Primer Synthesis Kinetics by *Escherichia coli* Primase on Single-Stranded DNA Templates†

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ABSTRACT: The kinetics of primer RNA initiation and elongation by *Escherichia coli* primase were measured. The single-stranded DNA template that was used to develop the system, d(CAGA-$(CA)_5$CTGCAAAGC), contained: (1) the preferred initiating trinucleotide d(CTG); (2) five residues 3′ to the d(CTG), the minimum required for efficient primer synthesis; and (3) a single guanine placed near the 5′-end to facilitate study of cytidine triphosphate analog incorporation at a unique site. The assay monitored radiolabeled nucleotide incorporation into the RNA primers. The various primers were separated by length using denaturing polyacrylamide gel electrophoresis. Different types of primers were observed when synthesis was monitored using γ- versus α-radiolabeled nucleotides as the probe. When [γ-^{32}P]-ATP incorporation was the probe, only primers initiated with ATP from the unique template thymine were observed. The sequences of these primers were complementary to that of the template. No primers shorter than a 12-mer accumulated, demonstrating that formation of the first phosphodiester bond was much slower than that of the next 10 phosphodiester bonds. The longest primer observed when monitoring [γ-^{32}P]ATP incorporation was 16 nucleotides long, the correct length for a primer completely template-directed and initiated at the unique thymine. Misinsertion of a noncognate nucleotide at the template's guanine indicated very poor nucleotide discrimination by this enzyme. When [α-^{32}P]UTP was the probe for primer synthesis, all primers synthesized were observed whether or not they were initiated with ATP. Under these conditions, "overlong" primers and the above-described template length-dependent primers were observed. The template length-dependent primers accumulated faster than the overlong primers, but, at long incubation times, the overlong primers became the dominant species. The overlong primers were not fully related to the template length-dependent primers since they were not initiated complementary to the template d(CTG). Nevertheless, the overlong primers did appear to arise as a consequence of the template length-dependent species since their length was double and they arose in the time course after the length-dependent species.

Primase from *Escherichia coli* is a single-stranded DNA-dependent RNA polymerase (Swart & Griep, 1993). Its biological role is to synthesize primer RNA during DNA replication (Kornberg & Baker, 1992; Marians, 1992; McMacken et al., 1987). At the replication origin (van der Ende et al., 1985), DnaB helicase unwinds the duplex DNA in conjunction with DnaA and DnaC proteins to allow primase to initiate leading-strand DNA synthesis in both directions from the origin. At each of the two replication forks, DnaB helicase unwinds the duplex DNA to create the single-stranded DNA that is the template both for DNA polymerase III holoenzyme on the leading strand and for primase on the lagging strand. Primase function is highly coupled to the other steps at the replication fork. For instance, nearly every time that primase synthesizes an RNA primer on the lagging strand, DNA polymerase III holoenzyme elongates from it to create the RNA/DNA heteropolymer called an Okazaki fragment (Zechner et al., 1992a). Equally important in establishing that primase plays a critical role during lagging-strand synthesis is that primase action correlates to the rate-determining step during Okazaki fragment synthesis (Wu et al., 1992c).

Okazaki fragments are an average of 1500 nucleotides with a distribution of lengths from 500 to 2000 nucleotides (Ogawa & Okazaki, 1980; Wu et al., 1992a; Zechner et al., 1992a). The average RNA primer length *in vivo* is 11 ± 1 nucleotides and is most often initiated with the sequence pppApG (Kitani et al., 1985; Yoda et al., 1988). *In vivo* studies have also shown that the template sequence which directs the initiation of primer synthesis consists of the trinucleotide sequence d(CTG) (Kitani et al., 1985; Yoda & Okazaki, 1991). From these data, it can be concluded that initiation most often begins complementary to the thymine of the template d(CTG) and then proceeds in the usual direction. Even though the guanosine in d(CTG) is apparently required for this site-directed initation, it does not serve as a template for primer synthesis.

With regard to leading-strand DNA initiation, most of what is known about primase function has been determined in the presence of other enzymes and proteins. Studies of bacteriophage single-stranded DNA (ssDNA)[1] replication have revealed two different types of initiation sites (Tanaka et al., 1994): the primosome assembly site (Shlomai & Kornberg, 1980; Wickner & Hurwitz, 1974) and the G4 origin initiation site (also called G sites) (Bouché et al., 1975). For both of these types of initiation sites, the ssDNA is coated with SSB

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as is most free ssDNA in the *E. coli* cell. The barrier to be overcome is that primase has lower affinity for ssDNA than does SSB (Swart & Griep, 1993), and, yet to synthesize an RNA primer it must bind the ssDNA. Primer synthesis that depends on the primosome assembly site involves the primosome, a complex of primase with DnaB helicase, DnaC, DnaT, PriA helicase, PriB, and PriC (Marians, 1992). PriA helicase binds to a specific hairpin (Shlomai & Kornberg, 1980) in the ϕX174 primosome assembly site, and then the complex migrates along the SSB-coated ssDNA in an ATPase-dependent manner (Lee & Marians, 1989). In an as yet undescribed manner, primase synthesizes RNA primers while the complex migrates. Any one of the primers can serve as the intiating primer for DNA synthesis. Initiation specificity at G4 origin-like sites involves ssDNA secondary structure (Hiasa et al., 1990; Lambert et al., 1987) and SSB (Sun & Godson, 1993). At the G4 origin, the secondary structure is such that SSB binding is phased and a single highly active d(CTG) initiation trinucleotide is exposed.

There are two *in vitro* systems that may resemble the action of primase at a replication fork: the general priming system and the rolling-circle DNA synthesis system. When DnaB helicase assists primase during primer synthesis on a ssDNA template, it is called general priming (Arai & Kornberg, 1979); the key to this system is the omission of SSB. The absence of SSB allows the binding of both DnaB helicase and primase. Like other helicases, DnaB helicase can translocate along ssDNA in an ATPase-dependent manner. Given that primase and helicase bind to one another, the primase can now bind to the ssDNA even at relatively low concentrations (Arai & Kornberg, 1981). The rolling-circle synthesis system involves an artificial replication fork. The minimum enzyme requirements have been established for this system (Wu et al., 1992a,b,c), and, in short, one needs DNA polymerase III holoenzyme, DnaB helicase, SSB, and primase to generate Okazaki fragments of the proper length and frequency on the lagging strand (Hiasa & Marians, 1994; Mok & Marians, 1987). Primase plays the central controlling role in the process. Primase is limited to action at the fork by its specific binding with DnaB helicase (Tougu et al., 1994) and by SSB which binds the free ssDNA created by the helicase.

