16*) that prompted us to study the activity of *E. coli* primase and its template sequence requirement in the absence of any DNA secondary structure or SSB and/or other proteins. By using this assay, we have addressed an important function of primase. We find that even though primase acting alone is very specific during initiation, its initiation specificity is greatly affected by the presence of DnaB helicase. When we studied the initiation specificity of primase from the three series of single-stranded DNA templates, the d(CTN), d(CNG), and d(NTG), where N denotes the altered base in the initiation trinucleotide, we found that the initiation specificity of primase broadened from d(CTG) for primase alone to being able to initiate from every trinucleotide tested although with a significant preference for d(CAG). This is important because the interaction between primase and DnaB helicase plays a crucial role in regulating the rate of lagging strand synthesis and controlling the movement of the replication fork (*24*). The interaction between these two proteins has also been demonstrated to stimulate the catalytic activity of primase in vitro (*18*−*20*).

The DnaB helicase-stimulated primase initiation preference for d(CAG) has interesting implications with regard to the recent *E. coli* chromosome sequence analysis (*36*). In a search for new sequence elements during the chromosome sequence analysis, it was discovered that 20 of the 25 most frequently found octanucleotide sequences contained a d(CTG) within the sequence. Furthermore, these octanucleotide sequences were preferentially found on what would be the leading strand template. Curiously, the octanucleotides occur with a sequence spacing that corresponded to Okazaki fragment sizes which suggested a role for the d(CTG) trinucleotide initiation specificity for free primase. But, Okazaki fragments are synthesized complementary to the opposite lagging strand template. It is to be noted that the complement of leading strand, Okazaki length-spaced d(CTG), would be lagging strand, Okazaki length-spaced d(CAG). The observation of a strict coordination between the completion of one Okazaki fragment and the initiation of the next (*7*) suggests that the solution to this conundrum is that the strand polarity of DnaB helicase places it on the lagging strand template (*22*) where it stimulates primer synthesis and broadens the primase initiation specificity to prefer d(CAG). As was suggested during the chromosome sequence analysis (*36*), the leading strand d(CTG)s are consistent with the initiation specificity of primase alone and they may serve as the initiation sequence during the special events of recombination and repair.

We have attempted to study the role of ATP in our DnaB helicase-stimulated primer synthesis assay. Even though the hexamer is able to hydrolyze any ribonucleoside triphosphate when single-stranded DNA is present (*19*, *34*), the general priming system is reported to have a specific requirement for ATP (*19*). However, we find that the presence or the absence of ATP has no significant effect in initiating primer synthesis from a 23 nucleotide long d(CTG)-containing template, which is almost the binding site size of DnaB helicase or from a $d(CA)_{17}$ template. On the other hand, in the absence of DnaB helicase, primase needs ATP to make the template-length-dependent primers from the d(CTG)-containing template (*20*) and is incapable of initiating from the $d(CA)_{17}$ template (data not shown). This suggests that ATP is not important for the helicase stimulation of primase activity but that it is important for template-sequence-dependent primer synthesis. This discrepancy with the earlier general priming results is likely to be that their assay was a coupled priming-DNA synthesis assay whereas we have assayed specifically for priming activity.

The goal of this study was to determine the initiation specificity of *E. coli* primase and investigate the possible role of DnaB helicase in the initiation of primer synthesis from a short single-stranded DNA template in vitro. Because we find that the initiation specificity of primase is significantly affected in the presence of DnaB helicase, we can hypothesize that the interaction of primase with additional proteins at the replication fork in *E. coli* may also be vital for affecting the initiation specificity of primase in vivo. This may be biologically acceptable as primers produced are removed later by RNaseH or DNA polymerase I. The interaction between primase and DnaB helicase could also play a significant role in restricting the length of the primers to 11 ± 1 nucleotides in vivo.

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