In a study to define the minimal ssDNA sequence in the G4 system that would direct site-specific primer initiation, only the sequence d(CTG) was found to be necessary when SSB was omitted from the reaction (Swart & Griep, 1993). Although it has not been proven rigorously that d(CTG) serves as the primer initiation sequence in the other systems described above, it has allowed us to develop an *in vitro* system for site-specific primer synthesis. We have chosen to develop this system to characterize the kinetics of site-specific initiation of primer synthesis. We find that formation of the first diribonucleotide is extraordinarily slow but that formation of the subsequent phosphodiesters bonds is much faster. This identifies the rate-determining step as either the rate of formation of the first phosphodiester bond or some step preceding it. The slow rate of formation of this first bond may be the mechanism by which primase controls the rate of lagging-strand synthesis.

[1] Abbreviations: dNTPs, 2′-deoxyribonucleoside 5′-triphosphates; ddNTPs, 2′,3′-dideoxyribonucleoside 5′-triphosphates; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; rNTPs, ribonucleoside 5′-triphosphates; SSB, *E. coli* single-stranded DNA-binding protein; ssDNA, single-stranded DNA.

MATERIALS AND METHODS

Chemicals. Radioactive end-labeling grade (7000 Ci/mmol) [γ-^{32}P]ATP and approximately 3000 Ci/mmol [α-^{32}P]-ATP, [α-^{32}P]GTP, [α-^{32}P]UTP, and [γ-^{32}P]GTP were purchased from ICN. Dideoxyribonucleotide triphosphates were purchased from Sigma, and the ribo- and deoxyribonucleotide triphosphates were purchased from Pharmacia. Glycogen was purchased from Boehringer Mannheim. Ultrapure formamide was purchased from ICN. Formamide was deionized (Blumberg, 1987) 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.

DNA. The oligodeoxyribonucleotide d(CAGA(CA)$_5$-CTGCAAAGC) was synthesized on an ABI DNA synthesizer using phosphoramidate methodology by the University of Nebraska−Lincoln DNA synthesis facility. It was purified using an 18% denaturing polyacrylamide gel, visualized by UV-shadowing (Efcavitch, 1990), cut from the gel, and electroeluted in a Little Blue Tank from ISCO. Oligonucleotide concentration was determined using approximate (±10%) calculated extinction coefficients (Borer, 1975); ϵ_{260} = 226.0 mM^{-1} cm^{-1}.

Proteins and Enzymes. Single-stranded DNA-binding protein was isolated according to a published protocol (Lohman et al., 1986). Primase was isolated as described previously (Swart & Griep, 1993) from a primase-overproducer 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 (Swart & Griep, 1993). The replication activity of primase was measured by the standard assay in which primer synthesis is coupled to DNA synthesis (Griep & McHenry, 1989). One unit of primase was defined as the amount needed to incorporate 1 pmol of (total) nucleotide/min into acid-precipitable DNA under conditions where all other components were saturating. Primase specific activity was 5.26 units/ng.

RNA Primer Synthesis Assay. The typical 25 μL reaction mixture contained 50 mM HEPES, 100 mM potassium glutamate, pH 7.5, 10 mM dithiothreitol, 10 mM magnesium acetate, CTP, UTP, and GTP each at 200 μM, 100 μM [γ-^{32}P]ATP (25 000−35 000 cpm/pmol), 200 nM ssDNA template, and 200 nM primase. Incubation was at 30 °C and typically for 10 min. Conditions were varied as noted in the text. Reactions were stopped by the addition of 30 μL of 3 M sodium acetate. The primed template was ethanol-precipitated with 30 μg of glycogen and 500 μL of cold 100% ethanol and incubed at −70 °C overnight. Samples were pelleted by centrifugation for 60 min at 14 000 rpm at 4 °C. The ethanol solution was decanted; the precipitate was washed with 80% ethanol and then centrifuged for 30 min at 14 000 rpm. The ethanol solution was decanted and the precipitate evaporated to dryness. The pellet was dissolved in 6 μL of a formamide loading buffer (95% formamide, 10 mM EDTA, 1 mg/mL bromphenol blue, and 1 mg/mL xylene cyanol).

The level of unincorporated radiolabeled nucleotides remaining in the sample was reduced about 10-fold by salty

ethanol precipitation. As was reported previously (Stayton & Kornberg, 1983; Swart & Griep, 1993; Zechner et al., 1992b), primers shorter than eight nucleotides were not observed during *E. coli* primase primer synthesis assays. A trivial reason would be that the salty ethanol precipitation did not lead to recovery of RNA polymers shorter than eight nucleotides. However, we have observed significant recovery of primers as short as dinucleotides in reactions containing ddNTPs which limit primer length (data not shown). The dinucleotide was visible even though there was sometimes considerable background from the unincorporated radiolabeled NTPs. This indicated that ethanolic precipitation of primers as short as a dinucleoside tetraphosphate would be observed by this technique if they had formed by a mechanism other than dideoxynucleotide termination. That there was very good recovery of these dideoxy-terminated dinucleotides also suggested that primase may remain bound to the "stalled" primers which, in turn, enhanced primer recovery during precipitation.

Polyacrylamide Gel Electrophoresis. RNA primer lengths and primer synthesis rates were evaluated by denaturing polyacrylamide gel electrophoresis. The electrophoresis buffer was 7 M urea, 18% polyacrylamide (1:20 bisacrylamide), 50 mM Tris, 50 mM boric acid, pH 8.3, and 2 mM EDTA (17 cm × 22 cm × 1.5 mm). Gels were prerun 1 h at 25 W; the samples were loaded and then run 3 h at 25 W. The gels were wrapped in plastic film and evaluated either by autoradiography or on a Molecular Dynamics PhosphorImager SF. For autoradiography, gels were exposed to Kodak XAR-5 film with a FisherBiotech L-plus intensifying screen at −70 °C overnight. For evaluation on the Molecular Dynamics PhosphorImager SF, gels were exposed to a Molecular Dynamics PhosphorImager screen overnight at room temperature. For quantification of primer synthesis, standards of known radioactive quantity were placed on the PhosphorImager screen along with the wrapped polyacrylamide gel, and primer synthesis was calculated from the resulting standard curve and the specific activity of the reaction solutions.

RESULTS

The short, ssDNA template used in most of the studies described below had the sequence d(CAGA(CA)$_5$-CTGCAAAGC). This template contained: (1) the preferred initiating trinucleotide d(CTG); (2) five residues 3′ to the d(CTG), the minimum required for efficient primer synthesis (Swart & Griep, 1993); and (3) a single guanine near the 5′-end to enable study of CTP, dCTP, and ddCTP incorporation at a unique site. The assay monitored the incorporation of radioactive nucleotides into the RNA primers of various lengths using high-resolution denaturing polyacrylamide gel electrophoresis. Whether or not the primer RNA sequence was complementary to the template sequence was verified by "four-lane" ddNTP incorporation; primase will incorporate ddNTPs to allow primer RNA sequencing (Swart & Griep, 1993). The sequences of the 23-nucleotide DNA template and its full-length (16 nucleotide) complementary RNA primer emphasize the locations of the phosphates. When [γ-^{32}P]ATP was used as the probe, only primer RNA with a 5′-ATP was visualized because the primer's initiating nucleotide was the only one to retain its γ-phosphate (and therefore label). In addition, initiation from only one position was possible because there was only one thymine in the template sequence which could serve as the initiation site for an adenine nucleotide. Primer yield was simple to quantitate in this case since each RNA primer had one radioactive nucleotide independent of its length:

RNA primer

3′-GpUpCpUpGpUpGpUpGpUpGpUpGpUpGpUp**GpAppp**

ssDNA template

5′-d(CpApGpApCpApCpApCpApCpApCpAp**CpTpG**pCpApApApGpC)

In contrast, when the α-labeled rNTPs, [α-^{32}P]GTP and [α-^{32}P]UTP, were used as probes of primer synthesis, all RNA primers were visualized regardless whether they were initiated by ATP. Because the α-labeled probes resulted in multiple sites of radiolabel incorporation per RNA primer, the correct primer sequence (and therefore length) needed to be considered so that the yield of primers could be calculated from the radioactivity incorporated per RNA primer length divided by the number of that nucleotide incorporated per primer length.

Optimal Conditions for Primer Synthesis. Standard buffer conditions were 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 100 mM potassium glutamate, 10 mM dithiothreitol, 200 μM each of CTP, GTP, and UTP, and 100 μM [γ-^{32}P]-ATP. During the trials that established the optimum conditions for primer synthesis, DNA template and primase concentrations were both 200 nM, and incubation was for 10 min at 30 °C.

There was no activity in the absence of magnesium acetate, optimum activity at 10 mM, and inhibition at higher concentrations. Potassium glutamate from 50 to 100 mM gave maximum primer synthesis activity. Higher glutamate concentrations were inhibitory such that activity was reduced to 50% at about 170 mM. Primer synthesis was not affected by altering the pH from 6.0 to 9.0. Temperature has very significant effects on primer synthesis, being highest at 30 °C, reduced at 25 and 37 °C, and not evident at 0 or 45 °C (data not shown). We also tested the temperature effect on primer RNA synthesis in the G4 origin system (M13Gori ssDNA coated with SSB). We confirmed that 30 °C was optimal for the synthesis of the 30-nucleotide-long primer (Rowen & Kornberg, 1978a). The major difference between the G4 origin system and our single-stranded DNA template system was that at 0 and 45 °C, even though the yields were much lower than at the intermediate temperatures, short RNA primers were synthesized (12−26 nucleotides long) rather than none.

The effects of rNTP concentrations on primer synthesis were slightly more complex. Due to the nature of the template used, each rNTP was involved in a different stage of primer synthesis. Initiation of primer synthesis from the trinucleotide start site d(CTG) required ATP, elongation required GTP and UTP, and 16-nucleotide primer synthesis required CTP. All four ribonucleotides showed maximal activity at 200 μM (data not shown). Apparent K_M's for ATP, GTP, and UTP were 30−50 μM while the apparent K_M for CTP was 24.5 μM (discussed later). Values of about 10 μM for each ribonucleotide have been reported for the G4 system (Rowen & Kornberg, 1978a) which might be explained by the different protein and DNA requirements.

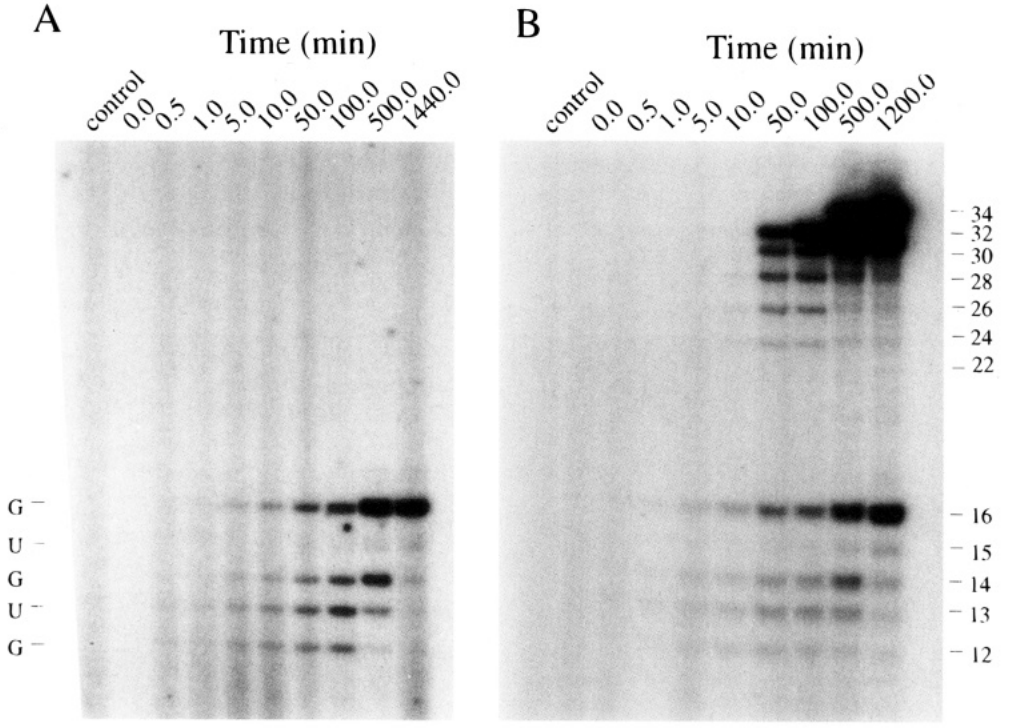

FIGURE 1: Primer synthesis time course using (A) [γ-^{32}P]ATP and (B) [α-^{32}P]UTP as probes. Primase (200 nM) and 200 nM template d(CAGA(CA)$_5$CTGCAAAGC) were incubated for the times indicated along the top of each gel image. The samples were then treated and electrophoresed, the imaging plate was exposed at 20 °C, and the resulting image was prepared as described under Materials and Methods. Along the left side of the image is the 3′-terminal sequence of the "template length-dependent primers", and along the right side are the RNA primer lengths for both "template length-dependent primers" and "overlong primers". The "control" reaction lacked primase.

Time Course for [γ-^{32}P]ATP Incorporation into RNA Primers. In the time course of [γ-^{32}P]ATP-initiated RNA primer synthesis directed by the template d(CAGA(CA)$_5$-CTGCAAAGC), a specific pattern of primer lengths was evident (Figure 1A). Even at the shortest incubation times, the shortest primer observed was 12 nucleotides. By 100 min, it was possible to detect primers that were 8 and 10 nucleotides in length, but their levels were so low that they represented much less than 1% of the primers synthesized. The lack of primers shorter than 8−12 nucleotides has been reported in priming systems that involved other proteins in addition to primase (Ogawa et al., 1983; Zechner et al., 1992a). That this same activity was observed in the presence of only primase indicated that it was an intrinsic property of primase. Each length species of primer accumulated at about the same rate until about 100 min of incubation. For incubations longer than 100 min, the 16-nucleotide primer accumulated at the expense of the shorter primers. This pattern of primer synthesis indicated a distributive mode for primers longer than 12 nucleotides. This pattern also indicated that the rate-determining step either was the first phosphodiester bond formation rate or preceded it.

The longest primer was 16 nucleotides and was the correct length for a primer that initiated at the unique thymine and reached the 5′-end of the template. In fact, ddNTP sequencing with primase confirmed that the [γ-^{32}P]ATP-initiated primers were complementary to the template (data not shown). Thus, the ATP-initiated primers were template length-dependent primers. In our previous study, we found that all of the [γ-^{32}P]ATP-initiated primers were equal in length to the template sequence plus one extra nucleotide (Swart & Griep, 1993). In that study, we used 2.0 μM primase and 2.5 nM template whereas in the current study both primase and template concentrations were 200 nM. At the much lower primase/template ratio in the current study, most of the RNA primers did not have the extra noncomplementary, 3′-overhanging nucleotide. This is consistent with other RNA and DNA polymerases which showed "complement plus a single 3′-overhang" activity only at very high enzyme/template ratios or at very long incubation times (Clark, 1988; Martin et al., 1988).

Primer Synthesis Kinetics for ATP-Initiated Primers (Also Appropriately Called Template Length-Dependent Primers). The primer synthesis time course was quantitated both for the amount of total primers synthesized and for the amount of 16-nucleotide primer synthesized (Figure 2A). Not every template directed the synthesis of a complementary [γ-^{32}P]-ATP-initiated primer. Even though the reaction was clearly saturated by 24 h (1440 min), the number of total primers per template was only 5.2%. Control experiments showed that the low yield was not due to loss of primase activity during the incubation. Primase retained full activity when incubated at 30 °C for as long as 2 days. As described below, the low yield is explained in part by the unfavorable association constant between primase and template. However, even with an unfavorable association constant, recycling of primase from a now-primed template to other unprimed templates should have eventually led to all of the templates being primed given the length of incubation time. There appeared to be two causes of the low yield of primer per template: each template was primed only once, and other types of primers were synthesized that were not initiated with ATP.

A kinetic model to describe ATP-initiated primer synthesis needed to incorporate the slow kinetics and the low yield. On the basis of the data presented above, we used a model in which it was assumed that the primase, template, and rNTPs were in rapid equilibrium and that the rate of the first

bond formed was the rate-determining step:

$$P + D_{r=0} + rNTPs \overset{K_d}{\rightleftharpoons} P{\cdot}D_{r=0}{\cdot}ATP{\cdot}GTP + rNTPs \xrightarrow{k} P{\cdot}D_{r=12-16} + PP_i$$

where P was primase, D_r was the ssDNA template with complementary r long RNA primer, K_d was the dissociation constant between primase and template in the presence of NTPs, and k was the formation rate of the first phosphodiester bond as measured by total primer synthesized. The slow formation rate of the first phosphodiester bond made this a good assumption and indicated that dissociation of the primase·template·NTP complex was much faster in comparison. The rate of total primers synthesized can be closely approximated by the kinetic equation:

$$[P{\cdot}D_{r=12-16}] \approx f_{ATP\ monomers}[P_0](1 - e^{-k_{obsd}t})$$

where $f_{ATP\ monomers}$ is the fraction of primase that synthesizes primers initiated with ATP, $[P_0]$ is the concentration of free primase at time zero, and k_{obsd} is the observed rate constant. As determined later, the primase and ssDNA concentrations were below the dissociation constant, and, therefore, not all of the primase was complexed with the ssDNA. Thus, the first-order catalytic rate constant, k, was calculated using the relationship $k_{obsd} = k[P{\cdot}D_0]/P_0$, where

$$[P{\cdot}D_0] = \{P_0 + D_0 + K_d - [(P_0 + D_0 + K_d)^2 - 4P_0D_0]^{1/2}\}/2P_0$$

in which K_d is the dissociation constant between primase and ssDNA in the presence of saturating amounts of ATP and GTP and D_0 is the concentration of DNA template at time zero. When the time course data were fit to this model using $f_{ATP\ monomers} = 0.052$ (taken from the 24 h time point), k_{obsd} was calculated to be 1.64×10^{-4} s^{-1}. It will be shown later that the dissociation constant was 720 nM. Using this value, the rate k was 0.00089 s^{-1}.

All RNA Primers Synthesized Could Be Observed by Monitoring [α-^{32}P]UTP Incorporation. Even though the [γ-^{32}P]ATP-initiated primers were complementary to the template, the low primer yields suggested either that not all templates were competent to be primed or that not all primers were initiating from the unique thymine in the template. Various attempts to enhance the fraction of templates competent to be primed proved fruitless. For instance, preheating the oligonucleotide templates for various lengths of time and in the presence of various salts such as magnesium did not result in greater primer yields, indicating that the templates were homogeneous in their ability to be primed. To test whether primers were being synthesized without ATP as the initiating nucleotide, we measured [α-^{32}P]UTP incorporation into RNA primers. The template d(CAGA(CA)$_5$CTGCAAAGC) contained multiple adenines which should direct primase to incorporate multiple radiolabeled uracils independent of where it was initiated.

Two species of primers were evident when using [α-^{32}P]-UTP as the probe of RNA primer synthesis (Figure 1B): "template length-dependent primers" and "overlong primers". The template length-dependent primers had the same pattern and migrated similarly to the species observed for [γ-^{32}P]-ATP incorporation (Figure 1A). Dideoxyribonucleotide

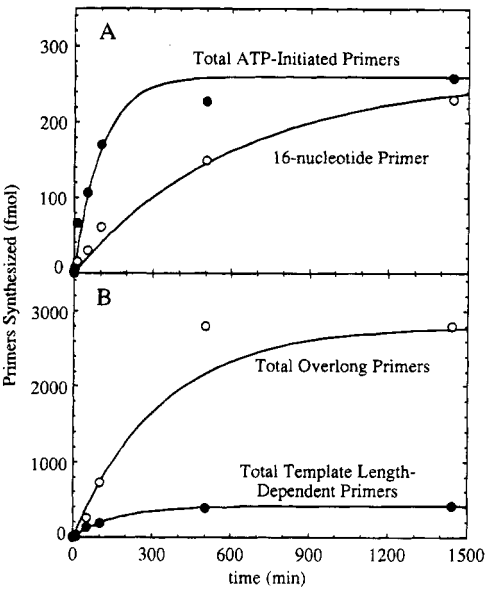

FIGURE 2: Primer synthesis kinetics for the incorporation of (A) [γ-^{32}P]ATP and (B) [α-^{32}P]UTP. The images from Figure 1 were quantitated as described under Materials and Methods. The data in part A were fit by the simple exponential equation described in the text using 2000 fmol of primase, 0.052 as the fraction of primase synthesizing these primers, and a rate of 1.64×10^{-4} s^{-1} for total primers and 2.72×10^{-5} s^{-1} for 16-nucleotide primers. In part B, the "template length-dependent primer" data were well fit by the simple exponential equation described in the text using 2000 fmol of primase, 0.084 as the fraction of primase synthesizing these primers, and a rate of 9.95×10^{-5} s^{-1}. However, the "overlong primer" data were not well fit by the single exponential equation using values of 0.56 as the fraction and 4.99×10^{-5} s^{-1} for the rate.

sequencing confirmed that the sequences of the template length-dependent primers were complementary to the template. As shown below, this primer species also accumulated according to the same time course as the primers observed using [γ-^{32}P]ATP (Figure 1A). Therefore, the template length-dependent primers were the same as the ATP-initiated primers already described. We have avoided the term "primer monomer" because this term has been used to describe an increment unit of primer length among a series of longer primers synthesized by eukaryotic primase on very long templates (Singh et al., 1986).

The origin of the overlong primers remains under investigation. A number of factors influence whether they are synthesized including the template sequence on the 3′-side of the initiating sequence d(CTG), the ratio of primase to template used, and the time allowed for synthesis. The initiating nucleotide was neither ATP nor GTP since primer synthesis carried out with either [γ-^{32}P]ATP or [γ-^{32}P]GTP as the probe did not lead to visualization of overlong primers. In fact, with [γ-^{32}P]GTP there were no template length-dependent primers observed, indicating that GTP never served as the initiating nucleotide for any type of primer synthesis. In contrast, overlong primers have been observed when using [α-^{32}P]ATP, [α-^{32}P]GTP, and [α-^{32}P]UTP, indicating that these three nucleotides were incorporated within its sequence. The longest overlong primer was 34 nucleotides, a significant excess over the 23-nucleotide template. Their limited maximum length suggested that they were somehow template sequence-directed. Possibly a signficant clue was that they were best observed when the concentration of primase was less than that of the template.

In fact, at long synthesis times using very low primase/template ratios, it was possible to visualize overlong primers that were two, three, and four times the length of the shortest ATP-initiated species. This observation suggested that the overlong primers were being generated as a result of one primase serially using ssDNA templates. In this case, primer synthesis would terminate when all of the templates have complementary RNA. The mechanism for the jump from template 1 to 2 and then 2 to 3 etc. may involve the ability of primase to add an extra nucleotide to the 3′-end of the RNA primer at long incubation times (Swart & Griep, 1993). If this overhanging 3′-nucleotide was adenine, it would explain why [α-^{32}P]ATP becomes incorporated into the overlong primers. A 3′-terminal adenine could assist the terminus of the primer in annealing to a second template at the d(CTG). Then, primer synthesis could continue for another incremental template length.

The ddNTP-determined sequence of the 3′-end of the overlong primers (Figure 1B) was 3′-r(GUGUGUGUGUGUGUGU) from lengths 34 to 19 (gel not shown). The overlong primer sequences at lengths less than 19 were difficult to obtain because of low overlong primer yield at lengths 18 and 17 and the prominent ATP-initiated primers at length 16 and less. Further compounding this problem was that the dideoxy sequencing reaction inhibited overlong primers better than template length-dependent primers. Nevertheless, from the sequence obtained, it was concluded that it was not strictly template sequence-dependent. The longest d(AC) stretch in the template was 12 nucleotides, too short to have coded for the 16-nucleotide 3′-terminus of repeating GU. Likewise, if the overlong primer sequence had been template sequence-directed, the sequence of the 3′-end of the overlong primers would have included a 3′-antepenultimate cytosine which was not observed. Polymerase slippage on the template is one mechanism that has been proposed to explain the template-dependent but not template sequence-complementary polymer synthesis observed by a variety of other RNA and DNA polymerases (Kunkel, 1990; MacDonald et al., 1993; Martin et al., 1988; Mikita & Beardsley, 1994) including possibly eukaryotic primase (Sheaff & Kuchta, 1994). The origin and nature of the overlong primers generated by *E. coli* primase remains an area of active study in our lab.

Template Length-Dependent and Overlong Primer Synthesis Kinetics. The kinetics of template length-dependent primer and overlong primer synthesis were determined for the case in which [α-^{32}P]UTP was used as the probe (Figure 2B). The femtomoles of total template length-dependent primer synthesized at each time was obtained by dividing the yield of uracil incorporated into each template length-dependent primer species by the number of uracils in that primer and then summing. At 20 h (1200 min), 8.4% of the templates had one template length-dependent primer, an additional 3.2% above that observed for [γ-^{32}P]ATP incorporation. Considering the number of corrections involved, this may be within error. Concerning radiolabel quantitation, we also did not account for degradation products that will accumulate during storage which would lead to an overquantitation of nucleotide incorporation. A final explanation might be that the increase was due to template length-dependent primers that migrated with similar lengths but which had not been initiated with ATP. When the kinetic data were fit to the equation described previously, k_{obsd} was

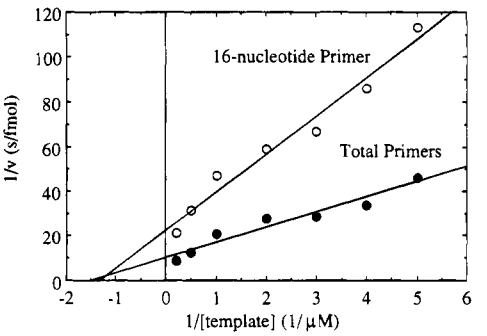

FIGURE 3: Template concentration effect on the rate of primer synthesis. The incorporation of [γ-^{32}P]ATP was monitored during a 10 min incubation of primase with rNTPs and various concentrations of the template d(CAGA(CA)$_5$CTGCAAAGC). Primer synthesis was linear during the first 10 min. Two data sets were averaged, and the double-reciprocal plot of the data is shown. The total primers synthesized data are well fit with a V_{max} of 10.2 fmol/s and K_M of 720 nM. The 16-nucleotide-long primer data are well fit with a V_{max} of 3.97 fmol/s and a K_M of 720 nM.

9.95×10^{-5} s^{-1} and k was 0.000538 s^{-1}. This rate was similar to that calculated for [γ-^{32}P]ATP incorporation, confirming that the template length-dependent primers were very similar in their properties to the ATP-initiated primers described above.

Determining the yield of overlong primers necessitated knowledge of the number of uracils incorporated per primer. Since it was only possible to establish the sequence of the 3′-terminal half of the primer, the uracil content of overlong primers had to be estimated. The uracil content in full-length overlong primer may be as low as 21% (7/34 uracils established from sequencing) or as high as 79% (27/34 if the non-guanosine nucleotides were to be all uracils). Assuming that half the nucleotides incorporated were uracil gave an estimate for the total yield of overlong primers of 0.56 overlong primer per template (Figure 2B). Overlong primer synthesis kinetics were not well fit by the single-exponential model, indicating the need for an additional kinetic step in the model. If the overlong primers are proven to be due to primase elongating a template length-dependent primer by an additional length of template, the extra step would include the ratio of primase to template among other factors. Unfortunately, not enough information about the nature of overlong primers existed to develop a two-exponential model.

Template Concentration Effect on ATP-Initiated Primer Synthesis. To determine the template kinetic constants for ATP-initiated, template length-dependent primer synthesis, a template concentration experiment was performed for [γ-^{32}P]ATP incorporation during a 10 min incubation. This incubation time was chosen because overlong primers began to be synthesized after this time and because template length-dependent primer synthesis was linear during this time. In addition, the template concentrations were in excess of primase to minimize the synthesis of the overlong primers. Double-reciprocal plots of the rate of primer synthesis versus template d(CAGA(CA)$_5$CTGCAAAGC) concentration indicated a K_M of 720 nM ssDNA template (Figure 3). Since the rate of primer synthesis was so slow, this K_M was equivalent to the K_d for this template. The K_M was the same for both the 16-nucleotide primer and total primer synthesis and represented formation of the primase−template−NTP complex that preceded the first phosphodiester bond forma-

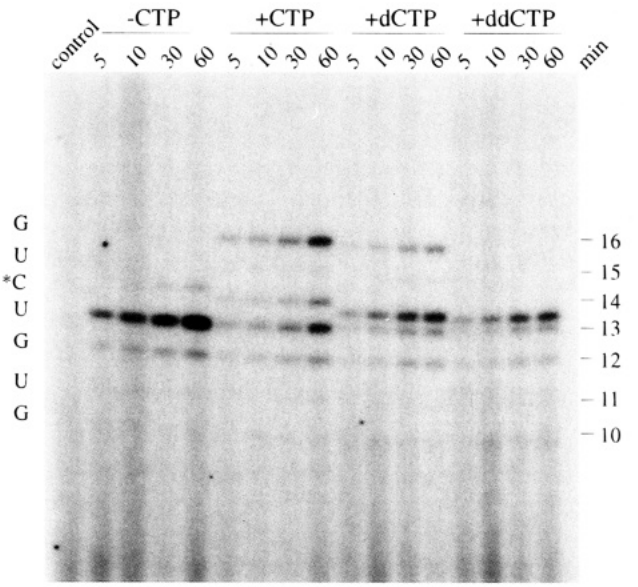

FIGURE 4: Time course for the incorporation of rCTP, dCTP, and ddCTP at position 14 in the primer. The incorporation of [γ-^{32}P]ATP into primers complementary to the ssDNA template d(CAGA(CA)$_5$-CTGCAAAGC) was monitored over the indicated lengths of time in the presence of 200 μM cytidine analog. The "control" reaction lacked primase. Along the left side is the primer sequence and along the right the length. The nucleotide incorporated at position 14 migrated differently depending on the structure of the 3′-terminal cytidine analog.

tion. The V_{max} for total primer synthesis was 10.2 fmol of primer/s. From this value, the turnover number for the first phosphodiester bond was calculated to be 0.00204 s^{-1}, slightly larger than the values determined during the kinetic analysis. At higher template concentration, the fraction of primase that synthesizes template length-dependent primers that initiate with ATP may increase. This would cause the primer synthesis rate measured in this assay to be larger than in the time course assays.

Site-Specific Insertion Rate. The template d(CAGA-(CA)$_5$CTGCAAAGC) was designed with a 5′-antepenultimate guanosine for use in single-site incorporation studies. The probe was [γ-^{32}P]ATP to simplify quantitation and to limit analysis to template length-dependent primer synthesis. Even though incorporation of dNTPs (Rowen & Kornberg, 1978b; Wickner, 1977) and ddNTPs (Swart & Griep, 1993) into ATP-initiated primers has been reported previously, a comparison of their relative rates of insertion was not reported. We observed that CTP and its analogs affected the lengths of the primers much more than their yields (Figure 4, Table 1).

In the absence of a cytidine triphosphate, the major primer observed was 13 nucleotides long (Figure 4). This was the primer length expected if it was synthesized one nucleotide short of the complementary template guanosine. Unexpectedly, we also observed that about 6% of the primers were 14 nucleotides long, the result of a misinsertion event. The misinserted nucleotide was not CMP since the primer synthesized under these conditions migrated slower than the 3′-cytosine-terminating primer in the adjacent "+CTP" lanes (Figure 4, compare the migrations of the 14-mers). In a Tris, borate, EDTA buffer, if the 3′-terminal nucleotide was either AMP, GMP, or UMP, it would be expected to decrease the electrophoretic mobility of the misterminated primer compared to a CMP (Frank & Köster, 1979).

Table 1: Effect of CTP Analogs on Site-Specific Insertion into a Primer[a]

condition	total primer (fmol)	frac$_{14+\ primers}$	$V_{ins\ 14th}$ (fmol/s)	$V_{ins\ 15th}$ (fmol/s)
no CTP	55.9	0.06 ± 0.02	0.0045	ND
rCTP	42.5	0.47 ± 0.06	0.030	0.023
dCTP	29.0	0.61 ± 0.03	0.057	0.0042
ddCTP	18.2	0.51 ± 0.06	0.019	ND

[a] The data analyzed are shown in Figure 4. Cytosine incorporation should generate a 14-nucleotide-long primer. The measurements made were as follows: yield of total primers synthesized; frac$_{14+\ primers}$, the fraction of primers that are 14 nucleotides or longer; $V_{ins\ 14th}$, the insertion velocity for the 14th nucleotide whether it was cytosine or not; and $V_{ins\ 15th}$, the insertion velocity for the 15th nucleotide. The frac$_{14+\ primers}$ remained relatively constant throughout the time course presented in Figure 4, so the four values were averaged and the standard deviation calculated. The insertion velocities were calculated using the running start insertion rates equation described by Goodman and co-authors (Goodman et al., 1993). Simply, the calculation we used was [(fmol of primers equal to or longer than the length being measured)/(fraction of total primers one nucleotide shorter than the one being measured)]/time. Since the amount of primers 15-nucleotides long was negligible, it was not possible to measure $V_{ins\ 16th}$. ND means not detectable.

Therefore, the terminal nucleotide's identity could not be identified according to its differential mobility. It was only possible to conclude that the possibly not optimized misinsertion rate was an order of magnitude slower than the optimum insertion rate for the correct nucleotide (Table 1).

In the presence of rCTP, the usual pattern of primer lengths was observed during the time course (compare Figure 1 with the "+CTP" lanes in Figure 4). The insertion velocities for the 14th and 15th nucleotides were very similar (Table 1), whereas the insertion velocity for the 16th nucleotide was much more rapid since virtually no 15-nucleotide primer accumulated. The migration of the 14- and 16-nucleotide-long primers in these lanes served as indicators for the electrophoretic mobility of primers with a ribocytosine at position 14.

In the presence of dCTP, primers of 12, 13, 14, and 16 nucleotides were again evident (Figure 4) except that there was more 14- and less 16-nucleotide primer. This was because the deoxyribonucleotide was inserted at a rate somewhat faster than a ribonucleotide but was difficult to incorporate as evidenced by the slow insertion velocity for the next nucleotide (Table 1). The ability of primase, an RNA polymerase, to incorporate 2′-deoxyribonucleotides was not unexpected since T3, T7, and SP6 RNA polymerases will incorporate 3′-dNTPs (Axelrod & Kramer, 1985; Htun & Johnston, 1992) and T7 RNA polymerase has been shown to incorporate 2′-dNTPs (Wyatt & Walker, 1989). Evidence that a deoxyribonucleotide was incorporated is that the 14- and 16-nucleotide-long primers migrated half nucleotide faster than those in the "+rCTP" lanes. The increased mobility of the 2′-deoxyribo-terminating primer was greater than that predicted by a simple decrease in molecular weight caused by the loss of a single oxygen. The differential mobility likely was compounded by differential interactions between the borate in the electrophoresis buffer and the ribo or deoxyribo termini of the polymers. A similar half nucleotide change in the mobility of RNA was observed when transcripts were terminated by a 3′-deoxyribonucleotide (Htun & Johnston, 1992) and can be seen in denaturing gels

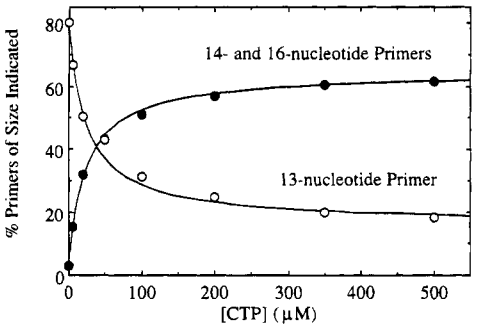

FIGURE 5: Effect of CTP concentration on the lengths of primers synthesized. The incorporation of [γ-^{32}P]ATP into primers was monitored during a 10 min incubation in the presence of various CTP concentrations. Two data sets were averaged to generate the plots shown. In the absence of CTP, the majority of primers were 13 nucleotides long. When CTP was present, the primers could be elongated past position 14, and primers of 14 and 16 nucleotides in length accumulated. The curves were generated using $[CTP]_{50\%\ effect} = 24.5\ \mu M$ as determined in double-reciprocal plots. The fitted equations were %(14- and 16-nucleotide) = 2.9% + 61.5%[CTP]/(24.5 μM + [CTP]) and %(13-nucleotide) = 80.0% − 64.0%[CTP]/(24.5 μM + [CTP]).

of RNA containing 2′-deoxyribonucleotides [Figure 1 of Wyatt and Walker (1989)].

In the presence of ddCTP, primers of 12, 13, and 14 nucleotides were evident (Figure 4). Again, the 14-nucleotide primers had slightly faster mobility than those ending in rCMP or dCMP although it migrated very close to that of the dCMP-terminated primers. No primers were synthesized with more than 14 nucleotides, consistent with the inability of polymerases to elongate from dideoxyribonucleotides.

CTP Concentration Affects the Primer Length in a Template Sequence-Dependent Manner. As stated above, the template d(CAGA(CA)$_5$CTGCAAAGC) was designed with a guanine in its third position for use in single-site incorporation studies. It was possible to demonstrate that full-length primer synthesis was dependent on rCTP concentration in the hyperbolic manner characteristic of an enyzme-catalyzed reaction (Figure 5). Primers longer than 13 nucleotides (14 and 16 nucleotides long) accumulated at the expense of the 13-nucleotide primer as the CTP concentration was increased. The apparent CTP K_M was 24.5 μM, about half that for the other three nucleotides.

DISCUSSION

E. coli primase is the slowest, weakest template binding and possibly most error-prone nucleic acid polymerase yet described (Table 2). Under optimal reaction conditions, the rate-determining step for primer synthesis occurs prior to the first phosphodiester bond formed. The rate of 0.00089 s^{-1} is even slower than that for eukaryotic primase which takes place at 0.0075 s^{-1} (Sheaff & Kuchta, 1994). Like eukaryotic primase though, its rate-determining step appears to occur at or prior to the first phosphodiester bond formed. The nature of the rate-determining step has not been discovered but may be an NTP-induced conformational change (Sheaff & Kuchta, 1994) or somehow related to the one-dimensional diffusion along the ssDNA template that must occur prior to initation site binding. Perhaps related is that the rate-determining step for transcription RNA polymerases occurs prior to chain initiation. The step occurs between promoter opening and the first phosphodiester bond formation although at a much faster rate than for the primases. In contrast, during polymer elongation by the catalytically faster DNA polymerases, the rate-determining step follows phosphodiester bond formation but precedes pyrophosphate release and translocation. Primase elongation kinetics for phosphodiester bonds 2−10 are also faster than its own initiation reaction and may involve this same elongation kinetic barrier. However, the elongation kinetic barrier is smaller than the initiation barrier and does not determine the overall rate of primer synthesis.

Template length-dependent primers accumulate earlier than the overlong primers and, because of this, probably represent the more biologically relevant primers. The template length-dependent primers but not the overlong primers are also of the correct length (11 ± 1) and begin with the preferred initiating nucleotide, ATP, to correspond to those observed *in vivo* (Kitani et al., 1985; Yoda & Okazaki, 1991; Yoda et al., 1988). The majority, if not all, template length-dependent primers are initiated complementary to the thymine on the template.

Table 2: Selected Polymerases, Their Synthetic Rates and Error Frequencies[a]

polymerase	max rate (s^{-1})	K_M or K_d (nM)	error freq	template	reference
RNA polymerases					
E. coli primase	0.00089	720	<0.1[b]	this study	
eukaryotic primase	0.0075	<100		poly(dT)	
	0.0075	<100		d(TCC)$_{20}$	Sheaff & Kuchta (1993)
			>0.01	d(TCC)$_{20}$	
			<0.01	d(ACC)$_{20}$ and d(CCCTCTCTCT)$_3$	Sheaff & Kuchta (1994)
E. coli RNA pol	0.100	1		duplex promoter	Hawley & McClure (1982)
			10^{-4}−10^{-5}	elongation	Springgate & Loeb (1975)
T7 RNA pol	0.83 s^{-1}	20		duplex promoter	Martin & Coleman (1987)
DNA polymerases (family A)					
E. coli DNA pol I	3.8 s^{-1}	2		oligo(dT)/poly(dA)	Bryant et al. (1983)
	50 s^{-1}	5		DNA-primed template	Kuchta et al. (1987)
T7 DNA pol	300 s^{-1}	18		DNA-primed template	Patel et al. (1991)
			10^{-5}−10^{-6}	single-site incorporation	Wong et al. (1991)

[a] The rates and error frequencies were determined over a range of differing conditions (pH 7.4−8.0 and 22−37 °C) and templates. Therefore, the values are only used comparatively. The maximum synthetic rates and minimum dissociation constants were chosen when a range was provided by the reference. The RNA polymerases include examples of primases, multisubunit bacterial transcriptases, and monomeric bacteriophage RNA transcriptases. Eukaryotic primase acts as part of a four-polypeptide complex that includes DNA polymerase α, the heterodimeric primase, and a phosphorylatable subunit. [b] The error frequency requires that the K_m and V_{max} for the misinserted nucleotide be known, the frequency being the ratio of V_{max}/K_m for the incorrect and correct nucleotides. The error frequency given for *E. coli* primase represents an upper limit since it has been calculated for the case in which the K_m's for the incorrect and correct nucleotide were the same.

There is a dichotomy in that primase is slow and yet very error-prone. Low fidelity may be tolerated because it may set the more accurate DNA synthesis machinery in motion. Primase stalling at a mismatch may stimulate the DNA polymerase's proofreading exonuclease to bind and then excise the mismatch. This could then be followed by transfer of the corrected primer to the DNA polymerase active site. Alternatively, after the complete Okazaki fragment has been synthesized, the incorporated primer mismatches may be a signal for removal by cellular repair enzymes or by the primer excision enzymes. Excision of primers with misincorporated nucleotides would prevent any permanent DNA synthetic errors. However, primase is capable of incorporating dNMPs, and if it misincorporated several consecutive dNMPs, full primer excision may be inhibited and DNA damage could become problematic. Primase activity then could make lagging-strand DNA synthesis more error-prone than leading-strand DNA synthesis.

Even though many family A DNA polymerases have 3′→5′ exonuclease "proofreading" domains, the transcription RNA polymerases which lack them are only about 10 times more error-prone (Table 2). In contrast, *E. coli* primase is many times more error-prone than *E. coli* RNA polymerase. Primase would appear to be designed for misincorporation. Although we did not attempt to stimulate misincorporation or misinsertion in this study (Figure 4), primase did not incorporate the misinsertion to a significant extent. This raises the possibility of misinsertion-induced stalling. We and others have found that primase will efficiently insert dNMPs but is slow to elongate from them (Rowen & Kornberg, 1978b; Swart & Griep, 1993; Wickner, 1977). It has already been suggested that primase stalling may stimulate the transfer of the primer to the DNA polymerase (Rowen & Kornberg, 1978b). We have also found that primer synthesis slows down after 12 nucleotides and this may represent yet another way in which primase may be recognized as being "stalled" by the DNA polymerase.

The slowing down of primer synthesis once the length reaches 12 nucleotides is not dependent of the distance to the end of the template since templates with longer 5′-flanking sequences showed the same effect (Swart, unpublished results). Thus, the shift of primase from fast to slow elongation is a result of distance from the d(CTG) rather than to the 5′-terminus. Structurally, primase may maintain contact with the initiating trinucleotide during subsequent primer elongation, and its active site flexibility would then limit primer length to the observed number of nucleotides beyond the d(CTG).

Primase has a moderate affinity, K_M = 720 nM, for the template used in this study. This affinity is 1 or 2 orders-of-magnitude less than that exhibited by other polymerases for their preferred templates (Table 2). One possibility for this low affinity relates to its function at a replication fork. Primase and hexameric DnaB helicase are capable of stimulating one anothers activities and may bind to one another. DnaB helicase has modest affinity for the ssDNA and dsDNA at the replication fork. Perhaps the ssDNA affinity and primer synthesis activity of primase are considerably enhanced by the presence of DnaB helicase. *In vivo* and *in vitro*, there is 1 primer synthesized in a directed manner every 1500 lagging-strand nucleotides, and the process continues for over 2 million nucleotides in *E. coli*. If the enhancement were several orders of magnitude, helicase could limit primase action to the replication fork because helicase's action is limited to the replication fork. In this way, DnaB helicase could influence the timing of primer synthesis initiation. Another factor limiting primer synthesis to the replication fork is that SSB, which is thought to coat the lagging-strand ssDNA, prevents primase from initiating RNA primers on the ssDNA (Swart & Griep, 1993). Thus, the low ssDNA affinity of primase limits primer synthesis to the replication fork.

The goal of this study was to determine the kinetic mechanism of site-specific initiating, sequence-dependent primer synthesis with the intent of creating a model for primase function. Knowing that the rate-determining step is the first phosphodiester bond formation or some step which precedes it suggests several mechanisms as to how primase regulates the initiation of Okazaki fragment synthesis (Wu et al., 1992c). However, the rate of primer synthesis by primase alone *in vitro* is too slow to be relevant *in vivo*. At the replication fork, there are many proteins with which primase interacts to coordinate the timing of primer synthesis initiation. By assessing the effects of these other proteins on primer synthesis kinetics, it should be possible to establish which protein(s) is (are) responsible for controlling the timing of Okazaki fragment initiation. It is also possible that several of the preprimosomal proteins interact directly with primase. The effect of these proteins on primase's affinity for the DNA template and rate of primer synthesis can now be more easily studied using our simplified assay system.

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